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Effect of storage temperatures on the pathogenicity of *Beauveria bassiana* formulations against the cotton leaf worm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae)

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Abstract :

The aerial conidia of the entomopathogenic fungus *Beauveria bassiana* served as the basis for the preparation of the wettable powder and inverted emulsion formulations. We evaluated the insecticidal efficacy of prepared conidia against the second-instar larva of the Cotton leafworm, *Spodoptera littoralis*, according to storage temperature in order to establish the ideal conditions for long-term storage.

The formulations' pathogenicity to *S. littoralis* larvae varied significantly, according to an analysis of mortality data. The formulation that best preserved the pathogenicity of conidia for a longer period of time was wettable powder. Formula kept at freezing conditions -10°C was more virulent comparing to formula kept at cooling conditions 4°C and at the room temperature (25°C). This finding implies that conidial pathogenicity is maintained at low and moderate temperatures.

Keywords:

Entomopathogenic fungus, *Beauveria bassiana*, Formulations, Conidia, *Spodoptera littoralis*

1. Introduction:

The Egyptian cotton leafworm (CLW), *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) is one of the most destructive pests of several crops such as cotton, corn, peanut, clover, vegetables, and various fruits in Africa, Asia and Europe [1, 2, 3].The cotton leaf worm is a destructive pest to about 112 host plants from different families in Egypt as well as in Mediterranean and Middle East countries [4, 5]. In addition to its direct damage, reducing photosynthetic area and reducing the marketability of vegetables and ornamentals [6].

The most effective control measure against cotton leaf worm has been achieved by using chemical insecticides [7]. Over the past 40 years, the intensive use of broad-spectrum insecticides against the cotton leaf worm had led to the development of resistance to many of them [8, 9]. Also, the continuous and unwise use of insecticides to control agricultural pests usually lead to adverse effects on beneficial insects, fish, and wildlife, hazards to man and animals by environmental pollution, and residues in foods [10, 2, 11]. Therefore, there is a great need to research alternative control agents with a new mode of action, safe to non-target organisms and environment components, and compatible with integrated pest management practices. Entomopathogenic fungi (EPFs) are common natural enemies of arthropods worldwide and are attracting attention as potential biocontrol agents. There are more than 700 species of EPFs [12, 13, 14]. Because they occur naturally, EPFs also have low environmental impact and are generally considered environmentally safe agents with low toxicity to mammals [15]. Temperature plays a significant role on the effectiveness of EFPs, especially high temperatures affect negatively conidial viability and germination [16, 15]. For example, *Beauveria bassiana* was found to be more effective against *Rhyzopertha dominica* and *Sitophilus oryzae* at 26 °C than at 30 °C [17]. High temperature reduces the viability of conidia in the field, a major obstacle to the successful application of entomopathogenic fungi in agriculture. Temperature is important for entomopathogenic fungi because it affects their metabolism by altering processes involving enzymes, toxins, spore germination, germ tube development, infiltration, colonization, and reproduction. To maintain the viability of conidia in the field, reduce costs and ensure biological control, another approach is to use formulated products that may provide benefits such as protecting conidia from radiation, increasing shelf live and facilitating storage, transport, etc. Jones and Burges [18] Among the commercial biopesticides currently on the market, about 25% of the sales are products formulated from oil dispersions, and the remaining 75% are sold as technical products only, without treatment or Add substances to improve, field persistence, efficient pest control, etc. [19]. Wettable powders (WP) are

usually composed of mineral materials such as clays and talc that can be ground into fine, sprayable powders. Powder formulations with integrated wetting agents, and often including drying materials, have been among the most favored of the formulations used by commercial developers. They are generally easy to handle, have excellent physical properties (preventing fungal propagules from settling and forming aggregations that may be difficult to re-suspend), typically support maximum shelf life of the formulated propagules, are light-weight (minimizing shipping costs), and exhibit low phytotoxicity [20]. Invert emulsion formulation of *B. bassiana* has a milky appearance and can provide the formulated fungus conidia with the water required for germination after application and they contain ingredients that are not harmful and can promote conidial germination and penetration of the insect host [21]. Therefore, this study aimed to prepare different formulations and evaluate the effect of different temperatures on the conidia of the formulated entomopathogenic fungus B. bassiana to examine their pathogenicity to the insect S. littoralis.

2. Materials and Methods

2.1. Culture conditions

The entomopathogenic fungus isolate, *B. bassiana* (AUMC 9896) was isolated in Bio-insecticide Production Unit, Plant Protection Research Institute and identified in Mycological Center, Faculty of Science, Assiut University [16]. This isolate was cultured on Czapek-dox agar (CZA) plates in several Petri dishes (9 cm in diameter) and were grown for 15 days at 25±1°C.

2.1.1. Preparing the wettable-powder formulation

A 250 ml conical flask containing 500 g of talc powder was used as the carrier, and it was sterilized for two days at 160 °C for an hour each day. Following sterilization, the carriers were combined with 5.0% gelatin to act as a sticker, 0.5% sodium glutamate to act as stabilizers, 0.2% Congo red to work as a UV protection, and 0.05% gentamycin to act as an antibiotic to fight germs. Conidia can be collected by gently scraping the surface of 14–15-day-old cultures with a sterile glass slide. 3.0 x 10^9 conidia/ml were thought to represent the approximate number of conidia in each plate.

Conidia from 10 plates were collected, and some of them were combined with powdered ingredients to create a wettable powder composition (unpublished data). This was calculated to be 2.5 X10^8 conidia per gram of powder. For three days, the mixture was allowed to thoroughly dray at room temperature (25 ^{0}C) into the sterile cabinet. The mixture was thoroughly ground by the mixer while cooling after it had dried completely. For the duration of the bioassays, the produced formulation was kept in sterile 50 ml falcon tubes and refrigerated at various temperatures (-10°C, 4°C, and room temperature).

2.1.2. Preparing the inverted emulsion (water-in-oil formulation)

The inverted emulsion formulation consisted of two phases: (a) water or aqueous phase comprising of a mixture of sterile distilled (45% w / w), glycerin (4.00% w⁄w), and water-soluble emulsifier (span 60: 6.00% w⁄w), and (b) oil phase comprising of a mixture of sunflower oil (43% w⁄ w) and oil-soluble emulsifier: Tween 20 (2.00% w⁄ w). For the preparation of this formulation, the ingredients of each one of the two phases were first mixed separately and then the two phases were combined in a 50: 50%ratio by adding the aqueous phase onto the oil phase to obtain a water-in-oil formulation, the two phases are mixed at a high speed (20,000–25,000rpm for 1.5min using a homogenizer). The high speed of mixing is necessary to ensure the homogeneity of the emulsion produced and to obtain longer stability [21].

2.1.3. Introduction of conidia of *B. bassiana* into inverted emulsion formulation

The conidia were harvested by scratching the 14-15 day old culture's surface gently with sterilized glass slid and then suspended in sterile de-ionized water. The conidial suspension was then standardized at 4.0 × 10^7 conidia ⁄ ml to get a final conidial concentration of 1.0 × 10^7 conidia/ml in the prepared formulation. Introduction of standardized conidial suspension into emulsion was performed during preparation of the ingredients by mixing them first with sterile de-ionized water which comprised 45% w/w of total volume of the emulsion. The fungal conidia concentration in the final emulsion was fixed at 1.0 × 10^7 conidia/ml. This is due to the fact the brought conidial suspension in the aqueous phase become standardized at 4.0 × 10^7 conidia/ml as decided by way of a haemocytometer. The prepared emulsion was held in darkish screw-caped glass bottles (500-ml capacity) and stored at different temperatures (-10°C, 4°C and room temperature) for the complete period of bioassays

2.2. Rearing of the Egyptian cotton leafworm, *Spodoptera littoralis*

Egg masses of the cotton leafworm, ***Spodoptera littoralis*** were gained from the Nuclear Research Center (NRC), the Egyptian Atomic Energy Authority (EAEA), Anshas area. Newly hatched larvae were kept in clean glass jars covered with a muslin cloth, where they fed on clean fresh castor bean leaves, ***Ricinus communis*** L., under laboratory conditions at 27±1.0ºC and 70.0±5.0% R.H. and a photoperiod of 12:12 hrs. (L: D) as described by [22, 23]. As larvae reached the 2nd instars, they were used in the experiments described below.

2.3. Pathogenicity of *B. bassiana* Formulation against the Larvae of *S. littoralis*

To assess the pathogenicity of ***B. bassiana*** against 2nd instars larvae of ***S. littoralis***; Samples of each formulation (WP and IE) were obtained at zero time. Blended 1g WP with 10ml sterile distilled water by vortex for 10 min (as crude of WP or 100% conic).

Four concentrations were prepared (100, 75, 50, and 25%) from (WP, IE) by distilled water. Fresh caster leaves and plastic containers were sprayed with each concentration of WP, IE, and distilled water as negative control. After air drying, the treated leaves were transferred into the contaminated containers and provided with 10 second instar larvae of ***S. littoralis*** each replicate (4 replicates). All containers were covered with muslin cloth for aeration [24] and maintained in an incubator at 27±1.0ºC and 70.0±5.0% relative humidity. The mortality was recorded daily, and accumulative larval mortality was determined at the end of the larval instar. The mortality percentages were corrected by Abbott's formula [25]. The virulence of ***B. bassiana*** formulations were stored for 7 months under different storage conditions was evaluated under laboratory conditions using the same technique as the pathogenicity test.

2.4. Statistical analysis

The mortality data for all treatments were analyzed by two-way analysis of variance (**ANOVA**) followed by comparison of means using Tukey's HSD test (**SPSS**) ($P < 0.05$).

3. Results

The obtained results reveal the efficiency of the two formulations of *B. bassiana* (Inverted emulsions and wettable powder) against the 2nd instar larvae. The percentage of larval mortality gradually increased as the concentrations of both formulations were elevated as shown in figures **Figures 1, 2, and 3**. Also, the effect of storage periods and the temperatures were examined on the storage day **(**Zero time**)**.

The inverted emulsions (IE) formulation exhibited a higher virulence on the larval mortality than wettable powder (WP) especially at the higher concentrations (75,100%) as shown in the all Figures. Whereas, IE formulation achieved 75 and 90 % larval mortality, while, WP formulation caused 72.5 and 87.5% at the mentioned concentrations respectively.

However, as storage periods and temperatures (-10 ^{0}C, 4 ^{0}C, and the room temperature) increased, the pathogenicity of both formulations (IE, WP) reduced. Among all different temperatures (-10 ^{O}C, 4 ^{O}C, and the room temperature) the larval mortality was significantly affected over the storage times among all the concentrations of formulations (WP, IE) as shown in all histograms in the three figures. under freezing conditions (-10 ^{0}C) and cooling conditions (4 ^{0}C), the infectivity of WP formulation persisted for the seventh months, causing 35% and 32.5% larval mortality at the highest concentration (100%) compared to that of 82.5% and 75% in the first month, respectively (**Histogram 4 in both Figures 1,2**). while, at room temperature 25°C, the infectivity of WP formulation persisted only for 5th month and the larval mortality at the maximum concentration (100%) considerably lowered to 22.5% from 62.5% in the first month, While the infectivity of IE formulation at

freezing (-10 ^{O}C) and cooling conditions (4 ^{O}C) persisted for the fourth month, significantly declining to 47.5% and 42.5% larval mortality at the highest concentration (100%)compared to 62.5% and 57.5% in the first month, respectively (**Histogram 4 in both (Histogram 4 in figure 3).**

Figures 1, 2), however, at room temperature (25°C), the infectivity of IE formulation persisted only for 2nd month and the larval mortality at the highest concentration (100%) significantly decreased to 35 % compared to 42.5 % in the 1st month

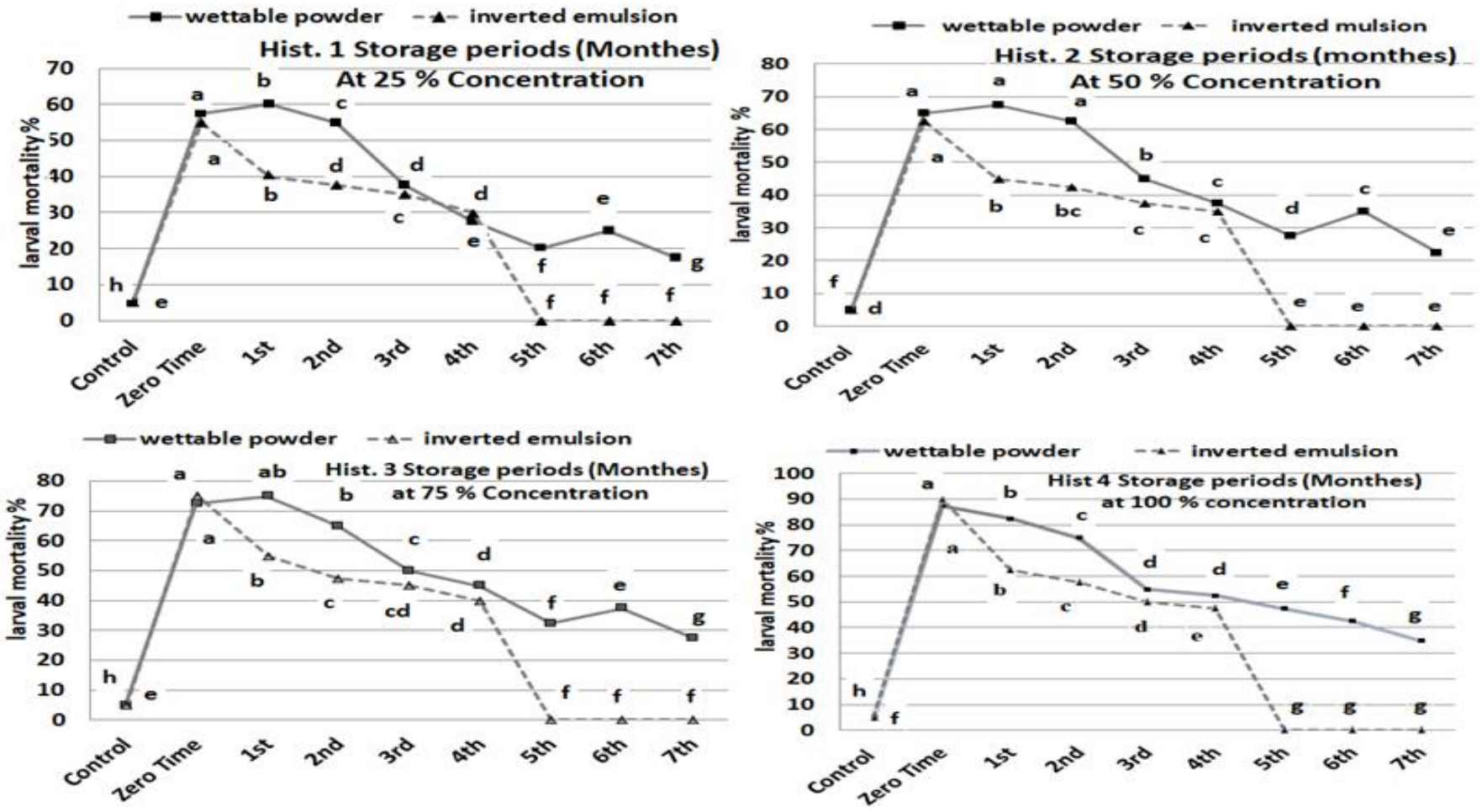

Fig. 1 larval mortality percentage of *Spodoptera littoralis* after treated with *Beauveria bassiana* wettable powder and inverted emulsion formulations in different storage times under freezing condition (-10 ^{O}C)

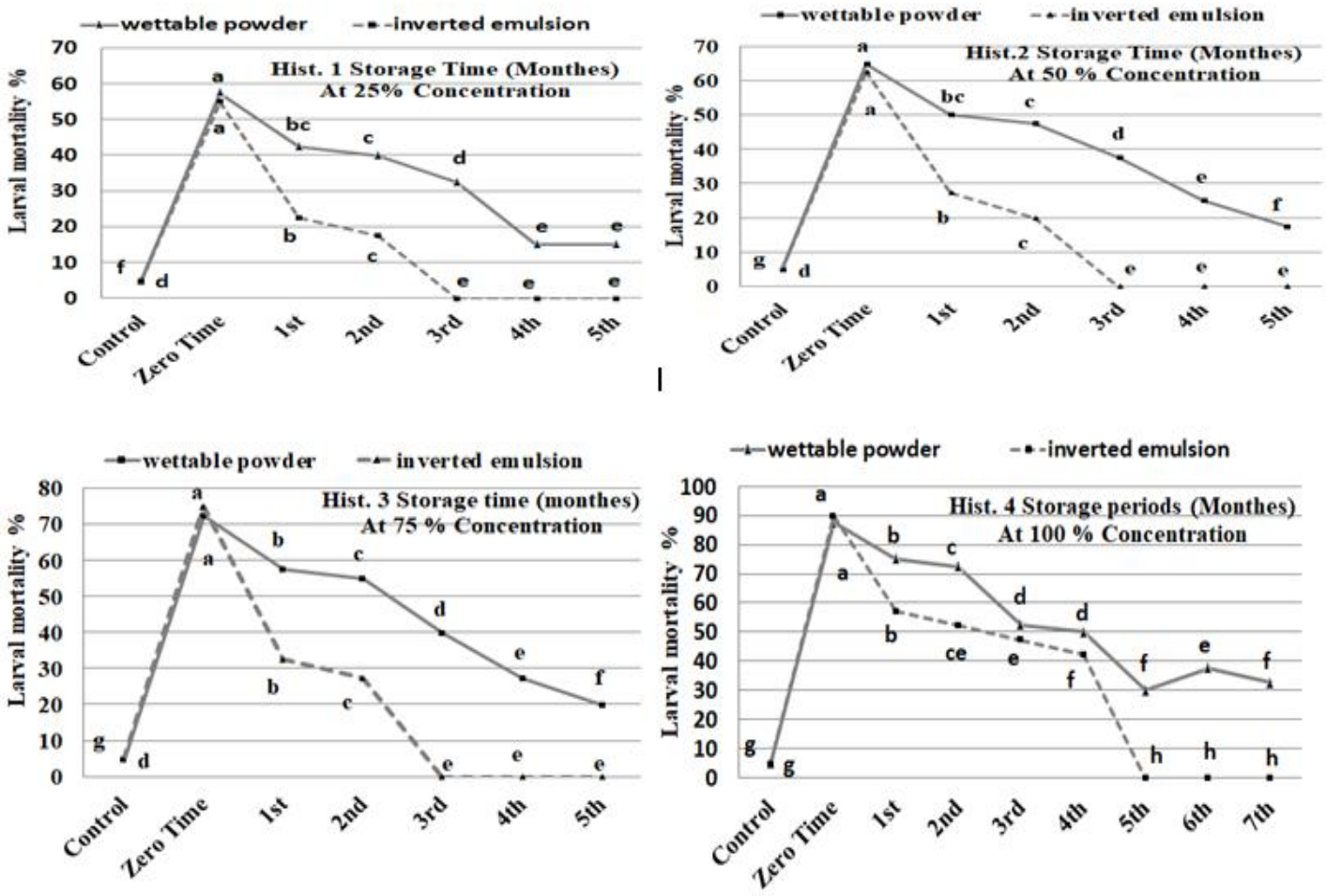

Fig. 2 larval mortality percentage of *Spodoptera littoralis* after treated with *Beauveria bassiana* wettable powder and inverted emulsion formulations in different storage times under cooling conditions (4 ^{O}C)

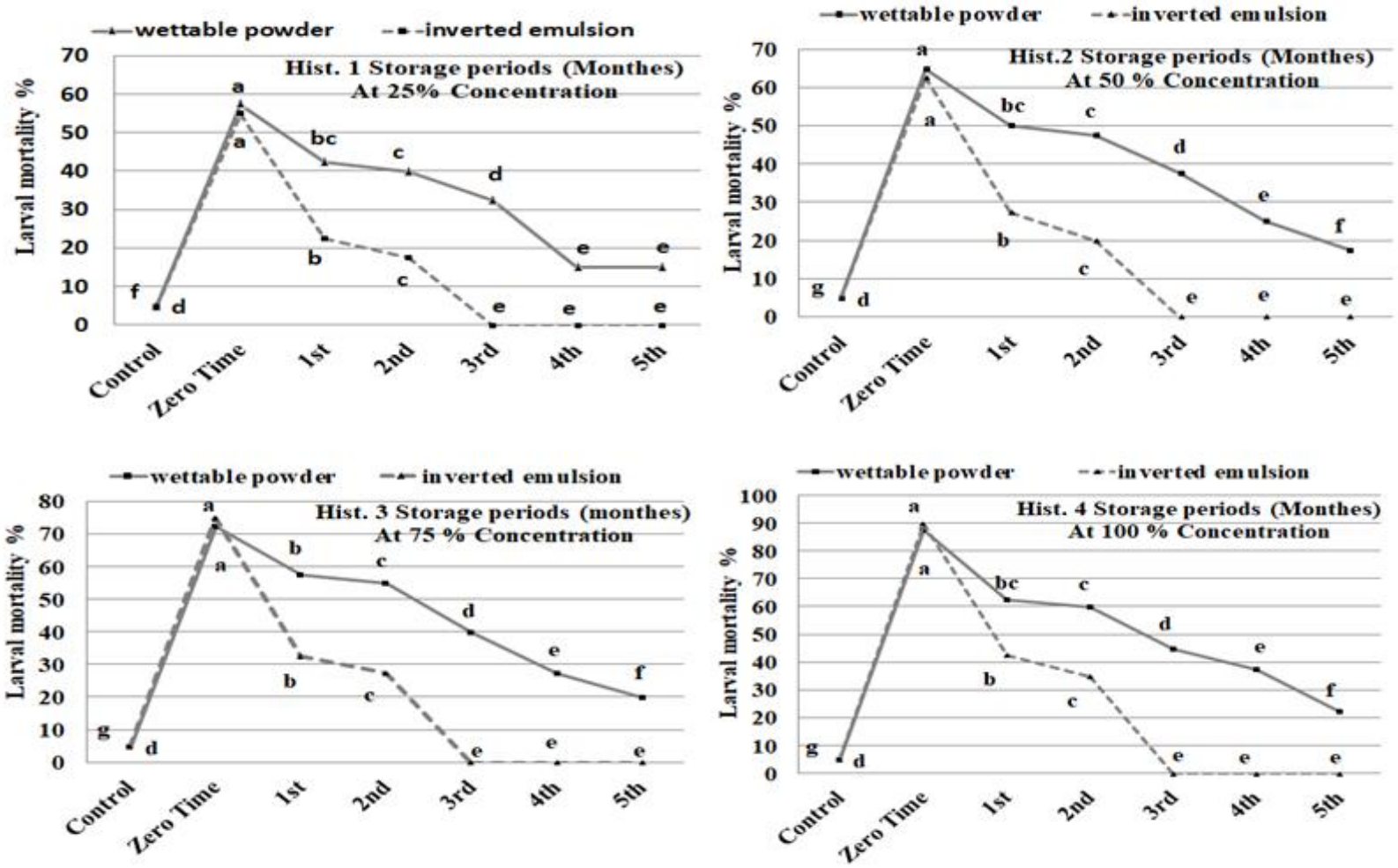

Fig. 3 larval mortality percentage of *Spodoptera littoralis* after treated with *Beauveria bassiana* wettable powder and inverted emulsion formulations in different storage times under room temperature

4. Discussion

The findings of our study show how certain formulations (the inverted emulsion and wettable powder) can increase the infectivity of entomopathogenic fungus spores to the *Spodoptera littoralis* 2nd instar larvae while they are stored. The inverted emulsion (IE) formulation had a greater effect than the wettable powder (WP) formulation on the *Spodoptera littoralis* larval mortality on the storage day (Zero time).

The investigation supports the findings of Batta [26], who noticed that *Beauveria bassiana* (strains 149 and Medea) formulated in invert emulsion has demonstrated a great efficacy against the adults of almond bark beetle, *Scolytus amygdali* achieving 100% adult mortality. *Nomuraea rileyi* formulated with sunflower oil achieved 93.2 percent total mortality against 3rd instar larvae of *Helicoverpa armigera*, while, talc-based WP formulation of *N. rileyi* caused 82% mortality of *S. litura*. Oils in different types of formulations have been shown to improve the effectiveness of mycoinsecticid [27]. These effects are typically related to oils' lipophilic characteristics. Oils are effectively bond to the hydrophobic surfaces of both fungal conidia and insect cuticle, making them extremely effective spray carriers and spray stickers. In addition, oils spread rapidly across insect cuticle, and this action transports conidia into protected recesses on the insect body, especially intersegmental regions, where moisture conditions may be favorable for germination and infection [27]. The amount of spore moisture is another factor that affects the ability to spores storage. The spore's moisture content up to 5% is necessary to survive. Using oil formulations were helped spore survival by maintaining moisture [28]. With regard to storage conditions the formulations stored at freezing and cooling condition were more efficient against 2nd instar larvae *Spodoptera littoralis* than those stored at room temperature. This may be due to the fact that cool maintaining condition caused the metabolic activity of conidia was decreased during storage time and this agent could be justified by higher spore viability and pathogenicity [29]. Chen et al. [28] revealed that both conidial germination and infection of host decreased with storage the temperature ranging from 15 to 35°C. Gindro and Pezet, [30], also, found general decrease of the energy level, O_2 consumption and aggressiveness was observed as the storage temperature increased. A shift from ATP to ADP and AMP was also observed, and the shift was larger in conidia stored at higher temperatures. Conidia stored at −80°C for 30 months still had 50% of their original energy charge, 70% respiration rate, 80% germination rate, and about the same aggressiveness (90%) as fresh spores. Our results also show the pathogenicity of WP formulation during storage condition lasted up to 7th months while invert emulsion lasted up to 4th months this was in agree

with Charnley and Collins, **[31],** who found some fungal biopesticides have a shelf life of less than six months even at low temperatures (2–6°C). Ramarethinam et al. [32] revealed that a talc based commercial formulation of *B. bassiana* can be stored for 7 months at temperature ranging from 20-32 ◦C. The differences in the pathogenicity and stability of WP and inverted emulsion formulations were related to kind of carrier materials. The selection of carriers based on their effect on viability maintainance of spores during formulation process. Talc powder was the best carrier to retain infectivity up to 6 months of storage, as was earlier reported in case of *Trichoderma longibrachiatum* [33, 34]., Sodium glutamate as a stabilizer and osmotic protector **[35, 36];** increased shelf life of fungal spores during drying period [37]. Congo red as UV-protectant [38], the best particle source for fixing the spores due to its solubility in water and its highly porous nature [39], gelatin as sticker [40], and increasing of susceptibility in powder formulation of *B. bassiana* [41].

5. Conclusion

Environmental safety and ecosystem stability considerations lead to the conclusion that the use of native isolates in a microbial control program is more convenient [42]. Also, mycoinsecticides may be most effective in pest managements programmes integrating beneficial arthropods, or in greenhouse crops where favorable environmental conditions (high humidity and low UV exposure) can be manipulated [43]. Commercialization of the entomopathogenic fungi as an alternative to synthetic insecticides demands the development of a formulation, which facilitates the fungus to survive under storage as well as in the field for a considerable length of time. It is seen that the wettable powder and oil based formulation used in the present study were proved very effective against *S. littoralis* larvae and hence, it can fit very well with the integrated pest management programmes. The mortality of the *S. littoralis* larvae was observed to increase with an increase in concentration of formulations, and during storage; most of the formulations lose virulence because of high temperature and decrease of nutrients.

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