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حفاظت از زنده‌مانی و بیماری‌زایی قارچ‌های بیمارگر حشرات، *Beauveria bassiana* و *Metarhizium anisopliae*، با فرمولاسیون در امولسیون معکوس روغنی

Preservation of viability and virulence of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* by formulation in an oil invert emulsion

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چکیده

با استفاده از لارو *Ephestia kuehniella* به عنوان میزبان، فرمولاسیون امولسیون معکوس (IE) و سوسپانسیون آبی (AS) قارچ‌های *Beauveria bassiana* (جدایه IR34-JS2) و *Metarhizium anisopliae* (جدایه IR41-TT1) در دمای نگه‌داری ۳ و یا ۲۵ درجه سلسیوس مقایسه شدند. در ابتدا، فرمولاسیون IE جدایه‌های JS2 و TT1 بر اساس مقادیر LC_{50}، ۱۳ و ۱۴ برابر بیش‌تر از فرمولاسیون AS زهرآگین‌تر بودند. مقادیر LC_{50} جدایه JS2 به ترتیب برای فرمولاسیون‌های AS و IE بعد از سه ماه نگه‌داری در دمای ۳ درجه سلسیوس حدود ۱/۳ و ۵/۶ برابر و ۱/۶ و ۷/۳ برابر بعد از سه ماه در دمای ۲۵ درجه سلسیوس افزایش یافت، در حالی که، این مقادیر برای جدایه TT1 به ترتیب معادل ۲/۱ و ۶/۸ برابر برای دمای ۳ درجه سلسیوس و ۳/۹ و ۱۵/۷ برابر در دمای ۲۵ درجه سلسیوس افزایش یافت. فرمولاسیون و دمای ذخیره‌سازی به طور قابل‌توجهی بر میزان تولید کنیدی پس از نگه‌داری تأثیر متقابل داشتند. رشد رویشی، کنیدیزایی و جوانه‌زنی کنیدی هر دو جدایه در دمای ۳ درجه سلسیوس بهتر از ۲۵ درجه در شرایط آزمایشگاهی حفظ شد. فرمولاسیون IE ظرفیت رشد رویشی را بهتر از فرمولاسیون AS در هر دو دما حفظ کرد و کنیدیزایی را در دمای ۳ درجه سلسیوس بهتر حفظ نمود، که البته این دما میزان جوانه‌زنی کنیدی‌های TT1 را در فرمولاسیون IE نیز بهبود بخشید. در دمای ۳ درجه سلسیوس، فرمولاسیون IE سرعت نسبی کشندگی را به همراه داشت که برای جدایه‌های JS2 و TT1 به ترتیب ۳/۵ و ۱/۳ برابر سریع‌تر از فرمولاسیون AS بود. می‌توان نتیجه‌گیری کرد که فرمولاسیون IE نسبت به فرمولاسیون AS برتری داشت و نگه‌داری آن در دمای ۳ درجه سلسیوس به حفظ فعالیت زیستی و بیماری‌زایی این قارچ ها کمک خواهد نمود.

واژه‌های کلیدی

Beauveria bassiana،
Ephestia kuehniella،
Metarhizium anisopliae،
شاخص‌های زیستی قارچی

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Abstract

Using *Ephestia kuehniella* larvae as hosts, invert emulsion (IE) and an aqueous suspension (AS) of *Beauveria bassiana* (isolate IR34-JS2) and *Metarhizium anisopliae* (isolate IR41-TT1) formulations were compared when stored for three months at either 3 °C or 25 °C. Initially, IE formulations of isolates JS2 and TT1 were 13- and 14-fold more virulent than AS formulations, based on their LC_{50} values. JS2 LC_{50} values increased 1.3- and 5.6-fold for the AS and IE formulations, respectively, after 3 months storage at 3 °C, and 1.6 and 7.3-fold after 3 months at 25 °C, whereas values for TT1 increased 2.1- and 6.8-fold for these formulations at 3 °C, and 3.9- and 15.7-fold at 25 °C. Formulation and storage temperature interacted significantly to affect conidial production post-storage. Vegetative growth, conidiogenesis, and conidial germination of both isolates were better preserved at 3 °C than at 25 °C *in vitro*. The IE formulation preserved vegetative growth capacity better than the AS formulation at both temperatures, and better preserved conidiogenesis at 3 °C, a temperature that also improved TT1 conidial germination in the IE formulation. At 3 °C, the IE formulation yielded a relative speed of kill that was 3.5- and 1.3-fold faster for the JS2 and TT1 isolates, respectively, than the AS formulation. It can be concluded that IE formulations were superior to AS formulations, and that storage at 3 °C will help preserve the biological activity and pathogenicity of these fungi.

Key words: *Beauveria bassiana*, *Ephestia kuehniella*, *Metarhizium anisopliae*, fungal bioindices.

Introduction

The need for environmental and human safety in agricultural pest control products has led to the development of biopesticide formulations that weaponize pathogens with improved efficacy and stability in various agricultural applications so as to enable their promotion as commercially viable alternatives to harmful synthetic pesticides. Entomopathogenic fungi (EPF), in particular, are increasingly the focus of widespread global interest as effective microbial biocontrol agents (de Faria and Wraight 2007; Senthil-Nathan 2015; Arthurs and Dara 2019). Among a great diversity of fungi pathogenic to insects, two species in particular, *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) have proven effective in diverse ecological contexts (Feng et al. 1994; Jaronski 2013; Kim et al. 2019). Until 2007, approximately 70% of commercially available EPF products employed various isolates of these two species (de Faria and Wraight 2007).

Considering EPF as a better alternative to chemical pesticides against several plant pests, the search for suitable methods to preserve fungal propagules and stocks is inevitable. Several methods have been developed for the preservation of EPFs (Batta 2003, 2016; Etheimine et al. 2013; Oliveira et al. 2015), but in general, Gallo et al. (2008) divided these methods into two metabolic groups based on their continuous or suspended metabolism. In the former the fungal growth is considered to be continued during the storage. Cool-condition storage at 5 to 8 °C and deep freeze at −20 °C in sterile water are among these methods which have demonstrated success in preserving long-term fungal storage (Pasarell and McGinnis 1992). Although storage in sterile water is known as an accessible and low-cost preservation method for most fungi, it has been proven that this method can cause serious damage to the biological, growth and morphological characteristics of fungi during storage (Borman et al. 2006). However, methods such as lyophilization and cryopreservation are considered to suspend fungal metabolism, but both require costly equipment (Nakasone et al. 2004). Therefore, in most laboratories, due to its high cost, the first method is followed in different techniques (Oliveira et al. 2015). Nevertheless, EPF-based

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biopesticides face many challenges related to formulation, mass production, and effective application in the field. Among these are problems associated with limited shelf life and the elucidation of physical conditions conducive to long-term storage of conidia without loss of viability (Fernandes et al. 2015; Rangel et al. 2015), problems that suitable formulation technologies could potentially solve. The selection of a suitable formulation can improve product stability and increase bioactivity (Gasic and Tanovic 2013). Many formulations of EPFs have been evaluated for application in either open agriculture or stored products, including crude suspensions and oil emulsions (Batta 2003; Batta and Kavallieratos 2018), encapsulates (Mishra et al. 2013), granules (Jaronski and Jackson 2008; Ali 2016; Kim et al. 2019), powders (Etheimine et al. 2013; Saeed et al. 2017), and oil emulsions containing diatomaceous earth (Luz et al. 2012).

Generally, the efficacy of EPF against pests tends to be much greater in the laboratory than in the field, due to more variable physical conditions in the latter. Thus, physical conditions, especially temperature, are important to consider when evaluating candidate formulations for use against specific targets, and for shelf life, as materials formulated for commercial sale may require storage for extended periods prior to use (Burges 1998).

Specific additives also have the potential to increase the activity of biopesticide formulations. For example, oil emulsions of EPF conidia often prove more effective than simple aqueous formulations (Prior et al. 1988; Bateman et al. 1993; Inglis et al. 1996; Batta 2016), improving shelf life and increasing their efficacy in dry environments (Bateman et al. 1993; Batta 2003). The use of oil as a carrier renders the formulation more lipophilic, enhancing its adhesion to insect cuticles and leaf surfaces, although the oil component may be absorbed by leaves, which can reduce the residual persistence of foliar applications (Behle et al. 2009). However, the oils alone may contribute insecticidal activity (Hidalgo et al. 1998), as well as improving the resistance of conidia to desiccation and facilitating the spread of conidia over leaf surfaces, this increasing the potential for EPF contact with the pest. An oil formulation can also promote the adhesion of fungal conidia to the insect integument, disrupt the epicuticlar wax layer, stimulate fungal germination, and improve fungal penetration of the cuticle (Bateman et al. 1993; Burges 1998).

In the present study, four plant-derived oils (sesame, sunflower, olive and mustard oils) were primarily evaluated *in vitro* to select one so compatible with two entomopathogenic fungi, *B. bassiana* and *M. anisopliae* after 30 days of storage at 25 ± 1 ℃. Accordingly, two EPF isolates were formulated as invert emulsions (water in oil) using sesame oil, derived from the seeds of *Sesamum indicum* L. (Pedaliaceae), and then stored at two temperatures for up to three months. These formulations were periodically evaluated for their virulence to larvae of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) to estimate their expected shelf life. In order to confirm the preservation of fungal viability following storage, key biological indices of both EPFs, (mycelial growth, conidiogenesis and conidial germination) were assayed before and after storage. Also, it can be mentioned that most of the farmers do not have much desire to keep the formulations in the refrigerated conditions which causes a sharp decrease in the biological activity of entomopathogenic fungi. Therefore, preparing a formulation with the lowest reduction in biological activity in non-refrigerated conditions over time provides better control of pests in the environment. This issue can be considered as another goal of the current study.

MATERIALS AND METHODS

Insect colony

A laboratory colony of *E. kuehniella* was established from eggs obtained from the insectarium of the Department of Plant Protection, Azarbaijan Shahid Madani University and held in a climate-controlled chamber set to 25 ± 1 ℃, 60 ± 5 % relative humidity (RH) and a 16: 8 (L: D) photoperiod. Egg cards were placed into cylindrical polypropylene containers (9 cm ht × 18 cm diam) covered with silk cloth for eclosion and rearing of larvae. Containers were each filled with a mixture of wheat flour and yeast (10: 1, wt: wt) maintained at a depth of three cm by adding new diet daily. When larvae completed development, pupae were collected by sieving the diet and placed into plastic funnels (17 cm diam, ca. 20 pupae / funnel) covered with fabric. Once adults emerged and females began oviposition, eggs were collected as they fell onto clean filter papers placed below the funnels; these eggs were then placed into clean rearing containers to start the next generation.

Fungal isolates

The IR34-JS2 isolate of *B. bassiana* and the IR41-TT1 isolate of *M. anisopliae* (abbreviated as 'JS2' and

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'TT1' hereafter) were obtained from the microbial repository of the Insect Pathology Laboratory, Plant Protection Department, Azarbaijan Shahid Madani University. In a previous study, these isolates were collected from the soils of Jiroft (Kerman Province, Iran) and Tabriz (East Azarbaijan Province, Iran) regions, respectively, and were analyzed phylogenetically (Alizadeh 2014). These isolates were preserved by a modified sterile filter paper method (Fong et al., 2000). Both fungi were cultured on potato dextrose agar (PDA) medium prepared by adding 10 g of dextrose and 7.5 g agar to 100 g of cooked mashed potatoes in a 500 ml flask. The volume was the raised to 500 ml by adding distilled water and the medium was autoclaved for 15 minutes at 121 °C, and then allowed to cool. Finally, the medium was poured into glass Petri dishes (9 cm diam) and allowed to solidify. Discs of fungal culture (5 mm diam) were cut using the wide end of a 1 ml disposable pipette tip, and each was placed upside down on the surface of medium in each dish and then incubated at 25 ± 1 °C, 60 ± 5 % RH and a 16: 8 (L: D) photoperiod.

Preparation of conidial suspensions

Suspensions of entomopathogenic fungi were made with conidia harvested from 15 d-old cultures. Aliquots of 10 ml of distilled water, each containing 0.03% Tween-80, were added to each Petri dish containing cultured fungi. The conidia were harvested by scraping the surface of the cultures with a sterile scalpel. The resulting fungal suspensions were filtered twice through silk cloth stretched over a sterile glass funnel and then poured into a sterile glass test tube (15.0 cm ht × 1.5 cm diam) and capped. Each test tube was vortexed for five minutes to prevent agglomeration of conidia, and conidia counts (no. per ml) of each suspension were determined using a Neubauer improved bright line hemocytometer (Simax, Kavalier, Czech Republic) (Borgio et al. 2011).

Oil selection and preparation of formulation

Prior to formulation preparation, four plant-derived oils (sesame, sunflower, olive and mustard oils) were evaluated *in vitro* for compatibility with *B. bassiana* JS2 and *M. anisopliae* TT1 isolates, based on three biological indices: mycelial growth, conidiogenesis and conidial germination. These preparations were all composed of oil (10 ml), 10 ml of conidial suspension (with a concentration of 5×10^8 conidia / ml), 2.5 ml of Tween-80 (0.03%), and the remainder (77.5 ml) distilled water. Each mixture was held separately in a 150 ml sterile polypropylene container at 25 ± 1°C and a photoperiod of 16: 8 (L: D) for 30 days; an oil-free aqueous fungal suspension was held as a control for each isolate. Three replications were conducted for each isolate and the experiment was repeated twice. Both isolates lost more than 50 percent of their viability after one month of storage in the oils, except for those prepared with sesame oil, whereas aqueous fungal suspensions lost more than 90 percent of their viability. Based on these observations, sesame oil was selected as the oil phase of invert emulsion.

The methods of Batta (2016) were followed, with slight modifications, to prepare aqueous suspension (AS) and invert emulsion (IE) formulations of each fungal isolate. The AS formulation was prepared as described above. The IE formulation consisted of sesame oil as the continuous phase (4.75 ml of sesame oil, plus 0.25 ml Tween-80 as an oil-soluble emulsifier), and water as the dispersed phase (4.5 ml of conidial suspension of each EPF isolate at a concentration of 5×10^8 conidia / ml of sterile distilled water, plus 0.4 ml of glycerin and 0.1 ml of Dehymuls FCE® as a water-soluble emulsifier). The ingredients of each phase were prepared separately under a laminar flow hood and then vortexed for five minutes. The two phases were combined in a 1:1 ratio by adding the dispersed phase to the continuous phase and then mixed together at 20,000 rpm for 1.5 min using a homogenizer. Aliquots of 10 ml of each fungal formulation were poured into 20 ml screw-top polypropylene vials and placed in a climate-controlled chamber set to one of two constant temperatures, either 3 °C or 25 °C, and a 16: 8 (L: D) photoperiod. However, to perform the monthly bioassays, two series of the vials were stored at these two temperatures for three consecutive months.

Assays of fungal viability

To evaluate mycelial growth of the fungi, a suspension of concentration of 3×10^6 conidia / ml was prepared from each isolate and 20 ml of PDA culture medium was poured into each of a series of sterile Petri dishes (9 cm diam, n = 3 per treatment). After solidification, a hole (5 mm diam) was made in the center of the medium in each dish using the wide end of a disposable pipette tip, and 50 µl of suspension was inoculated into this hole. The Petri dishes were then incubated at 25 ± 1 °C, 60 ± 5 % RH and a 16: 8 (L: D) photoperiod, and mycelial growth of the fungi was recorded 15 days later by taking two linear measurements across the hyphal mass perpendicular to one another and averaging them. This assay was repeated three times (Alves et al. 1998; Ummidi and Vadlamani 2014; Soleimani et

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al. 2022).

To evaluate fungal conidiogenesis, a concentration of 3×10^6 conidia / ml suspension was prepared. Disks of PDA medium (5 mm diam, n = 3 per treatment, with 3 replications) were cut from the 15 d-old cultures of each isolate/formulation combination as mentioned above. Each disk was placed in a test tube (15 cm ht × 1.5 cm diam) containing 10 ml of a 0.03% Tween-80 solution in distilled water and vortexed for 5 min to separate conidia from hyphae. The conidia concentration of the suspension was determined using a Neubauer improved bright line hemocytometer as previously mentioned. Conidiogenesis assay was repeated thrice (Ummidi and Vadlamani 2014; Soleimani et al. 2022).

To assess the germination, 10 µl of a 1×10^6 conidia / ml suspension of each isolate/formulation combination was inoculated on a patch of PDA medium (100 µl) on a sterile microscope slide (n = 3 slides per treatment) and placed in a sterile plastic Petri dish (as above). The dishes were then held in an incubator at same conditions as above for 24 h, whereupon the numbers of germinated and ungerminated conidia were counted under a compound microscope at 400x magnification. Conidia that produced germ tubes longer than the diameter of the conidium were considered to have germinated. This experiment was also repeated three times (Faraji et al. 2013; Hussain et al. 2015; Soleimani et al. 2022).

Insect bioassays

Fourth instar larvae of *E. kuehniella* (ca. 24 h post-molt) were used in all bioassays. After one, two, and three months of storage at the two temperatures, five-fold serial dilutions, ranging from 5×10^4 to 5×10^7 conidia / ml, were prepared from each formulation. In addition, LT_{50} values were estimated for each formulation at the end of each month using concentrations of the isolates which caused 80% mortality of fourth instar *E. kuehniella* larvae in a preliminary experiment (1×10^5 and 5×10^5 conidia / ml for JS2 and TT1 isolates, respectively).

For each bioassay, 280 µl of each formulation was poured into a 5 ml sterile polypropylene vial with a fine sprayer lid nozzle. The fourth instar larvae were placed individually in sterile Petri dishes and then sprayed with 280 µl / larva of each formulation. The larvae so treated (n = 3 replications of 36 larvae each) were gently removed using sterile forceps, and each was placed singly into the cavities of a 24-well polystyrene well plates (12.7 cm × 8.5 cm, with a well dimensions of 1.45 cm diam × 1.25 cm depth), each cavity lined with moistened filter paper using 50 µl of sterile distilled water. Sterile distilled water with 0.03% Tween-80 was used as a control. The plates were held in a climate-controlled growth chamber set to 25 ± 1 °C, 60 ± 5 % RH and a 16: 8 (L: D) photoperiod and mortality data were collected daily for up to seven days, by which time uninfected larvae had all pupated. Dead larvae were removed and each transferred singly to a sterile Petri dish (9 cm diam) lined with moistened filter paper and held under the same physical conditions for five days to confirm fungal infection. Each bioassay was repeated three times.

Calculations of formulation efficiency

Compatibility classification (Cc) for mycelial and reproductive fungal growth was calculated using the formula proposed by Alves et al. (1998). In this model the percentage of mycelial growth and conidiogenesis were calculated in relation to the control.

$$Cc\ (\%) = \frac{20(MG)+80(Cg)}{100} \quad (1)$$

where, MG and Cg are the percentage of mycelial growth and conidiogenesis after three months of storage, respectively, compared to those obtained before the storage. The compatibility rate of formulations with each fungal isolate was classified based on the Cc values obtained, as follows:

- Cc = 0.00-30.00: Completely incompatible (CI)
- Cc = 30.01-45.00: Incompatible (I)
- Cc = 45.01-60.00: Moderately incompatible (MI)
- Cc > 60.00: Compatible (C)

The relative activity (RA) of formulations was calculated using the following formula (Mehrvar 2012):

$$RA\ (\%) = \frac{LC_{50}(AS) - LC_{50}(IE)}{LC_{50}(AS)} \times 100 \quad (2)$$

Net relative activities based on concentration (NRA_C) and lethal time (NRA_T), were calculated by dividing the post-storage values by the pre-storage values

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(Mehrvar 2012):

$$NRA_C = \frac{LC_{50}\ (\text{after storage})}{LC_{50}(\text{before storage})} \quad (3)$$

$$NRA_T = \frac{LT_{50}\ (\text{after storage})}{LT_{50}(\text{before storage})} \quad (4)$$

The relative speed of kill (RSK) was calculated according to Shapiro and Argauer (2001) as follows:

$$RSK\ (\%) = 100 - \frac{LT_{50}(\text{treatment}) \times 100}{\text{The highest } LT_{50} \text{ value}} \quad (5)$$

Statistical analysis

Post-storage data were analyzed by 2-way ANOVA with 'formulation' and 'storage temperature' as independent factors. Means were separated by Bonferroni test (α = 0.05) whenever more than two groups were compared. LC and LT values were obtained by probit analysis using SPSS ver. 22 (SPSS 2020).

RESULTS

Fungal viability assays

Prior to storage, the TT1 isolate displayed greater mycelial growth than the JS2 isolate, although conidial production was lower, regardless of formulation (Table 1). The IE formulation yielded better conidiogenesis for both isolates compared to the AS formulation, although neither mycelial growth nor conidial germination were affected by formulation (Figure 1).

Table 1. Mean (± SE) mycelial growth (diameter of hyphal mass, in cm), conidiogenesis (no. conidia/ml ×10^6), and conidial germination (in percent) for the *Beauveria bassiana* JS2 isolate and the *Metarhizium anisopliae* TT1 isolate when initially formulated (prior to storage) as either an aqueous suspension (AS) or invert emulsion (IE). Values bearing different upper case letters were significantly different between formulations for a given isolate; values bearing different lower case letters were significantly different between isolates for a given formulation (Mycelial growth: $F_{1, 22}$ = 0.92; P = 0.35; Conidiogenesis: $F_{1, 22}$ = 2.40; P = 0.14; Conidial germination: $F_{1, 22}$ = 1.73; P = 0.20).

Variable	AS formulation$^{\Psi}$	IE formulation$^{\Psi}$	F	df	P
JS2 isolate					
Mycelial growth (cm)	4.03 ± 0.27Ab	4.54 ± 0.22Ab	2.14	1,10	0.174
No. conidia/ml × 10^6	4.11 ± 0.18Ba	4.78 ± 0.11Aa	9.78	1,10	0.011
Germination (%)	83.8 ± 3.7Ab	93.4 ± 3.7Aa	3.31	1,10	0.099
TT1 isolate					
Mycelial growth (cm)	7.05 ± 0.34Aa	7.93 ± 0.07Aa	6.58	1,10	0.028
No. conidia/ml × 10^6	2.30 ± 0.06Bb	2.90 ± 0.09Ab	30.90	1,10	< 0.001
Germination (%)	100.0 ± 0.0Aa	100.0 ± 0.0Aa	-		-

$^{\Psi}$Inoculation concentration = 3 × 10^6 conidia/ml.

The main effects of formulation ($F_{1, 20}$ = 38.45, P < 0.001) and storage temperature ($F_{1, 20}$ = 9.62, P = 0.006) on mycelial growth of the JS2 isolate were observed significantly, but without any interaction between these factors ($F_{1, 20}$ = 0.10, P = 0.616). There were also significant main effects of formulation ($F_{1, 20}$ = 17.66, P < 0.001) and storage temperature ($F_{1, 20}$ = 174.90, P < 0.001) on conidiogenesis by the JS2 isolate, and these factors interacted significantly ($F_{1, 20}$ = 6.20, P = 0.022). Conidial germination of the JS2 isolate was also affected by both formulation ($F_{1, 20}$ = 221.61, P < 0.001) and storage temperature ($F_{1, 20}$ = 51.28, P < 0.001), but without significant interaction between these factors ($F_{1, 20}$ = 1.71, P = 0.206).

Also, results revealed significant main effects of formulation ($F_{1, 20}$ = 30.52, P < 0.001) and storage temperature ($F_{1, 20}$ = 9.98, P = 0.005) on mycelial growth of the TT1 isolate, however their interaction was insignificant ($F_{1, 20}$ = 0.41, P = 0.532). There were also significant main effects of formulation ($F_{1, 20}$ = 34.36, P < 0.001) and storage temperature ($F_{1, 20}$ = 57.72, P < 0.001) on conidial production by the TT1 isolate, and these factors interacted significantly ($F_{1, 20}$ = 10.71, P = 0.004). Conidial germination of the TT1 isolate was also affected by both formulation ($F_{1, 20}$ = 56.15, P < 0.001) and storage temperature ($F_{1, 20}$ = 45.33, P < 0.001), but without significant interaction between these factors ($F_{1, 20}$ = 0.55, P = 0.466).

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The percentage of compatibility of AS and IE formulations with each of the fungal isolates showed that the IE formulation of the JS2 isolate was compatible (C) with 73.54 % at 3 °C (Table 2). The AS formulation of the JS2 isolate and the IE formulation of the TT1 isolate were both classified as moderately incompatible (MI) with the percentages of 56 and 55.25 %, respectively, at 3 °C. The AS formulation was also identified as an incompatible preservation method for the TT1 isolate at same temperature (Cc = 32.93 %). However, after three months of storage at 25 °C, both formulations were completely incompatible (CI) with both the isolates (Table 2).

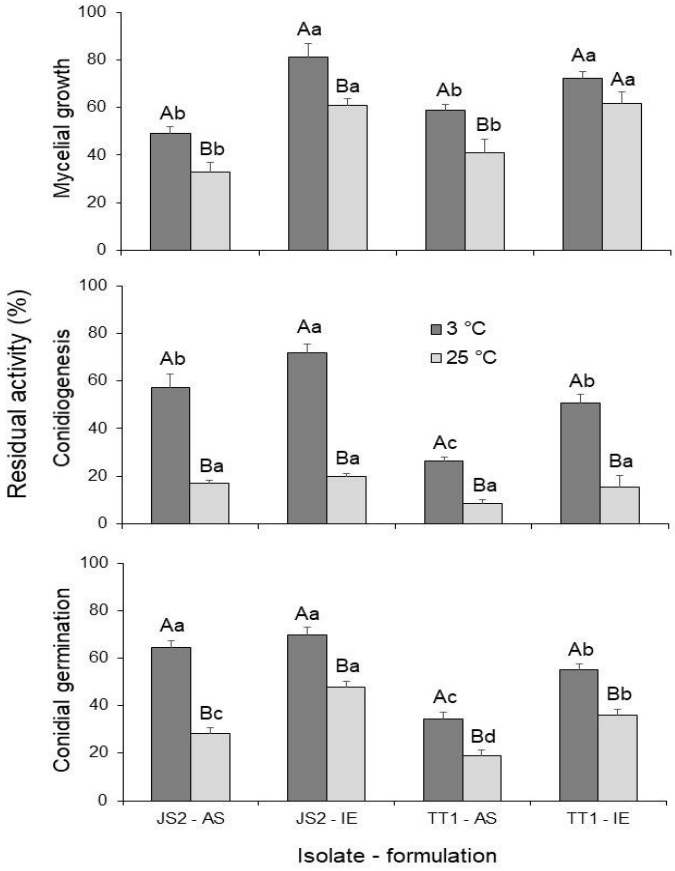

Figure 1. Mean (+SE) percentage reductions in mycelial growth (A), conidiogenesis (B) and conidial germination (C) of the entomopathogenic fungi, *Beauveria bassiana* (JS2 isolate) and *Metarhizium anisopliae* (TT1 isolate) after three months of storage at two different temperatures. Columns bearing different upper-case letters were significantly different among isolates, for a given temperature; columns bearing different lower-case letters were significantly different between temperatures for a given isolate. (ANOVA followed by Fisher' LSD test, $\alpha = 0.05$).

Table 2. Compatibility classification of the entomopathogenic fungi, *Beauveria bassiana* (JS2 isolate) and *Metarhizium anisopliae* (TT1 isolate) formulated as either an aqueous suspension (AS) or an invert emulsion (IE) over the time at two temperatures. Compatibility scores were calculated according to Alves et al. (1998) and rated as: C = compatible, MI = moderately incompatible, I = incompatible, CI = completely incompatible.

Fungal isolate	Formulation	Score (%): Rating after 3 months of storage	
		3 °C	25 °C
B. bassiana JS2 isolate	AS	56.00: MI	19.89: CI
	IE	73.54: C	28.15: CI
M. anisopliae TT1 isolate	AS	32.93: I	14.81: CI
	IE	55.25: MI	24.49: CI

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Table 3. LC_{50} values (no. conidia/ml), with their confidence limits (CLs), net relative activity (NRA_C), and percent relative activity over time (RA), when fourth instar *Ephestia kuehniella* larvae were exposed to the JS2 isolate of *Beauveria bassiana* formulated as either an aqueous suspension (AS) or an invert emulsion (IE) and stored at two temperatures for various periods.

Formulation	Storage (months)	LC_{50} (conidia/ml) (CLs*)	Chi-square (df = 5)	Slope ± SE	NRA_C	RA (%)
3 °C						
AS	0	1.08×10^5 ($5.66 \times 10^4 – 1.83 \times 10^5$)	0.75	0.75 ± 0.11	1.00	–
	1	1.21×10^5 ($6.85 \times 10^3 – 1.86 \times 10^5$)	4.38	1.19 ± 0.19	1.12	–
	2	1.30×10^5 ($7.92 \times 10^3 – 2.72 \times 10^5$)	5.97	0.85 ± 0.14	1.20	–
	3	1.44×10^5 ($1.08 \times 10^3 – 7.14 \times 10^5$)	15.05	0.70 ± 0.09	1.33	–
IE	0	7.87×10^3 ($2.88 \times 10^3 – 1.42 \times 10^4$)	3.33	0.92 ± 0.17	1.00	92.71
	1	2.20×10^4 ($1.97 \times 10^3 – 4.03 \times 10^4$)	0.71	0.60 ± 0.14	2.80	81.82
	2	3.66×10^4 ($2.15 \times 10^3 – 5.90 \times 10^4$)	4.91	1.02 ± 0.14	4.65	71.85
	3	4.43×10^4 ($1.38 \times 10^4 – 4.86 \times 10^5$)	9.74	0.81 ± 0.12	5.63	69.24
25 °C						
AS	0	1.08×10^5 ($5.66 \times 10^4 – 1.83 \times 10^5$)	0.75	0.75 ± 0.11	1.00	–
	1	1.17×10^5 ($4.25 \times 10^4 – 4.59 \times 10^5$)	3.53	0.94 ± 0.11	1.08	–
	2	1.40×10^5 ($2.81 \times 10^4 – 5.92 \times 10^5$)	4.65	0.84 ± 0.11	1.30	–
	3	1.71×10^5 ($1.06 \times 10^5 – 2.87 \times 10^5$)	0.55	0.48 ± 0.10	1.58	–
IE	0	7.87×10^3 ($2.88 \times 10^3 – 1.42 \times 10^4$)	3.33	0.92 ± 0.17	1.00	92.71
	1	2.90×10^4 ($4.89 \times 10^3 – 4.84 \times 10^4$)	9.54	1.07 ± 0.17	3.68	75.21
	2	3.40×10^4 ($9.38 \times 10^3 – 6.84 \times 10^4$)	3.09	0.95 ± 0.14	4.32	75.71
	3	5.73×10^4 ($1.82 \times 10^4 – 9.78 \times 10^4$)	4.84	0.57 ± 0.10	7.28	66.49

*CLs = confidence limits.

Formulations biological assays

After three months of storage at 25 °C in the IE formulation, the LC_{50} of the JS2 isolate increased 7.3-fold over its initial value, whereas it increased only 5.6-fold at 3 °C, a 30 % improvement (Table 3). A similar trend was evident for the IE formulation of the TT1 isolate, with LC_{50} values increasing 15.7- and 6.9-fold, respectively, after storage for three months at 25 °C and 3 °C (Table 4). IE formulations of the JS2 isolate also exhibited higher net relative activity based on concentration (NRA_C) compared to AS formulations at both temperatures, but this was true only for the TT1 isolate at 3 °C. The JS2 isolate exhibited somewhat faster rates of decline in relative activity (RA) over time than did the TT1 isolate, regardless of formulation (Figure 2A). Whereas both formulations of the JS2 isolate exhibited similar rates of RA decline at both storage temperatures, the RA of the TT1 isolate declined slightly faster when stored of 25 °C than when stored at 3 °C.

Before storage the LT_{50} value of the AS formulation of the JS2 isolate was 1.46-fold greater than for the IE formulation (Table 5), whereas for the TT1 isolate, it was only 1.03-fold greater (Table 6). After three months of storage, the net relative activity of IE formulation, based on time to death (NRA_T), remained almost 15 % higher than that of the AS formulation for the JS2 isolate, and almost 79 % higher in the case of the TT1 isolate. The relative speed of kill (RSK) of both isolates declined more rapidly at 25 °C than at 3 °C, and in both cases, the IE formulation retained a faster kill rate over time than did the AS formulation (Figure 2B).

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Table 4. LC_{50} values (no. conidia/ml), with their confidence limits (CLs), net relative activity (NRA_C), and percent relative activity over time (RA), when fourth instar *Ephestia kuehniella* larvae were exposed to the TT1 isolate of *Metarhizium anisopliae* formulated as either an aqueous suspension (AS) or an invert emulsion (IE) and stored at two temperatures for various periods.

Formulation	Storage (months)	LC_{50} (conidia/ml) (CLs*)	Chi-square (df = 5)	Slope ± SE	NRA_C	RA (%)
3 °C						
AS	0	8.53×10^5 ($5.27 \times 10^5 – 1.44 \times 10^6$)	1.49	0.88 ± 0.10	1.00	–
	1	1.04×10^6 ($8.77 \times 10^5 – 4.51 \times 10^6$)	3.43	0.98 ± 0.21	1.22	–
	2	1.58×10^6 ($7.32 \times 10^5 – 6.62 \times 10^6$)	5.49	0.99 ± 0.13	1.85	–
	3	1.82×10^6 ($8.58 \times 10^5 – 6.81 \times 10^6$)	5.60	0.76 ± 0.13	2.13	–
IE	0	5.99×10^4 ($3.59 \times 10^4 – 9.24 \times 10^4$)	1.34	1.09 ± 0.16	1.00	92.98
	1	1.16×10^5 ($8.85 \times 10^4 – 2.83 \times 10^5$)	1.72	1.34 ± 0.26	1.94	88.85
	2	3.11×10^5 ($9.46 \times 10^4 – 8.83 \times 10^5$)	5.46	1.31 ± 0.29	5.20	80.32
	3	4.10×10^5 ($1.92 \times 10^5 – 9.04 \times 10^5$)	6.28	0.78 ± 0.14	6.85	77.47
25 °C						
AS	0	8.53×10^5 ($5.27 \times 10^5 – 1.44 \times 10^6$)	1.49	0.88 ± 0.10	1.00	–
	1	9.47×10^5 ($4.05 \times 10^5 – 3.72 \times 10^6$)	7.73	0.68 ± 0.11	1.11	–
	2	2.01×10^6 ($1.88 \times 10^6 – 3.90 \times 10^6$)	0.48	1.13 ± 0.17	2.36	–
	3	3.36×10^6 ($1.97 \times 10^6 – 7.72 \times 10^6$)	4.34	0.89 ± 0.12	3.86	–
IE	0	5.99×10^4 ($3.59 \times 10^4 – 9.24 \times 10^4$)	1.34	1.09 ± 0.16	1.00	92.98
	1	1.27×10^5 ($9.89 \times 10^4 – 3.41 \times 10^5$)	1.46	1.63 ± 0.28	2.12	86.59
	2	3.23×10^5 ($1.05 \times 10^5 – 5.24 \times 10^5$)	3.70	0.92 ± 0.21	5.39	83.93
	3	9.39×10^5 ($6.99 \times 10^5 – 1.38 \times 10^6$)	3.64	0.63 ± 0.10	15.68	72.05

Discussion

After three months of storage, the residual biological activity of the different isolate/formulation combinations were compared with respect to mycelial growth, conidiogenesis, and conidial germination by expressing post-storage values as percentages of their pre-storage values (Figure 1). Storage of both formulations at 3 °C resulted in greater residual activity in all three biological indices than did storage at 25 °C, with the exception of the IE formulation of the TT1 isolate, which did not differ between temperatures. For both isolates, the IE formulation preserved mycelial growth capacity better than did the AS formulation, and the same was true for conidial germination of the TT1 isolate. The IE formulation also preserved conidiogenesis activity better than did the AS formulation when the isolates were stored at 3 °C, but formulation had no significant effect on conidial production after storage at 25 °C, which reduced it below 20% in all isolate/formulation combinations.

As a result, formulation of *B. bassiana* and *M. anisopliae* conidia as an invert emulsion using sesame oil in the continuous phase increased their virulence to fourth instar *E. kuehniella* larvae by 13-fold and 14-fold, respectively, relative to a conventional AS formulation.

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Table 5. LT_{50} values (no. days), with their confidence limits (CLs), and net relative activity (NRA_T) when fourth instar *Ephestia kuehniella* larvae were exposed to the JS2 isolate of *Beauveria bassiana* at a concentration of 1×10^5 conidia/ml and formulated as either an aqueous suspension (AS) or an invert emulsion (IE) and stored at two temperatures for various periods.

Formulation	Storage period (no. months)	LT_{50} (d) (CLs)	Chi-square (df = 5)	Slope ± SE	NRA_T
3 °C					
AS	0	5.48 (3.96 – 22.91)	15.01	0.42 ± 0.08	1.00
	1	6.42 (4.26 – 13.55)	8.27	0.44 ± 0.08	1.17
	2	8.36 (5.91 – 19.34)	9.37	0.25 ± 0.08	1.53
	3	8.91 (6.69 – 21.18)	13.39	0.43 ± 0.09	1.63
IE	0	3.76 (2.33 – 17.05)	15.08	0.36 ± 0.10	1.00
	1	3.92 (2.59 – 21.01)	15.06	0.49 ± 0.11	1.04
	2	6.11 (4.88 – 17.14)	13.42	0.27 ± 0.07	1.63
	3	7.03 (3.39 – 12.67)	6.99	0.53 ± 0.10	1.87
25 °C					
AS	0	5.48 (3.96 – 22.91)	15.01	0.42 ± 0.08	1.00
	1	5.83 (4.28 – 20.65)	13.64	0.77 ± 0.13	1.06
	2	8.27 (5.81 – 13.59)	3.94	0.36 ± 0.08	1.51
	3	9.66 (6.58 – 19.63)	7.03	0.18 ± 0.08	1.76
IE	0	3.76 (2.33 – 17.05)	15.08	0.36 ± 0.10	1.00
	1	5.28 (3.41 – 14.75)	7.79	0.40 ± 0.08	1.40
	2	7.99 (4.65 – 21.10)	13.26	0.31 ± 0.08	2.13
	3	8.38 (6.91 – 14.26)	6.96	0.26 ± 0.07	2.23

Furthermore, the IE formulation was superior to the AS formulation in conserving the pathogenicity of both fungal isolates over a three-month storage period, as indicated by lower LC_{50} values for *E. kuehniella* larvae exposed by spraying. Independent of formulation, the JS2 isolate *B. bassiana* appeared more virulent to *E. kuehniella* larvae than the TT1 isolate of *M. anisopliae*, both initially, and after three months of storage. However, the pathogenicity of *B. bassiana* diminished more rapidly during storage than did that of *M. anisopliae* at both tested temperatures, even when formulated as an IE, as evidenced by steeper slopes of decline in relative activity (Figure 2A). The decline in relative activity was less when the various formulations were stored at 3 °C compared with 25 °C, indicating that the lower temperature helped to preserve fungal pathogenicity. Relative speed of kill (RSK) estimates for IE formulations of the JS2 and TT1 isolates against *E. kuehniella* larvae were 3.5- and 1.3-fold better, respectively, than their corresponding AS formulations after three months of storage at 3 °C (Figure 2B). Same trend was also achieved with NRA_T values.

Batta (2003) estimated the half-life of *M. anisopliae* conidial viability at a storage temperature of 20 °C as ca. 4.6 months when formulated as an invert emulsion, compared to ca. 2.0 months when formulated as an aqueous suspension.

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Table 6. LT_{50} values (no. days), with their confidence limits (CLs), and net relative activity (NRA_T) when fourth instar *Ephestia kuehniella* larvae were exposed to the TT1 isolate of *Metarhizium anisopliae* at a concentration of 5×10^5 conidia/ml and formulated as either an aqueous suspension (AS) or an invert emulsion (IE) and stored at two temperatures for various periods.

Formulation	Storage period (no. months)	LT_{50} (d) (CLs)	Chi-square (df = 5)	Slope ± SE	NRA_T
3 °C					
AS	0	6.10 (5.56 – 8.13)	1.44	0.55 ± 0.09	1.00
	1	6.43 (3.38 – 19.41)	15.01	0.98 ± 0.21	1.05
	2	7.09 (4.06 – 21.55)	15.02	0.29 ± 0.09	1.16
	3	8.64 (6.33 – 23.61)	14.83	0.25 ± 0.07	1.42
IE	0	5.91 (5.18 –7.24)	3.52	0.39 ± 0.07	1.00
	1	6.06 (4.47 – 21.16)	14.77	0.21 ± 0.07	1.03
	2	6.34 (4.55 – 23.69)	15.08	0.31 ± 0.08	1.07
	3	8.33 (6.63 – 17.72)	8.24	0.24 ± 0.09	1.41
25 °C					
AS	0	6.10 (5.56 – 7.00)	1.44	0.55 ± 0.09	1.00
	1	7.37 (4.84 – 23.57)	15.08	0.70 ± 0.11	1.21
	2	9.03 (5.23 – 20.14)	9.78	0.41 ± 0.08	1.48
	3	9.71 (7.36 – 13.76)	3.62	0.37 ± 0.09	1.59
IE	0	5.91 (5.18 – 7.24)	3.52	0.39 ± 0.07	1.00
	1	7.69 (4.56 – 11.98)	7.50	0.37 ± 0.08	1.30
	2	7.86 (4.49 – 11.35)	7.25	0.31 ± 0.09	1.33
	3	8.82 (6.04 – 12.08)	4.83	0.43 ± 0.08	1.49

Prior et al. (1988) formulated an invert emulsion of *B. bassiana* conidia with coconut oil and showed that it remained highly infective to the cocoa weevil, *Pantorhytes plutus* (Coleoptera: Curculionidae) for up to 40 days. Although the authors did not address more prolonged storage, they inferred that high relative humidity contributed to the high virulence of the IE formulation observed after application in the field. Although high humidity is essential for rapid conidial germination on a suitable substrate, IE formulations reduce the humidity requirement for the infection process (Prior and Greathead, 1989), and can improve infectivity under low humidity conditions, because the virulence of conidia is better preserved in oil than in water (Bateman et al., 1993). The oil phase delays the desiccation of conidia, thus improving tolerance of high temperatures and dry conditions, and contributes to better adhesion of conidia to the lipophilic insect cuticle. Batta (2003) tested an invert emulsion of *M. anisopliae* conidia based on coconut and soybean oils against the red spidermite, *Tetranychus cinnabarinus* (Acari: Tetranychidae), and the tobacco whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae). When stored at 20 °C, fungal conidia had a half-life of 4.6 months in the invert emulsion, compared to 0.5 months for unformulated conidia stored in dry conditions at the same temperature. Marques and Alves (1996) formulated *B. bassiana* with sunflower oil and obtained 90% viability after eight months of storage at 20 °C. Luz and Batagin (2005) evaluated 11 types of plant oils in formulations of *B. bassiana* conidia against third instar nymphs of *Triatoma infestans* (Hemiptera: Reduviidae) and found that oil

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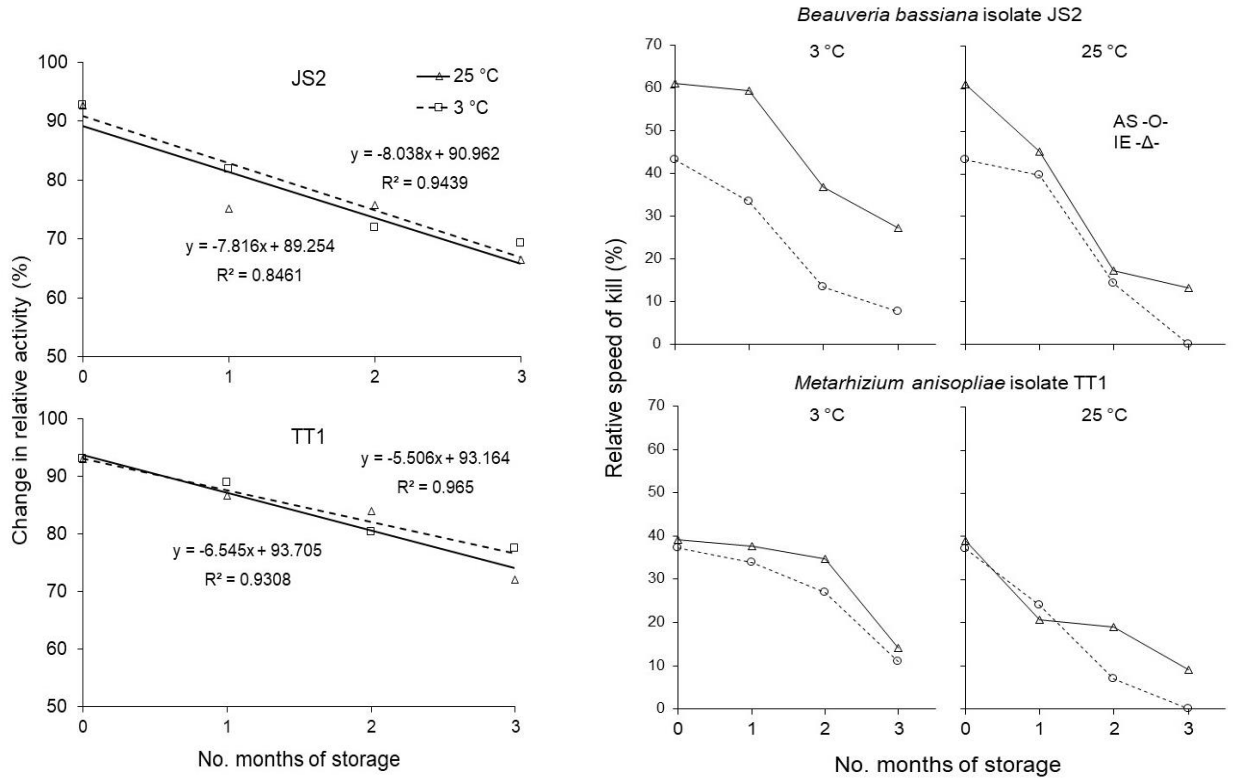
emulsions improved infectivity compared with an aqueous suspension, especially under low moisture conditions, and did not produce the repellency observed with pure oils. For example, an invert emulsion with 10% soybean oil was 2.2 times more virulent than its aqueous suspension. The compatibility classification (Cc scores) of both formulations showed a similar event with this study, where, the aqueous suspension was completely incompatible with both the isolates at 25 °C. However, the invert emulsion was compatible with *B. bassiana* JS2 isolate at 3 °C.



(A) (B)

Figure 2. (A) Changes in relative activity (RA) of invert emulsion (IE) formulations relative to aqueous suspension (AS) of the *Beauveria bassiana* JS2 isolate and the *Metarhizium anisopliae* TT1 isolate, when stored at either 3 °C or 25 °C over a period of three months. (B) Relative speed of kill (in percent) for aqueous suspension (AS) and invert emulsion (IE) formulations of the *Beauveria bassiana* JS2 isolate and the *Metarhizium anisopliae* TT1 isolate when stored at either 3 °C or 25 °C over a period of three months.

Luz and Batagin (2005) also found that fungal performance varied among oil types, with corn oil producing the best conidial germination after eight days, and sesame oil providing the maximum number of CFUs on medium after five days. Ummidi and Vadlamani (2014) evaluated eight plant oils in formulations of *B. bassiana* and *M. anisopliae* conidia and assessed their effects on germination rate, mycelial growth, and conidia production *in vitro*. Conidial emulsions made with four of these oils produced higher larval mortality in *Spodoptera litura* (Lepidoptera: Noctuidae) than simple suspensions, whereas others, including mustard and eucalyptus oils, proved toxic to the fungi at concentrations above 1%. The authors attributed the incompatibility of the latter to the presence of unsaturated fatty acids, such as oleic and linoleic acids, which have antifungal properties. It could be concluded that an IE of *B. bassiana* and *M. anisopliae* using sesame oil is a suitable formulation for storing and shipping these fungi for biological control applications. A storage temperature of 3 °C could be achieved with conventional refrigeration equipment and would further extend the viability of these fungi prior to application. Because different fungal isolates react differently to various plant oils and formulation components, further investigation is

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warranted to evaluate IE formulations that combine different plant oils with various entomopathogenic fungi to improve their pest control efficacy, and their residual biological activity following inevitable periods of storage.

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