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Effect of emulsifiable concentrate of *Metarhizium rileyi* (Farl.) Kepler, S.A. Rehner & Humber to third larval instar of fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae)

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Abstract *Metarhizium rileyi* (Farl.) Kepler, S.A. Rehner & Humber is a cosmopolitan entomopathogenic fungus effective against major insect pests of agricultural crops. In the Philippines, this fungus was successfully isolated from mycosed armyworm larvae collected in corn and onion fields. Our previous bioassays showed the virulence of crude fungal suspensions of *M. rileyi* isolate from onion armyworm (*Spodoptera exigua* Hübner) to *S. exigua* and its cross infection to fall armyworm (*S. frugiperda* J.E. Smith) and true armyworm (*Mythimna separata* (Walker)). This research work aimed to formulate this entomopathogenic fungus for increased efficacy and stability. Emulsifiable concentrate (EC) of *M. rileyi* was prepared using various liquid carriers. Exposure to EC formulations resulted to mycosis of third larval instar of *S. frugiperda*. Storage time and conditions affected the bioefficacy of the EC formulations. Pupation and emergence to adults were also affected. The efficacy and stability of these EC formulations are continuously being evaluated under storage conditions.

Keywords: Biological control agent, Entomopathogenic fungus, Pest management

Introduction

Fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) is a polyphagous pest with migratory behavior. In the first few months after its detection in the Philippines in 2019, this migratory pest was confirmed to be present in corn farms in 17 municipalities in 10 provinces (Navasero *et al*., 2019). As of December 2020, the pest has been infesting 20,457.54ha of corn fields in 70 provinces with about 26.61% estimated national damage (Cuaterno, 2020). Recently, this pest has also been reported

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infesting other major crops in the Philippines such as sugarcane (Ocampo, 2020) and rice (Valdez *et al*., 2021). This invasive pest has also been infesting various crops including weeds and grasses in Asia, Africa, and America (Rwomushana, 2019). Feeding of this lepidopteran insect pest leads to damages in fruit, inflorescence, leaves, stems, and whole plant. Damages in corn may range from window-paning of leaves at early whorl and destroyed silks and tassels to corn cob damages. *S. frugiperda* also has a competitive advantage over other insect pests primarily due to its cannibalistic nature. *S. frugiperda* has a competitive advantage over Asian corn borer, *Ostrinia furnacalis* (Guenée), in corn whorls and tassels (Zhao *et al*., 2022). Hence, management measures must be in place to prevent upshoot in its population.

Severe damages of *S. frugiperda* and other insect pests can be prevented by several measures in Integrated Pest Management (IPM). The Philippine Department of Agriculture recommended strategies from farmers level to the national level to mitigate this insect pest (Cuaterno, 2020). These strategies include methods under prevention/avoidance, monitoring, and suppression. Research for development and sustainability is also necessary such as capacity building and networking.

One of the agroecological measures to suppress pest population is through the use of biological control agents such as entomopathogenic fungi. These beneficial fungi have traits and modes of action making them effective biopesticides that can be developed to a more efficient and diversified use (Mantzoukas *et al*., 2022). In the Philippines, the potential of entomopathogenic fungi have been reported. An indigenous *Metarhizium rileyi* (Farl.) Kepler, S.A. Rehner & Humber isolate from *S. exigua* (Hübner) (onion armyworm) has a high efficacy against this host insect, *S. exigua* (Montecalvo *et al*., 2022a; Montecalvo and Navasero, 2020). This isolate also infected other lepidopteran species such as *S. frugiperda* (Montecalvo and Navasero, 2021). Another indigenous isolate of *M. rileyi* was recovered from mycosed *S. frugiperda* larvae and subsequently caused lethal infection (Montecalvo *et al*., 2022b). Bioassays showed their bioefficacy against these target lepidopteran pests with significant mortalities particularly in larval instars.

The production of viable and stable inoculum is critical in the utilization of entomopathogenic fungi. Oil-based formulations of fungal propagules allow infection of insect pests in an unfavorable condition for fungal development in the insect surface (Smith, 1997). Villamizar *et al*. (2004) developed two delivery systems of *M. rileyi* such as emulsifying concentrate with $1x10^{11}$ conidia/g and a wettable powder with $1x10^{9}$ conidia/g. Bhanu Prakash *et al*. (2015) discovered that incorporating sesame oil and sunflower oil in *M. anisopliae* (Mechnikov) Sorokin formulations were the most stable but

germination in all formulations was reduced to 60%. An emulsifiable concentrate (EC) formulation of *M. rileyi* using vegetable oil as carrier was also developed with 1.5 x 10^9 conidia/ml and remained viable after storage at 8℃ for 12 months (Grijalba *et al*., 2018).

Hence, this research work was carried out to prepare EC formulations of *M. rileyi* from *S. exigua* and evaluate its bioefficacy against *S. frugiperda*. This study determined the effect of these EC formulations on the mortality, growth, and development of *S. frugiperda*.

Materials and methods

Experiments were conducted at the National Crop Protection Center (NCPC), College of Agriculture and Food Science (CAFS), University of the Philippines of Los Baños (UPLB) from May to September 2022.

Mass rearing of S. frugiperda

The Biological Control Laboratory of NCPC, CAFS, UPLB maintains *S. frugiperda* population from Gonzaga, Cagayan, Philippines. To obtain significant number of third instar larvae for bioassays, adult moths were paired in mylar cages supplemented with sugar syrup and healthy corn plants. Egg masses laid in the plants were collected in sterile Petri plates lined with tissue paper. Upon hatching, neonates were transferred to sterile pans and fed with fresh corn leaves daily. Bioefficacy experiments were conducted using early third instar larvae.

Mass production and preparation of emulsifiable concentrates of M. rileyi

Mass production and bioefficacy testing were conducted at the Mycology Laboratory of NCPC, CAFS, UPLB. *M. rileyi* was subcultured in an artificial media. Dry spores were collected during full sporulation of the fungus using a sterile transfer needle. Dry spores of *M. rileyi* were mixed with sterilized liquid carrier containing organic oil and emulsifying agent. Spore concentration was determined using hemacytometer. The EC formulations were diluted to obtain desired conidial concentration for bioefficacy testing under laboratory condition. Samples of these EC formulations were incubated in refrigerator (4°C) and incubator (25°C, 75% relative humidity).

Laboratory bioassays against S. frugiperda

Fresh corn leaves were surface-sterilized and airdried prior to treatment. The effect of liquid carriers was determined by preparing spray suspensions

using the same volume of EC formulations to obtain 1 x 10^7 conidia/ml. Control set-up was treated with sterile distilled water. These treatments were mist sprayed to surface-sterilized corn leaves. After airdrying, these leaves were introduced to third instar larvae that were cultured singly per Petri plate. Fresh surface-sterilized leaves were fed to test larvae daily.

After 3 months of storage in refrigerator and incubator, the bioefficacy of the EC formulations (1 x 10^7 conidia/ml) was evaluated against third instar larvae of *S. frugiperda*. This experiment was carried out as previously described. Control set-up was treated with sterile distilled water.

Data gathering

The following data were gathered in bioassays: larval mortality, pupation, adult emergence, and larval and pupal period. Mycosis of larvae particularly those treated with *M. rileyi* was also observed.

Experimental design and statistical analysis

Experiments were laid out in completely randomized design (CRD). Treatments were replicated thrice with 10 test larvae per replicate. Analysis of variance was computed, and means were differentiated in statistical analysis software using Least Significant Difference (LSD) test at $P < 0.05$.

Results

Several bioassays were conducted to elucidate the insecticidal activity of the EC formulations of *M. rileyi* against *S. frugiperda* under laboratory conditions.

Bioassay of liquid carriers against S. frugiperda

The liquid carriers used in this study was assessed for possible effect on *S. frugiperda*. The various organic oils did not cause significant mortalities to the test insect (Figure 1). At 3 days after treatment (DAT), larval mortality ranged from 0 to 6.67%. Although there were mortalities recorded in other oils, the values were similar with the control and did not increase to 6.67% from 5 to 7 DAT. These findings suggest that the liquid carriers of *M. rileyi* had no insecticidal effect to *S. frugiperda*.

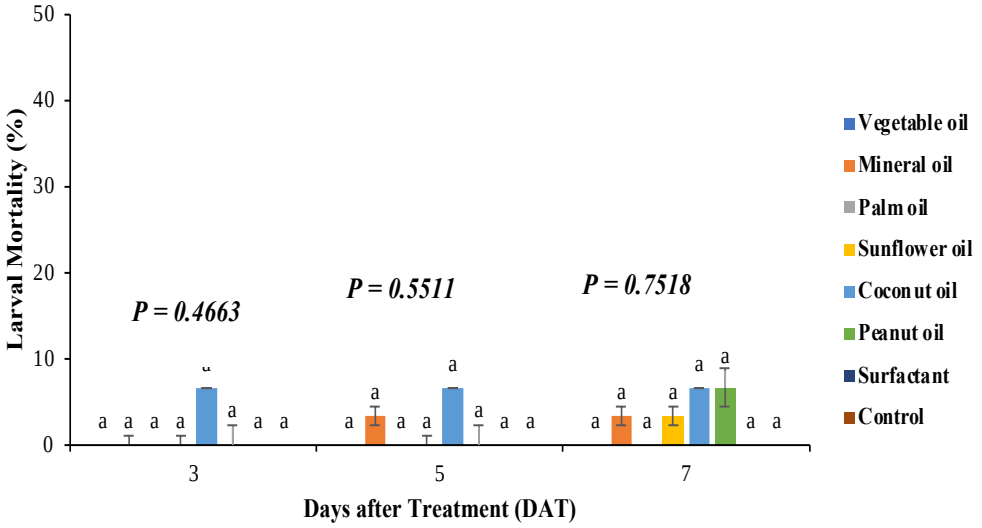

Figure 1. Effect of liquid carriers containing organic oil and emulsifying agent to third larval instar of *Spodoptera frugiperda* at 7 days after treatment. Group of means with the same letters are not significantly different at LSD (P<0.05). Bars represent standard error of the mean

Bioefficacy of EC formulations of M. rileyi against S. frugiperda

Phases of oil and emulsifying agent were evident in all EC formulations. During storage in refrigerator, coconut oil, palm oil, and vegetable oil solidified. Peanut oil, sunflower oil, and mineral oil do not solidify when stored in low temperature. When mixed with sterile distilled water, uniform suspension was observed in the spray suspensions.

The bioefficacy of formulated EC of *M. rileyi* was compared before storage and 3 months after storage in refrigerator (4°C) and incubator (25°C, 75% relative humidity) (Table 1). Mean larval mortalities were highest before storage (87.22%). Mortalities caused by EC formulations were lower after 3 months storage in refrigerator (17.49%) and incubator (46.59%). However, lethal time was shorter in 3 months storage in incubator at 6.47 days and refrigerator at 7.04 days as compared with 8.02 days before storage. Those larvae that succumbed to fungal infection had stiff larval body. Mycosis of the dead larvae was also evident signifying fungal infection due to *M. rileyi*. White fungal growth was observed and later turned to olive green as *M. rileyi* sporulated in the larval cadavers.

Table 1. Comparison of bioefficacy of emulsifiable concentrate of *Metarhizium rileyi* based on storage time and condition against third larval instar of *Spodoptera frugiperda*

Storage Time and Condition of Emulsifiable Concentrate* (EC)	Mean Larval Mortality (%)	Mean Lethal Time (Days)
Before storage	87.22a	8.02a
3 months storage in refrigerator (4°C)	17.47c	7.04ab
3 months storage in incubator (25°C, 75% relative humidity)	46.59b	6.47b

*Means with the same letters are not significantly different at LSD (P<0.05).

Figure 2a shows the total mortality caused by the formulated ECs against the third larval instar of *S. frugiperda*. Before storage, all EC formulations had similar effect (76.67 to 100%). However, mortalities generally declined after 3 months storage (10.00 to 62.22%). Among the EC formulations stored in refrigerator, *M. rileyi* in mineral oil and sunflower oil caused significant mortalities of 30.00% and 23.33%, respectively. Those stored in incubator had a varying result with *M. rileyi* in sunflower oil (62.22%), mineral oil (60.65%), peanut oil (58.89%), and palm oil (53.33%) inciting significant mortalities. These treatments also had the lowest reduction in virulence when larval mortalities were compared before and 3 months after storage. Considering the lethal time, a slight difference was observed before storage of EC formulations (6.16 to 9.02 days) (Figure 2b). Lethal time among the EC formulations in 3 months storage did not vary, which ranged from 3.75 to 9.50 days in refrigerator and 5.17 to 7.49 days in incubator.

The effect of EC formulations was also determined on growth and development of third larval instar of *S. frugiperda* (Table 2). The pupation (0 to 23.00%) was generally lower before the EC formulations were stored. High pupation was recorded at 70.00 to 90.00% due to lower mortalities in EC formulations stored in refrigerator. On the other hand, those EC formulations stored in incubator which incited significant larval mortalities had lower pupation (39.35 to 46.67%). Adult emergence was computed based on the number of emerging moths from pupated individuals. A slight difference on the adult emergence was observed regardless of storage time and condition. Larval period did not vary among the treatments and storage conditions. On the other hand, pupal period was similar among treatments before storage and 3 months in incubator. Storage in refrigerator had slight effect in pupal period.

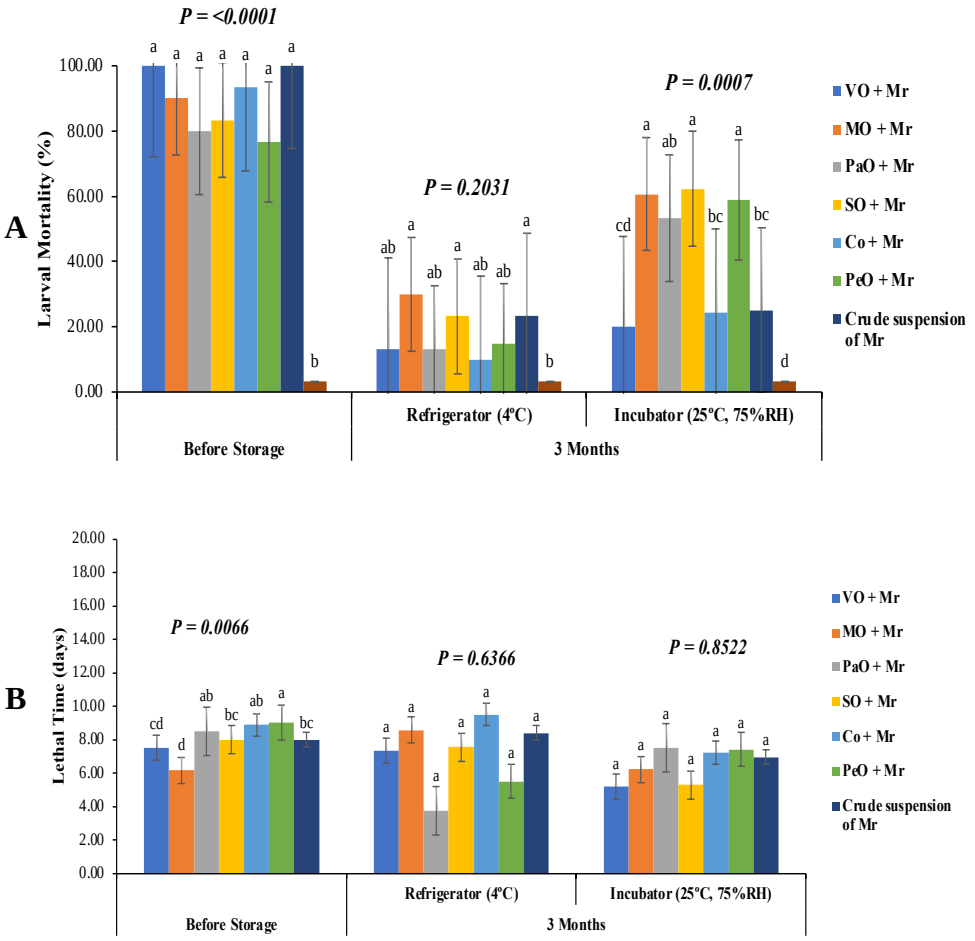

Figure 2. Varying bioefficacy of emulsifiable concentrates of *Metarhizium rileyi* against third larval instar of *Spodoptera frugiperda* as affected by storage time and conditions in terms of larval mortality (A) and lethal time (B). Mr: *M. rileyi*; VO: vegetable oil; MO: mineral oil; PaO: Palm Oil; SO: sunflower oil; CO: coconut oil; PeO: Peanut Oil. Group of means with the same letters are not significantly different at LSD (P<0.05). Bars represent standard error of the mean

Table 2. Effect of emulsifiable concentrates of *Metarhizium rileyi* on growth and development of *Spodoptera frugiperda*

Treatments*	Pupation (%)			Adult Emergence** (%)			Larval Period*** (days)			Pupal Period**** (days)		
		3 months			3 months			3 months			3 months	
	Before storage	4°C	25°C, 75% RH	Before storage	4°C	25°C, 75% RH	Before storage	4°C	25°C, 75% RH	Before storage	4°C	25°C, 75% RH
VO + Mr	0.00c	86.67ab	80.00a	0.00c	92.59ab	96.67a	-	9.83a	9.82a	-	6.43c	6.93a
MO + Mr	10.00bc	70.00b	39.35b	22.22bc	91.53ab	70.00a	9.67a	9.50a	11.00a	8.50a	6.70bc	7.75a
PaO + Mr	20.00b	86.67ab	46.67b	66.67ab	75.83b	95.24a	9.67a	9.20a	12.05a	7.50ab	6.93abc	6.50a
SO + Mr	16.67b	76.67ab	37.78b	100.00a	95.24ab	68.89a	10.00a	9.56a	10.22a	7.89ab	7.43ab	6.67a
CO + Mr	6.67bc	90.00a	75.56a	66.67ab	96.30a	90.74a	10.00a	9.94a	9.65a	7.00ab	7.17abc	7.35a
PeO + Mr	23.33b	85.19ab	42.22b	50.00ab	82.38ab	72.22a	9.67a	9.51a	10.67a	6.75b	7.08abc	7.33a
Crude suspension of Mr	0.00c	76.67ab	78.52a	0.00c	82.14ab	95.24a	-	9.91a	9.78a	-	7.04abc	7.97a
Control	96.67a	93.33a	93.33a	79.63ab	96.67a	96.67a	10.17a	9.19a	9.19a	7.65ab	7.63a	7.63a
Mean	**21.67**	**82.32**	**60.89**	**48.15**	**89.09**	**85.71**	**9.86**	**9.58**	**10.30**	**7.55**	**7.05**	**7.27**

*Mr: *Metarhizium rileyi*; VO: vegetable oil; MO: mineral oil; PaO: Palm Oil; SO: sunflower oil; CO: coconut oil; PeO: Peanut Oil.
**Adult emergence was calculated based on pupation.
***Larval period was determined based on the length of time the third larval instar reached pupation.
****Pupal period was computed from pupation to adult emergence.
Means in the same column with similar letters are not significantly different at LSD (P<0.05).

Discussion

This research work explored formulating a Philippine *M. rileyi* isolate from *S. exigua* as EC using liquid carrier with various organic oils and emulsifying agent that were further stored in different conditions. Oil-based formulations improve the persistence and insecticidal activity of entomopathogenic fungi (Bateman *et al*., 1993). The enhanced efficacy of oil formulation may be attributed to oils, which are excellent stickers that promote contact between the formulated active ingredient and the lipophilic insect cuticle as well as in increasing rain fastness on the waxy leaf cuticle of treated host plants (Prakas *et al*., 2015). Likewise, Devi *et al*. (2002) noted that emulsifiers like Tween and Triton X 100 aid in forming a stable and uniform suspension of spray fluid when diluted in water without the need for constant agitation during spraying. They further observed the field efficacy of *Nomuraea rileyi* (former scientific name of *M. rileyi*) conidia formulated in oil and applied as oil-in-water emulsion spray since the oil formulation forms a thin layer around the conidia protecting these fungal inocula from desiccation and ensuring better spread over the larval surface.

The first experiment aimed to determine if the liquid carriers had an insecticidal activity against *S. frugiperda*. Results indicate that the six organic oils do not cause lethal effect to *S. frugiperda* due to insignificant number of third instar larvae that were killed during the bioassay. Some researchers noted the insecticidal activity of oils particularly the essential oils against *S. frugiperda* (Santos *et al*., 2016).

Succeeding bioassays were conducted to compare the insecticidal activity of EC formulations against the third instar larvae of *S. frugiperda* before these formulations were stored and 3 months of storage in refrigerator and incubator. It was evident that storage conditions and time affected their bioefficacy wherein the freshly prepared EC formulations caused more epizootics. This study also showed that virulence of spores declined during storage but the lethal time was shorter in EC formulations stored in incubator. On the other hand, Grijalba *et al*. (2018) formulated a Colombian isolate of *M. rileyi* as EC and discovered its potential in controlling *S. frugiperda* infesting corn. This formulation had shelflives of 6 and 12 months at 18°C and 8°C, respectively.

Santos *et al*. (2012) noted that many factors affect the stability of the formulations such as the genotype of the microorganism used as active ingredient, formulation, temperature, relative humidity, and packaging. Loss of virulence of conidia in the refrigerator is due to the delayed germination process (Alves *et al*., 1996). Unformulated conidia and formulations of *B. bassiana* stored in refrigerator for 30 months had slow germination and low

virulence. However, other researchers had contrasting findings on the effect of storage conditions to the quality of the formulated product. Alves *et al*. (2002) observed that conidial viability of *M. anisopliae* var. *acridum* did not vary between period of storage, temperatures, and formulations in the first 15 weeks, however, conidial viability was significantly reduced over time with remarkable decline between 35 and 40 weeks of storage particularly at 27°C. Likewise, the viability of formulation of hyphomycetous fungi with vegetable, corn, sunflower, and canola oils did not vary at different temperatures such as 4, 17, and 26°C (Consolo *et al*., 2003). Amandeep and Neelam (2014) also observed that talc formulations of *B. bassiana* at refrigeration temperatures had minimum decrease in viable count since the fungus undergoes the stationary growth phase with decreased growth rate and low nutrient requirement resulting in longer viability of culture in low temperature than storing at 25°C. Alves *et al*. (2002) reported that the conidial viability of formulated *M. anisopliae* var. *acridum* remained high above 91% when stored at 10°C for 40 weeks. Jenkins and Grzywacz (2000) recommended 85% germination as minimum desirable biological activity for a bioinsecticide under controlled conditions.

This paper also presented that the various oils had varying performance as liquid carriers in the formulation. Likewise, EC formulations had higher efficacy than unformulated *M. rileyi*. Miscibility of oil in water suspension may also contribute in the bioefficacy of the EC formulations due to homogeneous mixture for treatment spray. Likewise, peanut oil, sunflower oil, and mineral oil which do not solidify appeared promising after three months of storage.

Vega-Aquino *et al*. (2010) noted that the type of oil used may affect the behavior of *M. rileyi* on target insect. Devi *et al*. (2002) also formulated *N. rileyi* and observed good miscibility of sunflower oil with Tween 80 and Triton X 100 resulting in a uniform dispersion of conidia in water in a spray fluid. They further observed that this formulation caused similar mortality with unformulated conidia against *S. litura*. In their field test, their formulation caused significant mortalities to *S. litura* larvae on rainy season groundnut (44.3%) and post-rainy groundnut (65.9%). Likewise, Smith (1997) also discovered that oil and emulsion formulations of *Paecilomyces fumosoroseus* had higher bioefficacy to whitefly instars as compared to aqueous formulations with surfactant.

On the other hand, Alves *et al*. (2002) noted that among the formulations of *M. anisopliae* var. *acridum*, peanut oil maintained conidial viability higher than 90% in 10°C and 27°C for 40 weeks. Prakash *et al*. (2015) discovered that *M. anisopliae* in gingelly oil and sunflower oil formulations had better viability as compared to other formulations tested. The (vegetal or mineral) oil used

affected the insecticidal activity of *M. rileyi* against *S. frugiperda* ranging between 90 and 100% (Vega-Aquino *et al*., 2010).

Furthermore, pH influences the quality of aqueous formulations (Aguirre *et al*., 2009). Villamizar *et al*. (2004) developed preformulated products of *N. rileyi* for control of *S. frugiperda*. High germination rates were recorded for EC (92%) and dispersible granule (87%), however, these formulations had low bioefficacy with 64% (EC) and 31% (dispersible granule). The pH and water activity were later identified as factors influencing the development and efficacy of *N. rileyi* (Aguirre *et al*., 2009).

In this study, unformulated *M. rileyi* caused 100% mortality but virulence declined during storage. Crude fungal suspension of the same isolate caused 76.67 to 90.00% mortality to third larval instar of *S. frugiperda* (Montecalvo and Navasero, 2021) while 16.98 to 50.21% using 1 x 10^7 conidia/ml and 42.13 to 92.13% mortality in 1 x 10^8 conidia/ml in third larval instar of *S. exigua* (Montecalvo *et al*., 2022a). The difference in results of the bioefficacy of the unformulated *M. rileyi* can be attributed to the varying time when the experiments were conducted, wherein there maybe variances in ambient temperature and relative humidity. The bioefficacy of the entomopathogenic fungi is influenced by conidial concentration, temperature, and relative humidity (Han *et al*., 2014).

We also demonstrated in this study that the EC formulations affected the survivors in terms of lowering the pupation and adult emergence. This was consistent with our previous findings that *M. rileyi* significantly affected growth and development of third larval instar of *S. exigua* in terms of larval duration, pupation, and adult emergence (Montecalvo and Navasero, 2020).

Based on our knowledge, this is the first attempt to formulate this entomopathogenic fungus in the Philippines. It should be noted that this study employed varying method and isolate in the formulation suggesting the differences in our findings with the previous results. Nevertheless, the efficacy and stability of these initial EC formulations under storage conditions are continuously being evaluated. Likewise, our current formulations will be continuously developed considering the microbiological, physical, chemical, and biological properties to ensure efficacy of formulated product. Optimization of the product is necessary such as adjusting the composition and process, and understanding the behavior of the microorganism to provide an optimal development conditions and insecticidal activity (Aguirre *et al*., 2009).

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