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RESEARCH ARTICLE

Evaluation of Different Liquid Formulations of Fungal Conidia of *Beauveria bassiana* and *Metarhizium anisopliae* Against *Bactrocera zonata*

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ABSTRACT

Beauveria bassiana and *Metarhizium anisopliae* are the most widely used entomopathogenic fungi (EPF) and have the highest rate of success stories over a variety of biopesticides. These EPF have some limitation related to their efficacy in areas with high temperature. The short shelf life of the biopesticides is the main barrier to their commercialization. This study was designed to find out certain liquid carriers which can retain the viability of the conidia of EPF. The research involved the bioassay of *B. bassiana* and *M. anisopliae* under an environment containing various formulations of vegetable oils and assessment of the impact of oil formulation on fungi's ability to cause mortality on pupae and adults of *Bactrocera zonata*. Conidia of *B. bassiana* and *M. anisopliae* added in oils such as Corn oil (*Zea mays*), Groundnut oil (*Arachis hypogaea*), Sunflower oil (*Helianthus Annuus*), Sesame oil (*Sesamum indicum*), Coconut oil (*Cocos nucifera*) and Soyabean oil (*Glycine max*) were used to prepare the formulations. All formulations were prepared by adding conidia in oil-in-water emulsions (W/O/W emulsions; the ratio of oil phase to water phase was 20:80) on the same day. These formulations were prepared on the same day and evaluated the efficacy on three different time periods: 1st day, 15th and 30th day. Difference in the adult emergence of the pupae and mortalities of the adults was observed. Different oils have different effects on maintaining the pathogenicity of the product. To obtain reliable findings, all treatments were replicated three times and one control. The comparison of mean of each treatment was compared using the ANOVA technique under a completely randomized design (CRD). Tukey HSD test was used for means of significant treatment at 5%. Aqueous suspension of EPF performed well on 1st day of preparation but lost its efficacy on 15th and 30th day. Evaluation of pathogenicity of the 30-day old formulations of *B. bassiana* showed maximum mortality 84.4% followed by *M. anisopliae* 82.2 % in sesame oil as compared to 2.2 % control. Significant differences were recorded for adult emergences of *B. zonata* at all intervals. Evaluation of 30-day old formulation showed poor suppression in adult emergence from pupae 15.6 % in sesame oil in comparison to 88.8 % in control. According to this study Sesame oil and Corn oil are recommended as best liquid carriers for production of commercial formulations of *B. bassiana* and *M. anisopliae*.

Keywords: *B. bassiana*, *M. anisopliae*, *B. zonata*, Liquid carriers, Liquid formulation.

INTRODUCTION

The peach fruit fly, *Bactrocera zonata* (Saunders) (Diptera: Tephritidae), is a serious pest of fruits and vegetables (Mahmoud *et al.*, 2024). *B. zonata* is considered as quarantine pest in all over the world

(Zhao *et al.*, 2024). In different regions of Pakistan, the most abundantly found fruit fly species is *B. zonata* (Majeed *et al.*, 2024). In many areas, control of *B. zonata* is problematic due to its behavior, feeding

nature and biological flexibility of different life stages and the absence of efficient broad-spectrum pesticides in the market (Papadopoulos *et al.*, 2024). Immediate knockdown effects of synthetic pesticides made them successful to manage fruit flies in developing countries (Ortolá *et al.*, 2024). These sprays not only cause mortality in fruit flies, but it also decreases the population of beneficial insect (Ramniwas *et al.*, 20224). Pesticide treatments raise production costs and leave hazardous residues in fruits, resulting in bio-magnification in humans (Oyugi *et al.*, 2024).

Biopesticide based on microorganism can be a best alternative to chemical insecticide and an effective component of integrated pest management (IPM) for management of various insect pests (Yasin *et al.*, 2020; Singh *et al.*, 2024). Entomopathogenic fungi (EPF) have ability to cause infection in insect when conidia come in contact with target insect (Ma *et al.*, 2024). Traditional formulations of Mycopenicid as treatments have been applied on insect pests in numerous crops (Bamisile *et al.*, 2021). Indeed, there are now more companies operating internationally because of the rising interest in microbial products. However, only a few numbers of Entomopathogenic fungi are commercially accessible or have been used in the field (Maina *et al.*, 2018). Nearly all the Mycoinsecticides registered in Brazil are based on the ascomycete fungus *Metarhizium anisopliae* s.l. (Metsch.) Sorokin and *Beauveria bassiana* s.l. (Bals.) Vuil (Ascomycota: Cordycipitaceae) (Oliveira *et al.*, 2018).

However, it is common to see products with poor quality and variable outcomes in the real world. Low quality and unpredictable outcomes are an issue that is partially explained by unfavorable field conditions for fungal infection and survival. The most significant abiotic parameters influencing the survival and pathogenicity of Entomopathogenic fungi are solar radiation, humidity, and temperature (Zimmermann *et al.*, 2008). To find out the impact of these abiotic variables on the biological parameters of the fungus *B. bassiana* and *M. anisopliae* in both laboratory and natural environments, multiple studies have been carried out (Huang and Feng, 2009).

In comparison to chemicals, the popularity of biopesticides is growing much more slowly, and there are now just 2% of them on the market (Dar *et al.*, 2019).

Entomopathogenic fungi based biopesticide are ecofriendly (Ginting *et al.*, 2020). Various formulating carrier materials are used for EPF. Longer shelf life is main concern in the development of the EPF based products (Sharma *et al.*, 2020).

Addition of carbohydrates improves germination and survival of conidia of EPF in the field conditions (Gautam *et al.*, 2020). In aqueous suspension water evaporate rapidly causing low moisture level resulting in reduce germination of the conidia (Gul *et al.*, 2014). Performance of oil based was recorded superior than aqueous suspension of EPF (Kaaya and Hedimbi, 2012). Oil based formulation have another advantage of protection from UV light (Kaiser *et al.*, 2019).

The objective of this study was to prepare formulations of *B. bassiana* and *M. anisopliae* that have longer persistence. Different liquid carriers have different abilities of sustain viability and pathogenicity of EPF against Fruit fly. Primary objective of the study was evaluation of liquid carriers with conidia of *B. bassiana* and *M. anisopliae* against *B. zonata*.

MATERIAL AND METHODS

Preparation of fungal culture: The cultures of EPF *B. bassiana* and *M. anisopliae* were obtained from the pre-maintained culture of Insect Pathology lab, Institute of Plant Protection MNS University of Agriculture Multan. A single spore purification technique, as reported by Noman *et al.* (2018) and Usman *et al.* (2024), was used to purify colonies on freshly prepared plates with potato dextrose agar (PDA) (Merck KGaA, Darmstadt, Germany). In autoclaved glass bottles, 39g PDA was added in 1 litre of sterile water. The solution was continuously stirred at 100°C for 3-5 minutes until the homogeneous medium was dissolved. To avoid contamination, the prepared media was sterilized at 121°C temperature and 15 psi for 15 minutes in the autoclave. After autoclaving, the media was left to cool down for some time (Yaqoob *et al.*, 2024).

Media pouring was carried out in the laminar air flow chamber and to avoid contamination the chamber was UV light treated for 15 min and the surface was sterilized with methylated spirit. A sterile autoclaved inoculation pin was used to inoculate *B. bassiana* and *M. anisopliae* spores/conidia on solidified PDA plates. These plates were sealed with Parafilm (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) and kept in incubator set at 25 °C under a 14:10 h L:D photoperiod.

Preparation of oil in water emulsions: Oil in water emulsions was prepared with non-ionic surfactant Tween-80. All percentages of oils are indicated as volume/volume. All components were sterilized and then maintained at 80°C for distilled water and 60°C for oils and Tween-80. Oil and surfactant Tween-80 were pipetted into the water while stirring at 200 rpm. The mixture was then stirred for 20 minutes at 200 rpm and room temperature.

Conidia were harvested by adding Tween-80 under vigorous magnetic stirring and kept in glass bottles. Hemocytometer was used to calibrate all conidial formulations. Concentration of 10^8 conidia ml^{-1} was inoculated in emulsified oils. Two batches of control were prepared with conidia in distilled water plus Tween-80 (no oil) and another with water and Tween-80 only (no oil and conidia). All formulations were retained overnight to allow conidia to be sensitized by the effect of liquid carriers. Then efficacy of these formulations was estimated in terms of pathogenicity against pupae and adults of the *B. zonata*.

There were six oils used: Corn oil (*Zea mays*), Groundnut oil (*Arachis hypogaea*), Sunflower oil (*Helianthus Annuus*), Sesame oil (*Sesamum indicum*), Coconut oil (*Cocos nucifera*) and Soyabean oil (*Glycine max*). Other liquid carriers were Foam gel, molasses, Water and Commercial products of *B. bassiana* and *M. anisopliae*.

Insect culture: Infested Mango fruits were collected from the orchard in Multan, Punjab, Pakistan. These infested fruits were placed in a container containing the sterilized soil at Insect Pathology Lab of the Institute of Plant Protection at MNS University of Agriculture Multan. To prevent contamination, muslin cloth was used to cover these containers. Pupation of fruit fly was started in the soil after completion their three larval instar inside these infested fruits. To obtain pupae from soil, soil was sieved after every three days. The pupa stage was sieved and kept in a screen cage (30 cm × 30 cm × 30 cm) a day before the adult emerged, allowing them to forage, mate, and lay eggs. The adults were fed with a mixture of sugar, protein hydrolysate (3:1) and water. All rearing conditions were provided to the culture of *B. zonata* for its successful growth. Light and dark periods of 16 D: 8 L and temperature of 25 ± 2 °C were maintained in the Lab.

Adult *B. zonata* Bioassay: The pathogenicity of *B. bassiana* and *M. anisopliae* against *B. zonata* was evaluated using several liquid carriers through contact treatment under lab conditions. There were eleven different treatments: molasses, corn oil, sunflower oil, sesame oil, groundnut oil, soya bean oil, coconut oil, foam gel, commercial product, aqueous spore suspension, and water. All formulations were prepared on same day and stored at room temperature 25 ± 2 °C for three different periods (1-day, 15-day and 30-day). Effectiveness of these formulations was assessed in terms of adult mortalities on the 5th day after application of the treatment. A plastic cage was sprayed by 2ml of liquid formulation of EPF and kept to dry, and then 15 adult fruit flies (3-day old) were entered. Control cages were treated with 2ml of mixture of water and surfactant. Each formulation was tested against adult fruit fly with 15 insects per treatment comprising of three replications (total of 45 insects / treatment). Treatments were replicated three times using a complete randomized design (CRD).

Pupae *B. zonata* Bioassay: The pathogenicity of *B. bassiana* and *M. anisopliae* was investigated against *B. zonata* 3-day old pupae using the contact assay under lab conditions. Adult emergences were recorded after treating with selected oil formulations: (Corn oil, Sunflower oil, Groundnut oil, Sesame oil, Soyabean oil, Coconut oil, Foam Gel, Aqueous spore suspension, Molasses, Commercial product, and Water with surfactant). On a single day, all formulations were developed and stored for three different periods (1-day, 15-days, and 30-days). The effectiveness of these formulations was assessed on the 5th day of each application. Three day old pupae were placed on sand after one minute of exposure in the liquid formulation of EPF. After application of treatment pupae were buried in the sand. In each treatment the number of newly emerged flies was recorded.

Field Application of liquid formulations of *Bb* and *Ma*: The field trial was carried out in a mango orchard located at Mango Research Institute Multan. There were five treatments (Conidia Bb-Ses, Conidia Ma-Ses, Protein Bb-Ses, Protein Ma-Ses and Commercial product) along with untreated control. Sesame oil was used to prepare liquid formulations. Out of four liquid formulations two were conidia and two were crude proteins of *B. bassiana* and *M. anisopliae* in sesame oil.

These formulations were applied in field and data were collected by using yellow sticky traps.

There were three intervals of data collection after application of treatments day-5, day-10 and day-15. Population reduction was monitor through adult catching data. Less adult catches refer to more efficacy of product. Experiment was conducted twice (2022 and 2023).

STATISTICAL ANALYSIS

Percent mortality among the treatment groups was compared with mortality in the untreated control group using Abbott's formula. The collected data was analyzed in STATISTIX 8.1 to determine significance of selected treatments. The data was run through Analysis of Variance under completely randomized design to compare the means at the 5% probability level. Tukey's HSD test was performed for multiple comparisons to differentiate among different treatments (Khan *et al.*, 2011).

RESULTS

Adult *B. zonata* Bioassay: The pathogenicity of *B. bassiana* and *M. anisopliae* was determined against *B.*

zonata adults using different liquid carriers through contact treatment under lab conditions. There were eleven treatments Corn oil, Groundnut oil, Sesame oil, Sunflower oil, Coconut oil, Soyabean oil, Foam Gel, Aqueous spore suspension, Molasses, Commercial product, and Water with surfactant.

Significant differences were recorded for mortality (%) of *B. zonata* adults at all intervals ($P < 0.5$) ($F_{10,22} = 70.83$, $\alpha = 0.05$). The mean mortality of *B. bassiana* with various liquid carriers against adult of *B. zonata* was observed in the following order: Sesame oil > Corn oil > Commercial product > Sunflower oil > Groundnut oil > Soyabean oil > Coconut oil > Molasses > Foam gel > Aqueous spore suspension. Evaluation of efficacy on 1-day old formulation shows maximum adult mortality 88.9 % when treated with sesame oil as compared to 6.7% in control. In 15-day old formulation, 86.4% adult mortality was recorded in the sesame treated *B. zonata* batches in comparison with 4.4% in control. Assessment of pathogenicity of the formulation on 30-day old formulation showed maximum mortality 84.4% in sesame oil as compared to control 2.2% (Figure 1).

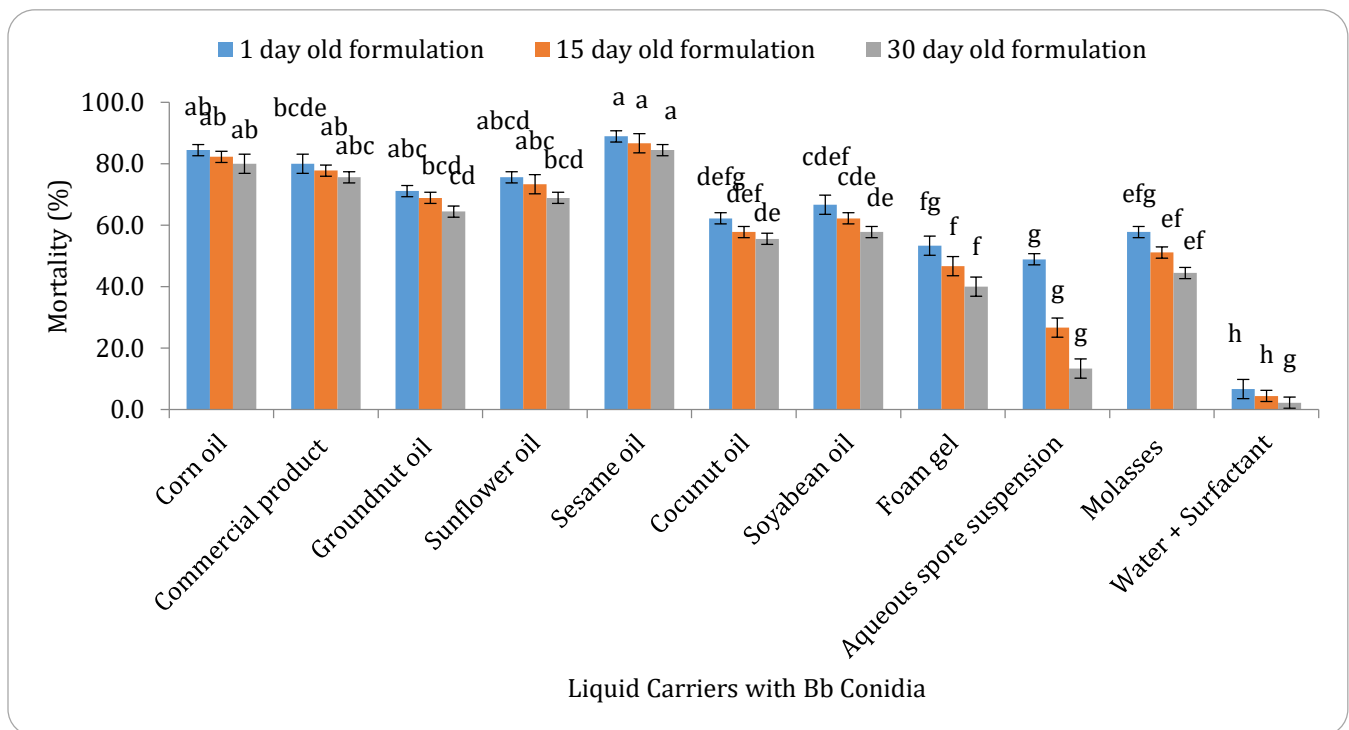


Figure 1. Adult mortality (Mean±SE) of *B. zonata* at different intervals when liquid formulations of conidia from *Bb* were applied to 3 days old adults under laboratory conditions

Similar trend was recorded in the term bioassay of *M. anisopliae* against the adult of *B. zonata*. Highest mortality

82.2 % in sesame oil was recorded in a pathogenicity test at 30th day as compared to 4.4% in control (Figure 2).

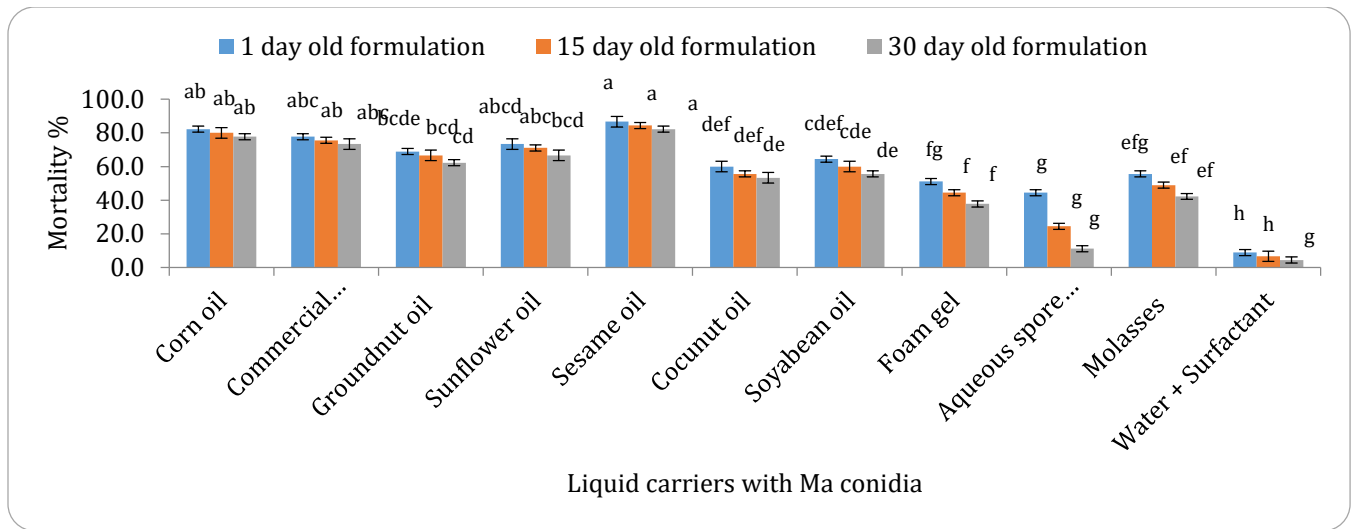


Figure 2. Adult mortality (Mean±SE) of *B. zonata* at different intervals when liquid formulations of conidia from *Ma* were applied to 3 days old adults under laboratory conditions

Pupae *B. zonata* Bioassay: The pathogenicity of *B. bassiana* and *M. anisopliae* was evaluated against 3-day old pupae of *B. zonata* using contact method under laboratory conditions. Formulation-based adult emergence was recorded as emergences were disturbed when treated with different formulations (Corn oil, Groundnut oil, Sesame oil, Coconut oil, Soyabean oil, Sunflower oil, Foam Gel, Molasses, Commercial product, Aqueous spore suspension and Water with surfactant). On a single day, all formulations were developed and stored for three different periods (1-day, 15-days, and 30-days). Less adult emergence was considered as more effectiveness of the formulation. The Suppression in adult emergence with various liquid

carriers was found in the following order: Sesame oil > Corn oil > Commercial product > Sunflower oil > Groundnut oil > Soyabean oil > Coconut oil > Molasses > Foam gel > Aqueous spore suspension. Significant differences were recorded for adult emergences of *B. zonata* at all intervals ($F_{10,22} = 59.74, P=0.000, \alpha=0.05$). Poor adult emergence of 8.9 % in sesame oil in comparison to 84.4 % in control was recorded in the bioassay of 1-day old formulation. Similarly, the 15-day old formulation shows 11.1 % adult emergence as compared to control 86.7%. Bioassay of 30-day old formulation of *B. bassiana* shows poor adult emergence 15.6 % in the sesame oil as compared to 88.8 % adult emergence in control (Figure 3).

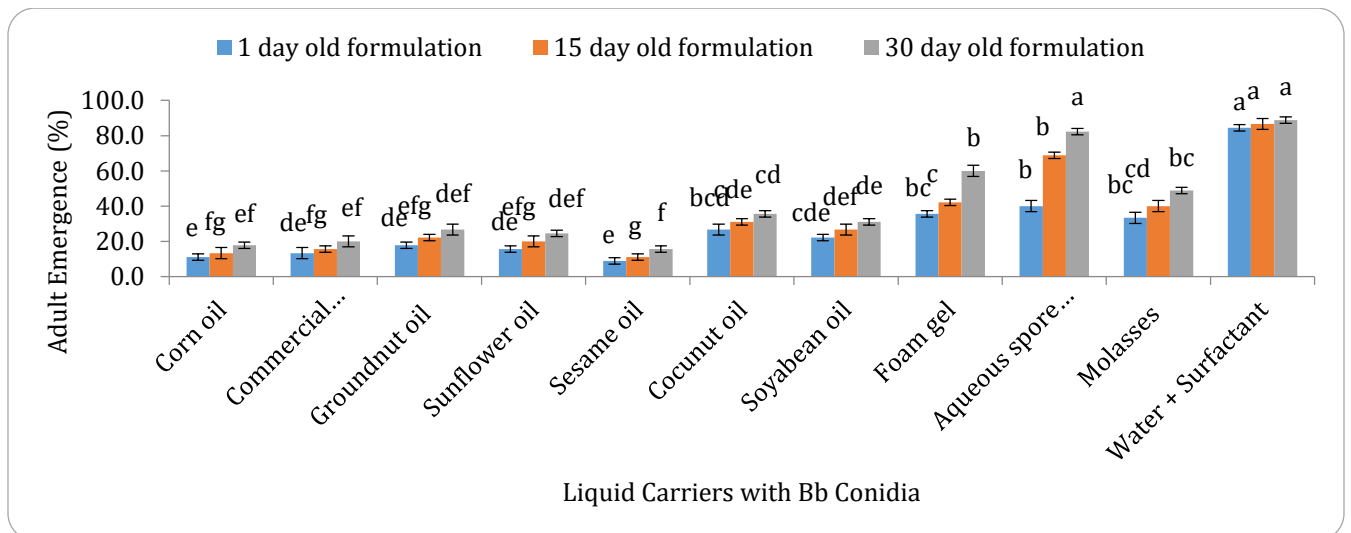


Figure 3. Adult emergences (Mean±SE) of *B. zonata* at different intervals when liquid formulations of conidia from *Bb* were applied to 3 days old pupa under laboratory conditions

Similar trend was recorded in the efficacy of formulations of *M. anisopliae*. The Suppression in adult emergence with various liquid carriers was found in the following order: Sesame oil > Corn oil > Commercial product > Sunflower oil > Groundnut oil >

Soyabean oil > Coconut oil > Molasses > Foam gel > Aqueous spore suspension. Poor adult emergence was observed 17.8% in sesame oil as compared to control 91.1% in the bioassay of formulations on 30th day of its preparation (Figure 4).

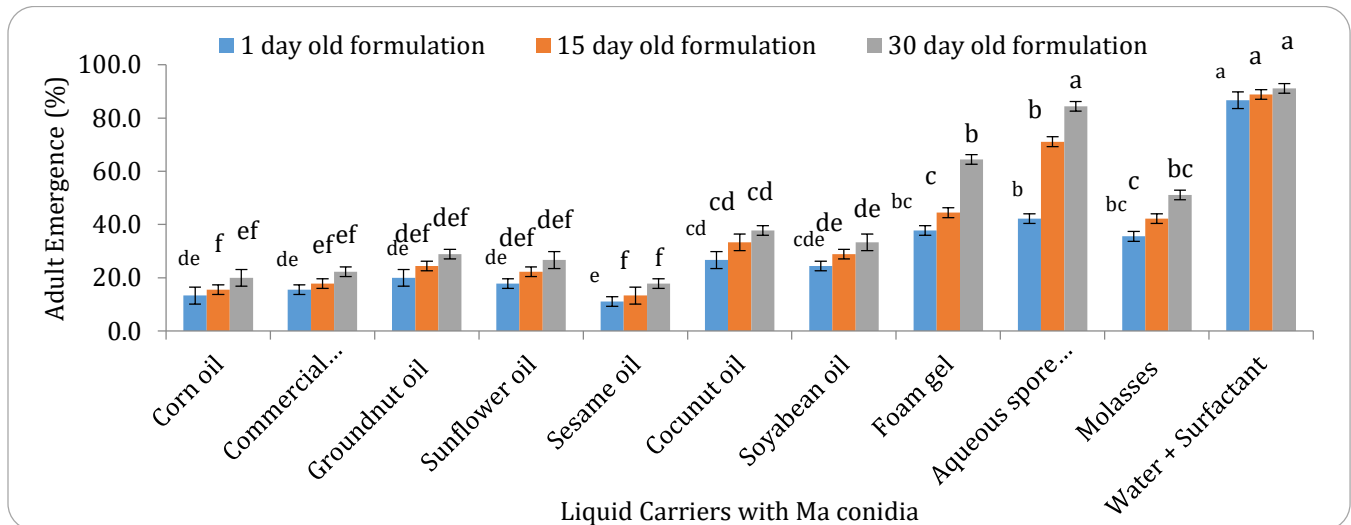


Figure 4. Adult emergences (Mean±SE) of *B. zonata* at different intervals when liquid formulations of conidia from *Ma* were applied to 3 days old pupa under laboratory conditions

Field Application of liquid formulation of *Bb* and *Ma*:

There were five treatments (Conidia *Bb*-*Ses*, Conidia *Ma*-*Ses*, Protein *Bb*-*Ses*, Protein *Ma*-*Ses* and Commercial product) along with untreated control. These formulations were applied in field and data were collected by using yellow sticky traps. There were three intervals of data collection after application of treatments day-5, day-10 and day-15. Population reduction was monitor through adult catching data. Less adult catches refer to more efficacy of product. Experiment was conducted 1st in 2022

and 2nd in 2023. Two years’ data was collected. Significant differences were recorded for adult population reduction of *B. zonata* ($F_{5, 18} = 2447.13, P=0.000, \alpha=0.05$). In 2022 population reductions was recorded as 88.94, 90.84 and 92.93 in Conidia *Ma*-*Ses* after 5th, 10th and 15th day of application of treatment. Trend of adult population reduction was recorded as following; Commercial product > Conidia *Ma*-*Ses* > Conidia *Bb*-*Ses* > Protein *Ma*-*Ses* > Protein *Bb*-*Ses* (Figure 5).

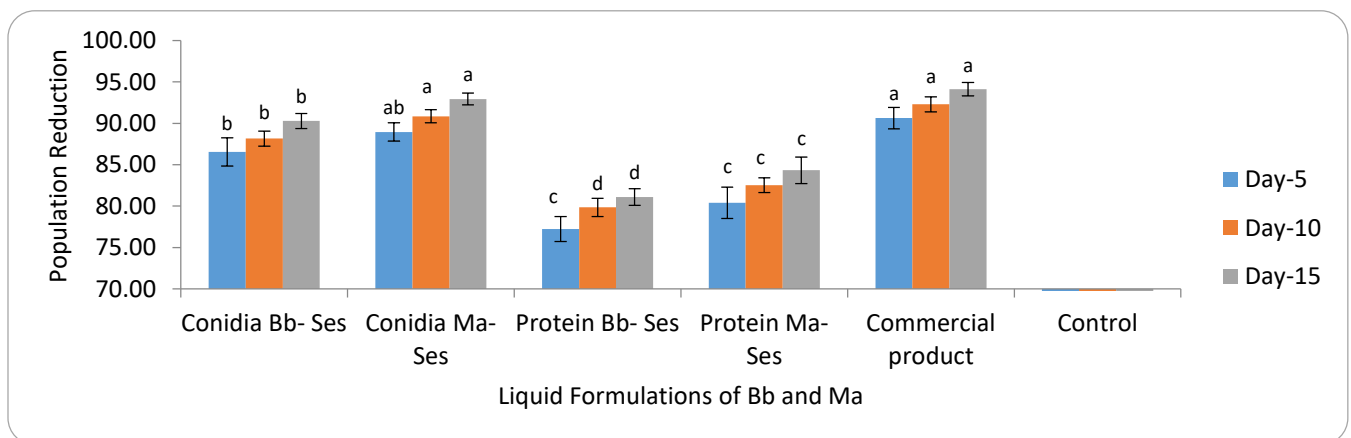


Figure 5. Adult population reductions (Mean±SE) of *B. zonata* at different intervals when liquid formulations of *Bb* and *Ma* were applied under field conditions in 2022

Significant differences were recorded for adult population reduction of *B. zonata* ($F_{5, 18} = 2544.35, P=0.000, \alpha=0.05$). In 2023 population reductions were recorded as 87.51, 89.19 and 91.58 in Conidia *Ma*- Ses after 5th, 10th and 15th

day of application of treatment. Trend of adult population reduction was recorded as following; Commercial product > Conidia *Ma*- Ses > Conidia *Bb*- Ses > Protein *Ma*- Ses > Protein *Bb*- Ses (Figure 6).

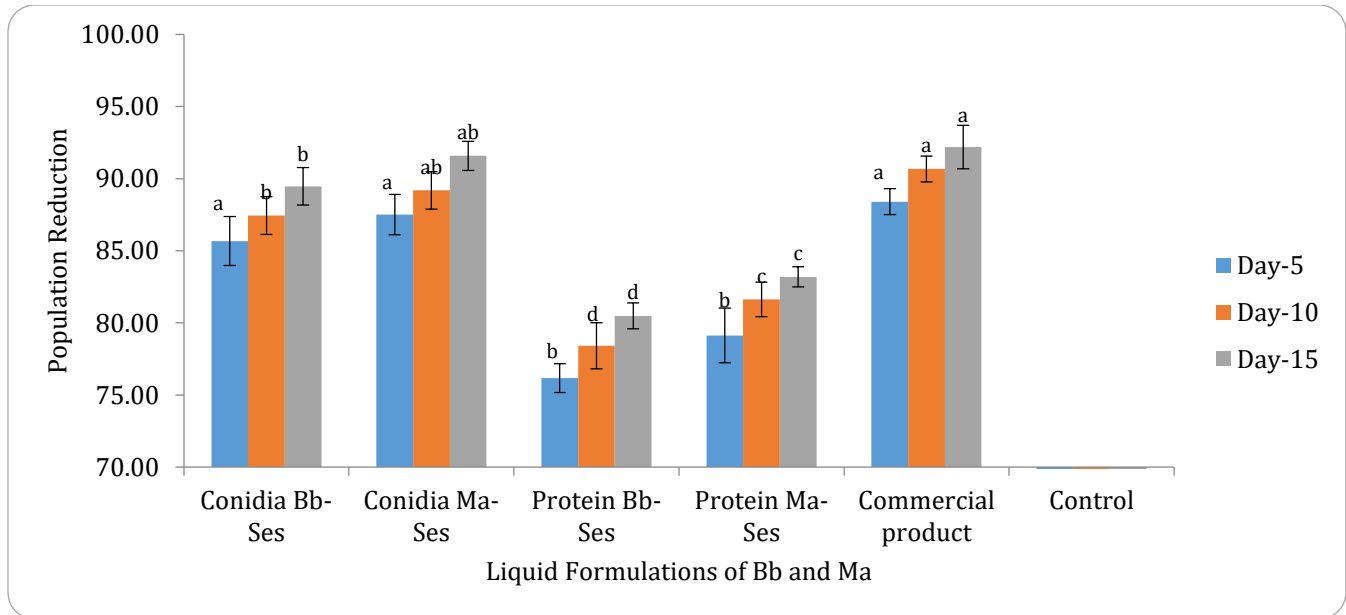


Figure 6. Adult population reductions (Mean±SE) of *B. zonata* at different intervals when liquid formulations of *Bb* and *Ma* were applied under field conditions in 2023

DISCUSSION

In this research, oil-based formulating agents were compared to water to make the formulation of a biological pesticide for the control of fruit fly. Conidia could recover from negative effects caused by some tested formulations and gave high pathogenicity levels, which could explain the significant interaction between liquid carriers and conidia of Entomopathogenic fungi (Sandhi *et al.*, 2024). It was useful to carry out 1st pathogenicity test after 24 hours of preparation of formulation (Li *et al.*, 2021).

The adherence and dispersion of conidia on the cuticle of insect hosts is facilitated by oil in water emulsion. Additionally, it reduces the negative impacts of abiotic variables such as heat stress and UV radiation and prevents conidia from desiccation in low humidity conditions. Additionally, Preparation of liquid formulations of conidia of *M. anisopliae* and *B. bassiana* has benefits for increasing shelf life, obtaining high mortality in insect hosts, and shielding conidia from damage during dilution (Gurpreet and Singh, 2018). The interaction type (additive, synergistic or antagonistic) always depends on target insect pest,

control agents, application time and infection dose (Nunes *et al.*, 2024). When two biocontrol perform separately in a combined infection, the interaction is additive while, if the interaction makes the blend of organisms more or less effective as compared with additive effect, interaction is either synergistic or antagonistic interaction (Basaid *et al.*, 2024). The same trend was exhibited in our result, which is an additive interaction on mortality of *B. zonata* was inflicted when EPF and oil liquid carriers were used. Although the mechanism for these positive interactions of combined applications is still unclear it can be hypothesized that one of the agents' act as sticker (oil liquid carrier) helping the conidia to remain attach with the body of the host, chances of mortality of insect increases (Lee *et al.*, 2023). Techniques of making oil-emulsions can improve effectiveness of these formulations. In laboratory bioassays, the wetting agent Tween 80 has been utilized to help hydrophobic conidia suspend (Qaderi and Safaie, 2023). These findings showed that conidia of *B. bassiana* can be formulated in vegetable oils as vegetable oil promote the viability of conidia (Fernandes *et al.*, 2015). Non-ionic surfactants are

considered as best surface-active agents, that gets their hydrophilic properties from non-ionizable groups like alcoholic hydroxyls, phenolic and carbonyl oxides of esters and amides (Muñoz *et al.*, 2022).

Non-ionic surfactants are mostly preferred in used for preparation of formulations due to lack of reactivity with ions found in hard water such as calcium, magnesium, and ferric ions (Smith *et al.*, 2022). This study showed that oil based formulation of *M. anisopliae* and *B. bassiana* are more effective. The less performance of aqueous suspension may be due to degradation during storage, less adhesion to the insect cuticle and sensitivity to the abiotic factors like evaporation. The shelf life, heat tolerance, and virulence against fruit fly were all greatly improved by this oil-based formulation. Sesame oil has unsaturated fatty acids that let it tolerate high temperatures, making it a superior carrier for shielding conidia from thermal stress.

These studies revealed that sesame oil can be used as a powerful carrier. Level of susceptibility and variable physiological conditions of *B. zonata* could lead to

inconsistency in mortalities. Maintaining viability of conidia during preparation, storage and application of formulation is another challenge. Mechanical stress, temperature fluctuations, UV radiations may effect conidia viability. There should be further research on exposure of formulations of EPF to UV radiations and to investigate which liquid carrier can shield the conidia from its detrimental effects. In field conditions pest management often involves integrated approaches of cultural, biological and chemical. The presence of other control measures in the field can interfere in the performance of these formulations. The result may explain that oil carriers, which have been demonstrated to alter the lipophilic insect cuticle and enhance conidial adhesion to Insect cuticle (Lei *et al.*, 2023).

CONCLUSION

Our research suggests sesame oil as a high-quality liquid carrier in the preparation of (oil in water emulsion) formulation of EPF that can maintain efficacy of conidia of *M. anisopliae* and *B. bassiana* up to 30 days of preparation of formulation.

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Contribution of Authors:

Mirza A. Qayyum	:	Concealed the idea, provided guidance in design of the study, critical revision of the article
Umer Sharif	:	Conducted the research trial, collected data, performed statistical analysis and give final shape to the article
Hasan Taha	:	Wrote the early version of the article
Shafqat Saeed	:	Some modification in the article and giving the instructions
Akhtar Hameed	:	Provide technical support, data analysis and interpretation
Muhammad Ishtiaq	:	Revised the draft and provide technical support