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Efficacy of Two Entomopathogenic Fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, Isolated from Eastern Saudi Arabia against the House Fly, *Musca domestica*

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Authors' Contribution
EAS and HMA conceived and designed the project. RSY collected fungi and prepared culture. EAS performed laboratory Bioassays, analyzed data and wrote the article. HMA and RSY reviewed the manuscript.

Key words
Entomopathogenic fungi, House fly larvae, Botanical oils

ABSTRACT

The house fly, *Musca domestica*, is not only a cosmopolitan but also a medically important insect acting as vector of some diseases. Entomopathogenic fungi, particularly *Beauveria bassiana* and *Metarhizium anisopliae,* and botanical oils have shown potential as synthetic insecticides alternative for house fly control. In the present work, local isolates from both fungi as well as their mixtures with essential oils were evaluated against house fly larvae under laboratory conditions. Batches of house fly larvae (25 individuals per replicate and 4 replicates per dose of fungi) were subjected to five doses from each fungus (10^1, 10^3, 10^5, 10^7 and 10^9 conidia/ml) in plastic cups for one min then transferred to a clean one. Both test and control cups were incubated for 7 days to determine $LC_{50, 90}$ and $EI_{50, 90}$. Joint action of these fungi with three botanical oils (celery, ginger and sesame) as well as influence of sublethal dose from both fungi on the larval development were also evaluated. *B. bassiana* was more potent than *M. anisopliae* in both larvicidal activity and inhibition of flies' emergence. Blends from fungi and essential oils exhibited synergistic effect but fungi mixture produced antagonistic effect. The development of *M. domestica* larvae was affected by sublethal dose from fungi. In conclusion, *M. anisopliae* is more efficient than *B. bassiana* and could be easily mixed with essential oils to either enhance larvicidal activity or utilize in integrated pest management. Furthermore, research on field evaluation and deleterious effects of environmental conditions on fungi capacity is required.

INTRODUCTION

The house fly, *Musca domestica*, is a worldwide insect causing annoyance, irritation and food spoilage. Furthermore, it is important vector transmitting many pathogenic diseases including anthrax, bacillary dysentery, cholera, infantile diarrhea, tuberculosis and typhoid to both human and animals (Lecuona *et al*., 2005; Förster *et al*., 2009; Barin *et al*., 2010). Accordingly, controlling this fly is crucial to avoid the previously mentioned diseases.

Synthetic insecticides were used to control the house fly (Cao *et al*., 2006) but due to extensive use of these chemicals, insecticide resistance as well as environmental and health hazards were evolved (Bell *et al*., 2010; Yadav, 2010). Globally, houseflies' resistance to synthetic insecticides became a big problem (Farooq and Freed, 2016) and directed the attention of the researchers to another safer alternatives exhibiting capacity in control such as entomopathogenic fungi (Zimmermann, 2007; Geden, 2012; Gul *et al*., 2014). Among the entomopathogenic fungi, both *Beauveria bassiana* and *Metarhizium anisopliae* are the most promising insecticides alternatives against both agricultural and medically important insects in addition to some important arthropods particularly ticks and mites which are human and animals ectoparasites (Immediato *et al*., 2015; Perinotto *et al*., 2017).

In addition to their environmental safety and lower/ negligible mammalian toxicity, entomopathogenic fungi showed great capacity in controlling house flies (Mishra *et al*., 2011; Khan *et al*., 2012; Acharya *et al*., 2015). Both *B. bassiana* (Bals.) Vuill., *M. anisopliae* (Metsch.) Sorok, were the most common fungi used in houseflies' management and produced rapid killing and high infection rates (Barson *et al*., 1994; Kaufman *et al*., 2005; Sharififard *et al*., 2011). Moreover, botanical derivatives could be also introducing another alternative to synthetic insecticides.

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Farooq and Freed (2016) mentioned that they exhibited capacity in repelling or controlling house fly whilst several other studies shown that they could be used against all the developmental stages of the house fly (Malik *et al*., 2007).

Recent studies have shown that botanicals could be added to entomopathogenic fungi in mixtures to synergize their potential for house fly eradication (Farooq and Freed, 2016). Ahmad *et al*. (2017) observed significant difference in larval mortality of *M. domestica* due to mixture of fungi and botanical oils except for mixtures of lower level of sub lethal doses (LC_{10} of fungi and LC_{10} of botanicals).

Unlike other bio insecticides, literatures revealed that data on the influence of sublethal doses of fungi on larval duration, pupal duration, pupation percentage, emergence percentage of flies and growth index are limited. The only study that was conducted by Ahmad *et al*. (2017) but for mixtures of fungi and botanical oils not for fungi alone.

Research is still in progress to find out which local isolates of the entomopathogenic fungi work effectively and can compete with synthetic insecticides. In accordance with the importance of housefly as medically and veterinary vector, the current study was carried out to investigate the efficacy of the entomopathogenic fungi *B. bassiana* and *M. anisopliae* isolated from Saudi Arabia against housefly larvae. Moreover, evaluating the effect of binary mixture from such fungi and some botanical oils as well as the effect of sublethal dose from both fungi on the development of larval stage.

MAERIALS AND METHODS

Collection, isolation, identification and purification of the entomopathogenic fungi

Both *B. bassiana* and *M. anisopliae* were the entomopathogenic fungi used in the present study. Soil samples were collected from Al-Ahsaa local livestock market during the month of August, 2017. The samples were collected in sterile glass tube for isolating the native strains of both entomopathogenic fungi in the laboratory based on morphological analysis. The fungus culture was purified by single conidia culture on potato dextrose agar (PDA) medium and subsequently sub-culturing was done according to method described by Dhingra and Sinclair (1995). Conidia were grown on PDA at 25 C in dark in standard Petri-dishes (90 mm diameter) for 10 days.

Spores' harvesting and suspension

The pure fungal culture was multiplied on PDA medium for 10 days. Spores were harvested by washing the dishes with pure water; subsequently the spore suspension was filtered through several layers of cheesecloth to remove mycelium. Spore concentration was determined with a haemocytometer under light microscope and adjusted to $1X10^1$ - $1X\ 10^3$ - $1X10^5$ - $1X10^7$ - $1X10^9$ spore/ ml.

House fly maintenance

The *M. domestica* flies were collected by flying insects net from livestock farms at King Faisal University, Al-Ahsaa, Eastern Saudi Arabia. Flies were transferred to insectary in Zoology department, College of Science, King Faisal University. Flies were reared in plastic cages (Collapsible insect rearing cages , Bug Dorm-1, from Bugdorm USA) measured 30× 30×30 cm^3 under laboratory conditions. Adults were feed on 10% glucose solution, while a soluble diet made from wheat bran and milk powder (1:2) soaked in white cotton inside colorless plastic jars 300 ml capacity was for egg laying and larval development. The colony was maintained at 28±2 °C, 70 ± 10 % relative humidity and photoperiod of 14L: 10D h.

Larvicidal bioassays

Immersion method was used to evaluate the infectivity of two entomopathogenic fungi, *B. bassiana* and *M. anisopliae*, against larvae of *M domestica* as mentioned by Farooq and Freed (2016).

Batches of 25 newly emerged fourth instar larvae were immersed for 1 min in 1 ml of the conidial at the desired concentration (1×10^6, 1×10^7, 1×10^8 spores/ mL) of the fungi inside 20 ml plastic cups. The control group was dipped in distilled water only. Excess water was removed by Pasteur plastic pipettes. Larvae were supplied with food (dried milk, wheat barn and water in white cotton balls) and incubated at 30 ± 2◦C, based on preliminary experiment, for 7 days. All treatments replicated 4 times for each concentration. Mortalities were recorded after the 7^{th} day and larvae that were immobile and did not respond to the needles counted as dead. Abbott's Formula (1925) was used for correcting observed mortalities in treatments if mortalities in control set exceeded 5% up to 10%.

Combined effect of fungi and essential oils

Three commercial botanical oils (celery, ginger and sesame) were used in the present results. A concentration of 10 % was freshly prepared from each oil before starting the experiments. One ml from desired oil was mixed in 10 ml distilled water and checked for one min whilst a concentration of $1x10^3$ conidia/ml from fungi was selected for preparing the mixture. The mixtures from both oils and fungi were prepared on bases of volume to volume whereas 4 ml from both oils and fungi were mixed together in a separate glass bottle and used in the treatments.

Same procedures used for the larvicidal bioassays were followed except that each group of larvae received 2ml from the mixture rather than 1 ml. larvae were

incubated at 30 ± 2◦C and mortalities were recorded daily up to 7 days.

To estimate the expected synergetic effect of fungl-oil mixture, the following formula (Farenhorst *et al*., 2010) was used:

Me = Mf + Mo (1 - Mf/100)

Where Me is expected mortality, Mf and Mo were the observed mortality percentage caused by the fungus and the oil separately. Positive Mfo - Me (observed mortality % for mixtures – expected mortality %) values were considered synergistic (Koppenhöfer and Kaya, 1998). This formula comparing mortality rates produced by fungal-oil mixture (observed) with the sum of mortalities produced by fungi and oils individually (expected).

Effect of sublethal dose of the fungi on the development of M. domestica *larvae*

Methodology used for determining the larvicidal activity was adopted to investigate the influence of both fungi on some biological parameters including larval duration, pupal duration, pupation percentage, pupal mortality, adult emergence percentage and growth index. One concentration, $4.5x10^4$ conidia/ml, was used from each fungus whilst control set received distilled water. The mortalities of both larval and pupal stages as well as emerged individuals were recorded on daily bases until the emergence of the last fly or the death of the last either larva or pupa.

Larval duration was estimated as the number of days since the starting of the experiment until they all reached the pupal stage whilst the pupal duration was estimated from pupal stage to adult emergence (Martinez-Tomas *et al*., 2009).

Growth index was calculated according to Saxena and Sumithra (1985).

Growth Index (GI) = percentage adult emergence / average developmental period (days).

The average developmental period is the sumition of larval duration and pupal duration.

Statistical analysis

Probit analysis was used to determine both LC_{50} and LC_{90} values whilst one-way ANOVA and the Tukey HSD post-hoc test were used for other data analysis at significance level of 5%. SPSS statistical package ver. 16 was used to perform statistical analyses.

RESULTS

Based on LC_{50} and LC_{90} of the tested entomopathogenic fungi against *M. domestica* larvae (Table I), larvicidal activity of these fungi could be arranged in the following descending order: *B. bassiana* (LC_{50} = 8.6 $x10^8$; LC_{90} = 8.9 $x10^{12}$ conidia/ml) > *M. anisopliae* (LC_{50} = 1.7 $x10^9$; LC_{90} = 6.9 $x10^{16}$ conidia/ml). Similarly, *B. bassiana* (IE_{50} = 1.5 $x10^8$; IE_{90} = 2.1 $x10^{11}$conidia/ml) was more potent than *M. anisopliae* (IE_{50} = 1.6 $x10^9$; IE_{90} = 5.08 $x10^{13}$ conidia/ml) in inhibiting flies emergence as has been shown in Table II.

Table I. LC_{50} and LC_{90} of fungi tested against newly moulted 4th instar larvae of *Musca domestica.*

Fungi	LC_{50} Conidia/ml	LC_{90} Conidia/ml	Chi square
Beauveria bassiana	8.6 $x10^8$	8.9 $x10^{12}$	113.99
Metarhizium anisopliae	1.7 $x10^9$	6.9 $x10^{16}$	82. 72

Table II. IE_{50} and IE_{90} of fungi tested against newly moulted 4th instar larvae of *Musca domestica.*

Fungi	IE_{50} Conidia/ml	IE_{90} Conidia/ml	Chi square
Beauveria bassiana	1.5 $x10^8$	2.1 $x10^{11}$	105.15
Metarhizium anisopliae	1.6 $x10^9$	5.08 $x10^{13}$	47.07

Table III. Synergistic effect of mixtures from fungi and essential oils against newly moulted 4th instar larvae of *Musca domestica*.

Mixtures/ Oils	Observed mortality Mean±SE	Expected mortality Mean±SE	Synergistic value Mean±SE
Beauveria bassiana			
Celery	94 ± 3.83	72.12 ± 6.24*	21.88 ± 9.6
Ginger	100 ± 0.0	69.04 ± 7.95*	30.96 ± 7.95
Sesame	100 ± 0.0	74.36 ± 8.74*	25.64 ± 8.74
Metarhizium anisopliae			
Celery	96 ± 4	77.08 ± 6.5*	18.92 ± 9.85
Ginger	96 ± 4	74.36 ± 8.06*	21.64 ± 11.06
Sesame	100 ± 0.0	79.76 ± 6ot.47*	20.24 ± 6.47
Beauveria bassiana* + *Metarhizium anisopliae			
	33 ± 5.7**	88.68±5.26*	-55.68±4.27**

*, In the same column means no statistical significance (P > 0.05); **, Significantly different from all other mixtures (P < 0.05) in the same column whilst no significant difference was found among mixtures of both fungi and oils (P > 0.05).

Data of blends from fungi and essential oils against 4th instar larvae revealed synergistic effect whilst antagonistic effect was produced when both fungi mixed together (Table III). The statistical analysis Tukey HSD post-hoc test indicated that synergism produced by fungal-

oil blends was significantly different from synergism produced by fungal blends (F=12.377; df=6; P < 0.05). Based on synergistic value (Table III), ginger oil come in the first order in synergistic action followed by sesame oil then celery oil and all oils exhibited better synergistic action when mixed with *B. bassiana* compared with *M. anisopliae*.

Results in Table IV indicated that the sublethal dose (4.5x104 conidia/ml) of both fungi influenced the development of *M. domestica* larvae. The influence of the sublethal dose of both *B. bassiana* and *M. anisopliae* on the larval mortality percentage of *M. domestica* was not statistically significant (F=3.838; df=2; P > 0.05). Contrarily, both fungi significantly influenced larval duration (F=7.929; df=2; P < 0.05), pupal mortality (F=13.972; df=2; P < 0.05), pupal duration (F=12.214; df=2; P < 0.05), average developmental period (F=11.870; df=2; P < 0.05), adult emergence (F=6.499; df=2; P < 0.05) and growth index (F=10.794; df=2; P < 0.05) compared to control.

Table IV. Effect of sublethal dose (4.5x10^4 conidia/ml) on the development of *Musca domestica* larvae.

	Control	*Beauveria bassiana*	*Metarhizium anisopliae*
Larval duration	4.25 ± 0.25^{ab}	5.25 ± 0.47^{a}	6 ± 0.0^{ab}
Larval mortality %	1^{a}	18 ± 3.46^{a}	12.75 ± 6.79^{a}
Pupal duration	6.25 ± 0.25^{ab}	4.25 ± 0.25^{b}	6 ± 0.40^{ab}
Pupal mortality %	3 ± 1.0^{ab}	8 ± 2.3^{ab}	55.75 ± 13.25^{b}
Average developmental period	10.5 ± 0.28^{a}	9.25 ± 0.47^{ab}	12 ± 0.40^{ab}
Adult emergence %	96 ± 1.6^{ab}	75 ± 3.4^{a}	55 ± 13.4^{ab}
Growth index	9.16^{ab} ± 0.3	8.05 ± 0.51^{ab}	4.51^{b} ± 1.1

^{a}Similar letters in the same row means no statistical significance (P > 0.05); ^{b}Significantly different (P < 0.05).

DISCUSSION

Present results revealed that the fungus *B. bassiana* is more potent larvicide (LC_{50} = 8.6 x10^8; LC_{90} = 8.9 x10^{12} conidia/ml) against *M. domestica* larvae than *M. anisopliae* (LC_{50} = 1.7 x10^9; LC_{90} = 6.9 x10^{16} conidia/ml). Present findings for *B. bassiana* and *M. anisopliae* isolated from Saudi Arabia are better than findings of Ibrahim *et al*. (2016) who found that the same fungi isolated from Egypt produced 7.5 and 1.25 larval mortality, respectively at a concentration of 10^{12}. These results indicate that the present Saudi fungal isolate is more potent and efficient than the Egyptian one. Contrarily to present findings, Mwamburi *et al*. (2010) stated that LC_{50} of *B. bassiana* isolates ranged between 10^3-10^5 conidia/ml and Mishra *et al*. (2011) indicated that *M. anisopliae* was more effective larvicide (LC_{50} = 4.1×10^8 conidia/ml) than *B. bassiana* (LC_{50} = 3.31×10^9 conidia/ml). Consequently, fungal strains may be responsible for differences in observed mortalities among house fly larvae (Weeks *et al*., 2017). Differences in findings among these studies could be due to adopting different methods (dipping or immersing and bait techniques) for evaluating capacity of these fungi against house fly larvae/adults (Weeks *et al*., 2017).

All mixtures produced significant larval mortalities compared with any of the agents acting alone but insignificant better synergistic action was produced by oils mixed with *B. bassiana* than *M. anisopliae*. Other similar investigations revealed the same synergistic action for blends of these fungi and some other botanical oils (Farooq and Freed, 2016; Ahmed *et al*., 2017). The high mortality percentage recorded after 24 h of utilizing mixtures could be due to the high synergism between these oils and fungi. Ahmad *et al*. (2017) reported that the synergistic action of these mixtures, entomopathogenic fungi with botanicals, could be exploited for integrated pest management (IPM) programs. In addition to their synergistic effect, it is advisable to use binary mixtures of fungal conidia with either mineral or botanical emulsified oils to avoid the deleterious effects of the environmental physical factors (Su *et al*., 2010; Mola and Afkari, 2012; Oliveira *et al*., 2018) because they protecting the conidia from such deleterious effects. In addition to their synergistic action, botanical oils can do other services for fungi. Out of seven vegetable oils, sesame showed highest effects on storage of *B. bassiana* conidia (Mola and Afkari, 2012). Corn oil was superior to sunflower and cotton seed oils in thermo tolerance of *B. bassiana* conidia (Su *et al*., 2010). Oliveira *et al*. (2018) mentioned that Oil-based formulations protect conidia from heat stress in water. They also protect entomopathogenic conidia from chemical fungicides and this advantage is being especially relevant for IPM programs whereas mycopesticides and chemicals are simultaneously sprayed (Lopes *et al*., 2011). Vegetable oils are also influencing the storage duration of entomopathogenic conidia (Mola and Afkari, 2012). Faria *et al*. (2009) reported that formulation of conidia of entomopathogenic fungi in pure (non-emulsifiable) paraffinic oil provided considerable protection from imbibitional damage.

From the present results, it is appeared that, both fungi particularly *M. anisopliae* have deleterious effects on the larvae when treated by sublethal dose nearly equal to half of the LC_{50}. Such deleterious effects included pupal mortality, the average developmental period, adult emergence and growth index. Unfortunately,

literatures revealed that studies in this aspect are very rare. Only one study mentioned the pupicdal activity of these fungi. *B. bassiana* and *M. anisopliae* caused pupal mortality percentages of 73.5 and 60.0 % at the highest concentration10^{12} Spores/ml (Ibrahim *et al*., 2016). Compared to the present study, present findings are better particularly *M. anisopliae* that produced pupal mortality percentages of 55.75 but at lower dose by approximately two thirds which again means the strong activity of the present strain of Saudi fungal isolates.

CONCLUSION

From these results it could be concluded that the fungus *M. anisopliae* is more efficient than *B. bassiana* and could be easily mixed with essential oils in order to either enhance their larvicidal activity or to be used in integrated pest management programs. Although these, a huge room for more research on many other disciplines are required including field evaluation and influence of environmental conditions on capacity of the entomopathogenic fungi either alone or in blends. Additionally, delivery mode in the field is another challenge. It needs more work to find convenient and more applicable mode that matches regulatory approval before commercialization.

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Statement of conflict of interest

The authors declare that they do not have conflict of interest.

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