Evaluating the virulence of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) and *Beauveria bassiana* (Ascomycota: Hypocreales) isolates to Arabian rhinoceros beetle, *Oryctes agamemnon arabicus*

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Abstract

Virulence of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* were tested against Arabian Rhinoceros Beetle, *Oryctes agamemnon arabicus* larvae. Four concentrations (1×10^5, 1×10^7, 1×10^9 and 1×10^{11} conidia/mL^{-1}) of two locally isolated entomopathogenic fungi spore suspensions were used in this study via larval direct spraying. Results revealed that both isolates can cause high mortality rate reaching 100% after 29 days. However, *Beauveria bassiana* scored higher mortality rate in short time especially at the concentration of 1×10^{11} conidia/ mL^{-1} with lethal time $(LT)_{50}$ 12.75 and LT_{90} 20.00; while, *Metarhizium anisopliae* caused the higher percentage of malformed adults. Moreover, both isolates affected insect's life cycle particularly in the pupal stage which was reduced remarkably by almost 50% in comparison with the control treatment.

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Key words: Biological control; date palm borer; Mycoinsecticide; *Metarhizium anisopliae*; *Beauveria bassiana*.

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Introduction

Date palms, *Phoenix dactylifera* L. are most economically important fruit trees in tropical and subtropical areas and are grown throughout large area spanning many countries including Iraq (Abdullah *et al*., 2010; Abul-Soad *et al*., 2011). One of the most important pests that infests date palm trees in Iraq and many other countries such as Iran, Saudi Arabia, Oman, Egypt, Tunisia, Libya and Qatar is palm tree borer that belongs to different species of genus *Oryctes* such as *O. agamemnon,* and *O. elegans* (Hussain, 1974; Dhiab *et al*., 1979; Bedford, 1980; F.A.O., 1982; Elwan & Al-Tameimi, 1999; Soltani, 2010). *Oryctes* species larvae can cause high yield losses, destroying trees and seedlings in oil palm, date palm, and coconut palm (Ragoussis *et al*., 2007; Al-Deeb *et al*., 2012). Borers and their larvae develop special breeding sites (tunnels) inside different plant parts such as root, stem, and crown making their control very difficult (Soltani, 2012). Rochat *et al.* (2004) mentioned that Arabian rhinoceros beetle causes direct and indirect damages exemplified by infesting young trees especially on apical bud providing eligible environment for secondary invaders. Controlling date palm borers by using insecticides is not efficient because borers spend long time hiding inside their feeding and breeding sites (Ragoussis *et al.*, 2007), making their control very difficult. Moreover, most of the employed insecticides are contaminant dangerous to both people and ecosystem.

The entomopathogenic fungi *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) and *Beauveria bassiana* (Ascomycota: Hypocreales) were used to control many arthropods pests such as beetles, aphids, plant hoppers, pear psylla, and termites (Milner & Soper, 1981; Liu *et al*., 1989; Samuels *et al*., 1989; Moorhouse *et al*., 1993; Puterka *et al*., 1994). They were tested against red palm weevil *Rhynchophorus ferrugineus* (Olivier) revealing high mortality rate among larvae reaching 100% after 6-7 days of treatment; moreover, treated eggs expressed lower hatching percentages (Gindin *et al*., 2006). *M. anisopliae* expressed high virulence against sugarbeet root maggot larvae as reported by Jonason *et al.* (2005). They were also applied to control brown-winged green bug, *Plautia stali* Scotto (Hemiptera: Pentatomidae) revealing efficient and successful outcome (Ihara *et al*., 2001).

In this investigation laboratory bioassays were conducted using four spore suspension concentrations (1×10^5, 1×10^7, 1×10^9, and 1×10^{11}) of the isolates *M. anisopliae* and *B. bassiana* that were isolated from Iraqi environment to evaluate their ability to control *O. agamemnon arabicus* larvae.

Materials and methods

Two locally isolated entomopathogenic fungi were used in this study: MARD 34 and 46 (Table 1); selected from Entomopathogenic

Fungal Isolates Bank at the Agricultural Research Directorate, Iraqi Ministry of Science and Technology.

Fungal isolation and purification

Entomopathogenic fungi *B. bassiana* (MARD46) was isolated using bait trap method (Zimmermann, 1986) with wax moth *Galleria mellonella* L. larvae. Soil samples were collected from 42 sites and from specific points according to global positioning system during two seasons of (2012 and 2013) and 31% of the collected samples expressed entomopathogenic fungi. Over 94 isolates were collected from the examined soil sources and sites. Results revealed that 75.5% of the isolated entomopathogenic fungi were *Beauveria bassiana* and 18.1% were *Metarhizium anisopliae* followed by *Lecanicillium lecanii* with 4.3%, *Paecilomyces lilacinus* and *Fusarium* sp. recorded the lowest frequency among other species with 1.1% as illustrated in Table 2 (Khudhair *et al.*, 2014). Soil samples were sieved to remove roots and big solid materials; then 40 g of soil was placed into plastic petri dish. Fourth instar *G. mellonella* larvae (5-6) were released in each petri dish followed by sealing them with parafilm and three replicates from each sample were used. Dishes were incubated in the dark at 25±2°C and 70% relative humidity (RH) for two weeks. Observations were taken every three days, and dead larvae were removed, washed with tap water and placed in a new sterile petri dish on a filter paper that wetted with sterile distilled water providing high level of humidity. Dishes were incubated in the dark to allow fungal mycelia to grow over dead larvae. *M. anisopliae* (MARD34) isolate was collected from infected date palm borer adult that revealed fungal growth. Fungal isolate was sub-cultured many times on full strength potato dextrose agar (PDA) medium. Strains were purified onto quarter-strength PDA plates which contain 100 μg streptomycin sulphate and 10 μg tetracycline hydrochloride mL^{-1}. Plates were incubated at room temperature and placed in the dark for 5 -7 days.

Single monoconidial strains selection was done via making local spore suspension by adding 3-4 drops of sterile distilled water on the fungal colony that were grown on the plate using flame-sterilised loop. Spore suspension was streaked onto 2% water agar media by using a flame-sterilised metal loop and plates were incubated under laboratory conditions for 24 h. A single germinated spore was transferred onto full-strength PDA media plate and incubated at ambient temperature according to Scott and Chakraborty (2010).

Recovered isolates were retested against *G. mellonella* larvae using 3 replicates each replicate contains 10 larvae with three spore suspension concentrations: 1×10^5, 1×10^7, and 1×10^9. The highest mortality rate was expressed by the two selected isolates in this study (MARD 34 and 46) reaching over 70% encouraged using them against *O. agamemnon arabicus*.

Borer culture

Borer larvae of *O. agamemnon arabicus* were collected from severely infested date palm trees from February to April 2014. Trees were cut into small pieces and early stages of larvae were collected from tunnels and kept in special plastic cages 30×20×22.5 cm. Larvae were reared at 25±2°C and 30% RH and fed on small pieces (5×10 cm) bunch stalks. Fresh stalk pieces were added regularly every two weeks to keep enough fresh food to the larvae. Larvae were kept until reaching the last larval instar for treatment, and then used for different treatments.

Bioassay test

Spore suspension was prepared by adding 5 mL of sterile distilled water to well developed colonies growth isolate Petri dish; then, by using a sterile metal scraper, fungal mycelia were scraped and the suspension was poured into a 50 mL Falcon tube after filtering the suspension through sterile miracloth (Billerica, MA, USA). The spore concentration was determined using haemocytometer and adjusted to 1×10^5, 1×10^7, 1×10^9 and 1×10^{11} conidia/ mL^{-1}. The four concentrations were applied separately by direct spraying on the larvae and their food. Three replicates (5 larvae each) were used for each treatment. Borer larvae in each replica were transferred into new sterilised cage 30×20×22.5 cm. The top of each cage was covered by cloth for ventilation and reducing humidity. Controls were sprayed with sterile distilled water only. Cages were kept under rearing room conditions 25±2°C and 70% RH and they were checked every three days to count dead larvae monitor their behaviour, and any noticeable morphological changes.

Statistical analysis

Probit analysis was used to determine the mean lethal time (LT_{50}). Mortality was transformed to arcsine transformation; then, data were subjected to analysis of variance using SPSS V. 20. Means were separated by using Duncan's multiple range tests.

Results

Survival percentages of date palm larvae after treating them with entomopathogenic fungal isolate MARD 46 spore suspensions revealed that the concentration 1×10^{11} inflict the highest mortality among larvae reaching 93.33 % after 19 days (LT_{50}=12.67 and LT_{90}=20.00, Figure 1), followed by the concentration 1×10^9 that recorded mortality of 66.66% at the same time (Figure 1). In addition, mortality reached 53.33% after 19 days using concentration of 1×10^7 and the lowest mortality was 40% at the concentration 1×10^5 after 19 days. All concentrations used decreased larval survivals with time progression reaching 0.0% at the end of the experiment (29 days) (Figure 1).

Figure 2 illustrates survival percentages of date palm borers' larvae during experiment duration (29 days) treated with different spore suspension concentrations of *M. anisopliae* (MARD 34). The results revealed that the highest mortality after 19 days was 66.66% at the concentration 1×10^{11} followed by the concentration 1×10^9 with 53.33% at the same time. The lowest mortality scored at the concentrations 1×10^5 and 1×10^7 reaching 46.66% after 19 days of the treatment. All concentration recorded decreased survival with time, reaching 0% at the end of the experiment (Figure 2).

Comparison between survival percentages results of different concentrations of the two used isolates MARD 34 and MARD 46 revealed that there were no significant differences (44.66 and 40%) between them at the lowest concentration. However, there was a significant dif-

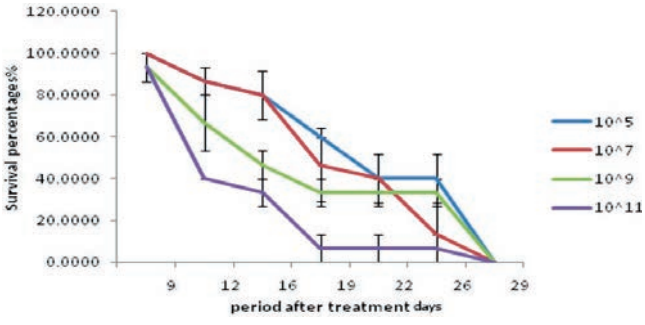

Figure 1. Survival percentages and error bar among date palm borer larvae *O. agamemnon arabicus* treated with the *B. bassiana* isolate MARD46 four spore suspension concentrations.

ference between the two isolates at the concentration 1×10^{11} especially the isolate MARD 46 which scored higher and faster survival reduction after 19 days in comparison with the isolate MARD 34.

Adults' malformations especially in the wings (number and degree) were recorded in almost all concentrations after 20 days of treatment (Table 3 and Figure 3). The highest malformed adults' number and degree of malformation were recorded at the concentration 1×10^{11} of the isolates MARD 34 especially after 27 days of treatment (Table 3); while, the lowest malformed adults' number and degree was recorded at the concentration 1×10^{11} of MARD 46 especially after 27 days of treatment.

Table 1. Isolates of *M. anisopliae* and *B. bassiana*.

Isolate code	Species	Location	Longitude	Latitude	Isolation source
MARD 34	*Metarhizium anisopliae*	Basrah Province	47° 77' East	30° 68' North	Infected adult of *O. agamemnon arabicus* Date palm and Citrus orchard
MARD 46	*Beauveria bassiana*	Dhi Qar Province Nasrea city	46° 48' East	31° 13' North	Date palm, orchard soil

Table 2. Sites of soil sources.

Province	Site	Locations	Longitude	Latitude	Soil source
Baghdad	Tarmia	3	44° 25' E	33° 64' N	Date palm
	Youssoufia	3	44° 43' E	33° 14' N	Date palm
	Kweresh	1	44° 42' E	33° 14' N	Date palm
	Khamisah	2	44° 39' E	33° 11' N	Date palm + Citrus
	Mahmoudia	3	44° 39' E	33° 11' N	Date palm + Citrus
	Al-Mada'in	3	44° 56' E	33° 15' N	Date palm + Citrus
	Sakhrijah	1	44° 39' E	33° 09' N	Date palm+ Citrus
	Abu Ghraib	3	44° 30' E	33° 17' N	Trefoil fields
	Tuwaitha	1	44° 30' E	33° 12' N	Sesame fields
Wasit	Suwayrah	3	44° 49' E	33° 23' N	Date palm + Citrus
	Aziziya	4	45° 06' E	32° 90' N	Date palm + Citrus
	Al-Numaniyah	4	45° 24' E	32° 35' N	Date palm + Citrus
	Al-Hafriyah	1	44° 84' E	32° 99' N	Date palm + Citrus
	Kut	3	45° 49' E	32° 30' N	Date palm + Citrus
	Dabuni	1	45° 21' E	32° 21' N	Wheat field
	Shethaif-Al Garbie	3	45° 11' E	32° 87' N	Date palm
	Brinaga	3	45° 05' E	32° 89' N	Date palm + Citrus
	Al Zubaidiya	3	45° 40' E	32° 31' N	Date palm + Citrus
Diyala	Diyala	2	44° 42' E	33° 63' N	Date palm + Citrus
	Al Khalis	1	44° 56' E	33° 93' N	Date palm + Citrus
Salah AL-din	Dujail	3	44° 27' E	33° 81' N	Grape orchards
	Balad	2	44° 43' E	34° 59' N	Grape orchards
Babil	Al-Mahawil	3	44° 39' E	32° 84' N	Date palm
Karbala	Hindiya	3	44° 19' E	32° 58' N	Date palm
	Hussainia	1	44° 06' E	32° 63' N	Date palm + Citrus
Amarah	Amarah	4	47° 14' E	31° 86' N	Date palm
	Ali Algharbie	3	46° 69' E	31° 26' N	Date palm
	Kumayt	1	46° 94' E	32° 10' N	Wheat field
Dhi Qar	Nasrea-AL-jueber	4	46° 40' E	30° 59' N	Date palm
	Syd Dkhyl	3	46° 48' E	31° 13' N	Date palm
	Nasrea-AL- Mbader	2	46° 44' E	31° 15' N	Date palm
Al-Qadisiyyah	Al Diwaniyah	1	44° 55' E	31° 59' N	Datepalm + Citrus
Al-Muthanna	Samawah	3	45° 17' E	31° 19' N	Date palm + Citrus
Al-Basrah	Al-Qurnah	3	47° 47' E	30° 95' N	Date palm
	Al-Haritah	2	47° 74' E	30° 67' N	Date palm
	Shatt al-Arab	4	47° 77' E	30° 44' N	Date palm + Citrus
	AL-Hota	1	47° 78' E	30° 63' N	Date palm + Citrus
	Abu Al-Khaseeb	5	47° 88' E	30° 47' N	Date palm + Citrus
	Nahar Khoz	1	47° 93' E	30° 46' N	Date palm + Citrus
Dohuk	Dohuk	5	43° 00' E	36° 52' N	Potato fields
Sulaymaniyah	Sulaymaniyah	5	45° 26' E	35° 33' N	Potato fields
Erbil	Erbil	3	44° 18' E	35° 70' N	Hills

Malformation features were determined according to wings normality, length, shape, colour, and in some cases the presence of wings; for instance, some adults were wingless and dead short time after emergence.

It can be seen from Table 4 that there were remarkable changes in the insect normal life cycle exemplified by reducing the duration of pupal stage that last from 9-12 days, in comparison with the control treatment which last 23-26 days. Most of the larvae transformed in pupae after 16 days from treatment at the isolate MARD 34; while, larvae from the isolate MARD 46 treatment turned to pupae after 19 days. Considerably, high number of the larvae and pupae were dead before reaching adult stage as illustrated in Table 4 through the reduction of survival percentage.

Discussion and conclusions

The present study emphasised that there was a significant effect of applying the two locally isolated entomopathogenic fungi *M. anisopliae* and *B. bassiana* recording a high level of reduction in survival percentage reaching 0.0% after 29 days of treatment. However, MARD 46 isolate (*B. bassiana*) inflected faster and higher reduction of longevity level among *O. agamemnon arabicus* larvae in comparison with the isolate MARD 34 (*M. anisopliae*). Ricaño *et al.* (2013) found that using more than one formula of *B. bassiana* can remarkably reduce survival and increase mortality rate among Red palm weevil larvae and adults. *B. bassiana* can increase mortality rate of *Helicoverpa armigera* larvae as well as mentioned by Agarwal (2012).

Most of the emerged adults from both isolate spore suspensions treatments revealed various degrees of severity of noticeable malformations particularly in wings. Nonetheless, higher number of malformed adults emerged after treating with MARD 34 isolate. This might be due to the shortening of some stage period *O. agamemnon arabicus* life cycle; for example the pupal stage lasts from 9-12 days in comparison with the control that last from 24-27 days. Hussein *et al*. (2013) demonstrated that treating *Spodoptera littoralis* (Boisd.) larvae with the entomopathogenic fungi *Isaria fumosorosea* (syn. *Paecilomyces fumosoroseus*) resulted in high number of malformed adults. Moreover, Sabbour and Abdel-Raheem (2014) found that exposing red palm wee-

Table 3. Malformed adults of *O. agamemnon arabicus* produced after treatment with the isolates MARD 46 and MARD34.

Days after treatment	Isolate MARD 34 Concentration			
	1×10^5	1×10^7	1×10^9	1×10^{11}
20 Days	-	-	+	+
23 Days	+	++	++	++
27 Days	++	+++	+++	++++
Isolate MARD 46				
20 Days	-	-	-	+
23 Days	+	-	++	++
27 Days	++	+	+++	-

+, low number and degree of malformed adults (1); ++, moderate (2-3); +++, high (4-6); ++++, the highest (6+); -, zero No.

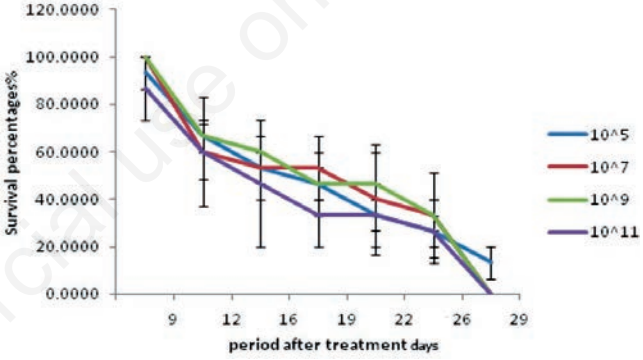

Figure 2. Survival percentages and error bar among date palm borer larvae *O. agamemnon arabicus* treated with the *M. anisopliae* isolate MARD34 four spore suspension concentrations.

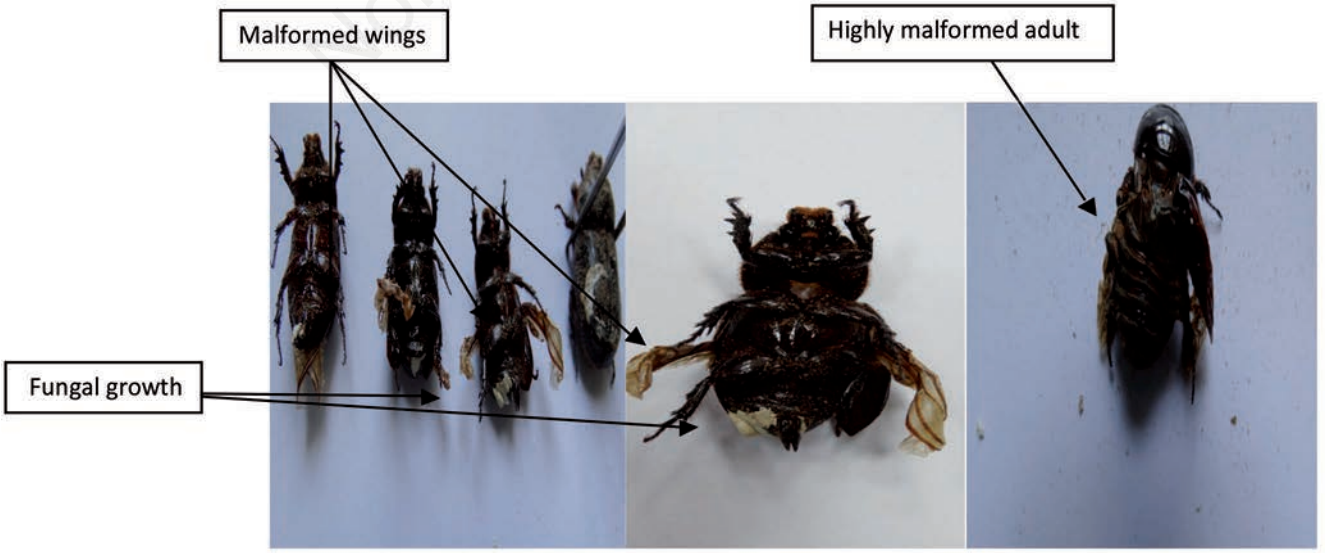

Figure 3. Malformed adults from both isolates MARD 34 and MARD 46.

Table 4. Changes in date palm borers' *O. agamemnon arabicus* life cycle after treating them with MARD 34 and 46 spore suspensions concentration.

Spore suspension Concentrations	MARD 46 isolate Percentage of date palm borer stages after treatment				
	16 Days	19 Days	22 Days	26 Days	29 Days
1×10^{5}	100% Larvae	46.6% Pupa	40% Pupa	26.3% Adults + 13.3% Pupa	100% Dead
1×10^{7}	100% Larvae	26.6% Pupa	40% Pupa	13.3% Adults	93.3% Dead
1×10^{9}	100% Larvae	33.3% Pupa	33.3% Pupa	33.33% Adults	100% Dead
1×10^{11}	6.6% Pupa	13.3% Pupa	6.6% Pupa	6.6% Pupa	100% Dead
Control	100% Larvae	100% Larvae	40.6% Pupa	86.6% Pupa	100% Pupa
MARD 34 isolate					
1×10^{5}	40% Pupa	33.3% Pupa	26.6% Pupa	13.3% Pupa+ 6.6% Adults	13.3% Adults + 6.6% Dead adults
1×10^{7}	60% Pupa	46.6% Pupa	33.3% Pupa	26.6% Adults	100% Dead adults
1×10^{9}	46.6% Pupa	46.6% Pupa	46.6% Pupa	26.6% Adults + 6.6% Pupa	100% Dead adults
1×10^{11}	33.3% Pupa	33.3% Pupa	33.3% Pupa	33.3 Adults	100% Dead
Control	100% Larvae	100% Larvae	40.6% Pupa	86.6% Pupa	100% Pupa

vil *Rhynchophorus ferrugineus* to *Isaria fumosorosea* isolates can cause high level of malformation among pupae.

Other recorded results were the significant change in date palm borer life cycle exemplified by the reduction of pupal and larval period in comparison with the control. Rahman *et al*. (2010) reported that exposing *Ostrinia nubilalis* to the combination of *B. bassiana* and *Nosema pyrausta* resulted in shorter lifecycle, and females oviposited fewer eggs in comparison with the non-exposed insects. Gindin *et al*. (2006) stated that treating red palm weevil *Rhynchophorus ferrugineus* with different formulations of *M. anisopliae* and *B. bassiana* can make oviposition period shorter and decrease females fertility.

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