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Study the pathogenecity of fungus *Beauveria bassiana* Balsamo, *Beauveria brongniartii* Saccardo and *Metarhizium anisopliae* Metsch on date horned beetle *Oryctes elegans* Prell larvae based on different bioassay methods

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Abstract: The date horn beetle *Oryctes elegans* Prell is one of the most important palm pest in many parts of the world and Iran. The aim of this study was to compare different bioassay methods for assess the pathogenecity of fungal *B. bassiana*, *B. brongniartii* and *M. anisopliae* on beetle larvae of the date horn beetle *O. elegans*. Five logarithmic doses including 5×10^7, 10^8, 5×10^8, 10^9, 5×10^9 spores/ml for immersion method, 10^8, 5×10^8, 10^9, 5×10^9 and 10^{10} spores/ml for oral method and 10^6, 5×10^6, 10^7, 5×10^7, 10^8 and 5×10^8 spores/ml for injection method had been used respectively. The results showed that *M. anisopliae* and *B. bassiana* with LC_{50} equal to 5.69×10^8 and 1.53×10^9 spores/ml on immersion method, *M. anisopliae* and *B. bassiana* with LD_{50} of 8.17×10^8 and 2.12×10^9 spores/ml and M. *anisopliae* and *B. bassiana*, with LC_{50} equal to 1.07×10^6 and 4.9×10^7 spores/ml had the highest and the lowest mortality rate based on immersion, oral and injection method respectively. The lowest LT_{50} occurred in concentration of 5×10^9, 5×10^8 and 10^{10} spores/ml of *M. anisopliae* equivalent to 3.61, 6.41 and 3.24 days based on immersion, oral and injection method respectively.
Keywords: *Oryctes elegans*, Pathogenic fungi, Bioassay methods

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is one of the most important fruit trees of Iran. This tree is attacked by several pests [2, 3, 18, 30]. The horned beetle (*Oryctes elegans* Prell) is a major pest of date palm planting in many parts of the world and Iran [10, 13]. Species of the genus *Oryctes* are attacked in natural conditions by various biological agents. Various entomopathogenic fungus including *Cordyceps* sp, *Metarhizium anisopliae* Metsch, *Beauveria bassiana* Balsamo, *Beauveria tenella* Sacc, *Beauveria brongniartii* Saccardo and *Plaecilomyces fumosoroseus* Apopka and *Spicaria rileyi* Farlow have been reported for genus *Oryctes* [23, 28].

Different Bioassays methods have been used to evaluate the effects of entomopathogenic fungi in the literatures. So, the pathogenicity evaluating should be designed based on fungi species, insect host species and objectives [7]. Entomopathogenic fungi bioassays were conducted to determine virulence of different strains, host range of the species, the ability of epizootic potential, effects of biotic and abiotic factors such as the age of the insect host, host plant, temperature, humidity and formulation [7].

The infection methods are depending on primarily inoculum type, size and the host insect body texture. The inoculation of insect diseases generally are performed directly such as dipping, spraying or indirectly such as the use of contaminated inoculum bait. Bioassay methods of Entomophtorales and Hyphomycetes fungi (such as *Beauveria* and *Metarhizium*) are different. Entomophtorales and Hyphomycetes are produced few sticky small projectile spores and lots of dry spores respectively. The insect infestations methods are included involve placing entomopathogen spores on anaesthetized insect body or their food source as leaf area. The entomopathogenic inoculum can be collected directly from infected insects, culture medium or dry mycelium and transferred to healthy insects [22].

Spray solution is widely used to infecting small insects with soft body tissue. Examples of this method application are honeybee infected by *Metarhizium flavoviride* Gams and Rozsypal, whitefly *Aschersonia aleyrodis* Webber and European corn borer *Ostrinia nubilalis* Hübner by *B. bassiana* [4, 11,12].

The insects are inserted in specific concentration of spore suspension at any defined period in immersion method. This method has been used in experiments *Verticillium lecanii* Zimmerman on aphids [16].

The insects spray method was used also for inoculation. Otherwise healthy insects walked on the surface of the spores mediums. This bioassay method was implicated for treatment *Blissus leucopterus* Say by *B. bassiana* and Japanese beetle *Popillia japonica* Newman by *B. bassiana* and *M. flavoviride* [19, 24].

The certain volume of inoculum is placed directly on the body surface of insect host in placing method of the inoculum. This method can be used for big size insects that holding them on hand does not injury them. This method of inoculation was used for treatment of termites *Copotermes formosanus* Shirak by *B. bassiana* [20].

The inoculum infected soil surface can be used for the insects that associated with soil in all or any life stages. This method was applicate for comparing the virulence of *B. bassiana* strains on walnut weevil *Curculio caryae* Horn [7].

The insect bioassay was sometimes conducted by walking on infected surfaces such as filter paper that it was infected by certain concentration of entomopathogenic fungus spores to acquire inoculum. This method of inoculating was used to treatment aphids *Myzus persicae* Sulzer and *Lipaphis erysimi* Kaltenbach by the fungus *M. anisopliae* [5].

The entomopathogenic inoculum can be directly added to insect's foods. This method used of digestive system infected fungi such as *Ascosphaera aggregate* Skou, but it can also be used for entomopathogenic fungi penetration from the external skin. Because mouthparts and insect body had been infected by fungal spores during feeding. This method was used to accessing the pathogenic effects of fungus *B. bassiana* on grasshoppers *Melanoplus sanguinipes* Fabricius the [17].

Most Entomophtorales species produced throwing spores. These spores are often short-lived and they weren't collectable and countable before application of them. The bioassay results in the fungus; the host directly using the culture medium is inoculated with spores or infected insect body. This bioassay method was used for infecting cicada *Empoasca fabae* Harris by fungi *Zoophtora radicans* Brefeld [29]. The special bioassay methods are designed for studying the aquatic insect pathogenic fungi such as genus *Lagenidiales* by inoculated water as insect habitat [6].

The aim of this study was to compare different bioassay methods for assessing the pathogenicity of entomopathogenic fungi *B. bassiana*, *B. brongniartii* and *M. anisopliae* on the date horned beetle *O. elegans* larvae.

MATERIALS AND METHODS

Insects rearing

Rearing of the *O. elegans* was continued in the captivity at the entomology laboratory of Date palm and tropical fruits research institute. The main culture of *O. elegans* was reared on fresh apical meristem tissue of Date palm. The culture started with specimens collected from highly infested palm tree farms at around Abadan city, Khuzestan province on the southwest of Iran. Restriction measures and care was performed during transportation and experimental work to prevent insects escaping. *O. elegans* stages were kept in pots (7 cm diameter and 10 cm height) having circular holes in the lid for ventilation. Cocoons were kept separately until emergence. Newly emerging adults were sexed, and then each couple of virgin females and males were kept separately in glass jars each containing 400gr apical meristem tissue of date palm for oviposition. The culture was maintained at 25±5ºC and 75±5 % RH. The laid eggs were collected daily by cutting Meristem tissue into small pieces, peeling and shredding with the aid of razor blade. Eggs were maintained under similar condition in Petri-dishes of 9-cm diameter with wet filter paper, until they hatched. Then they were provided with fresh Meristm tissue of date palm when transferring the larvae to pots.

Entomopathogenic fungi rearing

Three isolates of enomopathogenic fungi were obtained from Plant Protection research institute of Iran. The virulence of three isolates of *B. bassiana*, *B. brongniartii* and *M. anisopliae* to *O. elegans* adult was tested in the first screening. The fungal isolates used in this study (listed in Table 1) were obtained from soil or from *R. ferrugineus* larvae in Systan and Balochestan province, a hot climates province of Iran. Fungi were grown for 2 weeks at 25±2ºC on Sabouraud dextrose agar (Difco) under natural light. Conidia were harvested by surface scraping 14-day-old culture plates. Subsequently the spore suspension was filtered through several layers of cheesecloth to remove mycelium. Inoculums were suspended in 10-mL sterile distilled water containing 0.05% Tween 80 in universal bottles containing 3-mm glass beads. Conidial suspensions were vortexes for 5 min to produce a homogeneous suspension. Spore concentrations were quantified with a bright line haem cytometer. Serial dilutions were prepared to obtain the desired concentrations. Viability of conidia was determined before each bioassay by spread plating 0.1 mL of conidial suspension titrated at 5.0×10^6 conidia mL^{-1} on SDA plates. Sterile microscope cover slips were placed on each plate and plates were incubated at 26±2ºC and examined after 15–18 h. Percentage germination was determined from 100 spore counts at ×40 magnification. Each plate was replicated four times. Spore concentration was determined with a hemocytometer [13]. All the fungal isolates used in this study were obtained from the plant protection research institute of Iran (Table 1).

Table 1. Specifications of fungal species used against *O. elegans*

Species	Isolate	Origin of isolate	Locality/Country	%Germinations
Beauveria bassiana	IRAN 441C	*Rhynchophorus ferrugineus*	Saravan, Iran	92.7±1.4
Beauveria brongniartii	DEB1 013	Soil	Varamin, Iran	95.2±1.6
Metarhizium anisopliae	DEMID 01	*Rhynchophorus ferrugineus*	Saravan, Iran	98.6±2.1

Immersion Inoculation

Five logarithmic concentrations of each isolates include 5×10^7, 10^8, 5×10^8, 10^9 and 5×10^9 spores/ml had prepared to infect larvae after primary experiments were done and minimum and maximum concentrations were set for bioassays. 20 larvae were used for each replication. Tests were done in 6 treatments (different concentrations and control) and 4 replications. The larvae have been immersed in spore suspensions to infect them for 20 seconds. Then they have been cared the incubator on temperature 25± 1 °C and a relative humidity of 85 ±5% for two days after they had been leaved from spore. Similar treatment concentrations suspensions were separately prepared for each larva for ensure that all insects have been infected by similar concentration. Infected larvae have been cared on environment condition including 40±5% relative humidity, temperature 25±1°C and photoperiod (12D: 12L) for 14 days. Larvae were take cared in special cages that 400 g apex meristem tissues of Date palm were placed in them for feeding. The patient larvae were evaluated daily. Died larvae were collected and their body surface were sterilized by 2.5% sodium hypochlorite solution. Then the larvae have been placed in a moist desiccator until the fungus appeared on their bodies. Larvae mortality were recorded daily and cumulative mortality tables were prepared.

Oral inoculation

Five logarithmic concentrations of each isolates include, 10^8, 5×10^8, 10^9 5×10^9 and 10^7 spores/ml had prepared to infect larvae after primary experiments were done and minimum and maximum concentrations were set for bioassays. The Statistics and environmental experiment conditions were similar to previous experiments. In this method fungal spores were directly placed in into the end of digestive system foregut of the larval. For this purpose, one microliter of the spore suspension of the fungus pathogen isolates was placed in a larvae buccal cavity. The larvae put back and then the pipette was placed between the mandibles under the labrum for forced feeding (Fig. 1). This bioassay method was used for evaluating pathogenicity of bacterium Bacillus papillae on Japanese root beetle. Mortality of larvae has been recorded daily and cumulative mortality table has been prepared for 14 days.

Injection inoculation

Five logarithmic concentrations of each isolates include10^6, 5×10^6, 10^7, 5×10^7, 10^8 and 5×10^8 spores/ml had prepared to infect larvae after primary experiments were done and minimum and maximum concentrations were set for bioassays. The Statistics and environmental experiment conditions were similar to previous experiments**.** One microliter of the suspension was injected into larvae by using Hamilton syringe. At first the injection site (membrane between the second and third abdominal segment) was sterilized by using 75% alcohol. Then the needle were inserted homocell parallel to the outer surface of the larvae the body, so that it didn't injury to the internal organs of the larvae (fig 1). One microliter of carrying substance (water and Tween 60 (0.03 per thousand)) was injected into insects of control group [17, 18]. Larvae mortality were recorded daily and cumulative mortality tables were prepared for 14 days [8].

Statistical analysis

Percentage mortality data were arcsine transformed to normalize the data [14] after correcting for natural mortality [1]; angular values were then subjected to analysis of variance using the ANOVA procedure of SAS [25]. Lethal time and lethal concentration to 50% mortality (LT_{50} and LC_{50}) and LC_{90} mortality were estimated with repeated measures logistic regression using generalized estimating equations [27]. All analyses were carried out using GENMOD procedure of SAS [26].

Immersion Inoculation **Oral inoculation** **Injection inoculation**

Fig 1-Entomopathogenic fungi infection on the *O. elegans* larvae of by ingection, oral and immersion methods

RESULTS

In viability tests, germination of conidia ranged from 92.7% to 98.6% after 15–18 h (Table 1). The results showed that infect and cause disease in insect larvae studied by means of immersion, injection and oral is possible. All three strains of entomopathogenic fungi can cause disease on horned beetle (*O. elegans*) larvae. The muscardine symptoms including brown to black spots were found on the cuticle of larval body. The external signs of disease appeared on surface of cuticle larval 2 to 3 days after the larvae mortality. The body color of infected larvae by *B. bassiana*, *B. brongniartii* and *M. anisopliae* would change to yellowish pink, amber and light green respectively (fig 2).

B. bassiana ***B. brongniartii*** ***M. anisopliae***

Fig 2. The external signs of infected larvae after mortality

Entomopathogenic fungi lethal ability Based on immersion inoculation

The fungus *M. anisopliae* and *B. bassiana*, with LC_{50} equal to 5.69×10^{8} and 1.53×10^{9} spores/ml had the highest and the lowest mortality rate based on immersion method respectively (Table 2).

The 50 percent mortality time (LT_{50}) of *B. bassiana*, *B. brongniartii* and *M. anisopliae* isolates based on immersion inoculation are shown in table 3. LT50 values for the different concentrations of *M. anisopliae* varied from 3.61 to 7.54 days, for *B. brongniartii* from 4.06 to 10 days and *B. bassiana* from 5.09 to 10.13 days. The lowest LT50 occurred in concentration of 5×10^{9} spores/ml of *M. anisopliae* equivalent to 3.61 days and the highest on concentration of 5×10^{8} spores/ml of *B. bassiana* equivalent to 10.13 days

Entomopathogenic fungi lethal ability Based on oral inoculation

The fungus *M. anisopliae* and *B. bassiana* with LD_{50} of 8.17×10^{8} and 2.12×10^{9} spores/ml had the highest and the lowest mortality rate based on feeding method respectively (Table 4).

Table 2: Lethal concentrations values of fungus *B. bassiana, B. brongniartii* and *M. anisopliae* on the larvae of *O. elegans* by immersion method.

Fungi	LC_{50} (95% fiducially limits)	LC_{90}(95% fiducially limits)	(SE) ± Slope	(X^2)
B. bassiana	$1.53\times10^{9}(1.39\text{-}6.57)\times 10^{9}$	$6.19\times10^{11}(3.22\text{-}9.27)\times 10^{11}$	1.47 ±0.18	0.11
B.brongniartii	$9.87\times10^{8}(4.37\text{-}11.1)\times 10^{8}$	$3.16\times10^{11}(2.09\text{-}4.73)\times 10^{11}$	1.41 ±0.18	0.24
M.anisopliae	$5.69\times10^{8}(3.21\text{-}8.57)\times 10^{8}$	$2.50\times10^{11}(1.63\text{-}3.74)\times 10^{11}$	1.69 ±0.16	0.46

The 50 percent mortality time (LT_{50}) of *B. bassiana*, *B. brongniartii* and *M. anisopliae* isolates based on oral inoculation are shown in table 5. LT_{50} values for the different concentrations of *M. anisopliae* varied from 6.41 to 13.34 days, for *B. brongniartii* from 7.96 to 15.88 days and *B. bassiana* from 12.11 to 14.09 days. The lowest LT_{50} occurred in concentration of 10^{10} spores/ml of *M. anisopliae* equivalent to 6.41 days and the highest on concentration of 10^{9} spores/ml of *B. brongniartii* equivalent to 3.61 days Table5).

Table 3: 50 percent lethal time values of fungus *B. bassiana, B. brongniartii* and *M. anisopliae* on the larvae of *O. elegans* by immersion method.

Fungus	Concentrations(conidia/ml)	(95% fiducially limits) LT_{50}	(SE) ± Slope	(X^2)
B. bassiana	5×10^8	10.13 (9.06 – 11.6)	3.3 ± 0.14	1.8
	10^9	9.18 (8.11 – 10.61)	3.7 ±0.12	7.6
	5×10^9	5.09(4.56 – 5.65)	3.9 ± 0.11	3.6
B.brongniartii	5×10^8	10.0 (8.97 – 11.38)	3.4 ± 0.13	1.3
	10^9	9.1 (8.22 – 10.3)	3.5 ±0.12	8.1
	5×10^9	4.06(4.04 – 5.11)	4.1 ± 0.11	2.9
M.anisopliae	5×10^8	7.54 (6.87 – 8.32)	3.5 ± 0.13	4.04
	10^9	7.31 (6.61 – 8.63)	3.7 ±0.11	5.2
	5×10^9	3.61(3.18 – 4.02)	4.3 ± 0.12	3.7

Table-4: Lethal concentrations values of fungus *B. bassiana, B. brongniartii* and *M. anisopliae* on the larvae of *O. elegans* by oral method.

Fungi	LD_{50} (95% fiducially limits)	LD_{90}(95% fiducially limits)	SE) ± Slope(	) X^2 (
B. bassiana	2.12×10^9(1.93 - 2.28) $\times10^9$	2.34×10^{11}(2.17 - 2.45) $\times10^{11}$	3.2 ±0.14	4.96
B.brongniartii	1.05×10^8(1.01 - 1.71)$\times$ 10^9	1.78×10^{11}(1.16 - 1.91) $\times10^{11}$	0.96 ±0.16	1.09
M.anisopliae	8.17×10^8(6.54 - 9.32)$\times$ 10^8	5.16×10^{10}(4.92 - 7.73) $\times10^{10}$	1.82 ±0.43	0.78

Table 5: 50 percent lethal time values of fungus *B. bassiana, B. brongniartii* and *M. anisopliae* on the larvae of *O. elegans* by oral method.

Fungus	Concentrations(conidia/ml)	(95% fiducially limits) LT_{50}	SE) ± Slope(	)X^2(
B. bassiana	5×10^9	14.09 (13.11 – 15.46)	1.31 ± 0.4	6.54
	10^{10}	12.11 (11.79 – 14.95)	1.92 ±0.18	5.76
B. brongniartii	10^9	15.88 (14.91 – 16.24)	1.91 ± 0.18	5.44
	5×10^9	13.78 (13.06 – 14.78)	2.07 ± 0.17	4.62
	10^{10}	7.96 (7.31 – 9.21)	1.96 ±0.16	3.91
M. anisopliae	10^8	13.34(12.95 – 13.86)	2.4 ± 0.28	4.96
	5×10^9	11.91 (10.37 – 12.01)	2.08 ± 0.17	6.05
	10^{10}	6.41 (5.93 – 6.89)	2.07 ±0.16	2.71

Entomopathogenic fungi lethal ability Based on injection inoculation

The fungus *M. anisopliae* and *B. bassiana*, with LC_{50} equal to 1.07×10^6 and 4.9×10^7 spores/ml had the highest and the lowest mortality rate based on injection method respectively (Table 6).

The 50 percent mortality time (LT_{50}) of *B. bassiana*, *B. brongniartii* and *M. anisopliae* isolates based on injection inoculation are shown in table 7. LT_{50} values for the different concentrations of *M. anisopliae* varied from 4.02 to 4.69 days, for *B. brongniartii* from 4.38 to 7.08 days and *B. bassiana* from 4.89 to 9 days. The lowest LT_{50} occurred in concentration of 5×10^8 spores/ml of *M. anisopliae* equivalent to 3.24 days and the highest on concentration of 5×10^7 spores/ml of *B. bassiana* equivalent to 9 days.

Table-6- Lethal concentrations values of fungus *B. bassiana, B. brongniartii* and *M. anisopliae* on the larvae of *O. elegans* by injection method.

Fungi	LD50 (95% fiducially limits)	LD90(95% fiducially limits)	SE) ± Slope(	)X2 (
B. bassiana	4.9×10^7(3.5 - 5.4)$\times$ 10^7	8.45×10^9(1.49-10.81)$\times$ 10^9	2.68 ± 0.61	0.17
B.brongniartii	3.59×10^7(2.47 - 4.21)$\times$ 10^7	7.41×10^9(1.36 - 9.21)$\times$ 10^9	3.53 ±0.16	0.19
M.anisopliae	1.07×10^6(0.91 - 2.11)$\times$ 10^6	4.28×10^8(1.36 - 6.42)$\times$ 10^8	2.68 ±0.17	0.18

Table 7: 50 percent lethal time values of fungus *B. bassiana, B. brongniartii* and *M. anisopliae* on the larvae of *O. elegans* by injection method.

Fungus	Concentrations(conidia/ml)	(95% fiducially limits) LT_{50}	SE) ± Slope(	)X^2(
B. bassiana	5×10^7	9.0 (8.15-9.46)	0.32± 4.2	8.6
	10^8	5.25 (4.69-5.83)	3.9 ±0.24	7.8
	5×10^8	4.89 (4.34- 5.44)	3.96 ±0.22	7.06
B.brongniartii	5×10^7	7.08 (6.36 – 7.92)	3.76 ±0.24	2.75
	10^8	5.15 (4.58 – 5.73)	3.94 0.25	0.99
	5×10^8	4.38 (3.87 – 4.89)	4.03 ±0.25	3.09
M.anisopliae	5×10^7	4.69 (4.24 -5.14)	3.77 ±0.21	2.09
	10^8	4.02 (3.61 – 4.43)	3.89 ±0.23	3.01
	5×10^8	3.24 (2.58 – 3.86)	3.76 ±0.40	2.1

DISCUSSION

Comparison of different inoculation methods show that the intensity and speed date horned beetle larvae mortality was higher for studied fungi in injection method. This indicates that the low virulence of some fungi strains affected by lack of their penetrations ability to the cuticles of insect because there are certain factors in insect exoskeleton that excludes pathogen into homocell. The electrical charges of the cuticle surface reduce the connection of fungal pathogen enzymes that solving the cuticles. This issue affects the penetration speed and virulence of different fungus. Endoprotease enzymes are the most important facilitating factors for penetration entomopathoenic fungi into the cuticle have been separate in different species such as *B. bassiana* [9]. These enzymes differ in various species and strains just in electrical charge of them. According to studies cuticle soft and hard parts of surface cuticle have negative and positive electric charged respectively. So the relationship between electrical charged produced by isolate enzyme and different parts of the cuticle determine of the success of the fungus penetration to insect body [16].

Among the inoculation methods, oral method had less mortality effects. Because of existence of the antifungal substances in the insect gut. Nevertheless contact between the insect legs and mouthparts with infected bait during feeding can be obtained sufficient spore concentration for virulence. Polyvinyl pirolidon material (PVP) can absorb the antifungal phenols available in the insect gut. So the formulating fungal spores by PVP reduce antifungal effect and increasing the severity of diseases from the gastrointestinal [9].

The Pathogenicity of entomopathogenic fungi *Beauveria bassiana*, *Beauveria brongniartii* and *Metarhizium anisopliae* to adult *O. elegans* was tested in the laboratory. All the isolates tested were pathogenic to *O. elegans*. *M. anisopliae* isolate DEMID 01 had the lowest LC_{50} value of 5.69×10^8 conidia mL^{-1} and *B. bassiana* had the highest LC_{50} value of 1.53×10^9 conidia mL^{-1}. The LT_{50} values ranged from 6.05 to 10.89 days with *M. anisopliae* concentrations, from 6.45 to 10.12 days with *B. brongniartii* concentrations and from 7.21 to 11.62 days with *B. bassiana* concentrations [21].

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