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Preliminary study on virulence of some isolates of entomopathogenic fungi in different developmental stages of *Boophilus annulatus* in Iran

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Abstract: **To evaluate the virulance of 11 isolates of native entomopathogenic fungi as biocontrol agent of *Boophilus annulatus,* in this study, 4 three months old calves were used for tick rearing. Different developmental stages of the ticks, *Boophilus annulatus* were inoculated by 10^7 conidia/ml dilution of the fungal isolates in the presence of control groups. The mortality, egg hatchability and reproductive efficiency were determined in different treatments and control groups and the results were analized statisticaly. *Metarhizium anisopliae* strains DEMI001 and IRAN437C, *Beauveria bassiana* strain IRAN403C, and *Lecanicillium psalliotae* strain IRAN468C were the most virulent strains in comparison with their relative strains and caused 80 -100%, 20 - 80%, 0 - 40% and 0- 40% mortality for engorged females respectively. All 11 tested fungi reduced egg laying capability of the ticks several days before their death. The obtained data showed that the entomopathogenic fungi can affect all developmental stages of *Boophilus annulatus*, but their efficiency varies considerably according to the fungal species and strains. It is demonstrated for the first time the pathogenic effect of *Lecanicillium psalliotae* against *Boophilus annulatus*.**

Key words: *Boophilus annulatus*, entomopathogenic fungi, biological control.

Introduction

Boophilus annulatus is a one-host tick, known to feed on cattle around the Mediterranean Sea, Asia Minor and Central America (Onofre *et al*., 2001). It is one of the most important bovine tick species in northern part of Iran. It plays an important role as a vector of Babesiosis, an important disease of cattle. Tick control throughout the world is based mainly on the repeated use of chemical acaricides. Indiscriminate use of these tick controlling chemicals has resulted in problems related to environmental pollution, leaving residue in meat and milk, and the resistance development in the target species (Onofre *et al*., 2001).

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In the nature, predators, parasitoids, parasites and pathogens are known to influence tick populations (Hu *et al*., 1998; Samish and Rehacek, 1999). Entomopathogenic fungi are known to infect different tick species, among them *Beauveria bassiana* and *Metarhizium anisopliae* have received major considerations (Kalsbeek *et al*., 1995). The mechanism by which fungi infect arthropods isn't fully understood, but involves the production of some important enzymes such as chitinases, proteases, lipases and also fungal structures that penetrate the cuticle (St Leger 1995, St Leger *et al*., 1987, 1997).

As the first step towards using fungi as biocontrol agent, in the present study we determined the pathogenicity of 11 strains of *fungi including Metarhizium anisopliae* (3 strains), *Beauveria bassiana* (6 strains) and *Lecanicillium psalliotae* (2 strains) against various developmental stages of *Boophilus annulatus* for the first time in Iran. Also, we presented here the fungus *Lecanicillium psalliotae* as a novel biocontrol agent of *Boophilus annulatus*, in laboratory condition.

Material and Methods

Tick rearing: Adult *Boophilus annulatus* were collected from naturally infested cattle in Mazandaran province in the north of Iran. Female ticks were selected and maintained in the laboratory at 26°C and 70% RH in test tubes for laying eggs. After hatching of the eggs, tick larva were fed healthy 1-3 month-old holstein calves. Female ticks were collected from the experimentally infested calves and transferred to the laboratory to perform the subsequent experiment within 3-4 hours.

Fungal strains: 11 strains of entomopathogenic fungi, *Beauveria bassiana* (6 strains), *Metarhizium anisopliae* (3 strains) and *Lecanicillium psalliotae* (2 strains) were used in this study. They were provided from the fungal collection of the Department of Botany, Plant Pests and Diseases Research Institute, Tehran.

Preparation of conidial suspensions: The fungi were cultured on PDA (potato dextrose agar; E. Merck, Germany) in petri plates and were incubated for 2 weeks at 25°C. Conidia were harvested by washing the plates with an aqueous solution of 0.005% Tween 80.The conidial suspension was filtered through 4 layers of sterilized muslin to remove the fungal mycelia. Conidia numbers were determined using a Neubauer chamber and the concentration of conidia was adjusted to 1×10^7 conidia/ml. These suspensions were used as the source of fungal strains to study their virulences.

Experiment on ticks: The virulence of each fungal strain was tested by immersing 5 engorged *Boophilus annulatus* females to the conidial suspension for 3-5 sec. They were then transferred to petri dishes containing moist filter paper and incubated for 14 days at 25°C and 70% RH in the dark as described by (Gindin *et al*., 2001). For all fungal strains, a control group of five ticks was also treated in the same manner, except that they were immersed in 0.005 % tween 80 in sterile distilled water. Mortality rate was recorded daily and leaving cadavers in other dishes to observe the development of fungi on the ticks. The initial female weight (IFW) for each treatment was obtained by initial weighing of engorged female using an analytical scale, before putting them in petri dishes. The related egg laying weight (EW) was then calculated by subtraction of the weight of dead female from their initial female weight (IFW). The produced eggs from each treatment were placed in test tubes and incubated for 30 days to calculate the percentage eclosion (E%) by determining the percentage of hatched eggs. It is worth to mention that according to our experiences, every 100 mg of the tick eggs contains 1700 eggs. For each fungal treatment, the reproductive efficiency (RE) calculated by the equation of, RE= EW/IFW×E% and the percentage control (C%) that indicates the percentage of dead female ticks is calculated based on the below equation: C%= REc-REt / REc × 100, described by (Onofre *et al*.).

Experiment on Tick's Eggs: After sterilization of adult tick by immersing in ethanol 70% for 3 sec the engorged female ticks were transferred to sterile petri dishes to lay egg in 25°C and 70% relative humidity. Eggs (n=100) were placed in Petri dishes (50 mm diam.) containing filter paper that was previously impregnated with 0.5 ml of the fungal spores

Table 1. The mortality. Eclosion percentage(E%) , reproductive efficiency (RE) and control percentage (C%) of engorged Boophilus annulatus .

Fungus	Strain	Mortality rate (%)	Eclosion (E%)	Reperoductive efficiency **(RE)**	Control percentage (C%)
M. anisopliae	IRAN437C	100	0.50	0.17	99.00
M. anisopliae	IRAN715C	0	34.56	20.78	6.29
M. anisopliae	DEMI001	100	2.60	0.98	94.98
B. bassiana	IRAN403C	80	28.66	18.39	5.93
B. bassiana	IRAN428C	40	9.74	6.10	68.79
B. bassiana	IRAN789C	40	8.60	4.27	78.15
B. bassiana	DEBI001	40	8.30	4.96	74.62
B. bassiana	DEBI004	40	2.50	1.33	93.19
B. bassiana	DEBI009	60	22.72	10.96	43.93
L. psalliotae	IRAN468C	40	12.90	5.99	69.36
L. psalliotae	IRAN518C	20	39.00	22.20	13.55
Control	------	0	40.00	19.55	0

E%= egg hatchability. RE= reproductive efficiency calculated by: RE = Ew / Ifw× E%. C% represent the dead female ticks and calculated by this equation : C% = REc - Ret / REc × 100 (c= control, t = treated).

suspension (1×10^7 conidia/ml) and incubated at 25°C and 70% RH for 30 days. Percentage of eclosion (E%) was then evaluated for each treatment by direct examination (Gindin *et al*., 2001).

Experiment on Tick's Larva: Unfed tick larvae (n=100) were placed in Petri dishes (50 mm diam.) containing filter paper impregnated with 0.5 ml suspension of the fungal spores (1 107 conidia/ml) and incubated at 25°C for 7 days. In control group filter paper impregnated with 5 ml of aqueous solution of 0.005% Tween 80 in distilled water, was substituted.

Results

Entomopathogenic effects toward engorged females of B. annulatus: The Tested fungi differed in their virulence to engorged *Boophilus annulatus* females (Table 1). Mortality of treated females ranged between 0 and 100%, 14 days PI, whereas mortality in the control group was zero. The most virulent strains fungi were *Metarhizium anisopliae*, IRAN437C and DEMI001 which began to kill engorged females within 6-7 days PI. The highest mortality of females was observed within 6-10 days for *Metarhizium anisopliae* strain DEMI001, and 7-12 days for *Metarhizium anisopliae* strain IRAN437C. All treated ticks were killed in this period by these two highly pathogenic strains (see Table 2). *Beauveria bassiana* strains also killed the engorged females up to 80% within 6-12 days PI. Strains of *Lecanicillium psalliotae* were less virulent to females, killing a maximum of 20-40% of the treated ticks within 9-10 days PI. The growth of tested fungi on female ticks of *Boophilus annulatus* is shown in Figures 1-3.

Reproductive efficiency of infected females in all of treated groups compared with control groups were more and less reduced (see Table 1). *Metarhizium anisopliae* strains IRAN437C and DEMI001 and *Beauveria bassiana* strain DEBI004 were highly effective in reducing egg eclosion (E%) compared with the control groups.

Fungal virulance against eggs of *Boophilus annulatus*: Overall the strains of *Beauveria bassiana*, *Metarhizium anisopliae* and *Lecanicillium psalliotae* caused 35-100% mortality of treated eggs within 30 days PI. Egg hatchability was measured as 0-25 % after treatment with *Metarhizium anisopliae* strains, 27.5- 65% after treatment with *Beauveria bassiana* strains, and 17.5- 37.5 % after treatment with *Lecanicillium psalliotae* strains, compared with hatchability in the control group (Table 2). The infectivity of tested fungi toward *Boophilus annulatus* eggs is shown in Figures 4-6.

Table 2. Effect of different fungal strains(107 conidia / ml) on viability of Boophilus annulatus eggs and unfed larvae.

Fungus	Strain	Mortality rate (%)	
		Eggs (30 days PI)	Unfed larvae (7 days PI)
M. anisopliae	IRAN437C	90.0	71.5
M. anisopliae	IRAN715C	92.5	86.0
M. anisopliae	DEMI001	100	81.0
B. bassiana	IRAN403C	95	96.5
B. bassiana	IRAN428C	94.5	50.0
B. bassiana	IRAN789C	94.0	83.5
B. bassiana	DEBI001	87.05	84.5
B. bassiana	DEBI004	100	94.0
B. bassiana	DEBI009	96.5	70.5
L. psalliotae	IRAN468C	91.5	70.0
L. psalliotae	IRAN518C	92.5	80.0
control	-------	0	7.6

Three strains of *Metarhizium anisopliae*, six strains of *Beauveria bassiana* and two strains of *Lecanicillium psalliotae* caused 71.5-86.0%, 50 - 96.5% and 70-80% mortality in *Boophilus annulatus* larvae, respectively, at a concentration of 1×10^7 conidia/ml 7 days PI. The most virulant *Beauveria bassiana* IRAN 403C caused 96.5 % mortality in unfed larvae in this period. However, *Metarhizium anisopliae* strain IRAN 715C killed 86% of larvae within 7 days PI. *Lecanicillium psalliotae* strains showed considerable damage to unfed larvae of *Boophilus annulatus* (Table 2). Figures 7-9 show growth of all tested fungal genera on *Boophilus annulatus* larva in vitro.

Discussion

Although entomopathogenic fungi have been used widely for the control of agricultural and forest pests, little effort has been made to evaluate biocontrol potentials of entomopathogenic fungi against important arthropod vectors of human and animal diseases (Kaaya, *et al*., 1996). Based on our data, *Metarhizium anisopliae* strains IRAN437C and DEMI001 induced the highest mortality rate for *Boophilus annulatus* females respectively. That was followed by *Beauveria bassiana* strain DEBI004.*Lecanicillium psalliotae* strains, showed nearly equal virulent effects against eggs and larvae in comparison with *Beauveria bassiana* and *Metarhizium anisopliae* strains, while this effect for the adult ticks, was lower than two former species. *Lecanicillium psalliotae* strains used in this study were isolated from Ixodes sp. In the genus Lecanicillium, there are other important entomopathogenic species, e. g. *Lecanicillium muscarium* and *Lecanicillium longisporum*, which have been used as effective biocontrol agents against aphids and whiteflies. (Zare and Gams 2001, 2004). The genus Lecanicillium has been recently segregated from *Verticillium* on the bases of molecular, morphological and ecological criteria (Zare *et al*., 2000, Gams and Zare 2001). Regarding E%, *Metarhizium anisopliae* strain IRAN437C was the most effective fungus, however, there were considerable differences among the strains used, in relation to female reproductive efficiency.

Strains of *Beauveria bassiana* and *Metarhizium anisopliae* have been studied in other countries (Gindin *et al*., 2001, 2002). In our study, fungal suspensions containing 10^7 conidia/ml were highly efficient in the biological control of engorged *Boophilus annulatus* females. Onset of infection differs depending on the ability of the fungal strains to penetrate the tick directly through the cuticle. *Metarhizium anisopliae* could penetrate Boophilus microplus females through the integument

(Bitencourt, *et al*., 1995). In our study some strains specially *Metarhizium anisopliae* completed their full life cycle and formed conidiophores on B. annulatus females.

In an experiment in which *Boophilus annulatus* females were infected with two strains of *Metarhizium anisopliae* and two strains of *Beauveria bassiana* before laying egg, it was revealed that the highest mortality was 90%, at 7 days PI, and the lowest and the highest E% were 1.8% and 30.6%, respectively (Gindin, . G., *et al*., 2002). Our results showed the highest mortality rate of engorged females as 100%, at 10 days PI, for *Metarhizium anisopliae* strain DEMI001 that were obtained with a concentration of 10^7 conidia/ml. The lowest E% value (egg hatchability) was 0.5% for *Metarhizium anisopliae* strain IRAN437C and the highest E% value was 32.3% for *Metarhizium anisopliae* strain IRAN715C.

The preliminary results of the present study are that *Metarhizium anisopliae* strains IRAN437C and DEMI001 are highly efficient in biological control of engorged *Boophilus annulatus* females causing 80-100% mortality. *Beauveria bassiana* strains IRAN403C and DEBI004 are highly efficient in biocontrol of *Boophilus annulatus* larvae compared with Metarhizium and Lecanicillium strains. Staining of infected larvae with lactophenol-cotton blue revealed the hyphal formation and sporulation of the fungi inside and outside the infected larvae with the first signs of death, darkening and immobilization.

All the 11 fungal strains infected the eggs with nearly similar pathogenicity. Lecanicillium and Beauveria were successful in colonizing the egg surface, and prevent the hatching of the eggs, but could not penetrate the chorion layer. It is interesting to note that in contrast to lower entomopathogenic effects on engorged females, *Lecanicillium psalliotae* showed nearly similar or even more effects on eggs and larval stage of *Boophilus annulatus* as compared with *Metarhizium anisopliae* and *Beauveria bassiana*.

In general, the data presented here showed that entomopathogenic fungi can infect the various developmental stages of *Boophilus annulatus*, but their efficiency varies considerably according to the fungal species and strains. We demonstrated for the first time, the pathogenic effect of *Lecanicillium psalliotae* against *Boophilus annulatus*. This study was done as a preliminary screening on the virulence of the 11 strains of fungi against different developmental stages of *Boophilus annulatus*. Among these strains, 5 strains that had highest virulence against *Boophilus annulatus*, will be the subject of our future detail study to find out novel fungi suitable as biocontrol agents. Despite the efficacy of our fungal strains to control various developmental stages of *Boophilus annulatus* as an important cattle tick, evaluation of their effects at field conditions are needed before they can be use in designing of practical tick management program.

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بررسی اولیه اثرات برخی از قارچ‌های انتموپاتوژن بر روی مراحل مختلف رشد کنه بوافیلوس آنولاتوس در ایران

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چکیده

به منظور ارزیابی اثر ضد کنه‌ای یازده جدایه بومی قارچ‌های انتموپاتوژنیک (به عنوان عوامل بیوکنترل)، در شرایط آزمایشگاهی، در این بررسی چهار گوساله سه ماهه از نژاد هلشتاین به منظور کشت و تکثیر کنه بوافیلوس آنولاتوس مورد استفاده قرار گرفت کنه‌های تولید شده در گروه‌های مختلف تحت تأثیر 10^7 کونیدی در هر میلی لیتر از هر یک از جدایه‌های قارچی قرار گرفتند. میزان مرگ و میر، از تخم بر آیی نوزاد کنه‌ها و کفایت تولید مثلی این کنه‌ها در گروه‌های کنترل و درمان ارزیابی و نتایج مورد تجزیه و تحلیل آماری قرار گرفت. با واریاباسیانا IRAN 403C و جدایه لکانیسیلیوم پسالیوته IRAN 468C از حادترین جدایه‌ها بودند و به ترتیب باعث ۱۰۰-۸۰ درصد، ۸۰-۲۰ درصد و ۴۰-۰ درصد مرگ و میر در کنه‌های ماده خون‌خورده شدند. نتایج این بررسی نشان داد که قارچ‌های انتوموپاتوژنیک بر روی تمامی مراحل سیر تکاملی کنه‌های بوافیلوس موثرند و میزان این تأثیر به جدایه و گونه قارچ بستگی دارد. در این مطالعه برای اولین بار اثر ضد کنه‌ای قارچ لکانیسیلیوم پسالیوته علیه کنه بوافیلوس آنولاتوس گزارش شده است.

واژه‌های کلیدی: بوافیلوس آنولاتوس، انتوموفولوژی، کنترل بیولوژیکی، قارچ‌های انتموپاتوژن.

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