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Scanning Electron Microscopy (SEM) analysis and biological control of *Ixodes ricinus* using entomopathogenic fungi

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Abstract: In the present study, pathogenicity of four native strains of entomopathogenic fungus; *Metarhizium anisopliae*, was studied against adult stage of *Ixodes ricinus*. For this purpose a total number of 180 adult ticks were examined in triplicate. Thirty ticks for each strain and negative and positive controls were immersed in 2.4×10^7 fungal conidia/ml *in vitro*. Samples were incubated in separate Petri dishes at 26 °C and 70% relative humidity. Positive control groups were treated with Cypermethrin and negative controls were immersed in the same volume of sterilized distilled water. Mortality rate and fungal grow on ticks for each strain were reported in comparison with control groups. *M. anisopliae* IRAN 437 C showed the highest virulence in mortality and mycelium grow on ticks. Cypermethrin killed the ticks with higher potency than that of entomopathogenic fungi. Scanning electron microscopy showed the growth of *M. anisopliae* on the surface of tick bodies and penetration of fungal hyphae through tick cuticle. Taken together, results obtained from this study show potential of Iranian Entomopathogenic fungi as a biocontrol agents of *I. ricinus*. This is the first report demonstrates the mechanism of action of entomopathogenic fungi of the genus *Metarhizium* on ticks at electron microscopy level.

Key words: Biological control, *Ixodes ricinus*, *Metarhizium anisopliae*, entomopathogenic fungi

INTRODUCTION

Ticks are economically the most important pests of cattle and other domestic animals in tropical and subtropical countries. They are the vectors of a numerous pathogenic microorganisms including Protozoans (Babesiosis, Theileriosis), Rickettsiae (Anaplasmosis, Ehrlichiosis), Viral disease (e.g., Kyasanur Forest Disease reported from Karnataka State of India; Crimean-Congo Hemorrhagic Fever reported from Pakistan), Bacteria (e.g., *Pasteurella*, *Brucella*, *Listeria*, *Staphylococcus*) and also Spirochaetes (Jongejan & Uilenberg 2004).

Ixodes ricinus can be found on a wide variety of hosts, particularly mammals and birds but also reptiles (Gray and Khal 2001). The adult ticks feed mainly on large mammals such as cattle, sheep and deer, the larvae feed on small mammals (especially rodents), birds and reptiles, and the nymphs parasitize small- and medium-sized vertebrates. *I. ricinus* occurs in cool, relatively humid, shrubby or wooded areas. In addition to deciduous and mixed forests, it can be found in more open areas when the vegetation is dense and rainfall is abundant. This tick is endemic in most of Europe (with the exception of the Mediterranean region, which has a warm, dry climate). It also occurs as far south as the Caspian Sea and northern Iran, as well as in northern Africa and play an important role in transmission a number of pathogens Including *Babesia divergens* (Babesiosis), *Babesia bovis* in cattles, louping ill virus, tick born encephalitis virus, *Borrelia burgdorferi* (Lyme disease) and *Anaplasma phagocytophila* (Little 2008).

Although, economic losses due to ticks are mainly due to the diseases which they transmit (Garcia 2003), financial losses associated with nagging irritation and depreciation of the value of skins and hides (up to 20-30%) are also significant (Biswas 2003). In severely tick infested young cattle,

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sometimes ticks have been found in the oral cavity as well as in the stomach. They reach here as a result of constant licking induced by irritation. Since many years ago investigators have documented numerous potential tick biocontrol agents, including pathogens, parasitoids and predators of ticks (Kaaya 2003).

Application of chemical acaricides such as organophosphorus compounds (Malathion, Comaphous) and the carbamate carbaryl is the most common method for controlling tick populations (Rodriguez-Vivas et al. 2006), but they may be hazardous for the environment. Drawbacks to this strategy include environmental contamination (Pell et al. 2001), impacts on non-target organisms (Schulze et al. 2001), human health hazards due to chemical residues in food products (Ostfeld et al. 2006) and the development of resistance in ticks (Graf et al. 2004). These disadvantages have stimulated the search for alternative methods to control ticks.

Biological pesticides are natural, more environmentally friendly, potentially less expensive, and more effective than chemical pesticides, also problems with resistance are less likely to occur (Whipps & Lumsden 2001). Among biocontrol agents, entomopathogenic fungi received major attention in recent years (Briggs et al. 2006, Abolins et al. 2007, Tavassoli et al. 2008). One of the most pathogenic fungal species examined for pathogenicity against ticks under laboratory and field conditions is *M. anisopliae* (Ostfeld et al. 2006). It showed high pathogenic activity against the ixodid ticks *Amblyomma maculatum* and *Amblyomma americanum* (Kirkland et al. 2004), *I. scapularis* (Hornbostel et al. 2005), *Rhipicephalus appendiculatus* and *Amblyomma variegatum* (Kaaya and Hassan 2000) and *Boophilus microplus* (Alonso-Diaz et al. 2007). In Iran, some indigenous strains of *M. anisopliae*, *Beauveria bassiana* and *Lecanicillium psalliotae* have been isolated with promising results to control different life stages of *Rhipicephalus (Boophilus) annulatus* under laboratory conditions (Pirali-Kheirabadi et al. 2007). However, only few studies have been reported about the control of Ixodidae ticks by *M. anisopliae* or other entomopathogenic fungi (Zabalgogeazcoa et al. 2008).

The aim of this study was to introduce the safe and alternative way to control ticks, and also to find the natural and virulent strains of Iranian entomopathogenic fungi as promising candidate for tick biological control.

MATERIALS AND METHODS

Tick rearing

Adult *I. ricinus* was collected from naturally infested cattle in Mazandaran province, north of Iran. Ticks were transferred within 3–4 h and maintained in the laboratory at 26 °C and 70% relative humidity (RH) in test tubes for further study.

Fungal strains

Four entomopathogenic fungi including *M. anisopliae* strains Iran 437 C, DEMI 001, Iran 715 C and DEMI 002 used in this experiment were obtained from the fungal culture collection of Iranian Research Institute of Plant Protection, Department of Botany, Tehran, Iran (Table 1.).

Preparation of conidia suspension

The fungi were cultured on PDA (Potato Dextrose Agar; Merck, Germany) in Petri dishes for 2 weeks at 25 °C. Conidia were harvested by washing the plates with an aqueous solution of 0.005% sterile Tween 80. The conidial suspension was filtered through four layers of sterilized muslin to remove the fungal mycelia. Conidia numbers were determined using a Neubauer slide under the microscope and the concentration of conidia was adjusted to 2.4×10^7 conidia/ml. These suspensions were used as the source of fungal propagules.

Treatment of different developmental stages of *I. risinus* with conidial suspension

The virulence (Pathogenicity) of each fungal strain (2.4×10^7 conidia/ml) was tested by immersing ten engorged *I. ricinus* in conidial suspensions for 3–5 seconds per replication according to the method of Gindin et al. 2001. The experiment was carried out in three replicates and in total, 180 ticks were treated with the strains concentrations. A negative control group of ten ticks was immersed in the same volume of sterilized distilled water and three replicates were made for the controls and experimental groups. Like negative control a positive control group containing an acaricidal compound, Cypermethrin 15 ml/1 sterilized distilled water (Cypermethrin®, Aria Shimi, Zahedan, Iran) was also considered. Then ticks were transferred to Petri dishes containing moist filter papers and incubated for 20 days at 26 °C and 70% RH as described by Gindin et al. 2001.

Table 1. *Metarhizum anisopliae* strains used in the study

Strain	Original host	Origin
IRAN 437 C	*Chilo suppressalis*	Rasht, Iran (2001)
DEMI 001	*Rhychophorus ferrugine*	Saravan, Iran
DEMI 002	-	Noor, Iran
IRAN 715 C	*Caelifera*	Ahvaz, Iran (2001)

Mortality rate were recorded in 4, 8, 12, 16, and 20 days post treatment with fungal suspension and chemical acaricides.

Scanning Electron Microscopy (SEM)

For SEM analysis, ticks were fixed overnight at 4 ºC with 2% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. Post fixation was carried out in 1% (w/v) osmium tetroxide in the same buffer. The specimens were rinsed in buffer, dehydrated in series of 30–100% acetone solutions, dried at critical point in CO_2 (CPD 030 BALTEC), and coated with gold in a sputter-coater (SCD 050 BALTEC). The material was examined with Jeol JSM 5800 scanning electron microscope (SEM) at the Pasteur Institute of Iran, Tehran, Iran.

Statistical analysis

The data were expressed as the Mean ± SEM. Groups were compared using one-way ANOVA for repeated measurements. Student t-test was used for post hoc analysis. The software Sigmaplot version 12 was used for data analysis. A value of ($P < 0.05$) was considered significant.

RESULTS

Mortality rate of ticks exposed to fungal suspensions for four revealed that there were significant differences between *M. anisopliae* IRAN 437 C and other three fungal strains and also negative and positive controls (Cypermethrin). Positive control killed all the ticks in first four days and there were significant differences between positive control and treated groups as well. ($P < 0.05$) (Table 2). In four next days (eight days post treatment), the highest mortality rate was belonged to the strain Iran 437 C with 30% and minimum of mortality rate was 13.3 % in treatments by strain DEMI 002. There were significant differences between strain *M. anisopliae* Iran 437 C and *M. anisopliae* DEMI 002 and IRAN 715 C in killing activity against *I. ricinus* (Table 2). Maximum of 63.3% and minimum of 40% mortality was demonstrated by using *M. anisopliae* strains Iran 437 C and *M. anisopliae* strain DEMI 002 twelve days post inoculation, respectively and there was significant differences between *M. anisopliae* strain Iran 437 C and other fungal groups in this regard ($P<0.05$).

About 86.6% and 63.3% of ticks mortality was recorded treatment with *M. anisopliae* strains Iran 437 C and *M. anisopliae* strain DEMI 002 in Sixteenth days post treatment. Significant differences were observed between *M. anisopliae* strain Iran 437 C and *M. anisopliae* DEMI 002 and *M. anisopliae* Iran 715 C and between *M. anisopliae* DEMI 001 with *M. anisopliae* DEMI 002 ($P<0.05$) (Table 2). After twentieth day treatment, mortality rate of *I. ricinus* caused by *M. anisopliae* Iran 437 C, DEMI 001, Iran 715 C and DEMI 002 reached to 100 %, 93.3%, 90% and 83.3%, respectively. So the mortality rate increased with pass the time. In positive control groups (Cypermethrin), ticks were being killed early and faster than other groups and no mortality occurred in negative control groups even after twentieth days.

Metarhizium anisopliae IRAN 437 C was found to be the most virulent strain to adult stage of *I. ricinus*, followed by *M. anisopliae* DEMI 001 and *M. anisopliae* Iran 715 C. *M. anisopliae* strains IRAN 437 C and DEMI 001 had the most conidial growth on the killed tick's cuticles, respectively (Table 3).

Table 2. Mean of number of killed ticks by *Metarhizum anisopliae* in different times. Different lower cases (a-e) show the significant difference between rows of every columns (Values of $p < 0.05$ were considered significant).

Fungal strains	Mortality rate (killed ticks) (Mean ± SE %)				
	Day 4	Day 8	Day 12	Day 16	Day 20
DEMI 001	0.0 e	23.3 ± 5.7 ab	46.6 ± 5.7 b	80 ± 10 a	93.3 ± 5.7 a
DEMI 002	0.0 e	13.3 ± 5.7 b	40 ± 10 c	63.3 ± 11.5 b	83.3 ± 5.7 b
IRAN437C	5.7 ± 3.3 b	30 ± 0 a	63.3 ± 5.7 a	86.6 ± 5.7 c	100 ± 0 c
IRAN715C	0.0 e	16.6 ± 5.7 b	43.3 ± 11.5 c	70 ± 10 d	90 ± 10 a
Negative control	0.0 e				
Positive control	100% f				

Table 3. Mean of mycelium growth on killed ticks in different times. Different lower cases (a-e) show the significant difference between rows of every columns (Values of $p < 0.05$ were considered significant).

Fungal strains	Mycelia growth on killed ticks (Mean ± SE %)				
	Day 4	Day 8	Day 12	Day 16	Day 20
DEMI 001	0.0 e	6.6 ± 5.7 a	30 ± 0 a	5.7 ± 63.3 a	5.7 ± 86.6 a
DEMI 002	0.0 e	5.7 ± 3.3 b	30 ± 10 a	50 ± 0 b	15.3 ± 73.3 b
IRAN437C	0.0 e	20 ± 10 c	50 ± 10 b	80 ± 10 c	93.3 ± 5.7 c
IRAN715C	0.0 e	6.6 ± 5.7 a	26.6 ± 5.7 c	53.3 ± 11.5 d	83.3 ± 5.7 a
Negative control	0.0 e				
Positive control	0.0 e				

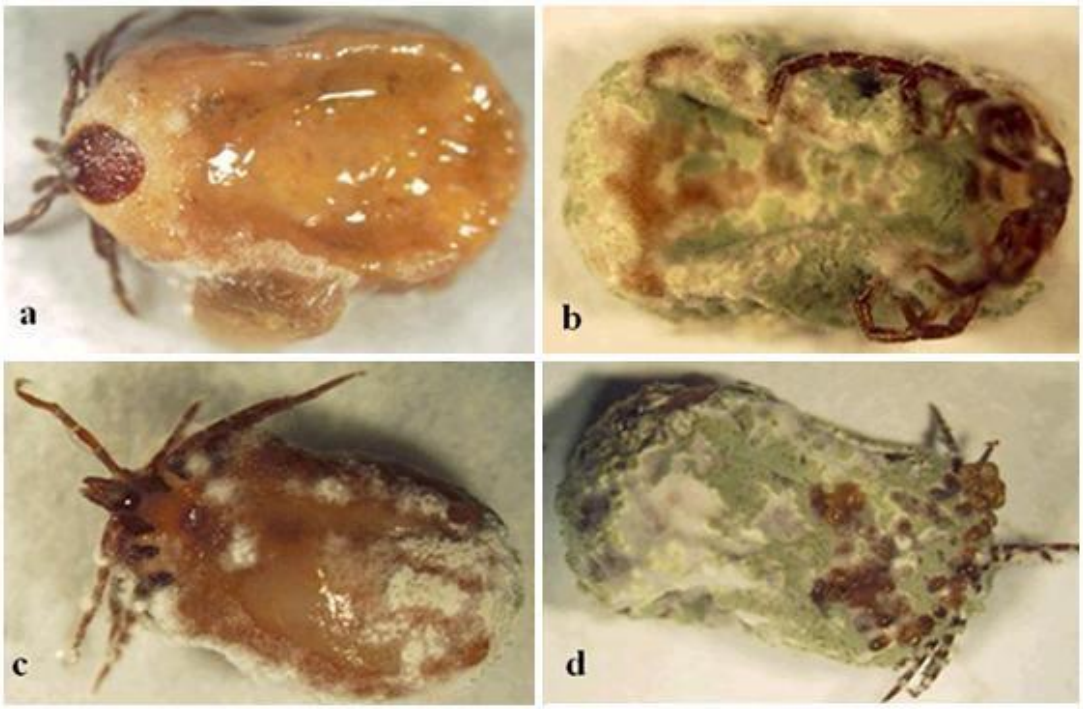

Fig. 1. Direct view of fungal mycelia growth on the cuticle of killed ticks. a. *Metarhizium anisopliae* DEMI 002; b. *Metarhizium anisopliae* DEMI 001; c. *Metarhizium anisopliae* IRAN 715 C; d. *Metarhizium anisopliae* Iran 437 C.

Figure 1 shows the mycelia growth of *M. anisopliae* on the cuticle of killed ticks.

A qualitative comparison of conidial binding, germination, and penetration of *M. anisopliae* on *I. ricinus* was performed using scanning electron microscopy of ticks infected throughout the time Day 20 when conidial germination occurred on ticks.

The conidia of *M. anisopliae* was generally spherical in shape. The fungus produced a thin amorphous mucilage layer that firmly adhered the conidia and germ tubes to the tick integument (Fig. 2). The first sign of conidia germination was germ-tube extrusion. Each conidium usually produced only one germ tube that penetrated to the tick cuticle (Fig. 2).

Examination of fixed samples indicated that conidial density and germination varied dramatically by body region. Within 72 h, most germinating conidia were found in the marginal groove and marginal body fold as well as around the anus and anal groove. In the early stages of infection comparatively few conidia were observed on the scutum, although patches of fungi could be found within the cervical groove and lateral carina. In several instances *M. anisopliae* were observed proliferating (in patches) on the cuticle surface of killed ticks, but several specimens contained hardly any germinating cells (Fig. 2).

DISCUSSION

Several authors have reviewed specific groups of natural enemies of ticks, including pathogens (Chandler et al. 2000), nematodes (Samish & Glazer 2001), parasitoids (Knipling & Steelman 2000), and predators (Samish & Alekseev 2001). Much effort has been applied to control pests by means of biological agents, often as part of integrated pest management (IPM) programs (Van Driesche & Bellows 1996). During the early 20th century, efforts were made to import parasitoids into the USA for tick control (Alfeev 1946). In addition, oxpeckers have been reintroduced into areas in Africa where these birds had become extinct (Couto 1994).

Results of this study show promising effect of entomopathogenic fungi as potential biocontrol agent against *I. ricinus.*

In intensive tick control programs in exotic and crossbreed dairy cattle in Africa, acaricides are applied as frequently as once per week (Norval et al. 1992). Small-scale farmers raise most of these dairy cattle where the family provides labor. Although spraying pastures with fungi may appear to create more labor for the family, this may not be the case for the following reasons: Firstly, horizontal transmission of infection from fungus-infected to uninfected arthropods has been observed (Backer et al. 1994). This often leads to fungal epizootic (Fargues & Remaudière 1977), especially in moist environments. Non-target organisms may also serve as secondary hosts on which fungal inoculum is maintained and propagated, thus promoting later infections in the target host populations (Goettel & Johnson 1992).

Over 700 species of entomopathogenic fungi have been reported, but only 10 species have been or are currently being developed for the control of insects (Butt et al. 2001). The most promising fungi are belonged to the mitosporic fungi.

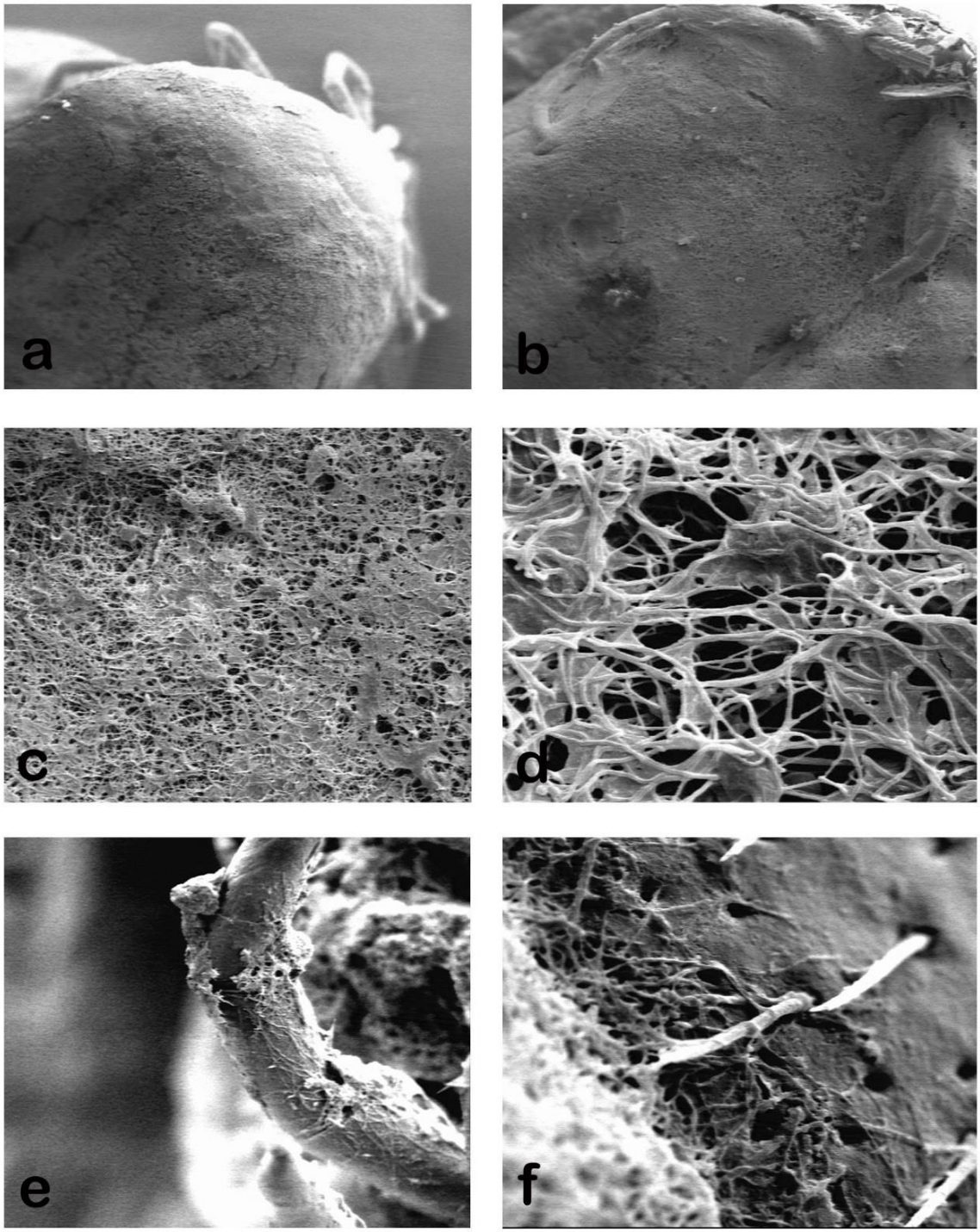

Fig. 2. Scanning electron microscopy (SEM) analysis of ticks contaminated with *Metarhizium anisopliae*. a–b. Growth of fungal mycelia on the cuticle of killed ticks which shows signs of fungal grow in dorsal and ventral surface of *Ixodes ricinus*, respectively (magnification ×25); c–d. Fungal grow with magnification × 100 (c) and × 500 (d); e. Fungus produces a thin amorphous mucilage layer and it firmly adheres the conidia and germ tubes to the tick integument; f. Each conidia usually produces only one germ tube that penetrates into the tick cuticle.

The ability of entomopathogenic fungi to penetrate the cuticle of arthropods, the ability of a strain to kill several stages of the same pest and the relatively specific virulence of a single strain to one or a small group of pests make them good candidates as biocontrol agents. However, fungi also have some disadvantages: they are slow in killing their host, they need high humidity to germinate and sporulate, they are susceptible to UV irradiation, and some strains can potentially affect non-target arthropods (Ginsberg et al. 2002). Mass production can be quite costly, and the limited shelf life of some products makes them

even more expensive. Many of these constraints can be addressed by advanced formulations. Most producers of fungal-based products suggest application methods similar to those used for chemical pesticides (Shelton and Roush 2000).

Taken together, results of the present study further substantiate the potential of entomopathogenic fungi of the genus *Metarhizium* as biocontrol agents of *I. ricinus*. This is the first report demonstrates the mechanism of action of entomopathogenic fungi on ticks at electron microscopy level.

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ارزیابی مکانیسم اثر میکروسکوپ الکترونی اسکنینگ و کنترل زیستی کنه *Ixodes ricinus* با استفاده از قارچ های انتوموپاتوژن

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چکیده: در مطالعه حاضر مکانیسم اثر چهار جدایه بومی قارچ های انتوموپاتوژن *Metarhizium anisopliae* در برابر مرحله بالغ کنه *Ixodes ricinus* مورد مطالعه قرار گرفت. بدین منظور تعداد ۱۸۰ کنه بالغ مورد آزمایش قرار گرفت. برای هر جدایه قارچی و همچنین برای گروه های کنترل مثبت و منفی ۳۰ کنه در نظر گرفته شد و کنه ها در سوسپانسیونی از $۲/۴ \times ۱۰^{۷}$ کونیدی در هر میلی لیتر شناور شدند این آزمایش به صورت سه بار تکرار در شرایط آزمایشگاهی انجام شد. هر کدام از گروه های مورد آزمایش در ظروف پتری جداگانه در دمای ۲۶ درجه سانتی گراد و رطوبت نسبی ۷۰٪ انکوباتور قرار گرفتند. گروه های کنترل مثبت با استفاده از سم سایپرمترین و گروه های کنترل منفی در آب مقطر استریل قرار گرفتند. حجم ها ی مورد نظر مساوی در نظر گرفته شدند. میزان مرگ و میر و رشد قارچ بر روی کنه های مورد آزمایش به صورت جداگانه برای هر جدایه قارچی در مقایسه با گروه های کنترل ثبت گردید. نتایج حاصله نشان داد جدایه قارچی *Metarhizium anisopliae* IRAN 437 C بیشترین حدت را در میزان مرگ و میر و رشد میسلیوم های قارچی بر روی کنه ها داشت. سم سایپرمترین با شدت بیشتری نسبت به قارچهای مورد مطالعه باعث تلفات در کنه ها شد. نتایج حاصل از ارزیابی مکانیسم اثر میکروسکوپ الکترونی اسکنینگ، رشد قارچ *Metarhizium anisopliae* روی سطح بدن و نفوذ میسلیوم های قارچی در کوتیکول کنه ها را نشان داد. نتایج به دست آمده از این مطالعه نشان از پتانسیل قارچ های بومی انتوموپاتوژن به عنوان یک عامل کنترل زیستی کنه *Ixodes ricinus* دارد. این مطالعه اولین گزارش در ایران است که نشان دهنده مکانیسم اثر قارچ انتوموپاتوژن *Metarhizium anisopliae* بر روی کنه با استفاده از میکروسکوپ الکترونی می باشد.

کلمات کلیدی: کنترل زیستی، *Ixodes ricinus*، *Metarhizium anisopliae*، قارچ های انتوموپاتوژن.

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