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OCCURRENCE OF ENTOMOPATHOGENIC AND OTHER OPPORTUNISTIC FUNGI IN SOIL COLLECTED FROM INSECT HIBERNATION SITES AND EVALUATION OF THEIR ENTOMOPATHOGENIC POTENTIAL

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ABSTRACT

A survey of entomopathogenic and other opportunistic fungi isolated from soil samples collected from insect hibernation sites in different habitats in Kurdistan region of Iraq was carried out during October to December 2009. By using dilution plate method, two entomopathogenic species (*Beauveria bassiana* (Bals.) Vuill.and *Isaria javanica* (Friedrichs & Bally) Samson & Hywel-Jones) were detected with isolation percentage (38.46%) each. Other opportunistic fungi such as *Alternaria alternata*, *Aspergillus flavus*, *A.niger*, *Penicillium glabrum*, *P. digitatum*, *Rhizopus stolonifer* and *Syncephalastratum racemosum* were also isolated. *B. bassiana* was the most virulent fungus and showed complete mortality (100%) on two aphid species *Hyalopterus pruni* Geoff. and *Aphis pomi* De Greer after six days of inoculation, followed by *I.javanica* with 66.67% and 75.59% mortality respectively. *I. javanica* was isolated for the first time from Iraq. A brief description along with photographs is provided for the newly recorded species.
Key words: Entomopathogenic fungi, Soil, Iraq.
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INTRODUCTION

Entomopathogenic fungi were occurred naturally as infections in insect or arachid hosts, and several of these fungi only occurred as infections in living hosts for a relatively short period of time during their life cycle. The remainder of the life cycle of these species presumably lurk as dormant propagules in the soil, in the vicinity of the dead host cadaver. Thus, the chances of finding good candidates to be used as biocontrol agents in these soils are very high (Olivares-Bernabeu and Lopez-Llorca, 2002).

Most fungi from the order Hypocreals are only known in their anamorphic life cycle, thus only mitosporic conidia are formed. The dead host cadavers will mostly fall to the ground, and thus, a reservoir of fungal material is present in the soil environment. Further, dispersal from cadavers as focal points presumably occur due to weather (wind and rain), soil manipulation and also insect activity (Meyling et al., 2006). Soil factors (temperature, pH, or organic content, relative moisture or mineral, organic or biotic factors) can affect fungal persistence and activity (Charnley, 1997).

In the laboratory, however, the conidia from hypocrelean entomopathogenic fungi can also germinate and grow on artificial media, and need to come in contact with susceptible host in

order to grow and proliferate successfully. These two methods of germination are manipulated for isolation of entomopathogenic fungi from the soil (Goettel and Inglis,1997).

In order to monitor the fate of applied fungal material in the soil, a selective media originally described by Strasser et al. (1996) were used for detection of survival *Beauveria* spp. *Metarhizium* spp., and *Paecilomyces* spp., Bacteria can be inhibited by the application of broad-spectrum antibiotics such as chloramphenicol, tetracycline, or streptomycin (Goettel and Inglis, 1997). However, to exploit the ability of the fungi to infect host, the insect bait method can be used (Zimmermann, 1986).

In the present work we have analyzed the presence of entomopathogens, mainly fungi, in soil samples collected from insect hibernation sites in different ecosystems of Duhok province such as natural forests of *Quercus rotundifolia,* agricultural soils and grape orchards and to evaluate their entomopathogenic potential on two aphid species.

MATERIALS AND METHODS

Sampling sites and Colleton of soil samples

Thirteen soil samples were collected from three insect hibernation sites in Duhok province,North Iraq during October to December 2009. The sites included fields with agricultural soil at Barway Bala (4 sampls), natural forests mainly *Qurcus rotundifolia* at Gara mountain (6 samples) and from grapevine yard at Siara Tooka (3 samples).

Soil samples about (500 g each) were taken from the depth of 0-10 cm with a trowel after removing litter or weed plants that insects hidden beneath then, placed in plastic bags and brought to the laboratory. Samples were subjected for fungal isolation within 2 days of collection.

Fungal isolation and identification:

Initial dilution was made by mixing 10g of soil with 90 ml of sterilized distilled water in 250 ml conical flask. Flasks were shaken for 3 minutes on an electrical shaker. Serial dilutions up to 10^4 were made in the same method. One ml. of 10^4 dilution was poured in each plate and mixed with 20 ml. of Potato dextrose agar medium (Himedia laboratories,Ltd. India) supplemented with 0.28 mg/l chloramphenicol to avoid bacterial growth. Six plates per replication were used. The plates were incubated at 25 C for 7 days. The isolates were purified and growing colonies were identified depending on their morphological characteristics of their reproductive structures with the aid of several taxonomic references (Samson 1974; Domsch *et al.*1980; Goettel and Inglis, 1997; Tzean *et al.,* 1997). Isolation percentage of a particular species in soil was calculated using the formula:

Isolation percentage = Number of positive soil samples for a particular species/ Total number of all samples × 100 (Abdullah and Mohamed, 2009).

Pathogenicity bioassay:

The pathogenicity trial was performed according to (Ali-Shtayeh *et al.*, 2002). The tested fungal isolates were grown on PDA plates for 10 days. Sterile water (5 ml.) was powered on each plate containing fungal colony to obtain spore suspension, adjusted their concentrations at 1 x 10^8 conidia/ ml. Twenty adults of each of two aphid species (*Hyalopterus pruni* (Geoff.)) and *Aphis pomi* (DeGreer) were sprayed with 10 ml of spore suspension for few seconds for each isolate and then transferred into a sterile plates containing two pieces of moistened filter papers and two host plant leaves. Plates were sealed with Parafilm to maintain the humidity and then incubated in darkness at 25 ^{o}C. Infected dead insects were

inspected and counted daily. Mortality percentage caused by each isolate was assessed after 2, 4 and 6 days.

The experiment was conducted as a completely randomized design with four replicates for each isolate. Differences between the treatments were determined by ANOVA and Duncan test at $P \leq 0.05$ with SAS software (SAS, 1999).

RESULTS

A total of 9 species assigned to 7 genera were recovered from 13 soil samples by the dilution method (Table 1). *Penicillium glabrum* was the most frequent species detected from all soil samples, followed by *Aspergillus niger* with 76.92% isolation rate .The two entomopathogenic fungi (*Beauvaria bassiana* and *Isaria javanica*) and *Rhizopus stolonifer* were each detected with 38.96% isolation rate. Other opportunistic fungi such as *Alternaria alternate*, *A. flavus*, *Penicillium digitatum* and *Syncephalastratum racemosus* were also isolated with isolation rates varying between 7.67% - 30.77%.

The present study revealed that entomopathogenic and other opportunistic fungi are common inhabitant of soil at insect hibernation sites, however, their diversity is relatively low as indicated by the isolation of two entomopathogenic species and seven opportunistic fungi.

Isaria javanica was isolated from Iraqi soil for the first time. The newly recorded species is described and illustrated.

Phenotypical characterization of *Isaria javanica* (Frieder. & Bally) Samson & Hywel-Jones. Mycol.Res.109, 588 (2005). Fig.1(A-B).

Colonies on PDA, growing slowly reached a radial of 4.6 mm in 14 days at 25 C, powdery to floccose, at first white becoming cream-coloured with age. Hyphae hyaline, septate, branched, smooth walled, 2-3um wide. Conidiophores erect, hyaline, simple or branched, up to 50 um long and 2-2.5um wide, forming verticillate branches with phialides in whorls of 2 to 3. Phialides 10-16 x 2-3 um, consisting of cylindrical basal portion tapering into a thin distinct neck. Conidia hyaline, smooth, one celled, fusiform, sometimes cylindrical, 5-6.5 x 2-2.3 um. Chlamydospores not observed.

The pathogenicity test (Table 2) showed that *B. bassaiana* was the most virulent species causing 100% mortality to both aphid species (*Hyalopterus pruni* and *Aphis pomi*) after six days, followed by *I. javanica* (66.7% and 75.6% mortality) to both aphid species respectively.

DISCUSSION

In our study we have isolated surviving entomopathogenic and opportunistic fungi from diversely soil environments. This indicates that these fungi can be naturally found close to phytophagous insects host. Most fungi found in Iraq during this work have been reported from other parts of the world (Vanninen,1995; Meyling and Eilenberg, 2006).

Regarding the entomopathogenic fungal species, *B.bassiana* was among the most frequently isolated fungi from soil at insect hibernation sites. This result is in agreement with several other studies, revealing that *B. bassiana* was encountered from a variety of agricultural and natural soils (Ali-Shtayeh *et al.*2002; Meyling and Eilenberg, 2006; Quesada-Movaga *et al.* 2007; Sun and Lin.2008; Sun *et al.*,2008). Moreover, the fungus seems has a wide distribution over the country and has been repeatedly isolated from different soils in Iraq

as well as from different insect hosts (Khalaf *et al.*1997, 1998; Assaf, 2007, 2009; Abdullah and Mohamed, 2009; Assaf *et al.*2011).

Isaria javanica (Frieder.&Bally)Samson & Hywel-Jones (formerly known as *Paecilomyces javanicus* (Frider & Bally) A. H. S. Brown&G.Smith) and was originally described by Friederich & Bally (1923) as *Spicaria javanica*. The species is isolated from diverse soils at insect hibernation sites for the first time in Iraq. The description of our isolate is in agreement with Samson (1974), Tzean *et al.* (1997) and Shimazu and Takastuka (2010). Our isolate of *I. javanica* did not form synnemata, however, Samson (1974) described that *P. javanicus* occasionally produces a few synnemata which was not reported by other authors (Brown and Smith, 1957; Tzean *et al.*1997; Shimazu and Takatsuka, 2010). Performance of pathogenicity test for our isolate on two aphid species (*H.pruni* and *A.pomi*) caused 66.7% and 75.6% mortality respectively. Most reported host insects for *I.javanica* are members of either Lepidoptera or Coleoptera (Tzean *et al.* 1997; Chen *et al.*2007; Hu *et al.* 2007; Spacht *et al.*, 2009; Shimazu and Tketsuko, 2010). The pathogenicity of the fungus was also proved on insects in Hemiptera (Scorselli *et al.*, 2008) and in Hymenoptera (Hu *et al.* 2011). The species was also reported as an entomopathogenic fungal endophyte being isolated from peduncles of coffe plants (Vega *et al.* 2008). Several species in the genus *Isaria* (formerly *Paecilomyces*) such as *I. farinosa* (Holmsk.) Fr. and *I. fumosorosea* Wize, are well known insect pathogens and frequently isolated from soil (Ali-Shtayeh *et al.*2002; Meyling and Eilenberg, 2006; Sun and Liu,2008; Hu *et al.*2010). *I. farinosa* have been previously reported from Iraq as *P. farinosus* on Sunn pest and aphids (Assaf, 2007, 2009).

Aspergillus flavus and *A.niger* isolated in the present study have previously been isolated as insect pathogens by several authors ((Sur *et al.*, 1999; Abdullah *et a*l.2001,2002; Sun and Liu, 2008; Abdullah and Mohamed, 2009 and Assaf *et al.*, 2011).

Several other fungal species including *Penicillium glabrum*, *P. digitatum*, *Alternaria alternata*, *Syncephalastratum racemosum* and *Rhizopus stolonifer* were detected with different isolation percentage. Though we considered these species as secondary colonizers, but these opportunistic fungi were proved their pathogenicity on different insect orders (Gunde-Cimerman *et al.*, 1998; Abdullah *et al.*2002; Ali-shtayeh *et al.*, 2002; Sun *et al.*, 2008 and Abdullah and Mohamed, 2009).

In conclusion, the present study provides the first report of *I. javanica* from Iraq as an entomopathogenic fungus, extending our knowledge of the occurrence and distribution of entomopathogenic fungi in Iraqi soil.

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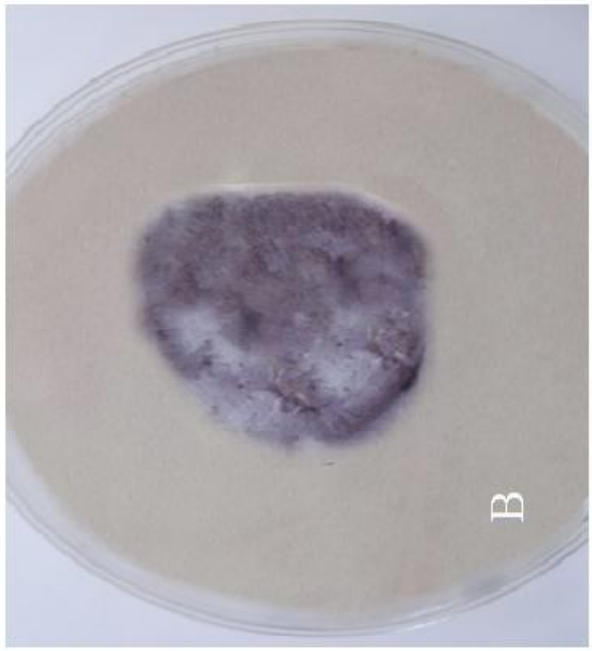
Table 1: Isolation % of entomopathogenic and opportunistic fungi isolated from soil samples.

Fungal species	N° positive samples	Isolation %
Alternaria altenata	1	7.69
Aspergillus flavus	2	15.38
A. niger	10	76.92
Beauveria bassiana	5	38.46
Isaria javanica	5	38.46
Penicillium digitatum	4	30.77
P. glabrum	13	100.0
Rhizopus stolonifer	5	38.46
Syncepalastratum racemosum	1	7.69

Table 2: Pathogenicity trial of fungal isolates on *H. pruni* and *A. pomi*.

Fungus species	Insect species	% Mortality		
		2 days	4 days	6 days
Control	*H. pruni*	0 e *	0 e	10 e
	A. pomi	5 de	10 de	15 de
B. bassiania	*H. pruni*	45 a	85 a	100 a
	A. pomi	31.58 ab	88.89 a	100 a
P. javanicus	*H. pruni*	20 bc	55 b	66.67 b
	A. pomi	26.32 bc	50 b	75.59 b
A. nigur	*H. pruni*	15 cd	25 c	27.78 cd
	A. pomi	5.26 de	22.22 cd	35.29 c
Penicillium glabrum.	*H. pruni*	15 cd	30 c	33.33 c
	A. pomi	21.05 bc	27.78 c	29.41 cd

* Means followed by the same letters in each column are not significantly different (P = 0.05).



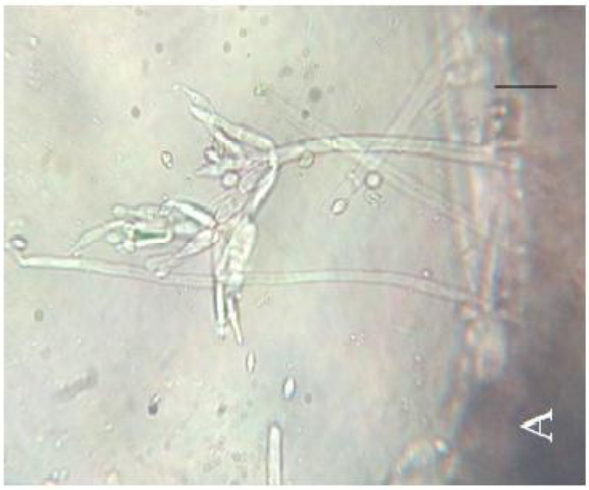


Figure.1: *Isaria javanica* (A) Phialides and phialoconidia; (B) Fourteen day old colony on PDA. Scale bar of A=10 um.

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تواجد الفطريات الحشرية والانتهازية فى ترب مواقع تشتية الحشرات وتقيم القابلية الامراضية

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الخلاصة

تم عزل الفطريات الممرضه للحشرات والانتهازيه من عينات تربه جمعت من اماكن تشتية الحشرات فى بيئات مختلفه فى كردستان العراق خلال الفتره من تشرين الاول- كانون الأول ٢٠٠٩. تم عزل نوعين منى الفطريات الحشرية

Beauveria bassiana (Bals.) Vuill.and *Isaria javanica* (Friedrichs & Bally) Samson & Hywel-Jones.

من التربة بأستخدام طريقة التخفيف وبتردد ٣٨,٦% لكل فطر. تم عزل فطريات اخرى انتهازية الامراضية مثل:

Alternaria alternata, *Aspergillus flavus, A.niger*, *Penicillium glabrum*, *P.digitatum*, *Rhizopus stolonifer* and *Syncephalastratum racemosum*.

الفطر *B. bassiana* كان الاكثر ضراوة واحدث نسبة قتل ١٠٠% على كلا النوعين من الحشرات. اظهر الفطر *I. javanica* نسبة قتل تراوحت مابين ٦٦,٦٧% و٧٥,٥٩% على كلا النوعين من الحشرات على التوالي. تم عزل وتسجيل النوع *I. javanica* لاول مرة في العراق. تم وصف النوع المسجل مع التوضيح بالصور الفوتوغرافية.