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Original article (Orijinal araştırma)

Occurrence of entomopathogenic fungi on insect pests of stored wheat and maize in Central and South Anatolia in Turkey

Türkiye'nin Orta ve Güney Anadolu Bölgesi'nde bulunan buğday ve mısır depolarındaki zararlılarda tespit edilen entomopatojen funguslar

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Summary

Naturally occurring fungal pathogens of stored-grain insect pests were identified and quantified using different insect sampling techniques in wheat and maize storage facilities in Central and South Anatolia in Turkey. Storage facilities were sampled by probing, trapping and visual inspection in five cities (Şanlıurfa, Kahramanmaraş, Adana, Mersin and Konya) from June to November 2013. Entomopathogenic fungi recovered from dead insects were identified to species level by sequencing the ITS1-5.8S-ITS2 region of the genomic DNA. Of the three species isolated, the majority were *Beauveria bassiana* (97 isolates), followed by *Purpureocillium lilacinum* (20 isolates). The third species, *Beauveria varroae* (9 isolates), is the first record on stored-product pest insects. Thirty-five isolates were from *Tribolium* spp., 29 from *Sitophilus* spp., 24 from *Cryptolestes ferrugineus,* 22 from *Rhyzopertha dominica,* 8 from *Oryzaephilus surinamensis,* 4 from *Trogoderma granarium,* 3 from *Latheticus oryzae* and 1 from a species of Cryptophagidae. The fungal infection of stored-grain pests did not vary significantly according to the time of sampling. A higher frequency of occurrence was recorded for Adana than the other cities and for *Tribolium* species than the other hosts. Grain samples taken by probing resulted in a higher frequency of fungal infection, but commodity type did not have a significant effect. The results demonstrated that (1) entomopathogenic fungi occurred at a low frequency, and (2) location, together with sampling technique, can affect their recovery. Further exploration of this ecosystem could yield important information for improving their use for management of stored-grain pests.

Keywords: Coleoptera, Hypocreales, microbial control, stored-product pests

Özet

Türkiye'nin Orta ve Güney Anadolu Bölgesi'ndeki buğday ve mısır depolarında tahıl zararlılarının doğal olarak bulunan fungal patojenleri, çeşitli böcek örnekleme yöntemleri kullanılarak belirlenmiş ve teşhis edilmiştir. 2013 yılı haziran – kasım aylarında beş ilde (Şanlıurfa, Kahramanmaraş, Adana, Mersin ve Konya) depolar, sonda kullanarak, tuzak yerleştirerek ve gözlemle örneklenmiştir. Ölü böceklerden elde edilen entomopatojen funguslar genomik DNAnın ITS1-5.8S-ITS2 bölgesi sekanslanarak tür seviyesinde teşhis edilmiştir. İzole edilen üç türün büyük çoğunluğu *Beauveria bassiana* (97 izolat) olup bunu *Purpureocillium lilacinum* (20 izolat) izlemiştir. Üçüncü tür ise *Beauveria varroae* (9 izolat)'dir ve depo zararlılarından ilk kayıttır. Tüm izolatların 35'i *Tribolium* spp., 29'u *Sitophilus* spp., 24'ü *Cryptolestes ferrugineus,* 22'si *Rhyzopertha dominica,* 8'i *Oryzaephilus surinamensis,* 4'ü *Trogoderma granarium,* 3'ü *Latheticus oryzae* ve birisi bir Cryptophagidae türünden izole edilmiştir. Depolanmış tahıl zararlılarında fungal enfeksiyonlar örnekleme zamanına göre önemli ölçüde değişmemiş, en yoğun olarak iller arasında Adana'da ve türler arasında da *Tribolium* türlerinde bulunmuştur. Sonda ile alınan tahıl örneklerinde fungal enfeksiyon daha yoğun tespit edilmiştir, ancak ürün cinsinin önemli bir etkisi belirlenmemiştir. Sonuçlar, entomopatojen fungusların düşük yoğunlukla dağılım gösterdiğini, ve lokasyon ile örnekleme tekniğinin fungus izolasyonunu etkileyebileceğini ortaya koymuştur. Bu ekosisteminde daha fazla çalışma yapılarak depolanmış tahıl zararlılarının mücadelesindeki kullanımlarını geliştirmek için önemli bilgilere ulaşılabilecektir.

Anahtar sözcükler: Coleoptera, Hypocreales, mikrobiyal mücadele, depo zararlıları

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Introduction

Cereals are important sources of nutrition for both humans and livestock throughout the world. These commodities are typically stored for various durations of time and require protection against insect and mite pests. Due to pest damage, unprotected grain storage usually leads to reduction in the weight and value of grain along with the germination decline of seeds (Moino et al., 1998; Padin et al., 2002; Haq et al., 2005; Stejskal et al., 2015). Although the use of synthetic insecticides to control stored-grain pest populations has been widespread (Athanassiou & Palyvos, 2006), the practice has been challenged due to various undesirable consequences including pest resistance to the chemicals (Arthur, 1996), residue accumulation in grain (Ferizli et al., 2005), and detrimental effects on humans and the environment (Michalaki et al., 2007). Therefore, efforts have been directed at evaluating nontoxic and environmentally-friendly techniques to control stored-grain pests. Entomopathogenic fungi have been considered as an alternative or complementary treatment (Moino et al., 1998; Michalaki et al., 2007; Sewify et al., 2014; Wakil & Schmitt, 2014) because of their natural occurrence, and low hazard towards human and the environment (Moore et al., 2000). Numerous studies have established the potential of entomopathogenic fungi as bioinsecticides against various insect pests of stored products (Cherry et al., 2005; Wakil & Ghazanfar, 2010; Shams et al., 2011; Barra et al., 2013; Khashaveh & Chelav, 2013; Sewify et al., 2014). Several other studies have also shown the potential of entomopathogenic fungi in combination with diatomaceous earth (Athanassiou & Steenberg, 2007; Athanassiou et al., 2008; Wakil et al., 2011; Riasat et al., 2011, 2013; Shafighi et al., 2014). The majority of research examining entomopathogenic fungi and stored-grain pests has been conducted with fungi isolated from sources other than the stored-grain pests themselves. Although the existence of entomopathogenic fungi in nature is well known, the extent and the manner of their distribution in stored-grain pest populations have received little attention. Odour et al. (2000) conducted a survey of *Beauveria bassiana* (Bals.-Criv.) Vuill. infections in pests of stored maize in Kenya, and Wakil et al. (2014) searched for entomopathogenic fungi infecting stored-grain insects in Pakistan. In the Mycopest Project, eight *B. bassiana* isolates were collected from UK grain stores (Wakefield et al., 2005). A better understanding of the natural occurrence of these fungal pathogens in their intended ultimate application ecosystem, grain stores, can be valuable in developing a fungus-based control strategy against stored-grain pests. To meet this objective, in the current study, naturally occurring fungal pathogens of stored-grain pests were identified and quantified from wheat (*Triticum aestivum* L. and *Triticum durum* Desf.) and maize (*Zea mays* L.) bulk stores in five cities in Turkey using different sampling techniques.

Materials and Methods

Sampling stored-grain insects

Insects were sampled from stored wheat and maize in five cities (Şanlıurfa, Kahramanmaraş, Adana, Mersin and Konya) in Central and South Anatolia, Turkey monthly from June to November 2013. In each month, a minimum of ten storage facilities in each city were sampled using three sampling techniques; (1) probing with a 2 m long metal grain probe in various sites of the bulk grains, yielding a total of 5 kg of grain, (2) trapping with five probe pitfall traps (Storgard WB Probe® II, Trécé Inc., Salinas, California, USA) in grain bulk, and (3) visual inspection of the facilities. Insect samples collected by trapping and visual inspection were placed in about 1 kg of grain stored in the facilities from which the samples were taken. Samples were put in plastic containers for transport back to the laboratory. Insects present in grain samples were separated by sieving (10, 18 and 35 mesh metal sieves, Retsch, Haan, Germany). Insect cadavers were taken and live insects were returned to the grain for incubation at 26±2°C, 65±5% RH in darkness for 1 month. Thereafter, the grain samples were sieved again to collect cadavers of those that had died during the incubation period. Following each examination, the insect cadavers were stored at 4°C until processed.

Isolation of fungi

Collected insects were identified according to taxonomic keys published by Gorham (1991) and Rees (2004). The collected cadavers were surface sterilized according to the procedure of Lacey & Brooks (1997) before incubation in humid chambers (sealed sterile Petri dishes lined with sterile damp filter paper) to promote fungal growth and sporulation on the surface of the cadavers. The chambers were kept at 26±2°C with a 16L:8D h photoperiod. The cadavers were checked daily and isolations were performed from those with fungal sporulation. Potato dextrose agar (PDA, Merck 1.10130, Darmstadt, Germany) supplemented with 0.6 g/L streptomycin sulfate and 10^5 IU/L penicillin was used for isolation

and PDA alone for subcultures. Once the purity of the cultures was ensured, lyophilized samples were deposited in the entomopathogenic fungal culture collection of the Department of Plant Protection, University of Kahramanmaraş Sütçü İmam, Turkey.

Identification of fungi

Morphological characteristics of fungi in cultures and on slides were combined with molecular techniques for precise identification to species (Humber, 1997; Luangsa-ard et al., 2011; Rehner et al., 2011). Fungal mycelia were grown in 1/4 strength Sabouraud dextrose broth plus yeast extract (0.5%) at 26±2°C on an orbital shaker for 4-6 days and subsequently harvested by filtration. The mycelial mat was lyophilized and 50 mg was ground into powder using a mortar and pestle. A CTAB procedure (Sirohi et al., 2013) with some modifications was followed to obtain genomic DNA. The powder was transferred to 1 ml CTAB buffer (10 ml 1M Tris HCL, pH 8.0; 28 ml 5 M NaCl; 4 ml 0.5 M EDTA; 2 g CTAB; completed to 100 ml with distilled deionized H_2O) and kept at 70°C for 75 min. After addition of 500 µl chloroform, the sample was further incubated for 7-8 min at 70°C, followed with 10 min centrifugation at 10,000 g. A 500 µl aliquot of the supernatant with 300 µl of isopropanol added was chilled at -20°C for 30 min before centrifuging for 10 min at 10,000 g. The precipitated DNA was washed using 200 µl of 70% ethanol, dried and resuspended in 50 µl of TE buffer (1 ml 1 M Tris HCl pH 8.0; 0.2 ml 0.5 M EDTA; completed to 100 ml with distilled deionized H_2O), and placed at -20°C until further processing. ITS1-5.8S-ITS2 sequences of the isolates were amplified using primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The PCR reaction mixture included 1 µl of each primer (20 pmol), 1 µl dNTP (1 mM), 0.5 µl Taq DNA Polimeraz (5 U/µl), 4 µl Taq buffer (10X), 32 µl distilled deionized H_2O. The DNA was denatured at 95°C for 5 min, followed by 35 cycles of amplification: 30 s at 95°C, 30 s at 50°C and 1 min at 72°C with final extension for 10 min at 72°C by using ABI Veriti Thermal Cycler 9902 (Applied Biosystems, Foster City, CA, USA). The PCR products were sent to MedSanTek (İstanbul, Turkey) for forward and reverse sequencing.

Statistical analysis

Forward and reverse sequences of each isolate were combined using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Isolates from the same species were aligned and those with the same sequences were grouped in clades. One isolate from each clade was chosen to represent the clade in the subsequent analyses. The Mega 6.06 (http://www.megasoftware.net) was used to align isolated sequences with representative sequences described by Rehner et al. (2011) for *Beauveria* and Luangsa-ard et al. (2011) for *Purpureocillium*. To determine the taxonomic positions of the fungal isolates, maximum likelihood and bootstrapping analyses were conducted using Mega 6.06. The trees were rooted using isolates from other genera of entomopathogenic fungi. Frequency of fungal infection was calculated for each host insect. Using Minitab16 (http://www.minitab.com/en-us/), contingency table analyses were employed to examine the effects of insect sampling time, site, host insects, sampling technique, examination time of the samples, and the commodities, from which samples were taken, on the variation of fungal infection. In order to avoid cells having expected frequencies <1, in the contingency table for host insects, *Sitophilus* and *Tribolium* spp. were pooled separately to genera, and the smallest two groups (*Latheticus oryzae* and Cryptophagidae) were excluded.

Results

A total of 126 fungi were isolated from 85,155 cadavers sampled from stored bulk grain from 5 cities and 87 sampling sites (61 wheat and 26 maize). Amplification of ITS1-5.8S-ITS2 region of genomic DNAs of *B. bassiana* (Bals.-Criv.) Vuill*., Beauveria varroae* Rehner & Humberand and *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson isolates produced 593, 594 and 620 bp sequences, respectively. Each *Beauveria* species had two sequences with only one nucleotide difference (Figures 1, 2) and thus each species was grouped into two clades. One isolate from each clade was chosen as a representative sample for further analyses. All of the *P. lilacinum* sequences were identical and one isolate was used as the representative in further phylogenetic analyses. Figures 3 and 4 illustrate the taxonomic positions within related genera. The sequences of representing isolates were deposited in NCBI GenBank with the following accession numbers; NCBI GenBank accession numbers for *B. bassiana* 151138, 54276, *B. varroae* 35727, 16787 and *P. lilacinum* 135233 were KU687110, KU687111, KU687112, KU687113, KU687114, respectively.

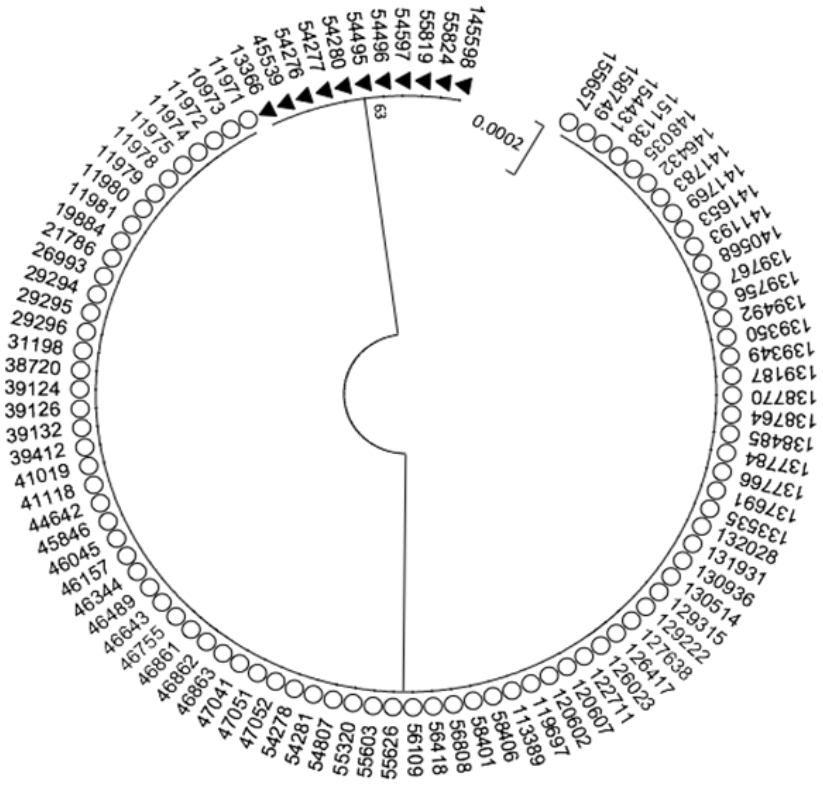

Figure 1. Two clades of *Beauveria bassiana* isolates based on ITS1-5.8S-ITS2 sequences; isolates marked with circles form clade Bb1 and those with triangles form clade Bb2.

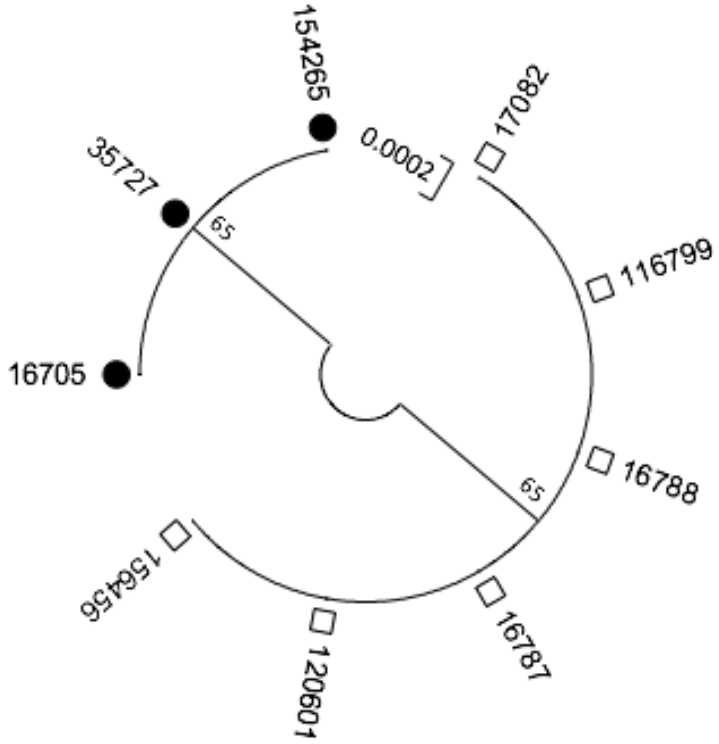

Figure 2. Two clades of *Beauveria varroae* isolates based on ITS1-5.8S-ITS2 sequences; isolates marked with filled circles form clade Bv1 and those marked with squares form clade Bv2.

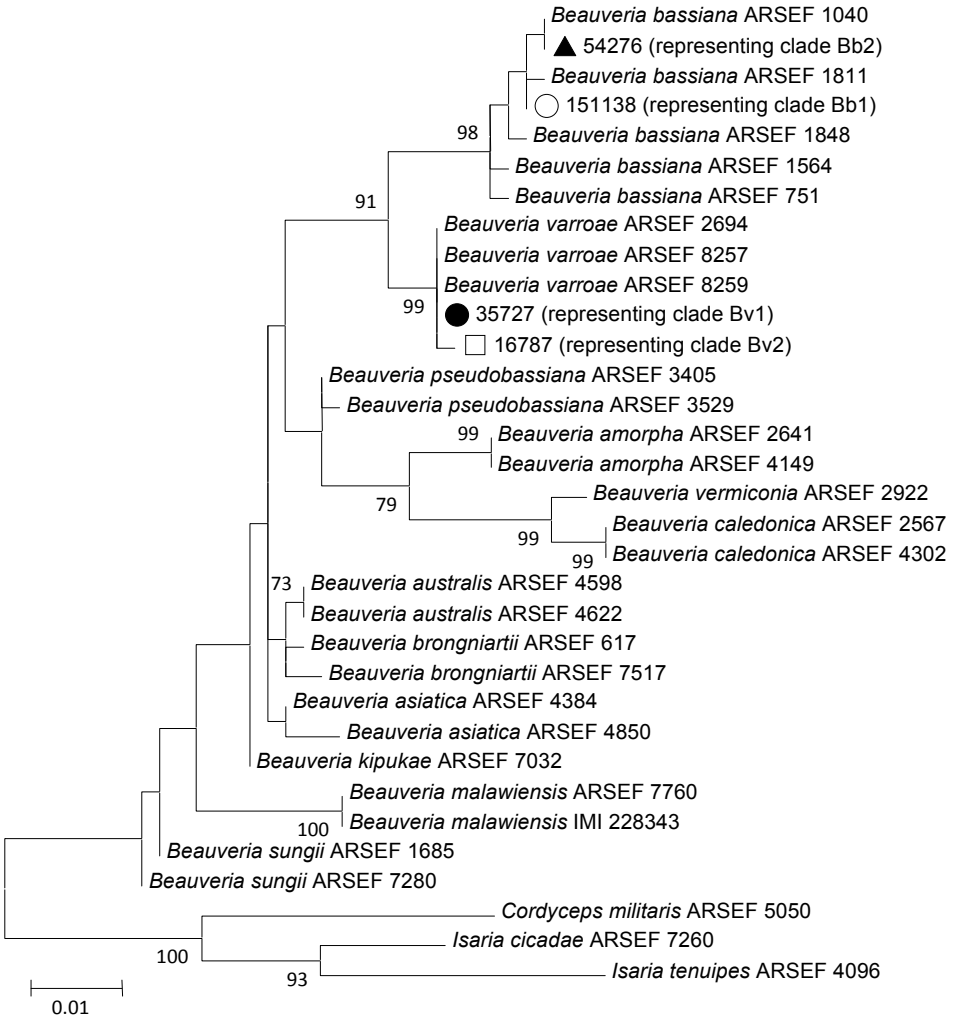

Figure 3. Phylogenetic position of isolates belonging to the genus *Beauveria* based on ITS1-5.8S-ITS2 sequences. Bootstrap values ≥70 are labeled.

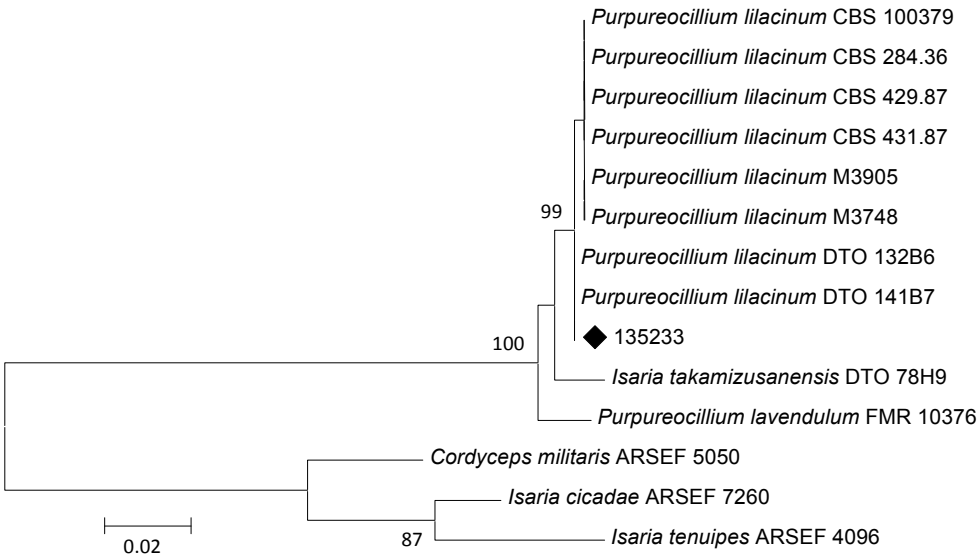

Figure 4. Phylogenetic position of isolates belonging to the genus *Purpureocillium* based on ITS1-5.8S-ITS2 sequences (Isolate 135233 represents all *Purpureocillium* isolates, which have the same sequences). Bootstrap values ≥70 are labeled.

Sampling parameters including fungal isolate identification, insect host, grain type, location and time of isolation are given in Table 1. The majority of isolates (97) were identified as *B. bassiana*, followed by *P. lilacinum* with 20 isolates and *B. varroae* with 9 isolates (Table 2). According to the insect host infection distribution, the highest frequency of *B. bassiana* isolation was from *Tribolium* spp., followed by *Sitophilus* spp., *Cryptolestes ferrugineus* and *Rhyzopertha dominica*. *Beauveria varroae* isolates were obtained only from *C. ferrugineus*, *R. dominica*, *Sitophilus oryzae* and *Trogoderma* specimens. The highest infection frequency of *P. lilacinum* was in *C. ferrugineus* followed by *R. dominica* (Table 2).

Table 1. Details of entomopathogenic fungal isolates recovered from collected stored-grain pests

Isolate number	Fungus species	Host insect species	Grain type	City	Sampling date
0973	*Beauveria bassiana*	*Tribolium castaneum*	wheat	Konya	18.06.2013
11971	*B. bassiana*	*T. castaneum*	wheat	Mersin	19.06.2013
11972	*B. bassiana*	*Sitophilus oryzae*	wheat	Mersin	19.06.2013
11974	*B. bassiana*	*S. oryzae*	wheat	Mersin	19.06.2013
11975	*B. bassiana*	*S. oryzae*	wheat	Mersin	19.06.2013
11978	*B. bassiana*	*T. castaneum*	wheat	Mersin	19.06.2013
11979	*B. bassiana*	*Rhyzopertha dominica*	wheat	Mersin	19.06.2013
11980	*B. bassiana*	*R. dominica*	wheat	Mersin	19.06.2013
11981	*B. bassiana*	*T. castaneum*	wheat	Mersin	19.06.2013
13366	*B. bassiana*	*S. oryzae*	wheat	Adana	20.06.2013
16705	*Beauveria varroae*	*Trogoderma* sp.	wheat	Konya	16.07.2013
16787	*B. varroae*	*Cryptolestes ferrugineus*	wheat	Konya	16.07.2013
16788	*B. varroae*	*R. dominica*	wheat	Konya	16.07.2013
17082	*B. varroae*	*Trogoderma granarium*	wheat	Konya	16.07.2013
19884	*B. bassiana*	*T. castaneum*	wheat	Mersin	18.07.2013
21786	*B. bassiana*	*C. ferrugineus*	maize	Adana	19.07.2013
26993	*B. bassiana*	*T. castaneum*	wheat	Konya	21.08.2013
29294	*B. bassiana*	*C. ferrugineus*	wheat	Adana	23.08.2013
29295	*B. bassiana*	*S. oryzae*	wheat	Adana	23.08.2013
29296	*B. bassiana*	*Oryzaephilus surinamensis*	wheat	Adana	23.08.2013
31198	*B. bassiana*	*T. castaneum*	maize	K.Maraş	27.08.2013
31304	*Purpureocillium lilacinum*	*S. oryzae*	mixed	K.Maraş	27.08.2013
35727	*B. varroae*	*R. dominica*	wheat	Konya	18.09.2013
35937	*P. lilacinum*	*C. ferrugineus*	wheat	Konya	18.09.2013
38720	*B. bassiana*	*T. castaneum*	wheat	Adana	20.09.2013
38813	*P. lilacinum*	Cryptophagidae	wheat	Adana	20.09.2013
39124	*B. bassiana*	*R. dominica*	wheat	Adana	20.09.2013

Table 1. (Continued)

Isolate number	Fungus species	Host insect species	Grain type	City	Sampling date
39126	*B. bassiana*	*S. oryzae*	wheat	Adana	20.09.2013
39132	*B. bassiana*	*T. castaneum*	wheat	Adana	20.09.2013
39412	*B. bassiana*	*R. dominica*	wheat	Adana	20.09.2013
41019	*B. bassiana*	*T. castaneum*	wheat	Şanlıurfa	27.09.2013
41118	*B. bassiana*	*C. ferrugineus*	wheat	Şanlıurfa	27.09.2013
41121	*P. lilacinum*	*T. castaneum*	wheat	Şanlıurfa	27.09.2013
44642	*B. bassiana*	*Sitophilus* sp.	wheat	Mersin	12.10.2013
45539	*B. bassiana*	*T. castaneum*	maize	Mersin	12.10.2013
45846	*B. bassiana*	*T. castaneum*	wheat	Adana	25.10.2013
46045	*B. bassiana*	*C. ferrugineus*	wheat	Adana	25.10.2013
46157	*B. bassiana*	*T. castaneum*	wheat	Adana	25.10.2013
46344	*B. bassiana*	*O. surinamensis*	wheat	Adana	25.10.2013
46489	*B. bassiana*	*C. ferrugineus*	wheat	Adana	25.10.2013
46643	*B. bassiana*	*T. castaneum*	wheat	Adana	25.10.2013
46755	*B. bassiana*	*S. oryzae*	wheat	Adana	25.10.2013
46861	*B. bassiana*	*C. ferrugineus*	wheat	Adana	25.10.2013
46862	*B. bassiana*	*C. ferrugineus*	wheat	Adana	25.10.2013
46863	*B. bassiana*	*T. castaneum*	wheat	Adana	25.10.2013
47041	*B. bassiana*	*Tribolium* sp.	wheat	Adana	25.10.2013
47051	*B. bassiana*	*S. oryzae*	wheat	Adana	25.10.2013
47052	*B. bassiana*	*S. oryzae*	wheat	Adana	25.10.2013
48423	*P. lilacinum*	*T. castaneum*	wheat	K.Maraş	20.10.2013
54276	*B. bassiana*	*T. castaneum*	wheat	Mersin	09.11.2013
54277	*B. bassiana*	*T. castaneum*	wheat	Mersin	09.11.2013
54278	*B. bassiana*	*T. castaneum*	wheat	Mersin	09.11.2013
54280	*B. bassiana*	*C. ferrugineus*	wheat	Mersin	09.11.2013
54281	*B. bassiana*	*S. oryzae*	wheat	Mersin	09.11.2013
54495	*B. bassiana*	*T. castaneum*	wheat	Mersin	09.11.2013
54496	*B. bassiana*	*S. oryzae*	wheat	Mersin	09.11.2013
54597	*B. bassiana*	*T. castaneum*	wheat	Mersin	10.11.2013
54807	*B. bassiana*	*Sitophilus* sp.	wheat	Mersin	10.11.2013
55320	*B. bassiana*	*C. ferrugineus*	wheat	Mersin	10.11.2013

Table 1. (Continued)

Isolate number	Fungus species	Host insect species	Grain type	City	Sampling date
55321	*P. lilacinum*	*C. ferrugineus*	wheat	Mersin	10.11.2013
55322	*P. lilacinum*	*C. ferrugineus*	wheat	Mersin	10.11.2013
55603	*B. bassiana*	*Latheticus oryzae*	wheat	Adana	23.11.2013
55604	*P. lilacinum*	*L. oryzae*	wheat	Adana	23.11.2013
55610	*P. lilacinum*	*O. surinamensis*	wheat	Adana	23.11.2013
55615	*P. lilacinum*	*C. ferrugineus*	wheat	Adana	23.11.2013
55626	*B. bassiana*	*R. dominica*	wheat	Adana	23.11.2013
55627	*P. lilacinum*	*R. dominica*	wheat	Adana	23.11.2013
55819	*B. bassiana*	*T. castaneum*	maize	Adana	23.11.2013
55824	*B. bassiana*	*S. oryzae*	maize	Adana	23.11.2013
56109	*B. bassiana*	*R. dominica*	wheat	Adana	24.11.2013
56418	*B. bassiana*	*C. ferrugineus*	wheat	Adana	24.11.2013
56717	*P. lilacinum*	*S. oryzae*	wheat	Adana	24.11.2013
56808	*B. bassiana*	*O. surinamensis*	wheat	Adana	24.11.2013
57202	*P. lilacinum*	*R. dominica*	wheat	Adana	23.11.2013
58401	*B. bassiana*	*R. dominica*	wheat	K.Maraş	16.11.2013
58406	*B. bassiana*	*T. castaneum*	wheat	K.Maraş	16.11.2013
59552	*P. lilacinum*	*Sitophilus* sp.	wheat	K.Maraş	16.11.2013
60778	*P. lilacinum*	*T. granarium*	wheat	Şanlıurfa	03.11.2013
113389	*B. bassiana*	*S. oryzae*	wheat	Adana	20.06.2013
116799	*B. varroae*	*T. granarium*	wheat	Konya	16.07.2013
119697	*B. bassiana*	*T. castaneum*	wheat	Mersin	18.07.2013
120601	*B. varroae*	*C. ferrugineus*	wheat	Adana	19.07.2013
120602	*B. bassiana*	*C. ferrugineus*	wheat	Adana	19.07.2013
120607	*B. bassiana*	*O. surinamensis*	wheat	Adana	19.07.2013
122711	*B. bassiana*	*Tribolium confusum*	wheat	K.Maraş	25.07.2013
126023	*B. bassiana*	*C. ferrugineus*	wheat	Konya	21.08.2013
126417	*B. bassiana*	*T. castaneum*	wheat	Konya	21.08.2013
127638	*B. bassiana*	*S. oryzae*	wheat	Mersin	22.08.2013
129216	*P. lilacinum*	*C. ferrugineus*	wheat	Adana	23.08.2013
129222	*B. bassiana*	*R. dominica*	wheat	Adana	23.08.2013
129315	*B. bassiana*	*S. oryzae*	wheat	Adana	23.08.2013

Table 1. (Continued)

Isolate number	Fungus species	Host insect species	Grain type	City	Sampling date
130514	*B. bassiana*	*T. castaneum*	wheat	Adana	23.08.2013
130936	*B. bassiana*	*R. dominica*	wheat	K.Maraş	27.08.2013
131931	*B. bassiana*	*S. oryzae*	wheat	K.Maraş	27.08.2013
132028	*B. bassiana*	*R. dominica*	wheat	K.Maraş	27.08.2013
133535	*B. bassiana*	*S. oryzae*	wheat	K.Maraş	27.08.2013
133630	*P. lilacinum*	*O. surinamensis*	wheat	K.Maraş	27.08.2013
135233	*P. lilacinum*	*R. dominica*	wheat	Şanlıurfa	28.08.2013
137691	*B. bassiana*	*R. dominica*	wheat	Mersin	19.09.2013
137766	*B. bassiana*	*S. oryzae*	wheat	Mersin	19.09.2013
137784	*B. bassiana*	*C. ferrugineus*	wheat	Mersin	19.09.2013
138485	*B. bassiana*	*O. surinamensis*	wheat	Mersin	19.09.2013
138764	*B. bassiana*	*C. ferrugineus*	wheat	Adana	20.09.2013
138770	*B. bassiana*	*Sitophilus* sp.	wheat	Adana	20.09.2013
139187	*B. bassiana*	*O. surinamensis*	wheat	Adana	20.09.2013
139349	*B. bassiana*	*R. dominica*	wheat	Adana	20.09.2013
139350	*B. bassiana*	*R. dominica*	wheat	Adana	20.09.2013
139492	*B. bassiana*	*C. ferrugineus*	wheat	Adana	20.09.2013
139756	*B. bassiana*	*C. ferrugineus*	wheat	Adana	20.09.2013
139767	*B. bassiana*	*T. castaneum*	wheat	Adana	20.09.2013
139988	*P. lilacinum*	*Sitophilus* sp.	wheat	Adana	20.09.2013
140568	*B. bassiana*	*C. ferrugineus*	wheat	Şanlıurfa	27.09.2013
141193	*B. bassiana*	*L. oryzae*	wheat	Şanlıurfa	27.09.2013
141653	*B. bassiana*	*R. dominica*	wheat	K.Maraş	20.09.2013
141769	*B. bassiana*	*Sitophilus granarius*	wheat	K.Maraş	20.09.2013
141783	*B. bassiana*	*R. dominica*	wheat	K.Maraş	20.09.2013
145598	*B. bassiana*	*T. castaneum*	maize	Mersin	12.10.2013
146432	*B. bassiana*	*S. oryzae*	wheat	Adana	25.10.2013
148035	*B. bassiana*	*T. castaneum*	wheat	K.Maraş	20.10.2013
151138	*B. bassiana*	*R. dominica*	wheat	Şanlıurfa	06.10.2013
154265	*B. varroae*	*S. oryzae*	wheat	Mersin	09.11.2013
154431	*B. bassiana*	*T. castaneum*	wheat	Mersin	09.11.2013
155657	*B. bassiana*	*T. castaneum*	wheat	Adana	23.11.2013
156456	*B. varroae*	*S. oryzae*	wheat	Adana	24.11.2013
158749	*B. bassiana*	*T. castaneum*	maize	K.Maraş	17.11.2013
160466	*P. lilacinum*	*R. dominica*	wheat	Şanlıurfa	02.11.2013

Table 2. Total number of insects examined and frequency of insects infected by entomopathogenic fungi

Host insects	Number of insects examined	*Beauveria bassiana* infections		*Beauveria varroae* infections		*Purpureocillium lilacinum* infections		Total fungal infections	
		Number of insects	Frequency (%)	Number of insects	Frequency (%)	Number of insects	Frequency (%)	Number of insects	Frequency (%)
Cryptolestes ferrugineus	18427	17	17.5	2	22.2	5	25.0	24	19.0
Oryzaephilus surinamensis	5954	6	6.2	0	0.0	2	10.0	8	6.3
Rhyzopertha dominica	16138	16	16.5	2	22.2	4	20.0	22	17.5
Sitophilus granarius	2069	1	1.0	0	0.0	0	0.0	1	0.8
Sitophilus oryzae	24144	18	18.6	2	22.2	2	10.0	23	18.3
Sitophilus spp.	826	4	4.1	0	0.0	2	10.0	5	4.0
Tribolium castaneum	11731	31	32.0	0	0.0	2	10.0	33	26.2
Tribolium confusum	2641	1	1.0	0	0.0	0	0.0	1	0.8
Tribolium spp.	62	1	1.0	0	0.0	0	0.0	1	0.8
Trogoderma granarium	1404	0	0.0	2	22.2	1	5.0	2	1.6
Trogoderma spp.	280	0	0.0	1	11.1	0	0.0	2	1.6
Latheticus oryzae	603	2	2.1	0	0.0	1	5.0	3	2.4
Cryptophagidae	76	0	0.0	0	0.0	1	5.0	1	0.8

Contingency table analyses revealed that fungal infection of stored-grain pests did not vary significantly with time of sampling within individual grain storage sites (Table 3). The occurrence of fungal infection, however, differed significantly within sampling sites, with the highest variation seen in Adana (X^2=7.11) where actual occurrence of infection was higher than the expected frequency (Table 4). In the follow-up chi-square test excluding Adana (X^2=2.22 and p=0.528) the variation was not significant between the other sites. There was also significant variation between fungal infection within host insect (Table 5), with the main source of variation came from *Tribolium* spp. (X^2=9.27) in which a higher than expected frequency was recorded. The follow-up chi-square test after elimination of *Tribolium* spp. indicated that the variation within the other host insects was not significant (X^2=2.62 and p=0.623). Insect sampling technique was another factor resulting in significant variation in the total observed fungal infections and in those recorded from the cadavers collected before and after incubation (Table 6). In all cases, the major source of variability was probing technique for which the frequency of fungus-infected insects was much higher than the expected. X^2 and p values of follow-up chi-square tests (probing eliminated) were 0.611 and 0.434 for cadavers collected before incubation, 1.260 and 0.262 after incubation, 1.850 and 0.174 for total infections, respectively. Fungus isolation from the cadavers separated from grain before incubation did not significantly differ from those cadavers obtained after incubation period regardless of sampling technique (Table 7). Similarly, the frequency of fungal infection in pests collected from wheat and maize did not vary significantly, regardless of the time of cadavers were collected from the grain samples (Table 8).

Table 3 Contingency table for the variation of fungal infections due to insect sampling dates (expected values are given in parentheses)

Sampling date	FI**	NI**	N_i	X^2
June	11 (8)	5063	5074	1.626
July	12 (13)	8558	8570	0.037
August	19 (23)	15195	15214	0.549
September	29 (31)	20849	20878	0.116
October	20 (24)	16103	16123	0.624
November	35 (29)	19261	19296	1.458
N_i	126	85029	85155	
X^2	4.404	0.006		4.411*

*p=0,492; **FI: individuals from which entomopathogenic fungi were isolated; NI: individuals not infected by entomopathogenic fungi.

Table 4 Contingency table for the variation of fungal infections due to insect collection sites (expected values are given in parentheses)

Collection site	FI**	NI**	N_i	X^2
Adana	57 (40)	27054	27111	7.112
Konya	11 (13)	8461	8472	0.189
Kahramanmaraş	17 (27)	17942	17959	3.456
Mersin	32 (33)	22576	22608	0.063
Şanlıurfa	9 (13)	8983	8992	1.396
N_i	126	85016	85142	
X^2	12.198	0.018		12.216*

*p=0,016; **FI: individuals from which entomopathogenic fungi were isolated; NI: individuals not infected by entomopathogenic fungi.

Table 5 Contingency table for the variation of fungal infections due to host insect species (expected values are given in parentheses)

Host insects	FI**	NI**	N_i	X^2
Cryptolestes ferrugineus	24 (27)	18403	18427	0.306
Oryzaephilus surinamensis	8 (9)	5946	5954	0.053
Rhyzopertha dominica	22 (24)	16116	16138	0.099
Sitophilus spp.	29 (39)	27011	27040	2.760
Tribolium spp.	35 (21)	14401	14436	9.265
Trogoderma granarium	4 (2)	1681	1685	0.971
N_i	122	83558	83680	
X^2	13.435	0.019		13.454*

*p=0,019; **FI: individuals from which entomopathogenic fungi were isolated; NI: individuals not infected by entomopathogenic fungi.

Table 6. Contingency table for the variation of fungal infections due to insect sampling technique, which were examined before and after one month of incubation (expected values are given in parentheses)

	Individuals obtained before incubation				Individuals obtained after incubation				Total individuals examined			
Sampling technique	FI**	NI**	N_i	X^2	FI**	NI**	N_i	X^2	FI**	NI**	N_i	X^2
Probe	23 (6)	4435	4458	43.919	10 (3)	1698	1708	19.802	33 (9)	6133	6166	62.578
Visual	44 (55)	38507	38551	2.121	36 (39)	24947	24983	0.302	80 (94)	63454	63534	2.09
Trap	9 (15)	10476	10485	2.337	4 (8)	4966	4970	1.89	13 (23)	15442	15455	4.264
N_i	76	53418	53494		50	31611	31661		126	85029	85155	
X^2	48.309	0.068		48.377*	21.96	0.034		21.996*	68.83	0.102		68.993*

*p<0,001; **FI: individuals from which entomopathogenic fungi were isolated; NI: individuals not infected by entomopathogenic fungi.

Table 7. Contingency table for the variation of fungal infections due to examination time of the grain samples taken by three different sampling techniques (expected values are given in parentheses)

	Probe sampling				Visual sampling				Trap sampling				Total (Probe+Visual+Trap)			
Time of examination	FI**	NI**	N_i	X^2	FI**	NI**	N_i	X^2	FI**	NI**	N_i	X^2	FI**	NI**	N_i	X^2
Before incubation	23 (24)	4435	4458	0.03	44 (49)	38507	38551	0.43	9 (9)	10476	10485	0.004	76 (79)	53418	53494	0.126
After incubation	10 (9)	1698	1708	0.08	36 (31)	24947	24983	0.66	4 (4)	4966	4970	0.008	50 (47)	31611	31661	0.212
N_i	33	6133	6166		80	63454	63534		13	15442	15455		126	85029	85155	
X^2	0.11	0.00		0.11*		1.08	0.00	1.08*		0.012	0.000	0.012*	0.338	0.000		0.338*

*respectively p=0.738, p=0.298, p=0.915, p=0.561; **FI: individuals from which entomopathogenic fungi were isolated; NI: individuals not infected by entomopathogenic fungi

Table 8. Contingency table for the variation of fungal infections due to type of sampled grains from which insects were collected before and after one month of incubation (expected values are given in parentheses)

	Individuals obtained before incubation				Individuals obtained after incubation				Total individuals examined			
Grain	FI**	NI**	N_i	X^2	FI**	NI**	N_i	X^2	FI**	NI**	N_i	X^2
Maize	5 (7)	5106	5111	0.705	2 (4)	2332	2334	0.772	7 (11)	7438	7445	1.466
Wheat	71 (69)	48312	48383	0.074	48 (46)	29279	29327	0.061	119 (115)	77591	77710	0.14
N_i	76	53418	53494		50	31611	31661		126	85029	85155	
X^2	0.778	0.001		0.78*	0.832	0.001		0.834*	1.604	0.002		1.607*

*respectively p=0.377, p=0.361, p=0.205; **FI: individuals from which entomopathogenic fungi were isolated; NI: individuals not infected by entomopathogenic fungi.

Discussion

The occurrence of entomopathogenic fungi in soil and in populations of pest insects from cropping areas has been examined in many studies; however, fungal pathogens of stored-product pest populations have received little attention particularly within the grain storage facilities themselves. Odour et al. (2000) recovered 26 *B. bassiana* isolates from insect pests of stored maize, Wakefield et al. (2005) obtained 8 *B. bassiana* isolates and Wakil et al. (2014) 26 fungal pathogens from stored-grain insects. This rather low occurrence is comparable to our 126 isolates, considering the high number of insects processed (frequency < 0.0015). These results, together with our observation that none of the insects collected from storage facilities showed any external evidence of fungal growth, suggest that entomopathogenic fungi occur at low frequency within stored-grain pest populations. This could be due to abiotic stress on the fungi, particularly low humidity, which could limit the ability of the fungus to persist in the stored grain, and/or a consequence of microbial defensive systems employed by stored-grain pests (Ortiz-Urquiza & Keyhani, 2013, 2015). The most commonly encountered fungal species was *B. bassiana* in both our study and that of Wakil et al. (2014). In addition, the frequency of host insect species from which pathogenic fungi were isolated was similar in our study and Wakil (2014). *Tribolium castaneum* was found to be the most frequently infected insect species. Thus although *T. castaneum* represented about 13.8% of the total insects examined, the frequency of fungal isolation from this insect accounted for about 24% of the total (31/126). *Tribolium* spp. are known to be difficult to kill with entomopathogenic fungi in laboratory bioassays (Rice & Cogburn, 1999; Padin et al., 2002; Akbar et al., 2004; Wakefield et al., 2005; Wakefield, 2006; Michalaki et al., 2006, 2007). This was recently shown to be due in part to the production of cuticular defensive secretions that inhibit growth of *B. bassiana* and other microbes (Pedrini et al, 2015). Wakil et al. (2014) also found differences in the number of fungal isolates with respect to the location where they had been sampled and a similar variation was noted in our study, especially in sites sampled in the city of Adana, which contributed most of the observed variation. Our work has provided a set of entomopathogenic fungi derived from the stored-grain pests directly. Future experiments comparing these isolates to wild types may provide clues as to whether these isolates have adapted to the stored grain environment and/or to the insects themselves or whether they represent low residual opportunistic infections. These data could have implications in developing isolates that may be more useful in stored grain pest control applications.

To the best of our knowledge, this is the first record of *B. varroae* infection in stored-product pest insects. This could be partially due to the technique used in previous studies to identify isolated *Beauveria* species, as some species cannot be easily distinguished according to their morphological features (Rehner et al., 2011). Another reason could be that there has been insufficient scrutiny of naturally occurring fungal pathogens of stored-product pests, indicating the importance of further investigation.

Odour et al. (2000) recommended using dead insects when collected rather than those that died during incubation in the laboratory. However, according to our results, isolates could also be obtained from insects that died during the one month of incubation. Incubation conditions may also alter the outcome, as Odour et al. (2000) incubated the samples under room conditions, whereas in this study the samples were incubated at 26±2°C, 65±5% RH in darkness. It is not clear if some live insects were already infected at collection or if they were infected during incubation. However, our data suggest a potential for increased isolation of resident fungal insect pathogens with incubation after sampling.

Sampling time did not affect the frequency of isolation of fungal pathogens, but the sampling techniques employed had a statistically significant effect. Collection of fungal infected insects by probing was more effective than the other techniques used. Assuming the insects that have died from fungal infection are distributed evenly in the bulk grain, probing can potentially allow their retrieval in a more representative manner, whereas the other techniques concentrated more on active insects and may therefore cause bias.

This and previous work have shown that stored-grain insects are naturally infected by entomopathogenic fungi in storage facilities but occur at low frequencies in the insect populations. A better understanding of this ecosystem for entomopathogenic fungi and their activities, together with a complete list of naturally existing insect pathogenic fungal species, may have important implications for developing microbial control strategies based on these fungi.

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