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Efficacies of Entomopathogenic Fungi from *Metarhizium*, *Beauveria* and *Isaria* on German cockroach, *Blattella germanic*a (L.) (Blattaria: Blattellidae)

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ABSTRACT

Blattella germanica is an important pest and able to transmit various pathogens and parasites of human, cause allergic reactions and food contamination. Due to risks and problems related to currently employed chemicals for their control, use of entomopathogenic fungi as alternative control approach has been under investigation. In this study, seven entomopathogenic fungi isolated from soil samples and five fungal isolates obtained from entomopathogenic fungal cultures have been tested against *B. germanica*. The fungi belong to the species *Beauveria bassiana* (3 isolates), *Metarhizium anisopliae* (4 isolates), *Isaria tenuipes* (1 isolate), *I. fumosorosea* (3 isolates), and *I. farinosa* (1 isolate). Conidial suspensions of all twelve isolates were applied at $1x10^7$ conidia ml^{-1} concentration to ten insects in each replication. Tests were carried out at 26±2°C, 65±5% relative humidity in darkness with three replications. Eight isolates were found effective on *B. germanica* with mortalities varying between 60.0% and 96.7% in 14 days. The results indicated that *Metarhizium* isolates had the highest efficacies (83.3-95.8%) followed by *Beauveria* isolates (70.8-79.2%) and the isolates of *Isaria* had the lowest efficacies (50.0-70.8%). These isolates were as effective as the previously reported isolates of the same species with higher efficacy values. The most effective two isolates of *M. anisopliae* (S8-2 and S11-6) appear to be worth investigating further for developing a microbial control agent against cockroaches.

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Metarhizium, *Beauveria* ve *Isaria*'ya Bağlı Entomopatojen Fungusların Alman Hamamböceğine, *Blattella germanic*a (L.) (Blattaria: Blattellidae), Etkinlikleri

ÖZET

Blattella germanica önemli bir zararlı olup insanların patojen ve parazitlerini taşıyabilmekte, alerjik reaksiyonlara ve yiyecek kontaminasyonlarına neden olabilmektedir. Mücadelesinde bugün için kullanılan kimyasallarla ilişkili riskler ve problemlerden dolayı alternatif yaklaşım olarak entomopatojen fungusların kullanımı araştırılmaktadır. Bu çalışmada, toprak örneklerinden izole edilmiş yedi ve entomopatojen fungus kültürlerinden beş entomopatojen fungus izolatı *B. germanica*'ya karşı test edilmiştir. Bu funguslar *Beauveria bassiana* (3 izolat), *Metarhizium anisopliae* (4 izolat), *Isaria tenuipes* (1 izolat), *I. fumosorosea* (3 izolat), ve *I. farinosa* (1 izolat) türlerinde yer almaktadır. Tüm izolatlara ait konidi süspansiyonları $1x10^7$ konidi ml^{-1} konsantrasyonunda her tekerrürde on böceğe uygulanmıştır. Testler 26±2°C, %65±5 nispi nemde karanlıkta üç tekerrürlü olarak yürütülmüştür. *B. germanica*' ya karşı etkili bulunan sekiz izolat 14 günde %60.0 ve %96.7 arasında değişen ölümlere neden olmuştur. Sonuçlar *Metarhizium* izolatlarının en yüksek etkinliğe (%83.3-95.8) sahip olduğunu, bunu *Beauveria* izolatlarının (%70.8-79.2) izlediğini ve en düşük etkinliğe *Isaria* izolatlarının (%50.0-70.8) sahip olduğunu işaret etmektedir.

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Bu izolatlar aynı türden raporlanmış önceki izolatlar kadar virülent olup daha yüksek etkinlik değerleri elde edilmiştir. *M. anisopliae*' nın en etkili iki izolatı (S8-2 ve S11-6) hamamböceğine karşı mücadele etmeni olarak geliştirilmek için üzerinde çalışılmaya değer mikrobiyal mücadele etmenleri olarak tespit edilmiştir.

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INTRODUCTION

Blattella germanica (L.) (Blattaria: Blattellidae), the German cockroach, is an important pest as it is able to transmit various bacterial and fungal pathogens and parasites of human being, and therefore spread serious diseases (Ahmad et al., 2011; Graczyk et al., 2005; Kassiri et al., 2018; Salehzadeh et al., 2007; Saitou et al., 2009; Wannigama et al., 2014). Furthermore, cockroaches cause allergic reactions by their secretions and remaining after death (Sheih et al., 2017; Pomes and Schal, 2020), and food contamination by various microorganisms that they carry (Yang et al., 2019). The primary control method of cockroaches depends mostly on chemical insecticides that poses risks to human and environment. Especially, chemical applications increase the concern for human health risk. Furthermore, the use of such insecticides has also been reported causing development of resistance in cockroach populations (Chang et al., 2009, 2010; DeVries et al., 2019; Fardisi et al., 2019; Wu and Appel, 2017; Zhu et al., 2016). All these have been directing researchers to explore alternative control methods of cockroaches, and biological control has been considered and studied as an alternative, safe and environmentally friendly technique (Suiter, 1997; Pereira et al., 2017; Pan and Zhang, 2020; Yang et al., 2021).

Several species of entomopathogenic fungi have potential for biological control of cockroaches, especially those belong to *Metarhizium* and *Beauveria* (Abedi & Dayer 2006; Hubner-Campos et al., 2013; Gutierrez et al., 2015, 2016; Lopes and Alves, 2011; Quesada-Moraga et al. 2004). *M. anisopliae, M. blattodeae, M. robertsii, M. frigidum, B. bassiana, Isaria fumosoroseus* and *Hirsutella thompsonii* have pathogenicity against cockroaches (Mohan et al., 1999; Pachamuthu et al., 1999; Montalva et al., 2016; Chaurasia et al., 2015; Zhang et al., 2018b). Although pathogenic effects of these fungi were demonstrated by assays conducted under laboratory conditions, there are few reported cases of such cockroach infections in nature (Montalva et al., 2016).

The main way of fungal infection initiation is penetration through the insect cuticle. However, *M. anisopliae* conidia were given to cockroaches per os by mixing in bait (Lopes and Alves, 2011) and Zhang et al. (2018a) added the conidia in water for ingestion. In both studies, cockroach mortalities were rather low, especially comparing to topical, dust or spray applications. There are studies showing the possibility of *M. anisopliae* transmission horizontally among a German cockroach population (Kaakeh et al., 1996, Quesada-Moraga et al., 2004). This was attributed to some behavioral features of the cockroaches like preference for humid places and aggregation (Kaakeh et al., 1996). This may also facilitate the spread of entomopathogenic fungi increasing the effect of fungal applications. This preference for places with high humidity by cockroaches is also in favor of germination of fungal conidia and sporulation of fungi on cadavers.

The utilization of entomopathogenic fungi combined with chemical insecticides and compounds has also been considered for controlling insect pests. Combination of *M. anisopliae* and some insecticides was more effective against *B. germanica* (Kaakeh et al., 1997; Pachamuthu et al., 1999; Pachamuthu and Kamble, 2000). Zurek et al. (2002) tested application of *M. anisopliae* and boric acid together and *B. germanica* mortalities increased while time to death decreased significantly. Dayer and Karvandian (2016) also found reduction of time to death by combining *M. anisopliae* and boric acid in baits. This synergism was found to be due to deleterious effect of boric acid on perithrophic membrane allowing *M. anisopliae* penetration and alteration in gut mycrobiome creating a better environment for *M. anisopliae* survival in *B. germanica* gut (Yang et al., 2021). All the authors highlighted promising potential of this combination for cockroach management.

All the previous studies show that entomopathogenic fungi have great potential for cockroach management either alone or in combination with other means and can reduce the use of hazardous chemicals that has been in use. In their review on biological control of *B. germanica*, Pan and Zhang (2020) emphasized the requirement for more studies including search for more virulent or better suited fungal strains of entomopathogenic fungi. In order to contribute to explore fungal strains, in this study, seven entomopathogenic fungi isolated from soil samples and five fungal isolates obtained from entomopathogenic fungal cultures have been tested against *B. germanica*.

MATERIALS AND METHODS

Insect culture

The *Blattella germanica* culture was maintained at 25±2°C and 60±5% relative humidity in darkness in a conditioned room. The insects were kept in large plastic containers (50 Lt in capacity) aerated by small holes on the lids. The upper inside of the containers were greased to prevent insect escapes. Paper egg cartons were placed inside the containers together with dog food and water, which were renewed as required in regular maintaining visits.

Fungal cultures

Five of the entomopathogenic fungal cultures (ARSEF 1512, 2488, 4045, 5855, 6646) were kindly provided by Dr Richard A. Humber from USDA ARS Collection of Entomopathogenic Fungal Cultures. The other seven fungal cultures (S7-1, S7-2, S7-3, S8-2, S11-2, S11-6, S12-1) were chosen from entomopathogenic fungal culture collection in our department. All these seven cultures were originally isolated from soil samples collected from Turkey. Isolates 6646, S7-2, 1512 belong to the species *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, isolates S12-1, S7-3, S11-6, S8-2 to *Metarhizium anisopliae* (Metschnikoff) Sorokin and isolate 2488 to *Isaria tenuipes* Peck, isolates S11-2, S7-1, 5855 to *Isaria fumosorosea* Wize, isolate 4045 to *Isaria farinosa* (Holmsk.) Fries. All the fungi are in Cordysipitaceae (Sordoriomycetes: Hypocreales). The fungi were grown on potato dextrose agar (PDA) in sealed Petri dishes at 25±2°C in darkness.

Preparing conidial suspensions

In order to collect conidia the fungal cultures on PDA were kept at 25±2°C in darkness until sporulation is completed (about 4 weeks). Ten ml of sterile 0.02% Tween 80 solution was added in each Petri dish and a conidial suspension was formed by dislodging conidia by the help of a glass spreader. The suspension was vortexed to disperse conidial clamps and then passed through a double layer of sterile cheese cloth to remove any debris from fungal cultures. The concentrations of these conidial suspensions were determined by counting conidia using a haemocytometer under a light microscope. By adding sterile 0.02% Tween 80 solution, the concentrations were adjusted to the required $1x10^7$ conidia ml^{-1} to be used in biological assays. Conidial viability was measured by a germination test for each suspension. A small amount of suspension was spread on 1.5% water agar and incubated in sealed Petri dishes at 25±2°C in darkness for 24 hours. Thereafter, spores were examined under a light microscope for germination. Conidia with germ tubes equal or longer than the spore length or diameter were considered germinated.

Pathogenicity tests

All twelve entomopathogenic fungal isolates were tested on German cockroach adults under controlled conditions. Viability of conidia were checked as described above and all the conidial suspensions utilized in bioassays had conidial germination ratio above 98%. The insects in control groups were treated with sterile 0.02% Tween 80 solution instead of conidium suspension. German cockroach adults were individually dipped into 20 ml of relevant conidial suspension at the concentration of $1x10^7$ conidia ml^{-1} for 10 seconds. They were placed in sterile glass jars (1 liter) with a small amount of food and water. As the purpose of bioassays were to demonstrate the pathogenic ability of the fungi, all treatments were kept at high humidity in the first day of experiments by placing them inside a sealed large plastic container (36x55x22 cm) with sterile distilled water. Thereafter, they were transferred to a conditioned room at 65±5% relative humidity.The bioassays were conducted according to complete randomized design with three replications. Each replication had ten mixed gender German cockroach adults. All the tests were carried out at 26±2 °C in darkness. In daily inspections food and water were added when required. Number of dead insects in treatments were recorded for two weeks starting the third day with two-day intervals and dead insects were removed.

After calculating mortality ratios, all data were transformed by using arcsine transformation. They were subjected to one way ANOVA and Tukey multiple comparison tests using Minitab statistics program. Furthermore, to allow comparison of only the effective isolates' pathogenicity levels, the related data were corrected for control mortalities according to Abbott's formula (Abbott, 1925) before further statistical analyses.

RESULTS AND DISCUSSION

Blattella germanica adult mortalities caused by the tested twelve entomopathogenic fungus isolates within 3-14 days post-treatment are presented in Tables 1-3. In treatments with four of the tested isolates (S12-1, 6646, 5855, 4045), the mortalities were constantly low and statistically not different from the control mortalities. The rest of the isolates generally caused an increasing mortality as post-treatment time was extended, and they all resulted in significantly high mortalities before the end of the experimental time. Especially the isolates in Table 1 required a longer initial time for significant cockroach mortality, while those in Table 3 started causing significant insect mortalities on the third or the latest fifth day.

Efficacies of entomopathogenic fungal isolates against *Blattella germanica* for the same duration were illustrated as corrected mortalities in Figure 1.

Table 1. *Blattella germanica* adult mortalities (%) (± s.e.) due to applications of first group entomopathogenic fungal isolates

Çizelge 1. Birinci grup entomopatojen fungus izolatlarının uygulaması sonucu Blattella germanica ergin ölümleri (%) (± s.h.)

Isolates	**Post-treatment time (days)** *Uygulama sonrası süre (gün)*					
İzolatlar	**3**	**5**	**7**	**9**	**11**	**14**
S 12-1	13.3±3.3 a	16.7±3.3 a	23.3±3.3 bc	23.3±3.3 bc	23.3±3.3 b	23.3±3.3 c
2488	13.3±3.3 a	26.7±8.8 a	40.0±5.8 abc	43.3±8.8 bc	56.7±6.7 ab	60.0±5.8 b
6646	16.7±8.8 a	20.0±5.8 a	20.0±5.8 c	20.0±5.8 bc	23.3±6.7 b	26.7±8.8 c
S 7-3	23.3±6.7 a	36.7±12.0 a	50.0±10.0 ab	53.3±6.7 ab	83.3±3.3 a	86.7±3.3 ab
S 11-6	6.7±6.7 a	30.0±0.0 a	40.0±5.8 abc	63.3±8.8 a	80.0±10.0 a	93.3±3.3 a
S 7-2	16.7±3.3 a	33.3±6.7 a	53.3±3.3 a	60.0±10.0 a	76.7±6.7 a	76.7±6.7 ab
Control *Kontrol*	6.7±3.3 a	10.0±0.0 a	16.7±3.3 c	16.7±3.3 c	16.7±3.3b	20.0±.0.0 c
F	1.17	2.3	6.56	7.65	12.67	24.88
P	0.374	0.093	0.002	0.001	0.000	0.000

- Different letters in each column represent statistically significant differences amongst insect mortalities according to Tukey multiple comparison tests, P≤0.05, n=3 (F and P values from ANOVA tests are presented in the last two rows; D.F.=6, 14).

- Tukey çoklu karşılaştırma testine göre her sütundaki farklı harfler böcek ölümlerinde istatistiksel önemli farkları belirtir P≤0.05, n=3 (ANOVA testi sonucu F ve P değerleri son iki satırda verilmiştir; S.D.=6, 14).

Table 2. *Blattella germanica* adult mortalities (%) (± s.e.) due to applications of second group entomopathogenic fungal isolates

Çizelge 2. İkinci grup entomopatojen fungus izolatlarının uygulaması sonucu Blattella germanica ergin ölümleri (%) (± s.h.)

Isolates	**Post-treatment time (days)** *Uygulama sonrası süre (gün)*					
İzolatlar	**3**	**5**	**7**	**9**	**11**	**14**
5855	6.7±3.3	13.3±3.3	23.3±3.3	33.3±6.7	33.3±6.7	33.3±6.7
4045	13.3±6.7	16.7±3.3	20.0±5.8	23.3±8.8	23.3±8.8	26.7±8.8
Control *Kontrol*	3.3±3.3	13.3±8.8	16.7±6.7	16.7±6.7	16.7±6.7	16.7±6.7
F	0.65	0.31	0.46	1.31	1.31	1.28
P	0.554	0.743	0.649	0.338	0.338	0.344

- According to ANOVA tests, there are not statistically significant differences amongst treatments in each column, n=3 (F and P values from ANOVA tests are presented in the last two rows; D.F.=2, 6).

- ANOVA sonucuna göre her sütundaki uygulamalar arasındaki farklar istatistiksel olarak önemli değildir, n=3 (ANOVA testi sonucu F ve P değerleri son iki satırda verilmiştir; S.D.=2, 6).

Table 3. *Blattella germanica* adult mortalities (%) (± s.e.) due to applications of third group entomopathogenic fungal isolates

Çizelge 3. Üçüncü grup entomopatojen fungus izolatlarının uygulaması sonucu Blattella germanica ergin ölümleri (%) (± s.h.)

Isolates	**Post-treatment time (days)** *Uygulama sonrası süre (gün)*					
İzolatlar	**3**	**5**	**7**	**9**	**11**	**14**
1512	30.0±5.8 a	63.3±3.3 a	63.3±3.3 ab	73.3±3.3 ab	76.7±3.3 ab	83.3±3.3 ab
S8-2	40.0±5.8 a	66.7±12.0 a	83.3±6.7 a	83.3±6.7 a	90.0±0.0 a	96.7±3.3 a
S 11-2	20.0±11.5 ab	46.7±12.0 a	70.0±5.8 ab	70.0±5.8 ab	73.3±3.3 ab	76.7±3.3 b
S7-1	13.3±3.3 ab	43.3±12.0 a	53.3±8.8 b	53.3±8.8 b	56.7±6.7 b	60.0±5.8 b
Control *Kontrol*	0.0±0.0 b	3.3±3.3 b	13.3±3.3 c	13.3±3.3 c	16.7±6.7 c	20.0±5.8 c
F	6.74	9.33	19.3	20.37	33.91	28.41
P	0.007	0.002	0.000	0.000	0.000	0.000

- Different letters in each column represent statistically significant differences amongst insect mortalities according to Tukey multiple comparison tests, P≤0.05; n=3 (F and P values from ANOVA tests are presented in the last two rows; D.F.=4, 10).

- Tukey çoklu karşılaştırma testine göre her sütundaki farklı harfler böcek ölümlerinde istatistiksel önemli farkları belirtir P≤0.05, n=3 (ANOVA testi sonucu F ve P değerleri son iki satırda verilmiştir; S.D.=4, 10).

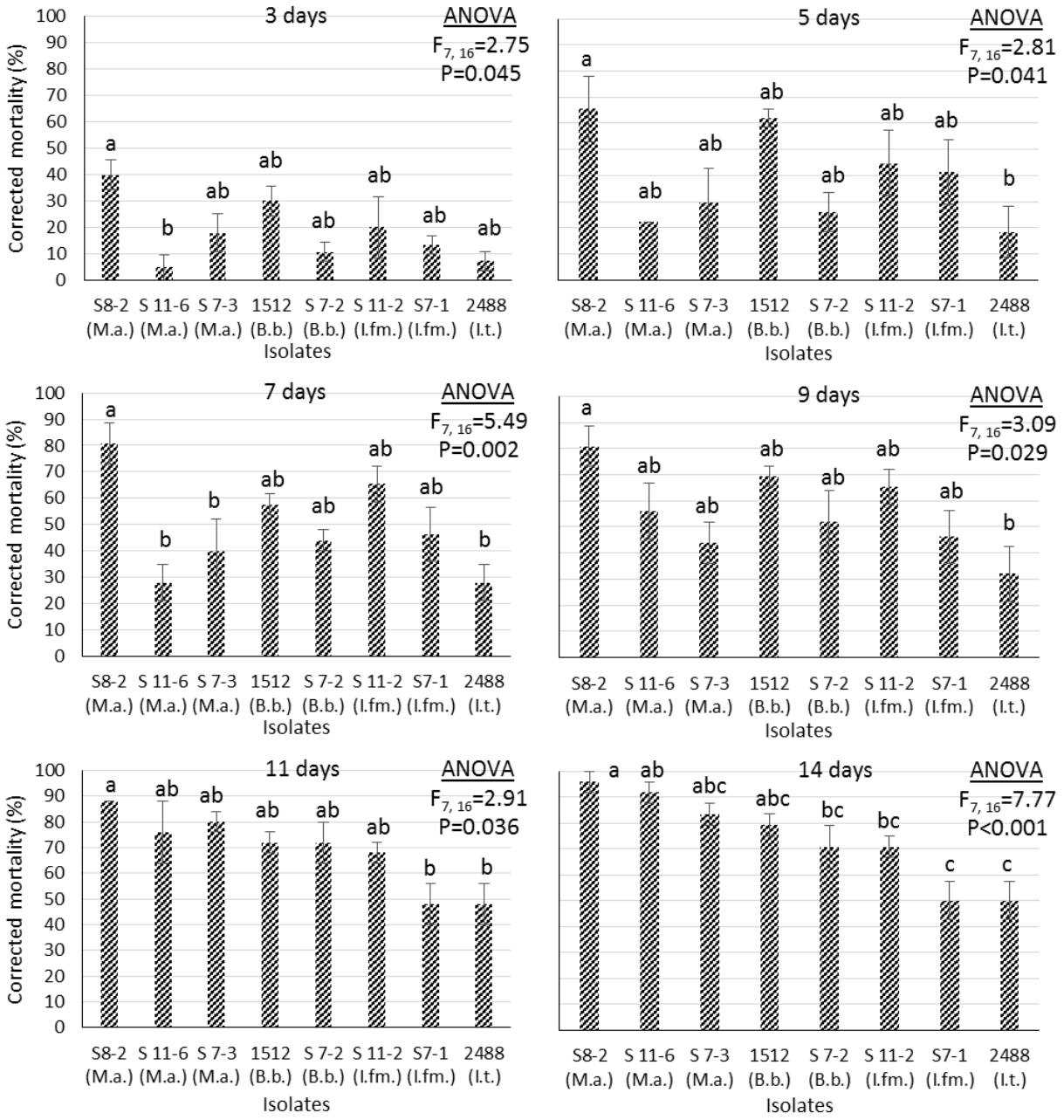

Figure 1. Corrected mortalities of *Blattella germanica* adults during two weeks after the application of entomopathogenic fungal isolates (Mortality corrections are according to Abbott's formula; bars represent standard errors; n=3; Different letters represent statistically significant differences amongst insect mortalities in each graph according to Tukey multiple comparison tests, P≤0.05) (M.a.: *Metarhizium anisopliae*, B.b.: *Beauveria bassiana*, I.fm.: *Isaria fumosorosea*, I.t.: *Isaria tenuipes*)

Şekil 1. Entomopatojen fungus izolatları uygulandıktan sonraki iki hafta süresinde Blattella germanica erginlerinin düzeltilmiş ölüm oranları (Ölüm oranlarının düzeltmesi Abbott formülüne göredir; barlar standart hatayı belirtir; n=3; her grafikteki farklı harfler Tukey çoklu karşılaştırma testine göre böcek ölüm oranları arasındaki istatistiksel önemli farkları belirtmektedir, P≤0.05) (M.a.: Metarhizium anisopliae, B.b.: Beauveria bassiana, I.fm.: Isaria fumosorosea, I.t.: Isaria tenuipes)

As above mentioned four isolates (S12-1, 6646, 5855, 4045) did not cause cockroach mortality higher than control at any time, these isolates were omitted from efficacy evaluation. In Figure 1, it is apparent that the efficacies of the eight remaining isolates increased in time with varying rates. The efficacies of different fungal isolates showed significant variation during the experiment (Figure 1). The efficacy of *M. anisopliae* S8-2 was constantly causing the greatest mortality with 95.8% on the 14th day. The differences

amongst the rest of the isolates, however, were not statistically significant until the 14th day. On the 14th day, along with the isolate S8-2, the isolate S11-6 showed significantly better efficacy than two *Isaria* isolates (S7-1 and 2488) did. At the end of the experiment, the efficacies of the tested isolates varried with corrected mortalities between 50.0% and 95.8%. This variation was due to differences at isolate level, as different isolates from the same species showed significant diversity. While one *M. anisopliae* isolate (S12-1) and one *B. bassiana* isolate (6646) had no effect on *B. germanica* adults, the other three *M. anisopliae* isolates (over 80% adult mortality) and, the other two *B. bassiana* isolates (70 to 80% adult mortality) had significant efficacies. Amongst the eight effective isolates, however, the corrected mortality values on the 11th and especially on the 14th days indicated that *Metarhizium* isolates had the greatest efficacies followed by *Beauveria* isolates and the isolates of *Isaria* had the least efficacies. The results of this study are in parallel with the results of previous studies, and the efficacies of some tested isolates can well be considered even better than previously tested entomopathogenic fungi. *Metarhizium anisopliae* has been one of the most tested fungal species on cockroaches. *Periplaneta americana* mortalities were 100% (Hubner-Campos et al., 2013) in 10 days and 56.23% in 48 hours (Chaurasia et al., 2015) after application of *M. anisopliae*. Pachamuthu et al. (1999) reported 86.3% German cockroach mortality in 21 days at the tested highest spore concentration of strain ESC-1. In other studies, *B. germanica* adult mortalities due to *M. anisopliae* applications reached 83.8% (Lopes and Alves, 2011), 93.3% (Gutierrez et al., 2014) and 83.33% (Zhang et al., 2018b) in 15 days. All these studies on German cockroach were carried out in similar ambient conditions with high doses but using different application methods. However, considering the doses together with delivery methods, the results are comparable with the ones reported in this study. German cockroach adult mortalities caused by *M. anisopliae* in the present study varied according to isolates and the most effective isolate S8-2 killed 96.7% adults in 14 days with corrected mortality of 95.8%. This isolate appear to be as effective as the previously tested isolates if not better. Effects of other species of entomopathogenic fungi on cockroaches have been reported in limited literature. *Beauveria bassiana* caused 75% mortality on *P. americana* in 10 days (Mohan et al., 1999). Gutierrez et al. (2014) reported 80% *B. germanica* mortality in *B. bassiana* treatment in 15 days. Hubner-Campos et al. (2013) tested two isolates of *B. bassiana* on *P. americana* nymphs and one isolate killed 58.3% and 81.7% in 10 and 25 days, respectively. In the same study, *Isaria farinosa* and *I. cateniobliqua* did not kill any insects, while a closely related fungus *Purpureocillium lilacinum* caused 18.3% mortality in 25 days. In the present study, more effective isolates of both *B. bassiana* and *Isaria* are found although their efficacies were lower than *Metarhizium anisopliae* isolates.

This study demonstrates interspecific and intraspecific variation in the virulence of different entomopathogenic fungi on German cockroach under controlled conditions. This finding supports the suggestion of Pan and Zhang (2020) to continue searching more effective isolates of entomopathogenic fungi as better isolates could always be encountered. Although high efficacies were recorded from different species, *M. anisopliae* seems to have superior isolates such as S8-2, S11-6 and thus should be considered for further studies to develop a microbial control agent to be utilized in the control of cockroaches, either alone or in combination with other means.

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Author Contributions

The authors declare that they have contributed equally to the article.

Conflicts of Interest

The authors declare no conflict of interest.

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