BRAZILIAN JOURNAL OF BIOLOGY®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

A comparative study on the virulence of entomopathogenic fungi against *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae) in stored grains rice

Um estudo comparativo da virulência de fungos entomopatogênicos contra *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae) em grãos armazenados de arroz

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Abstract

Entomopathogenic fungi (EPF) now a possible safer microbial control measure that could be considered as a substitute for chemical control of insect pests. Three EPF viz., *Metarihizium anisopliae, Isaria furnosoroseus* and *Beauveria bassiana* were evaluated for their virulence against the grubs of Khapra beetle, *Trogoderma granarium* (Everts) under laboratory conditions. The isolates were applied by two methods viz., diet incorporation and an immersion method with 3rd instar 20 grubs of *T. granarium* for each. The virulence of EPF was determined using percent mortality. Significantly higher mortality was observed in *M. anisopliae* applied through immersion (98.33%) and diet incorporation (93.33%) methods followed by *B. bassiana* (90.83 and 85.83%, respectively). The mortality caused by *I. furnosoroseus* was statistically lower in immersion and diet incorporation methods i.e. 81.67 and 73.33%, respectively. Based on the immersion method, all EPF were studied for multiple conidial concentration i.e., $1{\times}10^4$, $1{\times}10^5$, $1{\times}10^6$, $1{\times}10^7$ and $1{\times}10^8$ under the same in-vitro conditions. All the isolates were pathogenic to grub of *T. granarium* at the highest conidial concentration. *M. anisopliae* was proved the most effective virulent resulting in 98.33% mortality of the pest with LT_{50} 4.61 days at 1×10^8 conidial concentration followed by 90.83 and 81.67 percent mortality with 5.07 and 8.01 days LT_{50}, in the application of *B. bassiana* and *I. furnosoroseus,* respectively. M. *anisopliae* showed higher efficacy and could be considered as promising EPF for the development of myco-insecticides against effective biocontrol of *T. granarium*.

Keywords: bioassay, pathogenicity, microbial control, *T. granarium*, *B. bassiana*, rice grains.

Resumo

Os fungos entomopatogênicos (FPE) são agora a possível medida de controle microbiano mais segura, que pode ser considerada um substituto para o controle químico de pragas de insetos. Três EPF viz., *Metarihizium anisopliae*, *Isaria furnosoroseus* e *Beauveria bassiana* foram avaliados quanto à sua virulência contra as larvas do besouro Khapra, *Trogoderma granarium* (Everts) em condições de laboratório. Os isolados foram aplicados por dois métodos, a saber: incorporação de dieta e um método de imersão com 20 larvas de *T. granarium* de 3º ínstar para cada um. A virulência do EPF foi determinada usando a mortalidade percentual. Mortalidade significativamente maior foi observada em *M. anisopliae* aplicado pelos métodos de imersão (98,33%) e incorporação de dieta (93,33%), seguido por *B. bassiana* (90,83% e 85,83%, respectivamente). A mortalidade causada por *I. furnosoroseus* foi estatisticamente menor nos métodos de imersão e incorporação de dieta, ou seja, 81,67% e 73,33%, respectivamente. Com base no método de imersão, todos os EPFs foram estudados para múltiplas concentrações de conídios, ou seja, 1 × 104, 1 × 105, 1 × 106, 1 × 107 e 1 × 108 nas mesmas condições in vitro. Todos os isolados foram patogênicos à larva de *T. granarium* na maior concentração de conídios. *M. anisopliae* provou ser o virulento mais eficaz, resultando em 98,33% de mortalidade da praga com LT50 4,61 dias na concentração de 1 × 108 conídios seguido por 90,83% e 81,67% de mortalidade com 5,07 e 8,01 dias LT50, na aplicação de *B. bassiana* e *I. furnosoroseus*, respectivamente. *M. anisopliae* apresentou maior eficácia e pode ser considerada como um PFE promissor para o desenvolvimento de micoinseticidas contra o biocontrole efetivo de *T. granarium*.

Palavras-chave: bioensaio, patogenicidade, controle microbiano, *T. granarium*, *B. bassiana*, grãos de arroz.

1. Introduction

The khapra beetle, *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae) due to its voracious eating can withstand starvation for a longer time (Honey et al., 2017) which makes it one of the most destructive pests of stored-products in the world. It has been recognized as an A2 quarantine organism by European Plant Protection

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Received: April 7, 2021 – Accepted: June 9, 2021

Organization (EPPO, 1981) and ranked among the 100 worst invasive species worldwide (Lowe et al., 2000). Rice is one of the most important cash crops of Pakistan. It plays a major role worldwide as a rice exporter. Pakistan is exporting rice approximately 2 million tons annually, which is 10% of the world's trade. The export share of Pakistan from 2010-2014 suffered a rapid decline (2.2 billion dollars into 2.1 billion dollars) in the global market highlighting the constraints in the rice supply chain of the country. Continuous rejection of rice consignments among 2010-2014 was estimated 1000 million dollars loss by trading partners due to the presence *Trogoderma granarium* (Honey et al., 2017).

Detection of this notorious pest in rice consignments exported from Pakistan to other countries opened a new area for researchers to monitor the activity of this pest in stored rice (Green, 2011). The use of fumigants like methyl bromide and phosphine against *Trogoderma granarium* is the most primarily control method being used for many decades under storage conditions (Fields and White, 2002; Ahmedani et al., 2007a, b; Rajendran et al., 2008). Recent studies have revealed that methyl bromide is involved in the ozone depletion phenomenon and is designated as a major Ozone-depleting agent. This situation pushed many countries to minimize its usage under storage conditions (UNEP, 2010). Phosphine is also a very common fumigant being used for a long time but it gives moderate to poor control against *T. granarium* due to the development of resistance in insect pests against the fumigant (Bell et al., 1984; Rajendran, 2002). Furthermore, the application of different contact insecticides like malathion, pyrethrins and chlorpyriphos has been extensively used as a surface treatment on stored products (Khosla et al., 2005; Eliopoulos, 2013). But their effectiveness is also not reliable due to the fact that *T. granarium* has the ability to withstand adverse environmental conditions and they can hide in small cracks and crevices. Moreover, decreased persistence of some insecticides also lowers their effect after a certain period of time. Another major concern about the contact insecticide has been their residual effects on stored commodities that can harm stored products and humans involved with their handlings (Arthur, 2012). These concerns have led researchers to find out some alternate methods for the management of *Trogoderma granarium* (Traynier et al., 1994; Abdel-Ghany et al., 2015).

The public is getting aware of environmentally safe and sustainable control measures in pest management as well as the development of control strategies and measures based on low-risk pesticides in stored-product protection. One promising alternative is the application of entomopathogenic fungi (EPF) as microbial control (Brower et al., 1996; Scholler et al., 1997; Moore et al., 2000). Entomopathogens are naturally occurring, environmentally safe organisms that infect insects by contact. Insect fungal pathogens have a broad spectrum of hosts, can be mass-produced easily, rapidly and economically and can be applied with the same technical means as conventional contact insecticides (Rumbos and Athanassiou, 2017). *Beauveria bassiana* and *Metarhizium anisopliae* at different conidial concentrations have a wide host range and have been tested against most of the major stored-product insect pests other than *Trogoderma granarium* on rice (Sahayaraj and Namasivayam, 2008; Sabbour, 2015; Batta, 2012; Khashaveh et al., 2011). Five Iranian isolates of EPF compared to a commercially produced pathogens. The data demonstrate that the two Iranian isolates of *B. bassiana* and *M. anisopliae* have potential as biological control agents of *T. granarium.*

Keeping in view the above constraints, the present study was conducted to determine the virulence of *Metarhizium anisopliae*, *Isaria fumosoroseus* and *Beauveria bassiana*on the mortality of *T.granarium*on rice grains under laboratory conditions.

2. Materials and Methods

2.1. Mass culturing of insects

The study was conducted in the rearing laboratory at 30 °C with relative humidity 65%.The infested grain samples of stored rice grains were collected from different rice sellers of Tehsil Pasrur, District Sialkot and brought to the laboratory. These infested samples along with the insect were divided into two kg each and put into glass jars (4-kg capacity). The mouth of these jars was covered with Muslin cloth and kept in the laboratory under controlled conditions (25 ± 1 °C and RH 75% with 12:12 day: night regimes. The eggs were obtained by sieving the samples with a 60-mesh sieve for further rearing. Several eggs were poured each in ten glass jars containing 2-kg sterilized grains. Each jar was kept under controlled conditions to get same-aged grubs for further experimentation.

2.2. Source of fungal isolates

Three strains of entomopathogenic fungi namely *M. anisopliae* (strain Z3RAS), *B. bassiana* (strain GBBSTTS) and *I. fumosorosea* (strain RHZ4RAS) were used. These strains were imported from Agri Life Medak, District Hyderabad, Andara Pradesh, India. To prepare the conidial serial formulations, the conidia of all isolates were cultivated on PDA (Potato Dextrose Agar) under control conditions at 24 °C and incubate for 14 days. After that, the conidia were harvested in 2.5% Tween in 5 mL sterile distilled water. To read the number of fungal conidia a Neubauer hemocytometer (TIEFE 0.100 mm 1/400 9 mm) was used and serial concentrations of each isolate was prepared by dilution.

2.3. Initial screening of entomopathogenic fungi at higher concentration

Available strains of fungal isolates of *Metarhizium anisopliae* Z3RAS, *Isaria fumosoroseus* RHZ4RAS and *Beauveria bassiana* GBBSTTS were tested against *T. granarium* on rice (CV.Basmati 385) for single concentration i.e., $1x10^{8}$ conidia mL^{-1} for their virulence by immersion method (Mahdneshin et al., 2011) and food mix method. In both methods, the test insects and food were immersed for one minute into the conidial suspension of 5-mL and the excess of conidial excessive suspension was removed by putting the test insects on a sterilized paper. A total of 20 grubs of 3rd instar were used in each replication of each treatment. Treated insects in replicates were later transferred to 3.5 cm petri plates containing 20 gm insect-free grains. The petri plates were arranged in a Completely Randomized Design (CRD) replicated thrice and maintained under the standard environmental chamber conditions of 25 ± 1 °C and RH 75% with 12:12 day: night regimes. All plates were partially sealed with plastic solution tape to escape from the contamination of other insects. The grubs in the control treatment were treated with sterilized H_2O. Mortality data were recorded every 24 hours for 14 days.

2.4. Virulence of entomopathogenic fungi at multiple concentrations

Above mentioned Entomopathogenic Fungi were tested at four different concentrations of conidia of each fungus i.e. 1×10^{8}, $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ conidia mL^{-1} by using the immersion method under the same procedure as mentioned above. Mortality data were recorded every 24 hours for 14 days. Based on the percent maximum mortality of the pest, the LT_{50} was calculated by using Probit analysis (Han et al., 2017).

2.5. Statistical analysis

An analysis of variance (ANOVA) was used to determine significant differences within the tested isolates. Statistical analyses were performed using SAS statistical software 13.2.0 (SAS Inst.Inc., Cary, NC, USA). Probit analysis was used to calculate the mean survival times (MST) (Han et al., 2017).

3. Results

3.1. Fungal isolates tested by immersion methods at single concentration bioassay

The results (Figure 1) indicate significant difference among fungal isolates (P < 0.00, F=100, DF= 2, CV= 1.60). The maximum mortality (98.33%) of *T. granarium* was observed in the application of *M. anisopliae* in a single concentration of 1×10^8 and differed significantly from other Entomopathogenic Fungi (EPF). Significantly minimum mortality (81.67%) of the pest was recorded in the application of *I. fumosoroseus.* The application of *B. bassiana* had an intermediate effect showing 90.83 percent mortality of the pest and differed statistically from the effect of other EPF.

3.2. Fungal isolates tested by food mix method at single concentration bioassay

The results (Figure 2) reveal significant variation (P< 0.00, F=73.5, DF=2, CV = 2.45) among fungal isolates. The highest mortality (93.33%) of *T. granarium* was observed

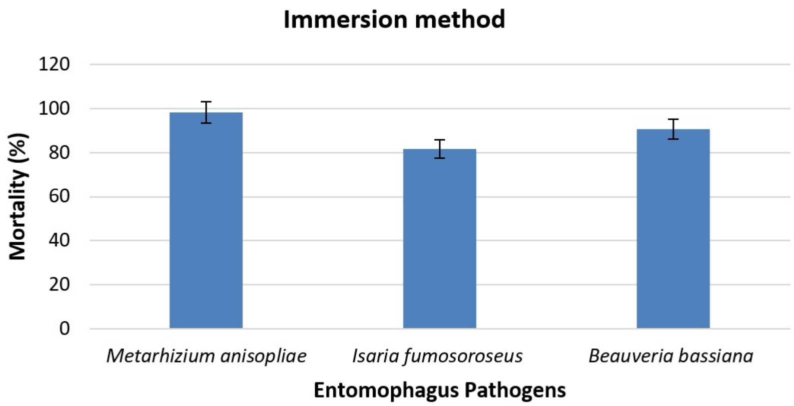

Figure 1. Mortality (%) of *T. granarium* grubs exposed to immersion method at $1x10^8$ conidia mL^{-1}. Different letters above the bars represent the significant difference at P=0.05.

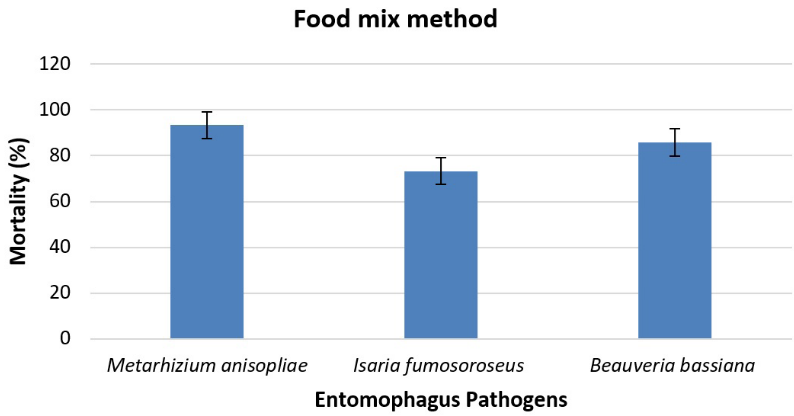

Figure 2. Mortality (%) of *T. granarium grubs* exposed to food mix method at $1x10^8$ conidia mL^{-1}. Different letters above the bars represent the significant difference at P=0.05.

in the application of M. anisopliae and differed statistically from other isolates. The minimum mortality was recorded to be 73.33 percent of the test pest in the application of *I. fumosoroseus.* The effect of *B. bassiana* was intermediate showing mortality of 85.33 percent of the pest and also differed statistically with those found in other isolates.

3.3. Comparison of immersion and the food mix fungus isolates

The results given in Figure 3 regarding the comparison of immersion and food mix method in different fungal isolates reveal that *M. anisopliae* had significantly maximum mortality of the pest in both application methods as compared to other isolates. The pest mortality was recorded to be 98.33 percent in the immersion method, whereas it was 93.33 percent in the food mix method and was at par statistically. The minimum mortality of the pest was observed in the application of *I. fumosoroseus* at both methods of application (Immersion, 81.67% and food mix, 73.33%) compared to other isolates. *B. bassiana* publicized 90.83% and 85.83% mortality of the pest in immersion and food mix method, respectively and categorized as intermediate. *M. anisopliae* was the most effective at both methods of application as compared to other isolates. Immersion method exposed higher mortality of the pest as compared to food mix method in all the isolate.

3.4. Multiple concentration bioassays

Fungus isolates viz., *M. anisopliae*, *I. fumasoroseus* and *B. bassiana* at various conidial concentrations viz., 1×10^4 to 1×10^8 were studied by emulsion method for their virulence against *T. granarium*. The results divulge that higher conidial concentrations exposed significantly higher mortality of the pest as compared to lower conidial concentrations in all the isolates (Figure 4, 5, and 6). The pest mortality reduced up to 43.33% from 98.33% in *M. anisopliae*, 33.33% from 81.67% in *I. fumosoroseus*

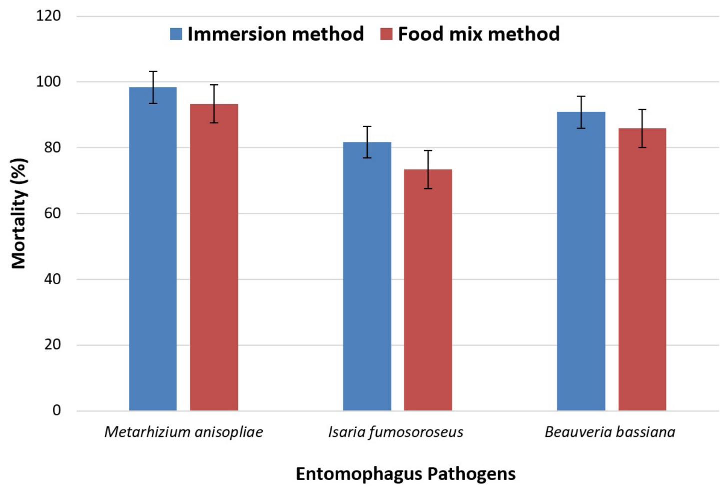

Figure 3. Mortality (%) of *T. granarium* grubs exposed to Immersion and Food mix method at a conidial concentration of 1x10^8. Different letters above the bars represent the significant difference at P=0.05.

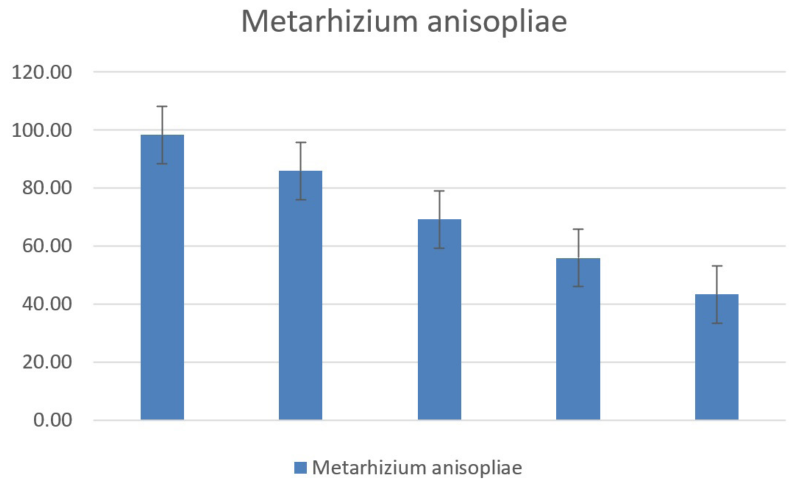

Figure 4. Mortality (%) of *T. granarium grubs* exposed to *Metarhizium anisopliae* at different concentrations. Different letters above the bars represent the significant difference at P=0.05.

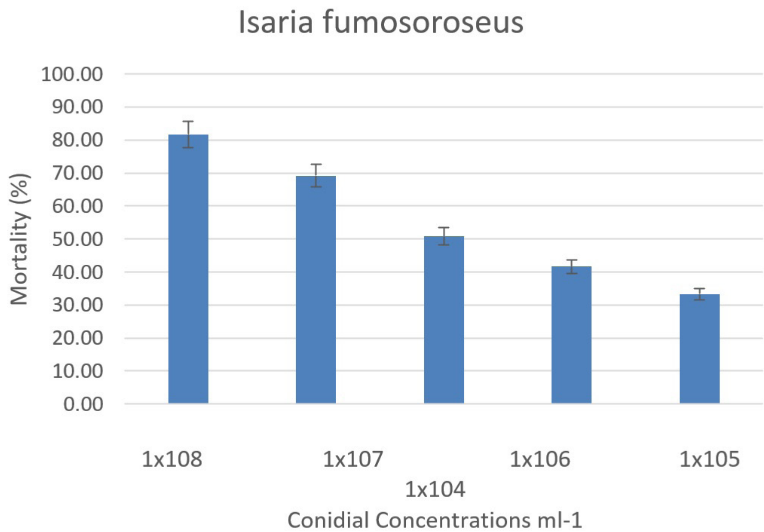

Figure 5. Mortality (%) of *T. granarium* grubs exposed to *Isaria fumosoroseus* at different concentrations. Different letters above the bars represent the significant difference at P=0.05.

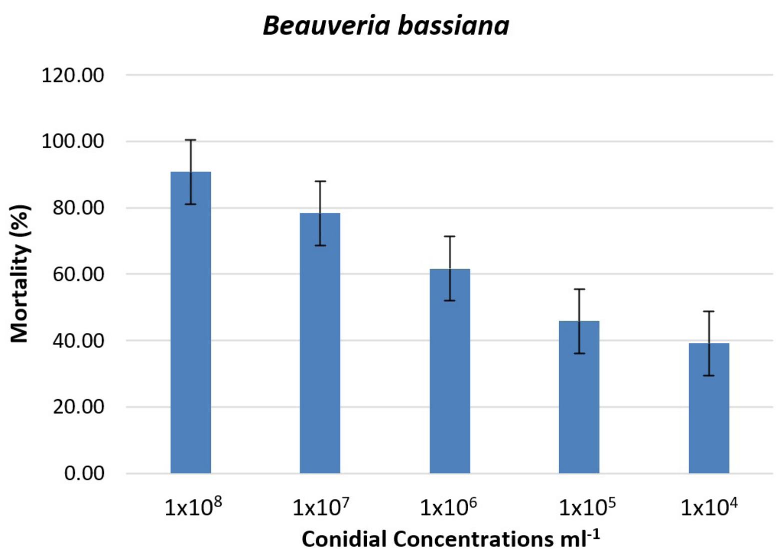

Figure 6. Mortality (%) of *T. granarium grubs* s exposed to *Beauveria bassiana* at different concentrations. Different letters above the bars represent the significant difference at P=0.05.

and 39.17% from 90.83% in *B. bassiana* in the conidial concentration of 1×10^4 and 1×10^8, respectively.

The results (Figure 7) regarding the comparison of various isolates at different conidial concentrations for their virulence against *T. granarium* reveal that *M. anisopliae* showed significantly higher mortality (98.33%) of the pest at higher conidial concentration i.e., 1×10^8 as compared to *I. fumosoroseus* (81.87% mortality) and *B. bassiana* (90.83% mortality). The virulence of all the isolates reduced significantly as the conidial concentrations decreased (P=0.027, F=2.73, DF= 8, CV =3.21). *M. anisopliae* found the most effective at all the conidial concentrations as compared to other isolates while *I. fumosoroseus* proved the least effective.

3.5. *LT_{50} at various conidial concentrations*

The results (Table 1) expose that *M. anisopliae* at conidial dose 1 x 10^8 was the most effective with minimum LT_{50} i.e. 4.61 days. The fiducial CI at 95% ranged from 4.19 to 5.02. The LT_{50} values increased on decreasing the conidial concentration in all the isolates. The application of *I. fumosoroseus* resulted in minimum effect (LT_{50} 8.01 days) on the pest as it inclined longer period for its survival with fiducial CI range at 95% was from 7.35 to 8.78 at the highest conidial concentration. The virulent *M. anisopliae* at the highest conidial concentration performed the best in controlling the pest as compared to *B.bassiana* (LT_{50}=5.53 days, CI range 5.07 to 6.00) and *I. fumosoroseus*. Therefore *M. anisopliae* is recommended at a conidial

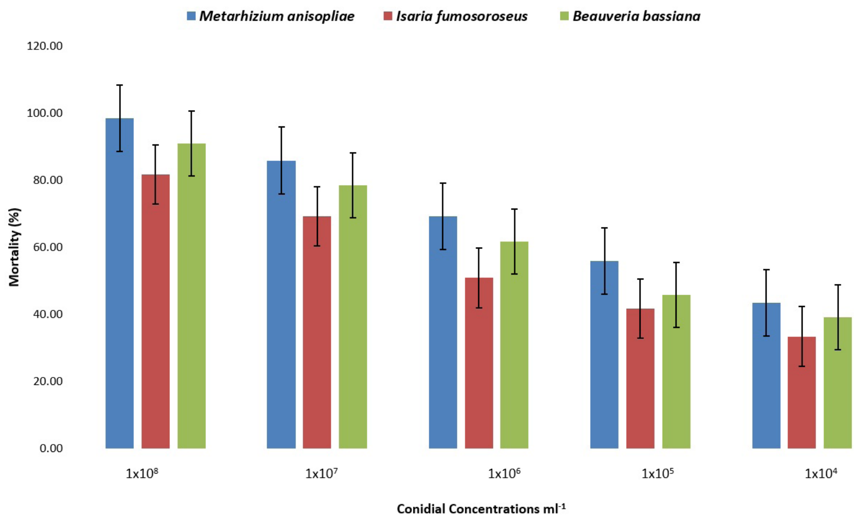

Figure 7. Mortality (%) of *T. granarium* grubs exposed to three different fungal strains at different conidial concentrations. Different letters above the bars represent the significant difference at P=0.05.

Table 1. Comparative LT_{50} at various conidial concentrations in different entomopathogenic isolates

Conidial Dose (mL^{-1})	*Metarihizium anisopliae*			*Isaria fumosoroseus*			*Beauveria bassiana*		
	Percentile	95% Fiducial CI		Percentile	95% Fiducial CI		Percentile	95% Fiducial CI	
		Lower	Upper		Upper	Lower		Upper	Lower
1 x 10^8	4.61±0.21	4.19	5.02	8.01±0.35	7.35	8.78	5.53±0.24	5.07	6.00
1 x 10^7	6.44±0.30	5.87	7.05	11.17±0.64	10.05	12.67	8.40±0.41	7.66	9.28
1x 10^6	10.47±0.65	9.35	11.97	15.71±1.23	13.70	18.77	13.35±1.00	11.69	15.78
1x10^5	16.43±1.55	13.95	20.45	21.72±2.25	18.20	27.70	18.72±1.82	15.83	23.45
1x10^4	22.89±2.81	18.63	30.64	28.79±3.87	23.05	39.87	25.11±3.08	20.44	33.62

CI = Confidence of interval.

dose of 1 x 10^8 mL^{-1} for the effective control of the pest understudy and suggested to incorporate virulent strain in IPM tactics.

4. Discussion

Entomopathogenic fungi are being developed globally to control the different insect pests and some microbial-derived products already existed commercially. Numerous studies have been conducted on the use of entomopathogenic fungi for the protection of stored commodities (Athanassiou et al., 2007; Hansen and Steenberg, 2007; Lord, 2001; Michalaki et al., 2006; Moore et al., 2000). Application of Entomopathogenic Fungi (EPF) as microbial control is a low-risk method for the management of stored grain insect pests (Brower et al., 1996; Scholler et al., 1997; Moore et al., 2000). Three entomopathogenic isolates viz., *Metarihizium anisopliae, Isaria fumosoroseus* and *Beauveria bassiana* were studied in vitro against *T.granarium* (Everts) on stored rice (CV.Basmati 385) at single conidial concentration i.e., 1 x 10^8 mL^{-1} by food mixed and immersion methods. *M. anisopliae* was significantly proved to be the most effective both at immersion (98.33% mortality) and food mixed (93.33% mortality) methods compared with other isolates.

Furthermore, the immersion method was more effective in all the virulence. *I .fumosoroseus* found least effective at both methods of application whereas *B. bassiana* categorized as intermediate and both EPF can be considered as potential microbial agents. These EPF isolates were further studied at multiple conidial concentrations i.e., 1 x 10^4 to 1x10^8 based on immersion method. The results of this study demonstrated significant variation among different fungal isolates. The grubs of *T. granarium* were susceptible to EPF isolates at higher concentrations. These findings in general can be compared with those of Kassa et al. (2002) and Batta (2005) who reported that treatment of stored grain pests with *M. anisoplie* and *B. bassiana,* were effective against stored grain insect pests. The results of the current study revealed *M. anisopliae* was the most effective followed by *B.bassiana* and *I. fumosoroseus* against *T. granarium*. The present findings are also supported by the findings of Smith et al. (1998). Moorhouse et al. (1993) reported that differences in a fungal ability to reduce the host defense mechanism lead to treatments with a higher LT_{50} as well prolong the insects' survival. The variation observed in the isolates, LT_{50} values likely reflect the physiological and genetic differences among isolates.

In the present study, the concentration was the critical parameter that determined the "speed of kill" of the exposed insect. Even though there was no prior study available on entomopathogenic infection against *T. granarium.* Dal Bello et al. (2006) reported that mortality within storage commodities in reference to storage pests can be induced by immunizing the use of different strains of *Metarihizium* and *Beauveria* sp. Todorova et al. (2002) reported that there is no reliable indicator for the virulence that shows the original host is specific for the specific fungus. It is also agreed that some fungus has a broad host range like *B.bassiana* and *M.anisopliae* and their pathogenicity also varies as per their host. Bidochka et al. (2000) reported an increasing trend in *B.bassiana* and *M. anisopliae* that pathogenicity drives by habitat selection. Thus, results from the present study indicate that screening of potential isolates should not be limited to those isolated from the original host. In the present study, Mortality of the pest was observed at all the concentrations of each isolate but this mortality is limited which ranged from 33.33% to 43.33% at lower concentrations and this low mortality is responsible for the slow development of spores which was the effect of low dose application. Similar findings were observed by Yoon et al. (1999). Furthermore, in the present dissertation, high conidial concentration caused the mortality of insects in a short time (4.61 to 8.01 days) whereas, a low conidial dose took a longer period (22.89 to 28.79 days). These findings can partially be in conformity with those of Hidalgo et al. (1998) who reported that the concentrations applied with high conidia rates caused mortality in a short time but on the other hand doses with low conidial concentrations may take two or more weeks to kill the test organisms. The test organism and host varied with those studied in the present experiment.

The present findings are in partial agreement with those of Mohammed et al. (2019) who tested five isolates of EPF compared to a commercially produced *B. bassiana*for their efficacy on fecundity and longevity of adult female of *T. granariun* and reported that 1st 2nd and 3rdinstars were more susceptible as compared to 4th 5th instar and adult to *B. bassiana* and *M. anisopliae* at 1 × 106 conidial concentration after 10 days of application. They also reported a 50% reduction in fecundity and 20% in the longevity of female adults. They suggested that both isolates have potential as biocontrol agents of *T. granarium*. The application method of the fungal formulation was the most important factor in its efficacy against *T. castanium* adults. Mortality was faster when the fungus was applied directly to the adults. This "speed of kill" is a crucial parameter in the use of entomopathogenic fungi for stored-grain protection, since fungi are generally slow-acting, and oviposition with concomitant progeny production is likely to occur before death (Moore et al., 2000). Previous studies provide interesting information on this issue. For example, Cherry et al. (2005) reported 100% mortality of *C. maculatus* adults dipped for 5seconds into a conidial suspension of *B. bassiana* or *M. anisopliae* after 6 and 8 days of exposure. Furthermore, it has been reported by (Kassa et al., 2002) that *S. zamias* adults which were dipped into certain isolates of *B. bassiana* suspension for 5 seconds suffer 100% mortality after only 4 days after application time. Nearly similar results were also found for *S. oryzae* treated with certain *B.bassiana* and *M.anisopliae*isolates. As for the application methods examined here, direct application (immersion method) of the fungus on insect bodies is more effective than contact of the insects with the fungal strains-applied on food substrate.

5. Conclusion

Isolates, *B. bassiana* and *M. anisopliae* at 1×10^8 conidial concentration applied through emersion method can be recommended as potential strains for further research to develop the myco-insecticides for the control *T. granarium* in rice storages. Lower concentration of the isolates resulting in increased survival period and decreased mortality of the pest. Further research should be needed for the production of biopesticides based on EPF for their specific control to stored commodity insects with the integration of EPF in advanced dissemination, control system and under the various environmental conditions.

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