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# Insecticidal Action of Several Isolates of Entomopathogenic Fungi against The Granary Weevil *Sitophilus granarius*

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**Abstract:** The insecticidal virulence of various entomopathogenic fungal isolates retrieved from soil samples was tested on adults of the granary weevil *Sitophilus granarius* (L.) (Coleoptera: Curculionidae). Bioassays were carried out in the laboratory where experimental adults were sprayed with 1 mL of conidial suspension ($10^8$ conidia/mL) from each isolate. Mortality was recorded at 7, 14, and 21 days after exposure. Mean mortality, mean lethal time, survival, and hazard effect were estimated for each isolate. Two isolates of *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae), one isolate of *Aspergillus insuetus* (Bainier) Thom & Church (Eurotiales: Trichocomaceae) and *Metarhizium anisopliae* (Metschinkoff) Sorokin (Hypocreales: Clavicipitaceae) resulted in the highest mortality (97–100%). The isolates with both the highest hazard effect and the lowest survival rate were *Aspergillus* sp. and *M. anisopliae*. Our results indicate that entomopathogenic fungi have the potential to become a very useful tool in reducing chemical applications in storage facilities.

**Keywords:** entomopathogenic fungi; virulence; *Sitophilus granarius* L.; Curculionidae; biological control; IPM

## 1. Introduction

Insects are major agricultural post-harvest pests causing serious damage to the quality, the quantity, and the commercial and agronomic value of various stored products [1]. Nowadays, stored product pest control is based mainly on the use of two broad categories of insecticides: residual insecticides and fumigants. Chemical strategies involve direct application of contact insecticides to grain and surface treatments on bag stacks, storage structures providing protection from invading pests, and fumigants used to control pests that are already infesting stored grain [2,3]. However, the development of insect resistance to many chemical insecticides, environmental pollution issues, human safety problems and the demands of consumers for residue-free products have led researchers to search for alternative non-chemical control means that do not leave residues on the product and are generally safe for the environment and human health [4,5].

*Sitophilus granarius (L.) (Coleoptera: Curculionidae)* belongs to the most severe storage pests of raw cereals in the world and its control consists mainly in the application of fumigants and residual insecticides [6]. The long-term use of these synthetic chemicals holds the risk that inadequate treatments could lead to resistance in pest populations, a fact that has already been reported for

Sitophilus species [7]. Resistance development coupled with public demand for residue-free food propels the exploration of alternative biological control methods.

Insect pathogens including entomopathogenic fungi, bacteria, viruses, protozoa, and nematodes offer many advantages such as high efficacy and compatibility with other IPM methods, and they are thus considered to be among the most promising alternatives to chemical-based insect control [8–12]. There is a plethora of studies available in literature focusing on the use of pathogens, mostly fungi, against stored product pests [13–19].

Entomopathogenic Fungi (EF) are naturally occurring microorganisms which are environmentally safe and have low mammalian toxicity [19,20]. Furthermore, they have the potential to develop on cadavers, thus reintroducing more inoculum into the system. Hence, while long term residual persistence is considered a drawback in the case of conventional insecticides, it is a desirable characteristic of EF [13].

The most commonly studied EF species against stored product pests is *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae). Several laboratory and field studies have demonstrated its efficient insecticidal action against postharvest insects [11,17,21–26]. Unlike *B. bassiana*, there are disproportionably fewer data on the use of other common EF species such as *Metarhizium anisopliae* (Metschinkoff), Sorokin (Hypocreales: Clavicipitaceae), and *Isaria fumosorosea* (Wize) (Hypocreales: Clavicipitaceae), despite the fact that there is strong evidence that they can be successfully used for the protection of stored grains against several insect pests [11,27–30].

Mycopesticides have been proven to have considerable potential for the management of insects while minimizing the adverse effects of insecticides, and they have accordingly been used worldwide to control various pests [31–34]. Although several stored product insects suffer high mortality as a result of pathogenic disease, the practical use of fungal pathogens as biological control agents in storage facilities has received very little attention [5,9].

As part of our project on the development and application of EF in the integrated pest management (IPM) of stored product pests, we investigated the potential of 27 fungal isolates (including *B. bassiana*, *M. anisopliae*, *I. fumosorosea*, and other entomopathogenic fungi) as biological control agents against the granary weevil *S. granarius*, a notorious pest of stored grain with worldwide distribution [6]. The granary weevil is a primary pest of grain since it is capable of infesting undamaged kernels [6]. Apart from grain (mainly wheat and maize) it can also attack other cereal products such as spaghetti. Very serious quantitative and qualitative losses may be caused including severe reduction in the weight and quality of grain as a result of the larvae feeding on the endosperm, secondary infestations by molds, bacteria and other insects, product contamination with frass, insect body fragments, and heating of the grain due to insects' action (hot spots) [1]. The fungal isolates were recovered from soil samples from Greece and Cyprus. The main purpose of our study is to enhance the application of safe biological means in stored product IPM.

## 2. Materials and Methods

### *2.1. Rearing of the Weevils*

The population of *S. granarius* was originally collected from infested wheat in Achaia (W. Greece) and reared for more than a year in the Plant Protection Institute of Patras, Achaia, Greece. Insects were maintained in glass jars (0.25 l capacity, Amiglass Athens Greece) containing 200 g of pesticide-free sterilized hard wheat (*Triticum durum* Desf. var. Mexa). The jars were covered with a sterilized muslin cloth and the adults were sieved out after two weeks. Insects were kept in a growth chamber (PHC Europe/Sanyo/Panasonic Biomedical MLR-352-PE), in controlled environmental conditions (25 ± 1°C, 65 ± 5% relative humidity, complete darkness).

### 2.2. Soil Samples

Soil samples were randomly collected from Glafkos (38°12′23.50″ N, 21°47′2.25″ E), Ayia (38°15′43.98″ N, 21°44′58.31″ E), Kastritsi (38°17′13.08″ N, 21°48′12.46″ E), and Zavlani (38°15′22.38″ N, 21°45′25.95″ E) in the prefecture of Achaia, Greece, and insect baits were used for the retrieval of fungal isolates (Table 1). The locations of the samplings were recorded with a GPS Garmin Etrex device. Once the surface litter was removed, a soil core borer was used to dig into a depth of 10 cm to extract the soil samples. These were then placed in plastic bags and kept at 4 °C until they were transferred to the laboratory stalls where they were placed on a rough cardboard for 24 h to reduce their humidity. This step was deemed essential as in conditions of excess humidity, the entomopathogenic nematodes of the soil attack the larvae of insect baits before the entomopathogenic fungi. Once it was drier, the soil was sieved and placed in Petri dishes where ten bait larvae or adults per species were also inserted. Each soil sample was tested three times; thirty individuals per species were consequently tested per soil sample. The samples were then left in special dark chambers at 25 ± 1 °C for 14 days.

**Table 1.** Isolates of various entomopathogenic fungal species which were tested in the present study. Coleopteran and Lepidopteran insect species were used as baits to retrieve the fungi. Fungal DNA sequences were matched with the Basic Local Alignment Search Tool (Blast ID Number).

| Fungal Species | Isolate | Bait | Collection Site | Blast ID Number |
|---|---|---|---|---|
| *Apophysomyces ossiformis* | B8B | *Rhyzopertha dominica* | Zavlani-W.Greece | 20140422CS9P1_A03_2016-04-27 |
| *Aspergillus* sp. | G11 | *Tribolium confusum* | Patra-W.Greece | 20140422CS4P1_D01_2016-04-27 |
| *A. insuetus* | D17 | *Tribolium confusum* | Zavlani-W.Greece | 20140422CS5P1_E01_2016-04-27 |
| *B. bassiana* | H20 | *Rhyzopertha dominica* | Glafkos-W.Greece | 20170105CS5P4_E01_2017-01-11 |
| | D | *Tribolium confusum* | Patra-W.Greece | 20170105CS5P1_E01_2017-01-11 |
| | 10T | *Tribolium confusum* | Glafkos-W.Greece | 20170105CS6P2_F02_2017-01-11 |
| | BD14 | *Tenebrio molitor* | Glafkos-W.Greece | 20170105CS6P3_G02_2017-01-11 |
| | ST 28/11 | Endophytic pepper plant | Patra-W.Greece | 20140422CS6P5_H02_2016-04-27 |
| | E20 | Endophytic strawberry plants | Patra-W.Greece | 20140422CS6P6_H02_2016-04-27 |
| | B18 | Endophytic potato plant | Patra-W.Greece | 20140422CS6P9_H02_2016-04-27 |
| | BB [a] | *Galleria mellonella* | Athens-C. Greece | 20140422CS6P8_H02_2016-04-27 |
| | B5/12 [a] | *Galleria mellonella* | Athens-C. Greece | 20140422CS6P10_H02_2016-04-27 |
| | ST19 | *Sesamia nonagioides* | Patra-W.Greece | 20140422CS6P11_H02_2016-04-27 |
| *Chaetomium* sp. | BD12 | *Rhyzopertha dominica* | Patra-W.Greece | 20140422CS8P2_H02_2016-04-27 |
| | BD2 | *Rhyzopertha dominica* | Zavlani-W.Greece | 20140422CS8P3_H02_2016-04-27 |
| *C. acropullum* | 2R | *Rhyzopertha dominica* | Patra-W.Greece | 20140422CS4P2_D02_2016-04-27 |
| *C. iranianum* | B17/3 | *Tribolium confusum* | Patra-W.Greece | 20140422CS8P1_H01_2016-04-27 |
| *C. truncatulum* | B4 | *Tribolium confusum* | Zavlani-W.Greece | 20170105CS7P1_G01_2017-01-11 |
| *I. fumosorosea* | I [a] | *Galleria melonella* | Ag. Stefanos-C. Greece | 20170105CS10P1_G01_2017-01-11 |
| *M. anisopliae* | Met A | *Rhyzopertha dominica* | Zavlani-W.Greece | 20140422CS3P2_C02_2016-04-27 |
| | Met B | *Plodia interpunctella* | Glafkos-W.Greece | 20140422CS3P3_C02_2016-04-27 |
| | B8A [a] | *Galleria mellonella* | Paramali-Cyprus | 20140422CS3P4_C02_2016-04-27 |
| | TH [a] | *Galleria mellonella* | Paramali-Cyprus | 20140422CS3P7_C02_2016-04-27 |
| *P. lilacinum* | B42 | *Plodia interpunctella* | Kastritsi-W.Greece | 20140422CS3P4_C02_2016-04-27 |
| *T. gamsii* | 1R, 4R, Z | *Rhyzopertha dominica* | Zavlani-W.Greece | 20140422CS7P1_G01_2016-04-27 |

[a] Isolates came from the Entomopathogenic Fungi (EF) cultures of the Benaki Phytopathological Institute. All other fungal isolates were collected from different regions in the prefecture of Achaia, Greece, using insect baits.

### 2.3. Isolation of Entomopathogenic Fungi

All infected larvae/adults were contained in Petri dishes on the nutrient Sabouraud Dextrose Agar (SDA) material. Alternatively, fungal conidia from the infected larvae/adults were cultivated on the same material. The Petri dishes were kept in a dark space, at 25 ± 1 °C and 65 ± 5% relative humidity, to enable the incubation of the fungi. The developed fungi were isolated again to avoid infestation and to achieve clear cultivation.

### 2.4. Lab Culture of Fungal Isolates

The fungal isolates that were tested during the present study are presented in Table 1. To prepare the appropriate suspensions, the isolates were grown in 9 cm Ø Petri dishes containing SDA and were left in the dark for 15 days at 25 ± 1 °C and 65 ± 5% relative humidity. The Petri dishes were sealed with Parafilm® American National Can, Chigago, USA) to avoid contamination. For each dose of the bioassays, fresh conidia were collected from the cultures after 15 days. Conidial suspensions were prepared by "scraping" the surface of the Petri dish using a sterile loop and transferring the conidia into a 500 mL glass beaker containing 50 mL of sterile distilled water plus 0.05% Tergitol® NP9 (Sigma–Aldrich, St. Louis, USA). The conidial suspension was panned across several layers of a sterile cloth and prepared by mixing the solution with a magnetic stirrer for 5 min [35]. The concentration of fungal conidia in the conidial suspension was determined using a Neubauer hemocytometer (WEBER SCIENTIFIC hemocytometer for cell counting) (Weber Scientific Inc., Hamilton Township, USA). Dilutions were prepared by adding 10 mL of the conidial suspensions to the desired quantity of sterile water, providing the final concentration of $10^8$ conidia per ml for all fungal isolates. The specific concentration was chosen given that it is a very common dose that has been widely used in a plethora of relevant studies. Conidial viability was >97% for all fungal isolates.

### 2.5. Bioassay

Adult weevils (1–2 weeks old) were used for experimentation. The evaluation of the virulence of each fungal isolate was carried out in Petri dishes with 10 adult beetles and 10 g of sterilized undamaged wheat kernels. Fifty adults were tested in each treatment (10 adults per replication for 5 replications per treatment). Adults of S. granarius were sprayed directly with 2.5 mL of conidial suspension containing $10^8$ conidia/mL of fungus, using a Potter spray tower (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, UK) at 1 kgf $cm^{-2}$. Untreated adults were used as control. All the insects were maintained under controlled conditions (25 °C, 65 ± 5% relative humidity, complete darkness), as described above. Petri dishes (control and treated adults) were checked after 7, 14 and 21 days and dead adults were counted and collected. 2.6. Pathogen Identification Methods

All dead weevils were immediately submerged in 95% ethanol for 1 min, washed in sterile distilled water for 5 min, allowed to dry, and then placed on moistened filter paper. The above-mentioned process was completed inside a laminar flow chamber (Equip Vertical Air Laminar Flow Cabinet Clean Bench, Mechanical Application LTD, Athens, Greece). Cadavers were kept at 25 °C and 65 ± 5% relative humidity, for 5–7 days in the dark, and those that showed hyphal growth characteristic of EF were recorded as infected. Dead Sitophilus adults were removed from the Petri dishes and superficial disinfestation with $NaOCl_2$ was applied to avoid fungal saprophytic growth. The sterilized dead adults were then kept individually in Petri dishes on a damp filter paper until mycelia appeared. Sitophilus cadavers showing external mycelia growth were examined using a microscope ZEISS Primo Star (Carl Zeiss Microscopy GmbH, Jena, Germany) at a 400x magnification, and fungi were determined based on the shape and size of hyphal growth [36–39].

The DNA sequencing process was also applied, adopting the method outlined by Rogers and Bendich [40]. The conidia were scraped off the surface of the dead *S. granarius* by using a sterile loop and transferring the conidia to PD Agar (In House technic). The genomic DNA (gDNA) was extracted applying universal primer sets ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAAC AAGG-3′), a fragment of the ITS spacer region was expanded. PCR reactions (30 µL) included 50 ng of template gDNA, 1.25 µL of each 10 pM oligonucleotide, 1 µL of 10 mM dNTPs, 1 µL of 2 U/µL Taq DNA polymerase (Minotech), 1.5 µL of $MgCl_2$, 2.5 µL of 10× PCR buffer. The PCR protocol for amplification of ITS regions includes 31 cycles at 94 °C for 60 s, at 55 °C for 60 s, and at 72 °C for 90 s, followed by a final elongation at 72 °C for 5 min. PCR products were kept at 4 °C. The quantity and quality of PCR products were resolved by gel electrophoresis using 2% agarose gel, which was stained with SYBR Safe DNA Gel Stain (Invitrogen) and visualized under UV

light (BIO RAD, Molecular Imager Gel Doc XR System). The amplified products were purified and sequenced in CeMIA SA, University of Thessaly.

### *2.6. Statistical Analysis*

In all cases, control mortality was very low (<2%) and, therefore, no correction was considered necessary for the mortality data. All values were arcsine transformed prior to analysis. Data were analyzed by two-way ANOVA using the general linear model of the SPSS 23.0 Windows (IBM Corp. 2015, New York, USA). In case of significant F values, means were compared using the Bonferroni test. The Probit analysis method was also selected to determine the median lethal time of *S. granarius* following the application of the pathogen concentrations. The Cox Regression method [41] was selected to determine the hazard effect of the isolates over *S. granarius*. It is a survival analysis regression model which describes the relation between the event incidence, as expressed by the hazard function, and a set of covariates. Comparison of survival distributions was obtained using Breslow (Generalized Wilcoxon) [42]. Comparison of Median lethal Time was performed using one-way ANOVA (Treatment as Factor).

Survival data are generally described and modeled in terms of two related probabilities, survival and hazard. The survival probability (which is also called "the survivor function"), S(t), is the probability that an individual survives from the time origin (e.g., beginning of treatment) to a specified future time, t.

The hazard probability is usually denoted by h(t) or λ(t) and refers to the probability that an individual who is under observation at a time t, has an event at that time. It represents the instantaneous event rate for an individual who has already survived by time t. Thus, while the survivor function reflects the cumulative non-occurrence of an event, the hazard function focuses on the occurrence of that event.

The mathematical expression of the Cox model is:

$$h(t) = h0(t) \times \exp\{b1x1 + b2x2 + \cdots + bpxp\}$$

where the hazard function h(t) is dependent on (or determined by) a set of p covariates (x1, x2, . . . , xp), whose impact is measured by the size of the respective coefficients (b1, b2, . . . , bp). The term h0 is called the baseline hazard and is the value of the hazard if all the xi are equal to zero (the quantity exp(0) equals 1). The 't' in h(t) reminds us that the hazard may (and probably will) vary over time.

## 3. Results

A total of 27 isolates were morphologically identified as they were retrieved from dead cadavers. The fungus which appeared on the cadavers of the 27 isolates was also confirmed by PCR. Fungal DNA sequences in the present work were matched with the Basic Local Alignment Search Tool (NCBI BLAST) (Table 1). Mycelial and conidial growth on cadavers suggested that recorded mortality was pathogen related. Observations of cadavers showed that external mycelium appears within the first 72 h after placing them on moist filter paper.

Mean mortality (%) of *S. granarius* is presented in Table 2, indicating high efficacy for many isolates by day 21 of the experiment. The several fungal isolates affected the survival time of the insect in diverse ways. All main effects and associated interactions between exposure time and fungal isolates, were significant for the mortality levels of *S. granarius* adults. (Table 3).

Two isolates of *B. bassiana* (H20 and 10T), *Aspergillus insuetus* (Bainier) Thom & Church (Eurotiales: Trichocomaceae) (D17) and *M. anisopliae* (Met A) caused the highest mortality, whereas *Chaetomium iranianum* Asgari and Zare (Sordariomycetes: Chaetomiaceae) (B17/3) as well as *M. anisopliae* (B8A) recorded the highest pest survival. As expected, the highest virulence to *S. granarius* was recorded after 21 days, while control mortality was lower than 2% even after the maximum exposure interval.

**Table 2.** Mean mortality (±SD) and Median Lethal Time of *S. granarius* adults exposed to various entomopathogenic fungal isolates. Means of the same column followed by the same letter are not significantly different (Bonferroni test, $p = 0.05$). Median Lethal Time was estimated by the Probit analysis and Means of the same column followed by the same letter are not significantly different (Bonferroni test, $p = 0.05$).

| Fungal Species | Isolate | Mean Mortality (%) | | | Median Lethal Time (days) (F = 5.230, df = 27, $p < 0.001$ |
|---|---|---|---|---|---|
| | | 7 days | 14 days | 21 days | |
| *Apophysomyces ossiformis* | B8B | 0.0 ± 0.0k | 3.3 ± 0.5j | 20.0 ± 1.7k | 20.4 ± 0.2a |
| *Aspergillus* sp. | G11 | 6.6 ± 0.5i | 20.0 ± 2.0e | 26.7 ± 2.0j | 19.1 ± 0.8a |
| *A. insuetus* | D17 | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 7.2 ± 0.2e |
| *B. bassiana* | BD14 | 10.0 ± 1.0h | 16.6 ± 2.1e | 16.6 ± 2.1l | 19.1 ± 0.8a |
| | D | 50.0 ± 2.0d | 70.0 ± 2.0c | 76.6 ± 2.5c | 12.6 ± 0.4c |
| | E20 | 0.0 ± 0.0k | 6.6 ± 1.1h | 36.6 ± 5.5g | 19.8 ± 0.3a |
| | H20 | 60.0 ± 2.6c | 100.0 ± 0.0a | 100.0 ± 0.0a | 9.8 ± 0.6c |
| | ST19 | 20.0 ± 1.0f | 23.3 ± 1.5e | 26.7 ± 1.1j | 17.9 ± 0.1b |
| | ST 28/11 | 13.3 ± 1.5g | 13.3 ± 1.5f | 16.7 ± 1.5l | 19.1 ± 0.8a |
| | 10T | 66.7 ± 3.2c | 86.6 ± 2.3b | 96.0 ± 0.5b | 10.5 ± 0.9c |
| | B18 | 23.3 ± 1.5f | 40.0 ± 3.0d | 53.3 ± 2.1f | 16.5 ± 1.1b |
| | B5/12 | 0.0 ± 0.0k | 3.3 ± 0.5j | 16.6 ± 0.5l | 20.3 ± 0.2a |
| | BB | 40.0 ± 2.6e | 46.6 ± 3.2d | 60.0 ± 4.4d | 14.9 ± 0.3b |
| *C. acropullum* | 2R | 10.0 ± 1.0h | 13.3 ± 1.5f | 16.6 ± 1.1l | 19.3 ± 0.9a |
| *C. iranianum* | B17/3 | 0.0 ± 0.0k | 6.6 ± 0.5i | 6.6 ± 0.5m | 20.5 ± 0.3a |
| *C. truncatulum* | B4 | 6.6 ± 0.5i | 10.0 ± 0.0g | 30.0 ± 1.7j | 19.8 ± 0.7a |
| *Chaetomium* sp. | BD2 | 0.0 ± 0.0k | 6.6 ± 1.4i | 13.3 ± 2.3l | 20.4 ± 0.2a |
| | BD12 | 10.0 ± 1.0h | 10.0 ± 1.0g | 44.0 ± 3.0h | 19.6 ± 0.8a |
| *I. fumosorosea* | I | 20.0 ± 1.7f | 36.6 ± 3.2d | 50.0 ± 4.4f | 17.1 ± 0.8b |
| *M. anisopliae* | Met A | 80.0 ± 2.6b | 100 ± 0.0a | 100 ± 0.0a | 8.4 ± 0.5d |
| | Met B | 53.3 ± 3.0d | 66.6 ± 4.2c | 70.0 ± 3.6c | 12.6 ± 1.2c |
| | B8A | 0.0 ± 0.0k | 3.3 ± 0.5j | 10.0 ± 1.0m | 20.4 ± 0.3a |
| | TH | 6.6 ± 1.1i | 20.0 ± 1.7e | 27.0 ± 1.5j | 19.1 ± 0.8a |
| *P. lilacinum* | B42 | 3.3 ± 0.5j | 3.3 ± 0.5j | 16.6 ± 1.5l | 20.1 ± 0.5a |
| *T. gamsii* | Z | 40.0 ± 3.4e | 60.0 ± 4.6c | 80.0 ± 3.4c | 11.4 ± 1.0c |
| | 1R | 50.0 ± 2.5d | 60.0 ± 3.5c | 63.3 ± 3.2d | 13.3 ± 1.2c |
| | 4R | 0.0 ± 0.0k | 0.0 ± 0.0k | 13.3 ± 2.3l | 20.4 ± 0.1a |
| Control | C | 0.0 ± 0.0k | 0.0 ± 0.0k | 1.0 ± 0.0n | 20.9 ± 0.0f |

**Table 3.** ANOVA parameters for main effects and associated interactions between exposure time and fungal isolates for mortality levels of *S. granarius* adults (error df 167).

| Source | df | F | $p$ |
|---|---|---|---|
| Exposure time | 2 | 30.844 | <0.05 |
| Fungal isolates | 27 | 5.323 | <0.05 |
| Exposure time × Fungal isolates | 54 | 4.435 | <0.05 |

Accordingly, in relation to the lowest median lethal time, this was estimated at 7.2 ± 0.2 days for *A. insuetus* (D17), 8.4 ± 0.5 days for *M. anisopliae* (Met A), 9.8 ± 0.6 days for *B. bassiana* (H20), 10.5 ± 0.9 days for *B. bassiana* (10T), 11.4 ± 1.0 days for *Trichoderma gamsii* (Z) Samuels & Druzhin (Hypocreales: Hypocreaceae), 12.6 ± 1.2 days for *M. anisopliae* (Met B) and *B. bassiana* (D), and 13.3 ± 1.2 days for *T. gamsii* (1R). In all other isolates, the median lethal time exceeded 15 days (Table 5).

**Table 4.** Survival and hazard effect of entomopathogenic fungi on *S. granarius* adults (Cox Regression method)(-2 Log Likelihood: 3998.475) (Chi-square: 414.314, df = 27, $p < 0.001$) * Survival effect: the probability that an individual survives from the time origin (e.g., beginning of treatment) to a specified future time; ** Hazard effect: the probability that an individual who is under observation at a time t, has an event at that time; *** Index values could not be estimated due to complete mortality.

| Fungal Species | Isolate | Exposure Time (Days) | Survival Table | |
|---|---|---|---|---|
| | | | Survival Effect * | Hazard Effect ** |
| *Apophysomyces ossiformis* | B8B | 7 | ***- | ***- |
| | | 14 | 0.967 | 0.034 |
| | | 21 | 0.800 | 0.223 |
| *Aspergillus* sp. | G11 | 7 | 0.933 | 0.069 |
| | | 14 | 0.800 | 0.223 |
| | | 21 | 0.733 | 0.310 |
| *A. insuetus* | D17 | 7 | 0.000 | 3.401 |
| | | 14 | ***- | ***- |
| | | 21 | ***- | ***- |
| *B. bassiana* | BD14 | 7 | 0.900 | 0.105 |
| | | 14 | 0.833 | 0.182 |
| | | 21 | ***- | ***- |
| | D | 7 | 0.500 | 0.693 |
| | | 14 | 0.300 | 1.204 |
| | | 21 | 0.233 | 1.455 |
| | E20 | 7 | ***- | ***- |
| | | 14 | 0.933 | 0.069 |
| | | 21 | 0.633 | 0.457 |
| | H20 | 7 | 0.400 | 0.916 |
| | | 14 | ***- | ***- |
| | | 21 | ***- | ***- |
| | ST19 | 7 | 0.800 | 0.223 |
| | | 14 | 0.767 | 0.266 |
| | | 21 | 0.733 | 0.310 |
| | ST 28/11 | 7 | 0.867 | 0.143 |
| | | 14 | ***- | ***- |
| | | 21 | 0.833 | 0.182 |
| | 10T | 7 | 0.333 | 1.099 |
| | | 14 | 0.133 | 2.015 |
| | | 21 | 0.033 | 3.401 |
| | B18 | 7 | 0.767 | 0.266 |
| | | 14 | 0.600 | 0.511 |
| | | 21 | 0.467 | 0.762 |
| | B5/12 | 7 | ***- | ***- |
| | | 14 | 0.967 | 0.034 |
| | | 21 | 0.833 | 0.182 |
| | BB | 7 | 0.600 | 0.511 |
| | | 14 | 0.533 | 0.629 |
| | | 21 | 0.400 | 0.916 |
| *C. acropullum* | 2R | 7 | 0.900 | 0.105 |
| | | 14 | 0.867 | 0.143 |
| | | 21 | 0.833 | 0.182 |
| *C. iranianum* | B17/3 | 7 | ***- | ***- |
| | | 14 | 0.933 | 0.069 |
| | | 21 | ***- | ***- |
| *C. truncatulum* | B4 | 7 | 0.933 | 0.069 |
| | | 14 | 0.900 | 0.105 |
| | | 21 | 0.700 | 0.357 |
| *Chaetomium* sp | BD12 | 7 | 0.900 | 0.105 |
| | | 14 | ***- | ***- |
| | | 21 | 0.567 | 0.568 |
| | BD2 | 7 | *** | ***- |
| | | 14 | 0.967 | 0.034 |
| | | 21 | 0.898 | 0.108 |
| *I. fumosorosea* | I | 7 | 0.800 | 0.223 |
| | | 14 | 0.633 | 0.457 |
| | | 21 | 0.500 | 0.693 |

**Table 5.** Survival and hazard effect of entomopathogenic fungi on *S. granarius* adults (Cox Regression method)(-2 Log Likelihood: 3998.475) (Chi-square: 414.314, df = 27, $p < 0.001$) * Survival effect: the probability that an individual survives from the time origin (e.g., beginning of treatment) to a specified future time; ** Hazard effect: the probability that an individual who is under observation at a time t, has an event at that time; *** Index values could not be estimated due to complete mortality.

| Fungal Species | Isolate | Exposure Time (Days) | Survival Table | |
|---|---|---|---|---|
| | | | Survival Effect * | Hazard Effect ** |
| *M. anisopliae* | Met A | 7 | 0.200 | 1.609 |
| | | 14 | ***- | ***- |
| | | 21 | ***- | ***- |
| | Met B | 7 | 0.467 | 0.762 |
| | | 14 | 0.333 | 1.099 |
| | | 21 | 0.300 | 1.204 |
| | B8A | 7 | ***- | ***- |
| | | 14 | 0.967 | 0.034 |
| | | 21 | 0.900 | 0.105 |
| | TH | 7 | 0.933 | 0.069 |
| | | 14 | 0.800 | 0.223 |
| | | 21 | 0.733 | 0.310 |
| *P. lilacinum* | B42 | 7 | 0.967 | 0.034 |
| | | 14 | ***- | ***- |
| | | 21 | 0.833 | 0.182 |
| *T. gamsii* | Z | 7 | 0.433 | 0.836 |
| | | 14 | 0.200 | 1.609 |
| | | 21 | 0.033 | 3.401 |
| | 1R | 7 | 0.500 | 0.693 |
| | | 14 | 0.400 | 0.916 |
| | | 21 | 0.367 | 1.003 |
| | 4R | 7 | ***- | ***- |
| | | 14 | ***- | ***- |
| | | 21 | 0.867 | 0.143 |

The survival effect of *S. granarius* adults was dependent on the hazard effect of the used isolate and the exposure time (Table 5). The highest hazard rate was recorded for the isolates of *B. bassiana* (H20), *A. insuetus* (D17) and *M. anisopliae* (Met A) at 7 days after exposure. The highest hazard rate was also recorded for *B. bassiana* (10T) but at 21 days after exposure. In contrast, the highest survival effect was recorded for the isolates *B. bassiana* (ST 28/11), *C. acropullum* (2R), *Chaetomium* sp (BD2), *M. anisopliae* (TH) and *T. gamsii* (4R) at 21 days after the exposure (Table 5).

## 4. Discussion

Entomopathogenic fungi are being developed worldwide for the control of insect pests and some products are already available commercially [43,44]. There is increasing evidence that habitat selection drives the pathogenicity of EF species [45]. Thus, results from our study indicate that screening of potential isolates should not be limited to those isolated from the original host.

*Sitophilus granarius* is one of the less studied storage pests as far as myco-biological control is concerned. Shams et al. [23] recorded 60 % mortality of *S. granarius* after 13 days, when weevils were immersed in a conidial solution of *B. bassiana*. They also estimated $LT_{50}$ at 10.45 days via probit analysis. When *S. granarius* adults were exposed to grain sprayed with a *B. bassiana* conidial suspension, the results demonstrated very low mortality (3%) [46].

Moreover, the insecticidal efficacy of EF is highly influenced by several other factors such as the insect's behavior, population density, age, nutrition and genetic information, environmental conditions, as well as the effect of host physiology and morphology on its sensitivity to biological control agents such as EF [47]. Therefore, the differences in insect susceptibility to EF could not be explained solely as a function of the applied conidial concentration [48].

Although significant variability in virulence was detected among various EF isolates, *B. bassiana* (H20 and 10T), *A. insuetus* (D17) and *M. anisopliae* (Met A) were generally the most virulent against *S. granarius*, with 10T causing significantly lower mortality (96%) than the other three mentioned isolates

(100%). These isolates caused the highest mortality within 7 days after inoculation, thus combining virulence and speed of action, which are the basic requirements for adequate effectiveness. The isolates with both the highest hazard effect and the lowest survival effect were *B. bassiana* (H20 and 10T), *A. insuetus* (D17) and *M. anisopliae* (Met A). The highest hazard effect indicates increased virulence or toxicity of the fungus over *S. granarius* adults, which conversely translates into the lowest survival effect of the fungus for the insect.

This is the first time that *A. insuetus*, *Apophysomyces ossiformis* P.C. Misra (Mucorales: Saksenaeaceae), *Purpureocillium lilacinum* (Thom) Luangsaard, Hou-braken, Hywel-Jones and Samson (Hypocreales: Ophiocordycipitaceae), *C. iranianum*, *Chaetomium truncatulum* Asgari and Zare, and *Chaetomium acropullum* X. Wei Wang (Sordariales: Chaetomiaceae) have been tested as potential biological control agents against an insect pest. Moreover, our study is the first attempt to test the EF species *T. gamsii* against a storage pest.

In general, eight EF isolates produced noteworthy mortality (>70%), whereas 11 isolates caused very low weevil mortality (<30%). There are a plethora of older and recent reviews reporting on EF treatments against stored product pests, with varying and often contradictory results. Cherry et al. [14] have also demonstrated that different isolates of *M. anisopliae* and *B. bassiana* can provide good control of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) by immersion bioassay at 12 days, whereby *B. bassiana* was reported to be more virulent than *M. anisopliae*. Khashaveh et al. [49] claimed that *B. bassiana* can be successfully used against stored wheat pests. Similarly, Wakefield et al. [50] reported that some *B. bassiana* isolates can achieve 100% mortality of *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) (organophosphate resistant strain) after 10 days of treatment with a dose of $1 \times 10^8$ conidia/mL. In older studies, mortality of stored grain pests reached 80–100% after 10–20 days [8,51].

Kassa [52] also reported that *B. bassiana* isolates were virulent against *Sitophilus zeamais* L. (Coleoptera: Curculionidae), but only at doses higher than $10^7$ conidia/mL and as such, variability among the different *B. bassiana* isolates was apparent. Hidalgo et al. [53] also pointed out that it is possible to achieve a useful level of control of *S. zeamais* by using formulated *B. bassiana* conidia. In laboratory experiments, Rodrigues and Pratissoli [54] evaluated the pathogenicity of *Beauveria brongniartii* (Saccardo) Petch (Deuteromycotina, Hyphomycetes) and *M. anisopliae* isolates against *S. zeamais* and *Acanthoscelides obtectus* Say (Coleoptera: Chrysomelidae). The first EF species caused 89% mortality to *S. zeamais* and 47% to *A. obtectus*, while *M. anisopliae* caused less than 50% mortality to both insects.

Batta [28] recorded high mortality of *Rhyzopertha dominica* F. (Coleoptera: Bostrichidae) after 7 days of treatment with *M. anisopliae*. Greater mortality of stored grain pests was achieved when these were inoculated with Beauveria rather than Metarhizium isolates [55,56]. In contrast, Dal Bello et al. [57] reported that treatment of *S. oryzae* with *M. anisopliae* was not effective.

In our experiment, median lethal time values indicate that the examined isolates are as effective as any other isolate reported in literature. Similar median lethal time values have been reported for various storage pests treated with EF species [17,23,49,55,58,59]. However, it should be mentioned that variation, not only in experimentation methods, fungal isolates and insect strains but also in median lethal time estimation methods, renders the direct comparison of these values impossible.

## 5. Conclusions

In summary, our results demonstrate that EF show promise against stored product pests and should therefore be further investigated as potential biological control agents and as a valuable component of stored product IPM. Considering the propensity of Sitophilus species to develop resistance to synthetic chemicals, the exploration of alternative biological control methods appears even more necessary. Although this is only a preliminary investigation into the use of EF, the fungal isolates we tested showed encouraging insecticidal effects which, however, need to be extensively followed-up. Future research steps include establishing the biosafety of these fungi for non-target organisms, examining

performance assurance in challenging environments, and the creation of formulations of enhanced persistence, longer shelf life, ease of application, and pathogen virulence.

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