

Article

The Effect of Grain Type on Virulence of Entomopathogenic Fungi Against Stored Product Pests

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Abstract: Fungal virulence is multifaceted and dependent on multiple abiotic factors. The present study represents an investigation of the effect of one such abiotic factor, that of the grain type, on the insecticidal action of three entomopathogenic fungal species, *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae), *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) and *Isaria fumosorosea* Wize (Hypocreales: Clavicipitaceae) on larvae of the three very common and destructive stored product pests: the khapra beetle (*Trogoderma granarium* Everts) (Coleoptera: Dermestidae), the confused flour beetle (*Tribolium confusum* Jacquelin du Val) (Coleoptera: Tenebrionidae) and the Mediterranean flour moth (*Ephestia kuehniella* Zeller) (Lepidoptera: Pyralidae). To this end, we selected four different grains, i.e., *Triticum aestivum* L. (Poales: Poaceae), *Oryza sativa* L. (Poales: Poaceae), *Arachis hypogaea* L. (Fabales: Fabaceae) and *Vicia faba* L. (Fabales: Fabaceae). Bioassays were carried out in the lab, where experimental grains were sprayed with 1 mL of conidial suspension (10^8 conidia/mL) from each isolate. Mean mortality, median survival time and weight loss of seeds were estimated for each species. Our results suggest that the differences in the efficacy of entomopathogenic fungi were dependent both on the isolates and the grain. The grain type as a factor is equally important to other abiotic factors.

Keywords: abiotic factor; grain type; *Trogoderma granarium*; *Tribolium confusum*; *Ephestia kuehniella*; entomopathogenic fungi

1. Introduction

The most common strategy for controlling insect pests in storage facilities is the application of residual or fumigant chemical insecticides [1,2]. However, this has led to the occurrence of many problems such as environmental pollution, various dangers for applicators and consumers and emergence of pest resistance [3,4]. All these negative side-effects drive researchers to search for alternative environmentally safe and residue-free control methods [3,5,6].

Entomopathogenic fungi are considered as potential candidates to replace chemical insecticides. Their potential pathogenicity has been the focus of a significant number of studies on the use of pathogens for stored product control [7–10]. Entomopathogenic fungi are microorganisms which are ubiquitous in nature and, as such, they are safe for the environment and of low toxicity to mammals [11–13]. Furthermore, they are able to develop on the cadavers, thus continuing to release

more inoculum into the system [14,15]. In this sense, the generated long-term effect of fungi is a desirable trait in biological control as opposed to the residual persistence of chemical pesticides [16].

Beauveria, *Metarhizium* and *Isaria* genera are listed among the most important microbial species which are used as biological control agents and which have been documented to act against a wide range of insect hosts [10,17–20]. The fungal species which have been the most studied for their action against stored product insect pests and which have produced promising results are *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae), *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) and *Isaria fumosorosea* Wize (Hypocreales: Clavicipitaceae) [9,21–25]. The extent of entomopathogenic fungal virulence is conditioned by the interaction of several factors which involve the time of the initial host infection, the time that the disease incubation period lasts, resulting in delayed host development, the rate at which the entomopathogenic fungus spreads and several other environmental factors which are known as being conducive to epizootics [26–28].

However, the input of other parameters such as the type of grain has been poorly studied. This factor might be related with the transmission of the pathogen to the host. Our objective was hence to investigate the effect of the grain type on the insecticidal action of three entomopathogenic fungal species, *B. bassiana*, *M. anisopliae* and *I. fumosorosea*, on larvae of the three very common and harmful stored product pests: the khapra beetle (*Trogoderma granarium* Everts) (Coleoptera: Dermestidae), the confused flour beetle (*Tribolium confusum* Jacquelin du Val) (Coleoptera: Tenebrionidae) and the Mediterranean flour moth (*Ephesia kuehniella* Zeller) (Lepidoptera: Pyralidae), on four different grains such as *Triticum aestivum* L. (wheat) (Poales: Poaceae), *Oryza sativa* L. (rice) (Poales: Poaceae), *Arachis hypogaea* L. (peanuts) (Fabales: Fabaceae) and *Vicia faba* L. (beans) (Fabales: Fabaceae). Our results are discussed within the framework of employing entomopathogenic fungi as biocontrol agents against stored product pests.

2. Material and Methods

2.1. Insect Rearing

Trogoderma granarium was reared on hard wheat, while whole wheat flour with 10% dried yeast was used for the rearing of *T. confusum*. The rearing of *E. kuehniella* was carried out in 24 × 45 × 30 cm cages. The frame, the rear side and the bottom surface of the cages were wooden (medium-density fiberboard, MDF), while the other two slanting sides were covered with a plastic grid. The front side of the cages was also covered with a plastic grid with a small cross-section which functioned as the entrance into their interior. In each cage, we placed 15 to 20 pairs of adults per insect species. The adult individuals were supplied with sugar water on 1 cm dental cotton balls which were placed in Petri dishes at the bottom of the cage. Inside the cages with *E. kuehniella*, sterile semolina and antibiotic, serving as artificial spawning bedding, were placed in transparent plastic containers. The containers remained in the cages between three and four days, and they were then transferred to a separate dark space until the insects completed their biological cycle. They had first been covered with tulle so that the newly hatched larvae did not escape. The larvae were placed in the transparent containers according to their age, to avoid overcrowding which would hinder both their development and food intake. The insects were in room conditions of 25 ± 1 °C, 60–70% humidity and photoperiod 16:8 hours light-darkness, for the duration of their development.

2.2. Entomopathogenic Fungi

We used strains of *B. bassiana* (strain name: GBBSTTS), *I. fumosorosea* (strain name: RHZ4RAS) and *M. anisopliae* (strain name: Z3RAS) which are local to the region of Achaia in Western Greece [10]. The conidial suspensions were prepared and the conidia germinated inside a laminar flow chamber (Equip Vertical Air Laminar Flow Cabinet Clean Bench, Mechanical Application LTD). To complete fungal sporulation, we cultivated all isolates on SDA (Sabouraud Dextrose Agar, OXOID LTD) and left them to incubate at 24 °C for 14 days. Fungal conidia were harvested in 2.5% Tween 80 and suspended

in 5 mL sterile distilled water. We used a Neubauer hemocytometer (TIEFE 0.100 mm 1/400 9 mm) to read the number of conidia. Serial dilutions (seven-spore concentrations) of each fungal isolate were performed to obtain the desired concentrations for each experiment. To establish the viability of conidia, we spread a drop of conidial suspensions on glass slides in Petri dishes lined with moistened filter paper. Three glass slides corresponded to each isolate (three replicates), and they were counted for germination after a 24-h period at 25 ± 2 °C. We considered as germinated those conidia whose germ tubes were at least equal to their width.

2.3. Bioassays

The grain varieties we used for the bioassays were: for *T. aestivum*, “Love”, for *O. sativa*, “Japonica”, for *A. hypogaea*, “Virginia”, and for *V. faba*, “Giant Prespon”. We placed the grains in a sterile 9 cm Petri dish and then sprayed with a 5 mL conidial suspension using a Potter spray tower (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, U.K.) at 1 kgf cm^{-2} . We let the fungal suspension drain through a sterilized 4 cm in diameter strainer. The treated grains were then moved to a Petri dish (9 cm) with a sterile loop. The Petri dishes contained each 10 g of the desired grain type. Control grains were treated in the same manner with ddH₂O. Ten third-instar larvae were used per replication, while each assay was replicated three times with a conidial suspension containing 10^8 spores mL⁻¹ of fungus. The larvae had been starved for 24 h. At the end of the bioassay, the content of the Petri dishes was re-weighed (Kern PCB 10000-1). All experimental insects were kept in conditions of 26 °C and $70\% \pm 5\%$ R.H. for 10 days. The mortality count was daily. Dead insects were transferred to different Petri dishes, on a filter paper impregnated with ddH₂O, where they were maintained separately for 10 days and monitored for signs of fungal infection. This process took place inside a laminar flow chamber (Equip Vertical Air Laminar Flow Cabinet Clean Bench, Mechanical Application LTD). The cadavers, which we collected at the indicated times, were immediately dipped in 95% ethanol for 1 min, rinsed with sterile distilled water for 5 min, left to dry naturally and finally placed on moistened filter paper. Dead larvae were kept in darkness at 25 °C for a 5- to 7-day period, and those that showed signs of hyphal growth typical of entomopathogenic fungi were noted as infected. We then confirmed the species with microscopic testing, using the shape and the size of hyphal growth on the cadavers as markers for the identification [29].

2.4. Statistical Analysis

Corrected percent mortality was calculated using Abbott’s formula [30] and, prior to analysis, values were arcsine transformed. Data were then analyzed by means of three-way Analysis of variance ANOVA using the general linear model of the SPSS (SPSS Inc., IL, USA, version 23). In case of significant F values, means were compared using the Bonferroni as post-hoc test. The Kaplan–Meier method was also selected to determine the median survival time of the insects from the applied concentration of pathogens and products. Comparison of median survival time was obtained using the Breslow test (Generalized Wilcoxon) (SPSS v.23.0).

We assessed the damage of the treated and untreated grains by taking samples from each Petri dish. Damaged grains (marked with characteristic perforations) and undamaged grains were counted and weighted. We calculated the percentage of weight loss following the FAO method [31] whereby:

$$\% \text{ Weight loss} = \frac{U_a N - (U + D)}{U_a N} \times 100$$

where: U = weight of undamaged fraction in the sample; N = total number of grains in the sample; U_a = average weight of one undamaged grain; D = weight of damaged fraction in the sample.

3. Results

Significant differences were detected among fungal isolates and the control (Table 1). Very low control mortality was recorded among all experimental larvae, while no fungal development was

detected on the dead control insects. The mean control mortality was calculated between 0 (wheat, beans and peanuts) and 3.33% (rice) for *T. granarium*, between 0 (wheat, beans and peanuts) and 3.33% (rice) for *E. kuehniella* and between 0 (wheat and rice) and 6.66% (peanuts) for *T. confusum*.

Table 1. Three-way ANOVA post-hoc (Bonferroni test to independent variable).

(A) Variable: Mortality			
Factor	df	F	Sig.
Insect	2	5.665	0.000
Product	3	14.818	0.000
Fungi	3	50.484	0.000
Insect * Product	6	1.438	0.196
Insect * Fungi	6	1.576	0.150
Product * Fungi	9	3.834	0.000
Insect * Product * Fungi	18	0.971	0.491
Error	1392		
Total	1440		
Corrected Total	1439		
(B) Variable: Seed Weight			
Factor	df	F	Sig.
Product	3	1.799	0.153
Insect	2	12.341	0.000
Fungi	3	106.072	0.000
Product * Insect	6	1.515	0.181
Product * Fungi	9	3.976	0.000
Insect * Fungi	6	1.294	0.267
Product * Insect * Fungi	18	1.217	0.264
Error	96		
Total	144		
Corrected Total	143		

* This symbol indicate interaction of the factors.

On peanut seeds, the mean mortality of larvae ranged between 60% (*I. fumosorosea*) and 76.67% (*M. anisopliae*) for *T. granarium*, between 66.67% (*B. bassiana*–*I. fumosorosea*) and 73.3% (*M. anisopliae*) for *E. kuehniella* and between 57% (*B. bassiana*) and 93% (*M. anisopliae*) for *T. confusum* (Figure 1). The strain of *M. anisopliae* was the most virulent to the *T. confusum* larvae, in comparison with the larvae of the other species.

On wheat seeds, the mean mortality of larvae ranged between 16.67% (*M. anisopliae*) and 53.33% (*I. fumosorosea*) for *T. granarium*, between 23.33% (*M. anisopliae*) and 43.3% (*I. fumosorosea*) for *E. kuehniella* and between 26.67% (*B. bassiana*) and 63% (*I. fumosorosea*) for *T. confusum*, and significant differences were detected among fungal isolates. *I. fumosorosea* was the most virulent strain to the *T. confusum* larvae, in comparison with the larvae of the other species.

On rice seeds, the mean mortality of larvae was calculated between 33.33% (*I. fumosorosea*) and 46.67% (*B. bassiana*) for *T. granarium*, between 26.67% (*B. bassiana*) and 46.67% (*I. fumosorosea*) for *E. kuehniella* and between 20% (*B. bassiana*) and 33.33% (*M. anisopliae*) for *T. confusum*, and significant differences were detected among fungal isolates. *I. fumosorosea* was the most virulent strain to *E. kuehniella* larvae while *B. bassiana* was the most virulent for *T. granarium*, in comparison with the larvae of the other species.

On bean seeds, the mean mortality percentage of larvae ranged between 40% (*I. fumosorosea*) and 46.67% (*B. bassiana* - *M. anisopliae*) for *T. granarium*, between 30% (*I. fumosorosea*) and 60% (*B. bassiana*) for *E. kuehniella* and between 26.67% (*B. bassiana*–*I. fumosorosea*) and 60% (*M. anisopliae*) for *T. confusum*, and significant differences were detected among fungal isolates. *M. anisopliae* was the most virulent

strain to larvae of *T. confusum*, while *B. bassiana* was the most virulent for *E. kuehniella*, in comparison with the larvae of the other species.

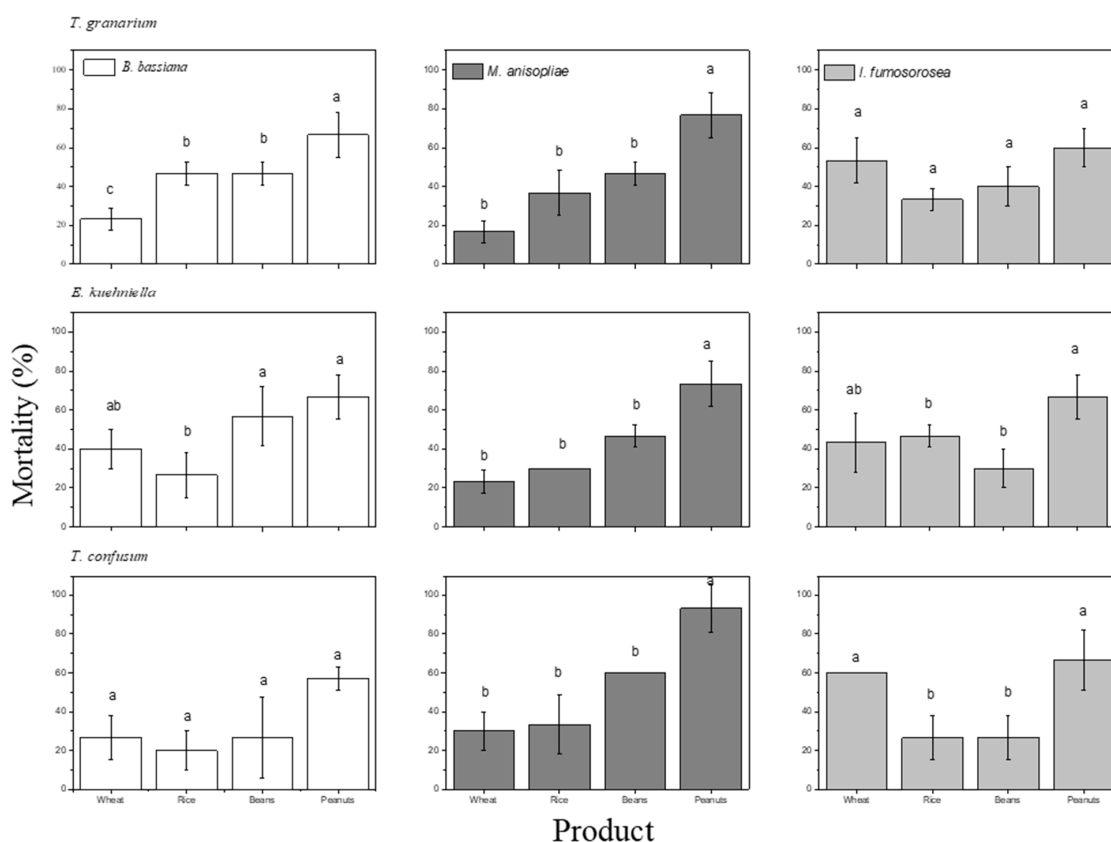


Figure 1. Mean larval mortality (% \pm SD) of *T. granarium*, *E. kuehniella* and *T. confusum* exposed for 10 days to rice, beans, peanuts and wheat grains treated with 10^8 spores mL^{-1} of *B. bassiana* (strain name: GBBSTTS), *I. fumosorosea* (strain name: RHZ4RAS) and *M. anisopliae* (strain name: Z3RAS). Mean \pm sd values with the same letter within a column are not significantly different ($p < 0.05$).

The median survival time was 7.3 days for the *B. bassiana* isolate, 6.9 days for the *M. anisopliae* isolate, 7.1 days for the *I. fumosorosea* isolate and 9.9 days for the control (chi-square = 195.7, $\text{df} = 3$, $p = 0.001$). In relation to the grain types used in our bioassays, the median survival time of all tested larval species was 8.2 days on wheat seeds, 7.8 days on rice seeds, 7.7 days on bean seeds and, finally, 7.1 days on peanut seeds (chi-square = 64.3, $\text{df} = 3$, $p = 0.001$). The overall median survival time was 8.4 days for *T. granarium* larvae, 7.2 days for *E. kuehniella* larvae and 8.4 days for *T. confusum* larvae (chi-square = 2.5, $\text{df} = 3$, $p = 0.284$).

The seed weight after infestation with *T. granarium* larvae was: for peanut seeds between 8.32 (control) and 9.76 g (*B. bassiana*); for wheat seeds between 8.48 (control) and 9.74 g (*M. anisopliae*); for rice seeds between 8.76 (control) and 9.59 g (*M. anisopliae*); for bean seeds between 8.92 (control) and 9.56 g (*I. fumosorosea*) (Figure 2). The seed weight after infestation with *E. kuehniella* larvae was: for peanut seeds between 8.66 (control) and 9.66 g (*M. anisopliae*); for wheat seeds between 9.06 (control) and 9.78 g (*M. anisopliae*); for rice seeds between 9.07 (control) and 9.72 g (*M. anisopliae*); for bean seeds between 8.88 (control) and 9.65 g (*M. anisopliae*) (Figure 3). The seed weight after infestation with *T. confusum* larvae was: for peanut seeds between 8.29 (control) and 9.54 g (*M. anisopliae*); for wheat seeds between 7.95 (control) and 9.64 g (*M. anisopliae*); for rice seeds between 9.12 (control, *B. bassiana*) and 9.60 g (*I. fumosorosea*); for bean seeds between 8.75 (control) and 9.52 g (*I. fumosorosea*) (Figure 4).

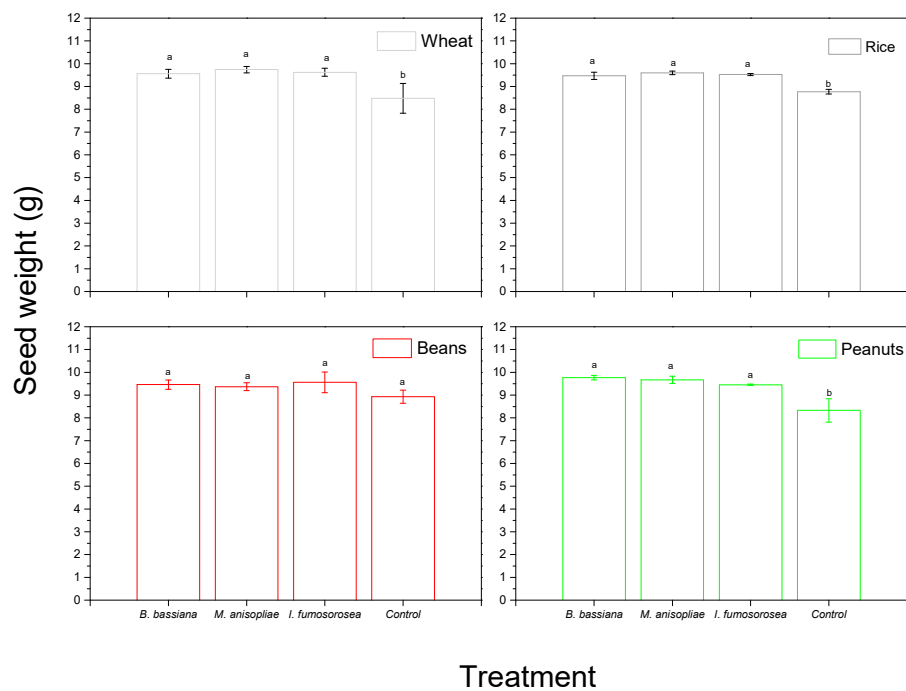


Figure 2. Seed weight ($\text{g} \pm \text{SD}$) of wheat, rice, beans and peanuts treated with 10^8 spores mL^{-1} of *B. bassiana* (strain name: GBBSTTS), *I. fumosorosea* (strain name: RHZ4RAS) and *M. anisopliae* (strain name: Z3RAS) after infestation with *T. granarium* larvae for 10 days.

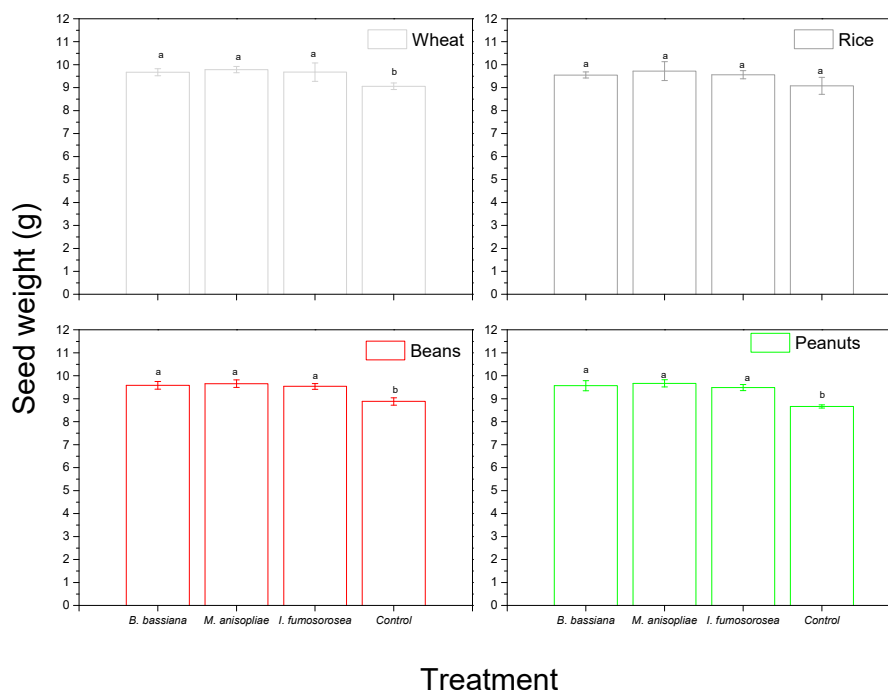


Figure 3. Seed weight ($\text{g} \pm \text{SD}$) of wheat, rice, beans and peanuts treated with 10^8 spores mL^{-1} of *B. bassiana* (strain name: GBBSTTS), *I. fumosorosea* (strain name: RHZ4RAS) and *M. anisopliae* (strain name: Z3RAS), after infestation with *E. kuehniella* larvae for 10 days.

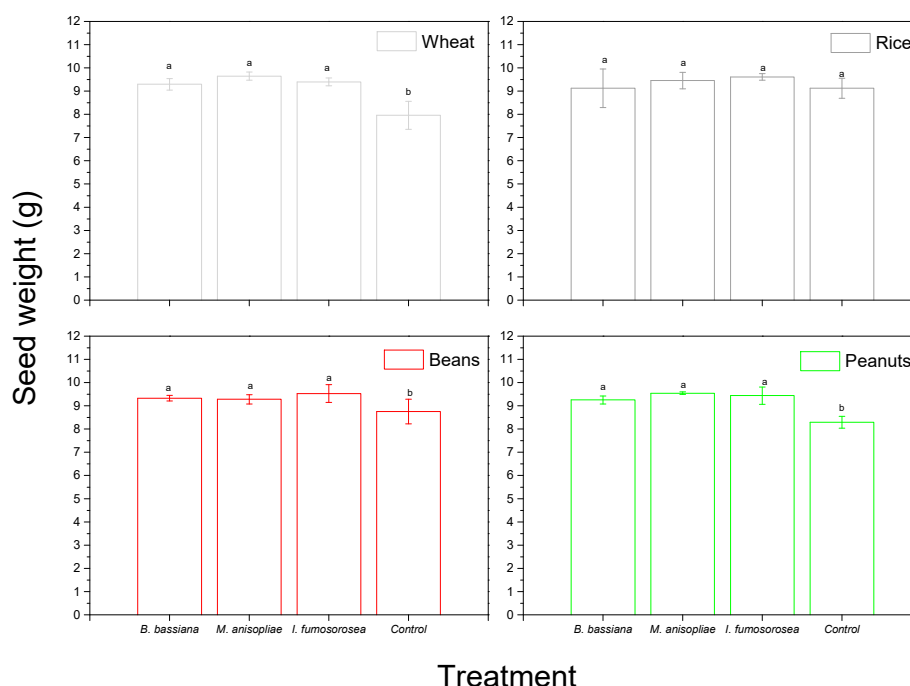


Figure 4. Seed weight ($\text{g} \pm \text{SD}$) of wheat, rice, beans and peanuts treated with 10^8 spores mL^{-1} of *B. bassiana* (strain name: GBBSTTS), *I. fumosorosea* (strain name: RHZ4RAS) and *M. anisopliae* (strain name: Z3RAS), after infestation with *T. confusum* larvae for 10 days.

In addition to the mortality of larvae which was caused by the entomopathogenic fungi, we also observed a decrease in product loss in all treatments (Table 1B). The mean percentage of loss of seeds infested with *T. granarium* larvae was: for peanut seeds, from 2.3% to 5.4% in treatments and 16.7% in the control; for wheat seeds, from 2.6% to 4.4% in treatments and 15.2% in the control; for rice seeds, from 4.0% to 5.3% in treatments and 12.3% in the control; for bean seeds, from 4.3% to 6.3% in treatments and 10.7% in the control.

The mean percentage of loss of seeds infested with *E. kuehniella* larvae was: for peanut seeds, from 3.3% to 5.1% in treatments and 13.8% in the control; for wheat seeds, from 2.1% to 3.3% in treatments and 9.4% in the control; for bean seeds, from 3.4% to 4.6% in treatments and 11.2% in the control; for rice seeds, from 2.8% to 4.5% in treatments and 9.2% in the control.

The mean percentage of loss of seeds infested with *T. confusum* larvae was: for peanut seeds, from 4.5% to 7.4% in treatments and 17.1% in the control; for wheat seeds, from 3.5% to 7.1% in treatments and 19.1% in the control; for bean seeds, from 4.7% to 7.2% in treatments and 12.5% in the control; for rice seeds, from 3.9% to 7.1% in treatments and 12.4% in the control (Figure 4).

4. Discussion

The results of the present study may be of increased interest as the environmental safety of an insecticide is today considered to be of paramount importance. Fungi have a specific role in pest control, having high persistence in the environment. Some entomopathogenic fungi infect a limited range of hosts, while other species have a wider host range, i.e., *M. anisopliae*, *I. fumosorosea* and *B. bassiana*. Although several published data exist regarding the efficacy of entomopathogenic fungi against stored-grain insect pests [22,23,32–34], there is a lack of references addressing the effect of the product type on the efficacy of these fungi.

The insecticidal degree of entomopathogenic fungi is interdependent with several factors such as the behavior of the insect, its age, nutrition, genetic profile, the density of its population, as well as the extent of host sensitivity to biological control agents, due to host physiology and morphology [35]. Therefore, it would not be accurate to attribute differences in the vulnerability of the storage beetle to

entomopathogenic fungi merely to the applied conidial concentration [36]. Our results point to the fact that variation in the efficacy of entomopathogenic fungi was a result of both the product and the isolates. On peanut seeds, our treatment of *T. granarium* with different species of entomopathogenic fungi indicated high levels of larval mortality 10 days after treatment. On wheat, rice and bean seeds, however, larval mortality was at moderate levels. The lowest overall mortality of *E. kuehniella* larvae was detected on rice seeds, while the highest was recorded on peanut seeds 10 days after treatment. The overall mortality of *T. confusum* larvae was the lowest on rice seeds and on peanut seeds, 10 days following treatment. It was reported that the treatment of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) with *B. bassiana* caused more than 80% mortality to adult insects which had been reared on three types of nutritional substrates, 21 days post-treatment [37]. Moreover, *M. anisopliae* isolates tested on wheat produced a significant rate of *E. kuehniella* larval mortality, exceeding 90%, 14 days after exposure [38]. By contrast, it was reported that the treatment of *E. kuehniella* with *M. anisopliae*, *B. bassiana* and *Lecanicillium lecanii* (Zimm.) Zare & W. Gams (Hypocreales: Cordycipitaceae) on wheat produced very low mortality rates (< 70%) [39]. A comparison of the cited studies with our own results suggests that different isolates and different products can have dissimilar effects on *E. kuehniella*, *T. confusum* and *T. granarium* larvae. Apparently, product is a favorable factor to be considered in the selection of suitable fungi for the control of serious stored-product pests, as suggested by the median lethal time of larvae in relation to the products. The median lethal time of larvae was the lowest on peanut seeds, and it was the highest on rice seeds.

Our results are certainly encouraging for the integrated control of *E. kuehniella*, *T. confusum* and *T. granarium* larvae, but the combined application of virulent fungal isolates with the suitable product may also be practically used to reinforce the control of other stored grain insect pests. It has been reported that treating stored wheat grains with *B. bassiana* resulted in a reduction of the total grain weight loss caused by *S. oryzae* (L.) infestation in the storage facility [40]. Grain loss related to storage insects has been studied mainly in short laboratory tests [41–43]. All the products which had been infested with *E. kuehniella*, *T. confusum* and *T. granarium* larvae but had not received treatment with entomopathogens were more extensively damaged by the larvae than the products which had been treated with entomopathogenic fungi. The mean fresh weight loss of products treated with the fungi was significantly lower than the corresponding mean fresh weight loss of untreated products. Along similar lines, the median survival time of untreated larvae was higher than that of treated larvae.

The use of entomopathogenic fungi could hold benefits for the environment and stored grain protection. After the death of the insect, the inoculum continues to be renewed as the cadaver still sporulates infectious agents [44]. Storage insects will thus contract entomopathogenic doses from the sporulating cadavers. In conclusion, the virulence and effectiveness of entomopathogenic fungi to control postharvest pests are not just a function of abiotic factors, such as temperature and relative humidity. The product as a factor has an equal share of significance, as do abiotic factors. Additional experiments are required to determine 1) how the product as a factor affects the efficacy of the fungi and 2) the extent to which the environment of the storage facility, including its entry points, affects the biological behavior of the insects as well as the pathogenicity of the entomopathogenic fungi.

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