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Control of Main Stored-Grain Insects with New Formulations of Entomopathogenic Fungi in Diatomaceous Earth Dusts

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Control of Main Stored-Grain Insects with New Formulations of Entomopathogenic Fungi in Diatomaceous Earth Dusts*

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Abstract

The present research is aimed at a formulation of the entomopathogenic fungi, *Beauveria bassiana* (Bal.) Vuillemin and *Metarhizium anisopliae* (Metch.) Sorokin, in two types of diatomaceous earth dusts, fossil shield and Silico-Sec, are then applied against the adults of three species of stored-grain insects: *Sitophilus oryzae* L., *Rhyzopertha dominica* Fab. and *Tribolium castaneum* Herbs. Effect of the treatment was assessed by comparing the mortality percentage of the adults of the three insect species exposed to the formulated fungi with that of the adults exposed to the unformulated fungi or the diatomaceous earth dusts or the undisturbed control. Results obtained from these exposures have indicated that treatment of the adults with the formulated fungi resulted in a significantly higher mean mortality percentage compared to the treatment with the unformulated fungi or the diatomaceous earth dusts or the undisturbed control. A synergistic interaction between the effect of fungal species and the diatomaceous earth dusts was shown. Viability of conidia of both fungal species in diatomaceous earth dusts was assessed by calculating the germination percentage of the conidia over time. Results indicated a small loss of mean germination percentage for formulated conidia of both fungal species versus a high loss of mean germination percentage for the unformulated conidia, thus the diatomaceous earth dusts used in the formulation of both fungi demonstrated a negligible effect on the viability of formulated conidia compared to the unformulated.

KEYWORDS: Rhyzopertha dominica, Sitophilus oryzae, Tribolium castaneum, Beauveria bassiana, Metarhizium anisopliae, Silico-Sec, fossil shield

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1. Introduction

The rice weevil (*Sitophilus oryzae* L., Coleoptera : Curculionidae), the lesser grain borer (*Rhyzopertha dominica* Fab., Coleoptera: Bostrichidae), and the red flour beetle (*Tribolium castaneum* Herbs., Coleoptera: Tenebrionidae) are considered the major stored-grain insect pests of cereals and pulses. *S. oryzae* and *R. dominica* are primary internal feeders of stored grains because they are able to attack whole, dried, sound grains of many cereals and pulses reducing them to empty shells, whereas *T. castaneum* is a secondary feeder of stored grains because it is able to attack the byproducts of grains such as flour and meal of cereals (Hagstrum et al. 1994, Elzinga 1997). These insects cause significant losses that may reach up to 10 % or more to stored grains and their byproducts, especially under hot dry storage conditions favorable to their development (25 to 35 ^{o} C and low RH) (Moino, Jr et al. 1998). Grain weight loss is caused by the feeding of larvae and adults of these insects so that the attacked grains are reduced to empty shells with little or no nutrient value, whereas qualitative damage is caused by product alterations such as loss of nutritional and aesthetic value, increased levels of rejects in the grain mass, and loss of industrial characteristics needed for preparation of breads and other products (Hagstrum and Flinn 1994, Moino, Jr et al. 1998).

Control of stored-grain insects in many countries is irregularly practiced to decrease their damage in storage facilities during warm months by chemical control with various types of insecticides such as Lindane, Malathion, Chlorpyrifos-methyl, Bioresmethrin, Resmethrin, Deltamethrin, Pirimiphos-methyl, Fenitrothion and Cyfluthrin (Bengston et al. 1975, Brun and Attia 1983, Arthur 1992, 1994; Lorini and Galley 1999) and fumigants such as Methyl bromide and Phosphine (Attia and Greening 1981, Tyler et al. 1983, Herron 1990, Zettler 1997, Zettler and Arthur 2000). In some countries, the control of these insects using other alternative means have been attempted to overcome the undesirable side-effects (e.g insecticide resistance) associated with the frequent use of chemical control (Champ and Dyte 1976, Zettler and Cuperus 1990, Arthur 1992). The use of entomopathogenic fungi as alternative means to chemical control is a novel approach to control insect pests of stored grain. Potential for this use has been reported by some investigators for the entomopathogenic fungus *Beauveria bassiana* (Bal.) Vuillemin (Deuteromycotina: Hyphomycetes) against the following stored-grain insects: rice weevil (*Sitophilus oryzae*), corn weevil (*S. zeamais*), granary weevil (*S. granarius*), lesser grain borer (*Rhyzopertha dominica*), red and confused flour beetles (*Tribolium castaneum* and *T. confusum*), *Oryzaephilus surinamensis*, and *Prostephanus truncatus* (Adane et al. 1996, Padin et al. 1996, 1997, 2002; Hidalgo et al. 1998, Moino, Jr et al. 1998, Rice and Cogburn 1999, Odour et al. 2000, Dal-Bello et al. 2001, Meikle et al. 2001, Lord 2001, Sheeba et al. 2001), and for another entomopathogenic fungus *Metarhizium anisopliae* (Metch.) Sorokin (Deuteromycotina: Hyphomycetes) against the following stored-grain insects: rice weevil (*Sitophilus oryzae*), lesser grain borer (*Rhyzopertha dominica*), and red flour beetle (*Tribolium castaneum*) (Dal-Bello et al. 2001, Batta 2004, 2005; Batta and Abu Safieh 2005, Michalaki et al. 2006).

The use of diatomaceous earth dusts, naturally occurring substances comprised of the fossilized remains of freshwater or marine unicellular diatoms, as alternative to the

conventional insecticides constitutes another novel approach to control insect pests of stored grain. Many investigators have reported the effective use of these dusts for suppressing the population of several stored-grain insects such as red flour beetle (*Tribolium castaneum*), confused flour beetle (*T. confusum*), the pulse beetle (*Callosobruchus maculatus*), *Oryzaephilus surinamensis*, *Sitophilus granarius* (Subramanyam et al. 1994, Quarles and Winn 1996, Golob 1997, Dowdy 1999, Arthur 2000, Flachsbarth et al. 2000, Mewis and Ulrichs 2001, Rigaux et al. 2001, Erb-Brinkmann and Straube 2002, Prasantha et al. 2002).

Up todate, very few studies have been reported on the use of diatomaceous earth dusts and entomopathogenic fungi combinations against certain species of stored-grain insects like *Tribolium castaneum*, *Rhyzopertha dominica* and *Oryzaephilus surinamensis* (Quarles and Winn 1996, Lord 2001, Waseem et al. 2004, Michalaki et al. 2006), but unproven efficacy at practical dose rates for these stored-grain pests has been shown during the above mentioned studies. This is attributed to the contradiction in moisture (humidity) requirements for the action of diatomaceous earth dusts and entomopathogenic fungi to reach a high level of effectiveness against the stored-grain insects. It is well-known that diatomaceous earth dusts are the most efficacious against stored-grain insects in non humid conditions (below 80 % RH) due to their action as desiccant of insect cuticle by abrading it and absorbing lipids from its epicuticle (Golob 1997, Appel et al. 1999, Erb-Brinkmann 2000, Mewis and Ulrichs 2001), whereas the entomopathogenic fungi are most efficacious against stored-grain insects in humid conditions or at higher levels of relative humidity where the conidia must adhere to, germinate on, and penetrate through the host cuticle (Moore et al. 1996, Sheeba et al. 2001, Batta 2005). However, the abrasion caused by the sharp-edged diatomaceous earth dust particles in the cuticle of treated insects is involved by the entomopathogenic fungi for penetration of the cuticle of attacked insects to grow in the hemocoel causing eventually the death of infected hosts. Therefore, the effectiveness of entomopathogenic fungi for the control of stored-grain insects should be enhanced when the conidia are combined with the diatomaceous earth dusts. In the present research, the efficacy of new formulations of two entomopathogenic fungi in two types of diatomaceous earth dusts was bioassessed against the adults of three species of stored-grain insects. Therefore, the objectives of the present research were: (i) to formulate the conidia of two species of entomopathogenic fungi, *B. bassiana* (strain 149) and *M. anisopliae* (strain Meta 1), in two types of diatomaceous earth dusts, fossil shield and Silico-Sec, (ii) to study the viability of conidia of both fungi in the formulations over time, (iii) to apply the formulated fungi against the adults of three species of stored-grain insects: *R. dominica, S. oryzae* and *T. castaneum*, and (iv) to evaluate the treatment effect (e.g. presence of synergistic interaction) with the formulated fungi for the control of stored-grain insects.

2. Materials and Methods

2.1. Insects used for bioassays

Adult insects of *S. oryzae* (strain RW10) and *R. dominica* (strain LGB5) collected from infested stored wheat grains were reared on healthy wheat grains (CV: Anbar) held in

cloth mesh covered plastic pots (15 cm diameter by 20 cm high) at 25 ± 2 ^{o}C, 75 ± 5% RH, 16:8 L:D cycle. Also, adult insects of *T. castaneum* (strain RFB8) collected from infested stored wheat flour were reared on healthy wheat flour (CV: Anbar) held in cloth mesh covered plastic pots under the same conditions as indicated above. The newly emerged adults (males and females) of the three insect species were used in bioassays.

2.2. Treatments and formulations of fungal species used for bioassays

Nine treatments of fungal species formulated or unformulated in diatomaceous earth dusts were applied in bioassays against the adults of the three species of stored grain insects. The treatments were: (1) *B. bassiana* conidia + fossil shield, (2) *B. bassiana* conidia + Silico-Sec, (3) *M. anisopliae* conidia + fossil shield, (4) *M. anisopliae* conidia + Silico-Sec, (5) *B. bassiana* conidia only, (6) *M. anisopliae* conidia only, (7) Silico-Sec only, (8) fossil shield only, and (9) undisturbed control. The fungal strains: Meta 1 of *M. anisopliae* and 149 of *B. bassiana* were obtained from "Galillee Regional Research and Development Center, Eilapoun, Israel" and "The Institute for Biological Control, Darmstadt, Germany", respectively. Each formulation contained a mixture of conidia of one of the two fungus species and one of the two diatomaceous earth dusts at 1:4 (w/w) ratio, respectively. The diatomaceous earth dusts are: fossil shield 90.0 (fine brownish powder contained amorphous silica particles with size ranged from 5 and 30 μm, a content of 73 % amorphous SiO2, a water content of about 2 %, in addition to 3% aerosil and other mineral compounds) and Silico-Sec (Kieselsaure R) (fine whitish powder contained amorphous silica particles with size ranged from 13 and 15 μm, a content of 96 % amorphous SiO2, a water content of about 2 %, in addition to other mineral compounds). They were obtained from "The fossil shield Co., Bein GmbH, D-36132 Eiterfeld, Germany" and "Agrinova GmbH, Hauptstr. 13, D-67283 Obrigheim/ Muhlheim, Germany", respectively. The comparative treatments used in bioassays were represented by the unformulated or unmixed conidia of *M. anisopliae* and *B. bassiana* and the two fungus-free diatomaceous earth dusts. The undisturbed control of each insect species has not received any treatment and used to check the natural mortality of the insect species, thus it was used to correct the mortality in the comparative and formulation treatments mentioned above. Conidia harvested from 14-day old cultures of *M. anisopliae* and *B. bassiana* grown on oat meal agar medium (OMA) plates were thoroughly mixed with the diatomaceous earth dusts using screw capped bottles. The formulations of *B. bassiana* and *M. anisopliae* in the two diatomaceous earth dusts contained $9.8x10^{9}$ conidia g^{-1} of formulation. This concentration was measured by suspending 0.5 g of each formulation in 100 ml of sterile distilled water (0.5 % w/v) held in screw-capped bottles followed by a vigorous shaking then making a dilution of 10-fold before counting the number of conidia microscopically using hemocytometer. The same stock of fungal conidia for each fungus was used for preparing the above mentioned treatments and formulations to ensure that no differences were present among the individual formulations and treatments. The initial water content measured on a dry weight basis was 5.6 and 6.1 % for the unformulated conidia of *B. bassiana* and *M. anisopliae*, respectively, but it was 3.1 and 3.8 % for the formulations of *B. bassiana* and

M. anisopliae in the diatomaceous earth dusts, respectively, according to the type of dust used.

2.3. Method for control of adults of stored-grain insects with *B. bassiana* and *M. anisopliae*

This method includes a treatment of *B. bassiana* and *M. anisopliae* conidia formulated in diatomaceous earth dusts to wheat grains or wheat meal infested with *S. oryzae*, *R. dominica* and *T. castaneum* adults. This treatment was performed by applying 0.20 g of each fungal formulation to 10.0 g of wheat grains (CV: Heteyia safra) for *S. oryzae* and *R. dominica* or 10.0 g of wheat meal (CV: Heteyia safra) for *T. castaneum*. The application rate of fungal formulations was equivalent to 2.0 % (w/w) of formulation to wheat grain or wheat meal, respectively. Applying of 0.16 g of each fungus-free diatomaceous earth dust and 0.04 g of each unformulated conidia was also performed in this treatment versus 0.20 g of fungal formulation for comparison. The application rate of diatomaceous earth dust and unformulated conidia was equivalent to 1.6 and 0.4 % (w/w), respectively. The treated wheat grain and wheat meal were held in a small, cloth-mesh covered plastic pots (9.5 cm diameter and 5.0 cm high). Ten newly emerged adults (5 males and 5 females) of *S. oryzae*, *R. dominica* and *T. castaneum* were introduced into each plastic pot immediately after treatment. Each plastic pot represented a treatment replicate and three replicates were used per experimental treatment. Nine experimental treatments were used (Section 2.2). Homogenous distribution of the formulations was done after application. All pots were kept at 25 ± 2 ^{o}C, 75 ± 5% RH, and 16:8 L:D cycle for 7 days before being evaluated for adult mortality. This mortality was shown by the lack of movement of treated adults within 5-minute period of continuous observation. Dead and living adults were counted 7 days after the treatment. The mortality percentage of *S. oryzae*, *R. dominica* and *T. castaneum* adults in each experimental treatment was calculated then compared. Corrected mortality percentage relative to the undisturbed control was calculated then compared to exclude the effect of natural mortality of adults on the efficacy of fungal treatment.

2.4. Method of testing conidial viability of fungal species formulated in diatomaceous earth dusts

Six treatments of *B. bassiana* and *M. anisopliae* were used in the viability test. They are: (1) *B. bassiana* conidia + fossil shield, (2) *B. bassiana* conidia + Silico-Sec, (3) *M. anisopliae* conidia + fossil shield, (4) *M. anisopliae* conidia + Silico-Sec, (5) *B. bassiana* conidia only, (6) *M. anisopliae* conidia only. The stock from which these treatments were taken was stored in tightly closed glass bottles at 25±2 ^{o}C in dark cabinet and sampled weekly for 12 successive weeks. Suspensions of 0.5 % (w/w) of each formulation or 0.1 % (w/w) of unformulated conidia of each fungus species were prepared in sterile distilled water and then diluted 10-fold. Samples of 100 µl were taken from the above diluted suspensions then spread in a thin layer on the surface of glass slides kept in Petri dishes under humid conditions (to prevent conidial desiccation) at 25 ± 2 ^{o}C for 24 hours, a time needed for conidial germination of the fungal species at 25 ^{o}C. Conidial germination was

assessed 24 hours after spreading by counting germinated (with germ tube) and non-germinated conidia of each sample in a randomly chosen field microscope using the magnification 400X. The average germination percentage of the counted conidia was calculated for each sample. The storage stability expressed by the loss of germination percentage was then calculated for samples of formulated and unformulated conidia. Four replicates representing 4 samples were set up for each sampling time of each fungal strain in each diatomaceous earth dust. Unformulated form of each fungal strain, in form of dry conidia harvested from cultures of the same strains, was used for comparison. Aqueous suspensions prepared from the unformulated form and contained the same concentration as that in the formulated conidia in diatomaceous earth dusts were used for viability comparison**.**

2.5. Statistical analyses

Data obtained on means of mortality percentage of the three stored grain insects in the different experimental treatments were analyzed statistically by ANOVA and means were separated by Duncan's Multiple Range Test (DMRT). Standard error of the means (SEM) was calculated then added to the means of mortality and germination percentage.

3. Results

3.1. Control of *S. oryzae*, *R. dominica* and *T. castaneum* adults with *B. bassiana* and *M. anisopliae*

Significant differences (P<0.05) were obtained between the treatments of *S. oryzae*, *R. dominica* and *T. castaneum* adults with *B. bassiana* and *M. anisopliae* (Tables 1, 2 and 3). Treatments with formulated conidia of *B. bassiana* or *M. anisopliae* in fossil shield or Silico-Sec resulted in a significantly higher mortality percentage (P<0.05) of *S. oryzae* or *R. dominica* adults (93.3 to 96.7 and 86.7 to 93.3 %, respectively, 7 days after treatment) when compared to the treatments with unformulated conidia of *B. bassiana* or *M. anisopliae* or with fossil shield or Silico-Sec alone or with the undisturbed control (16.7 to 76.7 and 20.0 to 73.3 %, respectively, 7 days after treatment) (Tables 1 and 2). Treatments with formulated conidia of *B. bassiana* in fossil shield or Silico-Sec or formulated conidia of *M. anisopliae* in fossil shield dust resulted in a significantly higher mortality percentage (P<0.05) of *T. castaneum* adults (86.7 to 96.7 %, 7 days after treatment) when compared to the treatments with formulated conidia of *M. anisopliae* in Silico-Sec or unformulated conidia of *B. bassiana* or *M. anisopliae* or fossil shield or Silico-Sec alone or with the undisturbed control (13.3 to 83.3 %, 7 days after treatment) (Table 3).

Similar significant differences (P<0.05) among the above mentioned treatments were obtained when the corrected adult mortality percentage of the three insect species relative to mortality percentage of the undisturbed control were compared (Tables 1, 2 and 3). However, no significant differences between the mean mortality percentage of adults of the three insect species were obtained when adults of these species were treated with fossil shield only or Silico-Sec only (Tables 1, 2 and 3). Similar non-significant

differences between the treatments with the unformulated conidia of *B. bassiana* or *M. anisopliae* were obtained when applied against *R. dominica* or *T. castaneum* adults, but the differences were significant (P<0.05) when applied against *S. oryzae* adults (Tables 1, 2 and 3).

Comparison of the treatment efficacy of the unformulated conidia of the entomopathogenic fungi, *B. bassiana* and *M. anisopliae*, with that of the diatomaceous earth dusts, fossil shield and Silico-Sec, against adults of the three insect species indicates that the diatomaceous earth dusts were more effective than the unformulated conidia of entomopathogenic fungi. This is because mean mortality percentage of *S. oryzae* and *T. castaneum* adults treated with the diatomaceous earth dusts were significantly different (P<0.05) from the mean mortality percentage of the same insect species treated with the unformulated conidia of the entomopathogenic fungi. However, the differences were not significant for the above treatments when applied against *R. dominica* adults (Table 1, 2 and 3).

Table 1: Effect of treatment with *Beauveria bassiana* and *Metarhizium anisopliae* on adult mortality of rice weevil, *Sitophilus oryzae*, infesting wheat grains (CV: Heteyia safra) stored at 25 ± 2 ^{o}C, 75 ± 5% RH, 16:8 L:D cycle

Treatments	Mean mortality percentage of adults 7 days after treatment (±SEM)	Corrected mean mortality percentage of adults 7 days after treatment relative to the undisturbed control (±SEM)
Undisturbed control	16.7 ±4.6 a [1)]	
Fossil shield only [2)]	76.7 ±9.4 d	60.0 ±8.7 c [1)]
Silico-Sec only [2)]	73.3 ±8.8 cd	56.6 ±4.3 bc
B. bassiana conidia only	63.3 ±7.6 c	46.6 ±4.2 b
B. bassiana + Fossil shield	96.7 ±4.9 e	80.0 ±9.1 d
B. bassiana + Silico-Sec	93.3 ±5.5 e	76.6 ±5.3 d
M. anisopliae conidia only	50.0 ±8.7 b	33.3 ±6.2 a
M. anisopliae + Fossil shield	96.7 ±4.3 e	80.0 ±7.8 d
M. anisopliae + Silico-Sec	93.3 ±7.2 e	76.6 ±4.9 d

[1)] Means of adult mortality percentage or means of corrected adult mortality percentage relative to the undisturbed control within each column followed by different letters are significantly different at P<0.05 according to ANOVA and Duncan's multiple range test (DMRT).

[2)] Diatomaceous earth dust used only

Table 2: Effect of treatment with *Beauveria bassiana* and *Metarhizium anisopliae* on adult mortality of lesser grain borer, *Rhyzopertha dominica*, infesting wheat grains (CV: Heteyia safra) stored at 25 ± 2 °C, 75 ± 5% RH, 16:8 L:D cycle

Treatments	Mean mortality percentage of adults 7 days after treatment (±SEM)	Corrected mean mortality percentage of adults 7 days after treatment relative to the undisturbed control (±SEM)
Undisturbed control	20.0 ±0.3 a[1)]	
Fossil shield only [2)]	70.0 ±7.8 b	50.0 ±8.1 a[1)]
Silico-Sec only [2)]	73.3 ±6.1 b	53.3 ±5.6 a
B. bassiana conidia only	70.0 ±8.9 b	50.0 ±0.4 a
B. bassiana + Fossil shield	86.7 ±3.7 c	66.7 ±2.9 b
B. bassiana + Silico-Sec	86.7 ±3.6 c	66.7 ±3.9 b
M. anisopliae conidia only	73.3 ±5.1 b	53.3 ±6.4 a
M. anisopliae + Fossil shield	93.3 ±7.3 c	73.3 ±6.2 b
M. anisopliae + Silico-Sec	90.0 ±0.2 c	70.0 ±7.9 b

[1)] Means of adult mortality percentage or means of corrected adult mortality percentage relative to the undisturbed control within each column followed by different letters are significantly different at $P<0.05$ according to ANOVA and Duncan's multiple range test (DMRT).

[2)] Diatomaceous earth dust used only

Table 3: Effect of treatment with *Beauveria bassiana* and *Metarhizium anisopliae* on adult mortality of red flour beetle, *Tribolium castaneum*, infesting wheat meal (CV: Heteyia safra) stored at 25 ± 2 °C, 75 ± 5% RH, 16:8 L:D cycle

Treatments	Mean mortality percentage of adults 7 days after treatment (±SEM)	Corrected mean mortality percentage of adults 7 days after treatment relative to the undisturbed control (±SEM)
Undisturbed control	13.3 ±6.9 a [1)]	
Fossil shield only [2)]	73.3 ±10.5 cd	60.0 ±8.4 bc [1)]
Silico-Sec only [2)]	70.0 ±9.6 c	56.7 ±2.8 b
B. bassiana conidia only	26.7 ±3.0 b	13.4 ±6.3 a
B. bassiana + Fossil shield	96.7 ±3.3 d	83.4 ±7.2 d
B. bassiana + Silico-Sec	86.7 ±3.5 d	73.3 ±5.7 d
M. anisopliae conidia only	20.0 ±6.8 ab	6.7 ±4.4 a
M. anisopliae + Fossil shield	90.0 ±1.0 d	76.7±4.0 d
M. anisopliae + Silico-Sec	83.3 ±5.1 cd	70.0±1.3 cd

[1)] Means of adult mortality percentage or means of corrected adult mortality percentage relative to the undisturbed control within each column followed by different letters are significantly different at P<0.05 according to ANOVA and Duncan's multiple range test (DMRT).
[2)] Diatomaceous earth dust used only.

3.2. Viability of *B. bassiana* and *M. anisopliae* conidia in diatomaceous earth dusts

Over a 12-week sampling period of viability of *B. bassiana* conidia formulated in the two diatomaceous earth dusts, the loss of mean germination percentage that expresses the storage stability was amounted to 9.3% when formulated in fossil shield (decreased from 81.7 to 72.4 %) and 5.8% when formulated in Silico-Sec (decreased from 85.6 to 79.8 %) in comparison with the loss of 31.8 % when the unformulated *B. bassiana* conidia were stored under the same conditions over the same sampling period of viability (decreased from 87.2 to 55.4 %) (Table 4).

Similar trend of viability was obtained when *M. anisopliae* conidia were formulated in fossil shield and Silico-Sec (14.7 and 8.6 % loss of mean conidial germination percentage, respectively) versus the unformulated *M. anisopliae* conidia stored under the same conditions (27.6 % loss of mean conidial germination percentage) (Table 4). This indicates a high storage stability of *M. anisopliae* and *B. bassiana* conidia when mixed with the diatomaceous earth dusts in comparison with a low storage stability of the unmixed or unformulated conidia of both fungal species. Thus, mixing conidia of the two fungi with the diatomaceous earth dusts improved their storage stability in comparison with the unformulated (dry) conidia.

Table 4: Viability of *Beauveria bassiana* (strains 149) and *Metarhizium anisopliae* (strain Meta 1) in two types of diatomaceous earth dusts, Fossil shield and Silico-Sec, stored in tightly closed glass bottles at 25 ± 2 °C for 12 successive weeks

Time after mixing conidia with diatomaceous earth dusts (in weeks)	Mean germination percentage of conidia after mixing with dusts[1)]					
	B. bassiana (strain 149)[2)]			*M. anisopliae* (strain Meta 1)[2)]		
	Conidia+ Fossil shield	Conidia+ Silico-Sec	Conidia only (unmixed)	Conidia+ Fossil shield	Conidia+ Silico-Sec	Conidia only (unmixed)
0	81.7±2.1	85.6±1.9	87.2±2.4	78.8±1.1	80.2±1.6	81.4±2.3
1	80.8±1.5	85.1±1.2	85.5±2.5	76.1±2.4	79.6±2.0	80.1±2.0
2	79.6±1.8	84.9±1.9	84.3±1.9	75.9±1.2	78.1±0.9	78.2±1.8
3	79.1±0.9	84.3±1.8	81.1±2.9	74.3±1.4	78.0±0.4	76.9±2.1
4	78.1±1.2	83.8±0.9	78.4±3.1	73.2±1.5	77.5±1.4	74.1±2.6
5	77.4±1.3	83.7±0.6	73.6±3.9	72.3±1.3	77.1±0.7	72.1±2.5
6	76.1±1.0	82.8±1.2	70.4±2.7	72.1±0.5	76.4±1.2	68.9±3.8
7	75.3±1.6	81.9±1.8	69.7±1.6	71.4±1.1	75.1±1.0	66.8±2.0
8	74.8±1.2	81.2±1.0	68.1±1.3	69.3±1.6	74.2±1.8	61.2±3.8
9	74.1±0.7	80.6±1.1	66.3±2.4	68.6±1.0	74.0±0.6	59.6±3.3
10	73.8±1.0	80.1±0.9	62.4±4.1	66.4±2.3	72.9±2.2	57.4±2.8
11	73.6±0.8	80.1±0.5	60.8±3.1	65.3±0.8	72.0±0.3	55.1±2.7
12	72.4±1.1	79.8±1.2	55.4±5.0	64.1±1.3	71.6±0.5	53.8±1.9

[1)] Conidia of fungal species were mixed with the dust carriers at a ratio of 1:4 (w/w), respectively. Suspensions of 0.5 % (w/w) of each mixture or 0.1 % (w/w) of unmixed conidia of each fungus species in sterile distilled water were prepared and then diluted 10-fold to be used in the viability test.

[2)] Four replicates representing 4 samples of 100 µl of mixed or unmixed diluted conidial suspensions per sample time were used. Means of germination percentage were presented as mean ± standard error of the mean (SEM).

4. Discussion

Results of the present research show that the mortality of *S. oryzae*, *R. dominica* and *T. castaneum* adults exposed to the formulations of *M. anisopliae* and *B. bassiana* conidia in two diatomaceous earth dusts, fossil shield and Silico-Sec, at a rate of 2.0 % (w/w of formulation to wheat grain, respectively) was significantly higher ($P<0.05$) than the mortality of adults of the same species exposed to the unformulated conidia of the same fungi or the diatomaceous earth dusts alone. This indicates the effectiveness of treatment with the formulated entomopathogenic fungi in the diatomaceous earth dusts against the target stored-grain insects. Also, a synergistic interaction between the effect of fungal species and diatomaceous earth dusts was statistically proven in the present research

under constant experimental conditions (25 ± 2 ^{o} C, 75 ± 5% RH) because significant differences were obtained in the mortality of adults treated with the formulated fungi in the diatomaceous earth dusts in comparison with the adult mortality due to treatment with the diatomaceous earth dusts alone or entomopathogenic fungi alone. This synergism could be explained by the increase of diatomaceous earth dust efficacy with the increase in temperature (over 20 ^{o} C) and with the decrease in relative humidity (below 80 % RH) since these dusts usually provide a long-term control under unhumid conditions. Many investigators have reported that low levels of relative humidity and high levels of temperature, within the above mentioned limits, accelerate the desiccating effect of diatomaceous earth dust during application, thus its efficacy increases (Golob 1997, Erb-Brinkmann 2000, Mewis and Ulrichs 2001). Moreover, the efficacy of entomopathogenic fungi increases with the increased levels of temperature and relative humidity, so that these fungi, under our constant experimental conditions (25 ± 2 ^{o} C, 75 ± 5% RH), become more able to utilize the abrasions caused by the applied diatomaceous earth dust particles for more penetration into cuticle of the target host insects thus causing more insect mortality (Hidalgo et al. 1998, Rice and Cogburn 1999, Odour et al. 2000, Dal-Bello et al. 2001, Lord 2001, Batta 2004 and 2005, Batta and Abu Safieh 2005, Michalaki et al. 2006). This demonstrates why the experimental conditions of temperature and relative humidity that were used in the present research are favorable for enhancing the effectiveness of both fungi and diatomaceous earth dusts.

It is noteworthy to mention that the synergistic effect shown in the present research could be used for improving the efficacy of previous formulations of entomopathogenic fungi and diatomaceous earth dusts, thus enhancing their performance as insect control agents by reducing the application rate of each one when applied in a combined form for the control of insects. Decreasing this rate for diatomaceous earth dusts during application against stored-grain insects is important for lessening some of the unacceptable effects that may be caused by these dusts at higher effective use rates. For example, decreasing their effect on physical properties of treated grain represented by reducing the flow characteristics of bulk grain in bulk storage and in handling facilities and reducing the dust (silica) inhalation problems when used in closed areas (Golob 1997).

It is well-known that the diatomaceous earth dusts are classified as desiccant dusts and exercise their desiccating effect on target insects by abrading their cuticle due to contact with the sharp-edged silica (silicon dioxide) particles of the dusts following application, so that the wax layers of insect cuticle is destroyed leading to desiccation or water loss then death of treated insects (Golob 1997, Appel et al. 1999, Erb-Brinkmann 2000, Mewis and Ulrichs 2001). However, in the present research, no significant desiccating effect was exercised by the diatomaceous dust particles on the conidia of entomopathogenic fungi when mixed together because a negligible effect of diatomaceous earth dusts was shown on viability of the conidia mixed with these dusts and sampled for viability over a 12-week sampling period. In addition, the use of formulations of entomopathogenic fungi in diatomaceous earth dusts for the control of stored-grain insects as demonstrated in the present research may offer the following advantages over the conventional insecticides: (i) There is no development of physiological resistance by the treated insects because the action of diatomaceous earth

dusts is not dependent on a metabolic pathway (Subramanyam et al. 1994), but it may be possible for insects to develop a behavioral response to the dust and avoid contact, (ii) As a major component of these formulations, the diatomaceous earth dusts have a very low mammalian toxicity (e.g. the commercial diatomaceous earth dust "Insecto" has the acute oral rat $LD_{50} > 5000$ mg Kg^{-1}), and in USA, diatomaceous earth dusts are recognized as "safe products" by the US Food and Drug Administration (Subramanyam et al. 1994), (iii) Ingredients of these formulations are natural products with white or brown layers and they are easy to apply. They have also low price in comparison with the storage insecticides, (iv) Amorphous silica which is the active component of the diatomaceous earth dusts used in the present formulations is an approved direct food additive, it is harmless for human and mammals and exempt from tolerance requirements (Erb-Brinkmann 2000), (v) Entomopathogenic fungi which are the other component of the present formulations have potential to persist in the environment through secondary cycling and may be redistributed within a store by the movement of the insects themselves through walking on grains or by flight at higher temperatures.

The application rate of entomopathogenic fungi formulated in diatomaceous earth dusts is usually lower than that of diatomaceous earth dusts when used alone due to the synergistic interaction that may occur between them as described earlier. The recommended application rate for diatomaceous earth dusts is dependant upon the source and origin of the dust (marine or freshwater), the species of the stored-grain insect intended to be controlled, and method of application or exposure conditions. This rate is varying and may reach up to 30 Kg ton^{-1} for the older formulations of unproven efficacy at practical dose rate for stored-grain pests (Arthur 2000, Erb-Brinkmann 2000, Erb-Brinkmann and Straube 2002, Stathers et al. 2002). The newer formulations of diatomaceous earth dusts are more effective than the older products and can be used at lower rates. However, at higher rates, diatomaceous earth dust can potentially decrease the bulk density and flow rate of treated grain (Arthur 2000) and these effects on physical properties of treated grain are largely dose dependent and can be reduced by the decrease of application rate.

In conclusion, both types of diatomaceous earth dusts used in the present research showed statistically synergistic interaction with *B. bassiana* and *M. anisopliae* on adults of *S. oryzae*, *R. dominica* and *T. castaneum* under constant experimental conditions (25 ± 2 ^{o} C and 75 ± 5% RH) and application rate of 2.0 % (w/w of formulation to wheat grain). Therefore, control performance of these insects using combinations of entomopathogenic fungi and diatomaceous earth dusts was enhanced in this study. These results may provide a basis for a least-toxic approach of both materials to control stored-grain insects and for efficacy-enhancing formulations of entomopathogenic fungi and diatomaceous earth dusts. One important use of these new formulations is to be a main part of IPM strategy of stored-grain insects and to indicate the lower dose that could be commercially effective.

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