Available online at www.sciencedirect.com
SCIENCE DIRECT
Crop Protection 25 (2006) 1087–1094
Crop Protection
www.elsevier.com/locate/cropro

Effect of the combined use of *Metarhizium anisopliae* (Metschinkoff) Sorokin and diatomaceous earth for the control of three stored-product beetle species

N.G. Kavallieratos[a,*], C.G. Athanassiou[b], M.P. Michalaki[b], Y.A. Batta[c], H.A. Rigatos[d], F.G. Pashalidou[d], G.N. Balotis[e], Ž. Tomanović[f], B.J. Vayias[b]

[a]*Laboratory of Agricultural Entomology, Department of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, 8 Stefanou Deltastr., 14561, Kifissia, Attica, Greece*
[b]*Laboratory of Agricultural Zoology and Entomology, Agricultural University of Athens, 75 Iera Odosstr., 11855, Athens, Attica, Greece*
[c]*Laboratory of Plant Protection, Department of Plant Production and Protection, Faculty of Agriculture, An-Najah National University, P.O. Box 425 (Tulkarm), West Bank, Palestine, Israel*
[d]*Department of Organic Farming, Technological Educational Institute of Ionian Islands, 28100, Argostolion, Cephalonia, Greece*
[e]*Institute of Agricultural Sciences, 182 Kifissias Avenue, 15124, Amaroussion, Attica, Greece*
[f]*Institute of Zoology, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia and Montenegro*

Received 31 May 2005; received in revised form 1 December 2005; accepted 21 February 2006

Abstract

Laboratory bioassays were conducted in order to evaluate the use of the entomopathogenic fungus, *Metarhizium anisopliae* (Metschinkoff) Sorokin (Deuteromycotina: Hyphomycetes), against adults of three stored-grain beetle species, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae), *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae). Two fungal preparations were compared, a conidial suspension and a conidial powder. These were applied to wheat, at three dosages, 8×10^6, 8×10^8 and 8×10^{10} conidia/kg of wheat, alone, or in combination with the diatomaceous earth (DE) formulation SilicoSec® (Biofa Gbmh, Germany), applied at 0.5 g/kg of wheat. Adult insects were exposed to treated wheat for 24 h, 48 h, 7 d and 14 d. The mortality of *R. dominica* adults after 14 d of exposure to the treated substrate was 100% and 96% at the dosages in combination, for the suspension and the powder, respectively. The respective figures without DE were 94.4% and 74.6%. In contrast, against *S. oryzae* adults, the application of conidial suspension combined with DE was not as effective as the application of DE alone. Adult mortality of *S. oryzae* increased notably on wheat treated with the conidial powder. Similarly, the conidial suspension, with or without DE, was not as effective against *T. confusum* as the conidial powder. The progeny production of *R. dominica* on wheat treated with the highest suspension dosage, with or without DE, was significantly lower than that for the other aqueous fungal dosages. Moreover, significantly less progeny were produced on wheat treated with the highest dosage of powder conidia combined with DE, in comparison with the other treatments. In contrast, *S. oryzae* progeny production was notably reduced only in wheat treated with the highest dosage of fungal spore powder mixed with DE. No *T. confusum* progeny were found on wheat treated with the highest dosage of both fungal preparations combined with DE.

Keywords: *Metarhizium anisopliae*; Diatomaceous earth; *Rhyzopertha dominica*; *Sitophilus oryzae*; *Tribolium confusum*

1. Introduction

Resistance to traditional insecticides used for insect control in stored products and the demand for residue-free food, has prompted researchers to evaluate other alternative control methods. These methods include the application of insect growth regulators, phytochemicals, inert materials, controlled environments and biological control. There are two major components for the biological control of stored-product pests. The first is the use of

*Corresponding author. Tel.: +30 2102 128015; fax: +30 2108 077506.
E-mail address: nick_kaval@hotmail.com (N.G. Kavallieratos).

0261-2194/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cropro.2006.02.009

predators and parasitoids and the second is the use of insect pathogens, such as fungi, bacteria, protozoa, etc. The second option may have certain advantages over the use of predators and parasitoids such as (a) pathogens can be applied with the same equipment used for the application of insecticides while the application of beneficial insects is more specialized and (b) insect fragments in food is a matter of controversy, even if fragments come from predators or parasitoids.

Among the pathogens, entomopathogenic fungi are the most promising alternatives to traditional pesticides. The fungus conidia attach and penetrate through the insect's cuticle, causing the insect's death. Entomopathogenic fungi are naturally occurring organisms, environmentally safe and with low mammalian toxicity (Cox and Wilking, 1996). *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes) has been tested with success against several stored-product insect species in both laboratory and field tests (Rice and Cogburn, 1999; Moore et al., 2000; Lord, 2001; Dal-Bello et al., 2001, Padin et al., 2002; Stathers, 2002; Wakefield et al., 2002; Akbar et al., 2004) and it has also been registered to be used as a stored food protectant in the United States (Moore et al., 2000). However, there is evidence that another fungus, *Metarhizium anisopliae* (Metschinkoff) Sorokin (Deuteromycotina: Hyphomycetes) is also effective against stored-product insects (Batta, 2004, 2005). This fungal species is a good model for bioassay-response tests, as it produces large numbers of conidia, which can easily be harvested.

One of the basic drawbacks in using entomopathogenic fungi is the need for formulated conidia, which although increases the effectiveness, it may also increase the cost of mass production of a fungal preparation. So far, several inert materials have been evaluated as carriers for conidial preparations, and some of them increase fungal adhesion to the insect's cuticle (Akbar et al., 2004).

The use of diatomaceous earths (DE) is another promising alternative to conventional insecticides. DEs are the fossilized remains of phytoplanktons (diatoms), which occurred during the Eocene and Miocene periods. They exhibit insecticidial properties given that DE particles inactivate the epicuticular lipids of the insects' cuticle and thus causing internal water loss and death through desiccation (Ebeling, 1971; Korunic, 1998; Mewis and Ulrichs, 2001). Similar to entomopathogenic fungi, DEs have natural origin and low mammalian toxicity (Golob, 1997; Korunic, 1998). Moreover, they can be easily removed from the treated grain and applied with approximately the same technology as conventional insecticides. Several DE formulations are registered for use as grain protectants and have been successfully used against a wide range of stored product species (Korunic, 1998; Subramanyam and Roesli, 2000). According to several reports (Lord, 2001; Akbar et al., 2004; Lord, 2005) the combined use of DE with fungal preparations of *B. bassiana* demonstrated a higher insecticidial effect than that it was exploited when fungal preparations were used alone. Moreover, other types of inert dusts such as charcoal, oven ash, chalk powder, charcoal and wheat flour have also been evaluated as carriers of *M. anisopliae* conidia fungal preparations and it was suggested that some of them can prolong their shelf-life (Batta, 2004).

In the present study we examined the potential of using a DE formulation in combination with *M. anisopliae*, formulated as a conidial suspension and a conidial powder. As test insects we used three major stored product beetle species, the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae), the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and the confused flour beetle, *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae). *R. dominica* and *S. oryzae* are primary pests, and can easily infest sound grain seeds while *T. confusum* is a secondary pest, which means that this species infests only damaged or broken seeds (Aitken, 1975) as well as milled products such as semolina or flour. Apart from the mortality caused by the application of *M. anisopliae* and DE to the exposed individuals of these species, the capability for progeny production in the treated substrate was also evaluated.

2. Materials and methods

2.1. Test insects

Adults of *R. dominica*, *S. oryzae* and *T. confusum* were used in the tests. The *R. dominica* and *S. oryzae* adults used were taken from a culture that was kept in the laboratory on whole wheat at $27\pm1\,^{\circ}C$ and $65\pm5\%$ r.h. *T. confusum* adults were taken from a culture kept on wheat flour plus 5% brewers yeast (by weight) at $28\pm1\,^{\circ}C$ and $65\pm5\%$ r.h. All cultures had been kept for more than 5 years at the Laboratory of Agricultural Entomology, Department of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, Kiffissia, Attica, Greece. All individuals used in the tests were <2 weeks old.

2.2. DE formulation

The DE formulation used in the experiments was SilicoSec® (Biofa GmbH, Münsingen, Germany). SilicoSec®, is a relatively new DE formulation of freshwater origin containing 92% SiO_2, 3% Al_2O_3, 1% Fe_2O_3, and 1% Na_2O with average particle size between 8 and 12 μm. The DE sample was stored in the laboratory at ambient conditions, until the beginning of the experiments (approximately 3 weeks).

2.3. Fungal preparations

The *M. anisopliae* isolate used in the tests was strain Meta 1 (Batta, 2003, 2004, 2005). It was obtained from the Galilee Regional Research and Development Center (GRRDC) in Eilapoun, Israel. This strain was first isolated

from an infected individual of *Harpalus caliginosus* (F.) (Coleoptera: Carabidae) (Batta, 2003). The principal Meta 1 culture was stored as a freeze-dried sample, at the Laboratory of Plant Protection, Department of Plant Production and Protection, Faculty of Agriculture, An-Najah National University, Tulkarm, Palestine, Israel. The fungus was first isolated on Sabouraud's culture medium then subcultured on plates with oat meal agar (OMA) medium for the mass production of the fungus conidia. During conidial production, incubation of the culture plates was carried out at 20 ± 1 °C and 16 h illumination per day. After 14 days of incubation, the fungal conidia were collected by scraping away the conidial layers formed on the plate's surface using a sterilized scalpel. Two fungal preparations were produced, a conidial suspension and a conidial powder.

For the preparation of the suspension, the fungal conidia were added to 100 ml sterile distilled water, in a screw capped bottle and stirred vigorously on a shaker at 10,000 rpm before being filtered through tulle. There was no need to add a wetting agent to disperse the conidia in the suspension as this could be achieved by vigorous shaking. The concentration of fungal conidia in the homogenous conidial suspension was determined using a haemocytometer. Three concentrations of conidial suspension were applied: 8×10^6, 8×10^8 and 8×10^{10} conidia/kg of grain. A standard amount of wheat (1 kg, var. "Mexa") was treated by spraying with the appropriate concentration of conidia using a CLK-608 sprayer of 450 ml capacity (China Kunli Plastic Sprayer & Bottle Manufacturer). The moisture content of the grain as determined before carrying out the experiments by a Dickey-John moisture meter (Dickey-John Multigrain CAC II, Dickey-John Co., USA) was $11.2 \pm 0.3\%$. Spraying was carried out in a tray, on which the 1 kg of grain was previously spread into a thin layer. The treated grain was then left for approximately 30 h to dry, in a controlled environmental chamber at 26 °C and 65% r.h. Then the moisture content of the grain was again determined and its value was increased by $0.2 \pm 0.04\%$ in comparison with the moisture content before spraying. The DE formulation was then added to some of the treated wheat at the rate of 0.5 g/kg of wheat and the treated wheat was then manually shaken for approximately 15 min to achieve equal distribution of the dust.

For the preparation of the conidial powder, the scraped off fungal conidia were thoroughly mixed with the DE formulation. The ratio of both fungal conidia and DE was adjusted to obtain the same three concentrations of fungal conidia in the mixture as in the aqueous fungal suspension. These fungal preparations were added to 1 kg of wheat grains, and then thoroughly mixed to achieve a homogenous distribution.

For both fungal preparations 1 kg of wheat grains was treated with either of the three *M. anisopliae* dosages alone or the DE alone, the combination of both, plus a series of untreated wheat grains which served as control.

2.4. Bioassays

Two tests were carried out, one with the conidial suspension and the other with the conidial powder. For each experiment, six samples, of 50 g each, were taken from each lot of treated wheat, and each sample was placed in a small cylindrical glass vial, which was closed, apart from a hole at the top of the vial (1.5 cm in diameter) which was covered with organtine for sufficient aeration (Athanassiou et al., 2005b). Six additional vials, containing untreated wheat were used as a control. Thirty *R. dominica* adults were placed into each vial. The same procedure was followed with *S. oryzae* and *T. confusum* adults. Each species was treated separately. The jars were then placed in incubators, set at 26 °C, 65% r.h. The desired relative humidity was maintained by using a saturated salt solution of sodium bromide, as recommended by Greenspan (1977). Dead adults were counted after 24 h, 48 h, 7 d and 14 d of exposure in the treated substrate and in the control vials. Temperature and humidity during the experiment were monitored using HOBO digital recorders (HOBO H8, Onset Computers, USA).

2.5. Progeny production counts

After the 14 d mortality count all adults (dead and alive) were removed from the vials, and the vials were left in the incubators at the same conditions for an additional period of 60 d. The number of emerged individuals of each species was then counted. Only adults were recorded in the case of *R. dominica* and *S. oryzae*, since its larvae develop inside the grain kernels, while in the case of *T. confusum*, in addition to the number of adults, the number of immatures was also recorded.

2.6. Data analysis

The mortality counts were corrected using Abbott's (1925) formula. The data were arcsine transformed prior to analysis. All data were analyzed, separately for each experiment, species and exposure interval, using the GLM Proceedure of SAS (SAS Institute, 1995), with insect mortality as the response variable and fungal/DE treatment as the main effect. For the progeny production counts, the same procedure was followed, with the number of progeny as the response variable. Progeny production in the control vials was not included in this analysis, since a preliminary ANOVA revealed that, in all cases, the number of offspring produced in the control vials was significantly higher than the respective figures in the treated vials ($P < 0.0001$). In this way, differences in progeny production among treatments were indicated in the analysis. In all cases, means were separated by using the Tukey–Kramer (HSD) test, at $P = 0.05$ (Sokal and Rohlf, 1995).

3. Results

3.1. Mortality of R. dominica adults

In the first experiment with the conidial suspension of *M. anisopliae*, significant differences in *R. dominica* mortality were noted between treatments at each exposure interval (Table 1). Generally, after 24 h of exposure, the mortality levels were significantly higher in the *M. anisopliae*/DE treatments than in the *M. anisopliae* only treatments; however, the highest mortality level was noted on wheat that was treated with DE alone. At the 48 h exposure interval, significantly more adults were dead in wheat treated with the highest *M. anisopliae*/DE dosage combination and in wheat treated with DE alone in comparison with the other treatments. Seven days of exposure resulted in a significant increase in *R. dominica* mortality in all treatments, but only in the case of the highest fungal dose combined with DE was the mortality 100%. Even after 14 d of exposure, mortality did not reach 100% in any of the other treatments.

In the second experiment with the conidial powder, as above, significant differences were also found in adult mortality between treatments (Table 2). After 24 h of exposure, significantly more adults were dead in wheat treated with the highest fungal/DE dose combination and in wheat treated with DE alone, in comparison with the other treatments. This trend was also evident at the 48 h and 7 d mortality counts. After 14 d of exposure, significantly more *R. dominica* adults were dead in wheat treated with the highest fungal dosage when combined with DE, in comparison with the other treatments. Generally, at all exposure intervals, adult mortality in the three fungal treatments combined with DE was significantly higher than the respective figures at the three dosages of *M. anisopliae* alone. In addition, control mortality of *R. dominica* adults did not exceed 6.0%.

Table 1
Mean mortality (±SE) of *Rhyzopertha dominica* adults after 24 h, 48 h, 7 d or 14 d of exposure in wheat treated with three dose rates (expressed as conidia/kg of wheat) of *Metarhizium anisopliae* conidia suspension with or without the addition of DE, or with DE alone, (in all cases $N = 36$; $df = 6$, 35; means in the same exposure interval followed by the same letter are not significantly different; Tukey–Kramer HSD test at $P = 0.05$)

Treatment	Exposure interval			
	24 h	48 h	7 d	14 d
8×10^6 conidia/kg	12.2±1.7a	14.4±1.5a	33.3±2.8a	50.1±3.6a
8×10^8 conidia/kg	24.4±1.7b	31.1±1.4b	57.8±3.7b	76.7±6.8b
8×10^{10} conidia/kg	35.6±1.7c	42.2±4.6c	85.6±3.9c	94.4±1.9d
DE alone	47.8±5.1d	63.3±4.4e	83.3±5.6c	86.8±4.7bc
8×10^6 conidia/kg + DE	30.0±3.3c	31.7±1.6b	61.0±2.4b	80.0±2.8b
8×10^8 conidia/kg +DE	35.7±1.9c	53.9±2.7d	80.1±3.6a	92.2±6.8cd
8×10^{10} conidia/kg + DE	42.0±2.8d	69.1±5.1e	100.0±0.0d	100.0±0.0e
F	23.7	24.1	50.3	15.7
P	<0.0001	<0.0001	<0.0001	<0.0001

Table 2
Mean mortality (±SE) of *Rhyzopertha dominica* adults after 24 h, 48 h, 7 d or 14 d of exposure in wheat treated with three dose rates (expressed as conidia/kg of wheat) of *Metarhizium anisopliae* conidia powder with or without the addition of DE, or with DE alone (in all cases $N = 36$; $df = 6$, 35; means in the same exposure interval followed by the same letter are not significantly different; Tukey–Kramer HSD test at $P = 0.05$)

Treatment	Exposure interval			
	24 h	48 h	7 d	14 d
8×10^6 conidia/kg	1.1±0.7a	2.4±1.5a	8.9±2.6a	40.3±3.4a
8×10^8 conidia/kg	2.3±1.5a	6.7±3.2a	17.8±5.1b	53.3±4.5b
8×10^{10} conidia/kg	16.7±3.2b	46.7±4.9c	50.6±1.9c	74.6±1.9c
DE alone	44.4±2.9d	64.4±2.5d	81.3±4.9e	83.9±4.3d
8×10^6 conidia/kg + DE	17.8±2.2b	36.9±2.1b	44.9±3.9c	63.0±4.9b
8×10^8 conidia/kg +DE	27.8±2.3c	46.9±4.4c	71.6±5.3d	84.4±8.1cd
8×10^{10} conidia/kg + DE	43.3±1.9d	60.2±3.2d	87.9±6.0e	96.0±3.1e
F	53.6	41.3	45.8	13.1
P	<0.0001	<0.0001	<0.0001	<0.0001

Table 3
Mean mortality (±SE) of *Sitophilus oryzae* adults after 24 h, 48 h, 7 d or 14 d of exposure in wheat treated with three dose rates (expressed as conidia/kg of wheat) of *Metarhizium anisopliae* conidia suspension with or without the addition of with DE, or DE alone, (in all cases $N = 36$; $df = 6$, 35; means in the same exposure interval followed by the same letter are not significantly different; Tukey–Kramer HSD test at $P = 0.05$)

Treatment	Exposure interval			
	24 h	48 h	7 d	14 d
8×10^6 conidia/kg	0.0±0.0a	0.0±0.0a	3.3±1.2ab	8.9±1.9a
8×10^8 conidia/kg	0.0±0.0a	0.0±0.0a	3.4±2.1ab	11.4±2.0a
8×10^{10} conidia/kg	0.0±0.0a	1.1±0.7ab	6.7±2.2bc	24.4±3.6b
DE alone	0.0±0.0a	16.8±2.4c	93.8±0.8e	97.9±0.9e
8×10^6 conidia/kg + DE	2.1±0.7b	1.1±0.7ab	2.2±0.7a	52.2±9.1c
8×10^8 conidia/kg +DE	2.2±0.7b	2.2±0.8b	6.5±0.7c	62.2±10.8c
8×10^{10} conidia/kg + DE	7.8±1.9c	15.6±2.5c	24.4±1.9d	83.3±6.5d
F	12.9	28.2	560.8	27.4
P	<0.0001	<0.0001	<0.0001	<0.0001

3.2. Mortality of S. oryzae adults

Significant differences were noted between treatments, in the tests using the fungal suspension at all exposure intervals (Table 3). Twenty-four hours after the introduction of the *S. oryzae* adults in the treated substrate, the average mortality was extremely low and dead adults were found only in wheat treated with the three *M. anisopliae*/DE dose combinations. This trend continued at the 48 h exposure interval, when the mortality was still low, with the

Table 4
Mean mortality (±SE) of *Sitophilus oryzae* adults after 24 h, 48 h, 7 d or 14 d of exposure in wheat treated with three dose rates (expressed as conidia/kg of wheat) of *Metarhizium anisopliae* conidia powder with or without the addition of with DE, or DE alone, (in all cases $N = 36$; $df = 6$, 35; means in the same exposure interval followed by the same letter are not significantly different; Tukey–Kramer HSD test at $P = 0.05$)

Treatment	Exposure interval			
	24 h	48 h	7 d	14 d
8×10^6 conidia/kg	0.6±0.5a	7.8±2.5a	27.8±1.8a	54.6±2.2a
8×10^8 conidia/kg	1.1±0.9a	14.6±3.2b	55.6±1.9c	81.4±2.4c
8×10^{10} conidia/kg	6.7±4.0a	30.3±3.9d	67.6±7.9d	82.3±7.1c
DE alone	1.1±0.8a	15.6±5.1b	91.3±2.5e	95.2±1.9d
8×10^6 conidia/kg + DE	1.7±0.9a	16.7±3.6b	32.3±4.6a	66.8±5.6b
8×10^8 conidia/kg +DE	2.8±1.1a	17.7±4.7b	45.6±6.1b	67.9±6.9b
8×10^{10} conidia/kg + DE	6.7±4.2a	24.6±3.2c	98.9±0.7f	100.0±0.0e
F	2.0	3.5	36.2	11.3
P	0.0871	0.0076	<0.0001	<0.0001

Table 5
Mean mortality (±SE) of *Tribolium confusum* adults after 24 h, 48 h, 7 d or 14 d of exposure in wheat treated with three dose rates (expressed as conidia/kg of wheat) of *Metarhizium anisopliae* conidia suspension with or without the addition of DE, or with DE alone, (in all cases $N = 36$; $df = 6$, 35; means in the same exposure interval followed by the same letter are not significantly different; Tukey–Kramer HSD test at $P = 0.05$)

Treatment	Exposure interval			
	24 h	48 h	7 d	14 d
8×10^6 conidia/kg	0.0±0.0a	1.2±0.8b	1.5±0.7a	1.1±0.7a
8×10^8 conidia/kg	0.0±0.0a	1.1±0.7b	1.8±0.8a	2.2±0.7ab
8×10^{10} conidia/kg	1.1±0.7a	5.5±0.7c	6.7±1.2b	7.8±0.7c
DE alone	5.6±1.9b	13.1±5.1d	26.4±6.2c	58.9±8.6e
8×10^6 conidia/kg + DE	0.0±0.0a	0.0±0.0a	1.2±0.7a	3.3±1.3b
8×10^8 conidia/kg +DE	0.0±0.0a	0.0±0.0a	1.1±0.7a	3.6±1.2b
8×10^{10} conidia/kg + DE	0.0±0.0a	3.3±1.2c	6.7±1.2b	24.4±4.3d
F	7.6	3.9	11.5	32.8
P	<0.0001	0.0040	<0.0001	<0.0001

exception of the highest fungal/DE dosage combination as well as to the grain treated with DE alone. At the 7 d exposure interval approximately 94% of the exposed adults died in the wheat treated with DE alone, while mortality was still low in the other treatments. After 14 d all adults were dead in the DE-treated grain. Moreover, at this exposure, significantly more adults were dead in wheat treated with the highest *M. anisopliae*/DE dosage combination, in comparison with the other *M. anisopliae* suspensions, with or without DE.

In the case of the conidial powder, as above, significant differences were also noted between treatments and mortality levels were higher than those caused by the suspension (Table 4). At the 48-h exposure interval, significantly more adults died in wheat treated with the highest dosage, with or without DE. After 7 d of exposure, significantly more adults were dead in wheat treated with the highest fungal dose/DE combination, as compared with the other treatments. This trend was also evident at the 14 d exposure interval, when all *S. oryzae* adults were dead in the highest dosage in combination with DE. Moreover, *S. oryzae* adult mortality was nil and did not exceed 3.0% to the untreated grain that served as control.

3.3. Mortality of *T. confusum* adults

In the tests using the conidial suspension, significant differences were found between treatments, with the exception of the 24-h exposure interval (Table 5). After 48 h of exposure significantly more adults were dead in wheat treated with DE alone, in comparison with the other treatments. Moreover, at the 7-d exposure interval more than 26% of the exposed adults died in wheat treated with DE alone, while in the other treatments mortality did not exceed 6.7%. Finally, after 14 d of exposure approximately 60% of *T. confusum* adults were dead in the DE-treated wheat. In contrast, for the fungal treatments, mortality was <10%, with the exception of the highest fungal/DE dosage combination.

Table 6
Mean mortality (±SE) of *Tribolium confusum* adults after 24 h, 48 h, 7 d or 14 d of exposure in wheat treated with three dose rates (expressed as conidia/kg of wheat) of *Metarhizium anisopliae* conidia powder with or without the addition of DE, or with DE alone, (in all cases $N = 36$; $df = 6$, 35; means in the same exposure interval followed by the same letter are not significantly different; Tukey–Kramer HSD test at $P = 0.05$)

Treatment	Exposure interval			
	24 h	48 h	7 d	14 d
8×10^6 conidia/kg	2.2±0.8a	2.2±0.9a	7.9±2.5a	33.3±2.1a
8×10^8 conidia/kg	3.3±1.2a	5.6±0.7b	18.9±1.9b	37.8±3.1a
8×10^{10} conidia/kg	17.9±2.8c	20.3±2.4d	47.9±4.6d	74.4±3.7c
DE alone	7.9±2.2b	15.9±4.4cd	29.0±2.4c	61.0±2.8b
8×10^6 conidia/kg + DE	6.7±1.2b	10.1±2.1c	21.1±7.1bc	66.7±5.3bc
8×10^8 conidia/kg +DE	13.3±2.2c	20.1±3.2d	33.7±6.5c	68.9±6.7bc
8×10^{10} conidia/kg + DE	25.6±3.9d	37.9±1.9e	93.4±1.2e	100.0±0.0d
F	19.2	26.5	49.7	43.5
P	<0.0001	<0.0001	<0.0001	<0.0001

In the tests using the conidial powder, significant differences were noted among treatments at all exposures periods examined and adult mortality was higher than the corresponding mortality for the suspension (Table 6). After 24 and 48 h of exposure, significantly more adults were dead in wheat treated with the highest *M. anisopliae*/DE

dosage combination in comparison with the other treatments. Moreover, after 7 and 14 d of exposure, adult mortality in the case of the highest fungal dosage in combination with DE was 93.4 and 100%, respectively. In addition, control mortality for *T. confusum* adults was lower than 4.5%.

3.4. Progeny production

Significant differences were noted in progeny production in tests using the conidial suspension between treatments for all three species (Table 7). For *R. dominica*, significantly fewer offspring were recorded in vials containing wheat treated with DE alone, compared to the highest dosage or in combination with DE, than with the other treatments. For *S. oryzae*, progeny production in the wheat treated with DE alone was significantly reduced (1.3/vial), compared with the other treatments. In contrast, progeny production in wheat treated with the other treatments was high, especially in the case of the three doses of *M. anisopliae* alone. Finally, for *T. confusum*, no progeny were found in vials containing wheat treated with the highest fungal dosage, when combined with DE, while extremely few progeny were found in vials containing wheat sprayed with the highest fungal dosage.

For the conidial powder, significant differences were noted between treatments for progeny production counts, for all species (Table 8). In the cases of *R. dominica* and *S. oryzae*, significantly less F_1 individuals were recorded in wheat treated with the highest fungal/DE dosage combination than with the other treatments. The same holded for *T. confusum*, where no progeny was found in wheat treated with the highest fungal dosage in combination with DE.

Table 7
Mean number of *Rhyzopertha dominica*, *Sitophilus oryzae* and *Tribolium confusum* offspring produced (±SE)/vial in wheat treated with three dose rates (expressed as conidia/kg of wheat) of the *Metarhizium anisopliae* aqueous suspension with or without the addition of DE, or with DE alone, 60 d after the removal of the parental adults; the progeny production in the control vials was 14.7±4.4, 21.9±4.9 and 6.9±1.8 individuals/vial for *R. dominica*, *S. oryzae* and *T. confusum*, respectively (in all cases $N = 36$; $df = 6, 35$; within each species, means followed by the same letter are not significantly different; Tukey–Kramer HSD test at $P = 0.05$)

Treatment	Species		
	Rhyzopertha dominica	*Sitophilus oryzae*	*Tribolium confusum*
8×10^6 conidia/kg	12.1±2.6c	31.7±5.9c	5.3±1.5c
8×10^8 conidia/kg	5.6±0.9b	29.7±5.8c	1.7±0.7b
8×10^{10} conidia/kg	1.3±0.3a	28.1±5.6c	0.3±0.2a
DE alone	2.1±0.9a	1.3±0.5a	3.3±1.0b
8×10^6 conidia/kg + DE	6.3±1.2b	20.3±5.9bc	6.2±2.1c
8×10^8 conidia/kg +DE	5.7±0.8b	16.3±4.7b	1.6±0.7b
8×10^{10} conidia/kg + DE	1.1±0.4a	14.6±3.4b	0±0a
F	14.3	4.6	11.9
P	<0.0001	0.0015	<0.0001

Table 8
Mean number of *Rhyzopertha dominica*, *Sitophilus oryzae* and *Tribolium confusum* offspring produced (±SE) in wheat treated with three dose rates (expressed as conidia/kg of wheat) of the *M. anisopliae* conidia powder with or without the addition of DE, or with DE alone, 60 d after the removal of the parental adults; the progeny production in the control vials was 15.1±3.7, 26±4.4 and 7.1±0.9 individuals/vial for *R. dominica*, *S. oryzae* and *T. confusum*, respectively (in all cases $N = 36$; $df = 6, 35$; within each species, means followed by the same letter are not significantly different; Tukey–Kramer HSD test at $P = 0.05$)

Treatment	Species		
	Rhyzopertha dominica	*Sitophilus oryzae*	*Tribolium confusum*
8×10^6 conidia/kg	5.1±1.1d	5.9±1.4c	4.3±1.0d
8×10^8 conidia/kg	2.8±0.7c	1.9±0.8b	2.1±0.4c
8×10^{10} conidia/kg	2.5±0.4c	2.0±0.6b	1.0±0.3b
DE alone	3.2±1.0cd	2.1±1.1b	2.3±0.8cd
8×10^6 conidia/kg + DE	4.1±0.5d	1.5±0.4b	3.3±1.0cd
8×10^8 conidia/kg +DE	1.5±0.3b	1.6±0.4b	3.7±1.3cd
8×10^{10} conidia/kg + DE	0.3±0.2a	0.7±0.2a	0±0a
F	8.2	5.8	3.5
P	<0.0001	0.0003	0.0084

4. Discussion

One of the most interesting results of the present study is the different efficacy recorded between the conidial suspension and the conidial powder. The powder proved more effective than the suspension, in the case of *S. oryzae* and *T. confusum*. However, for *R. dominica* the suspension appeared to be more effective than the powder. Hence, insect species should be seriously taken into account when conducting similar experiments or planning control strategies, which include the use of entomopathogenic fungi preparations, against stored products' pests. These results are in agreement with recently obtained results by Batta (2005) who used the same fungal strain in an aqueous suspension (fungal conidia incorporated in invert emulsion) and powder (fungal conidia mixed with wheat flour). The author reported that mortality of *R. dominica* adults reached 93.3% and 86.7% after 7 d of exposure in chickpea grains treated with the two aforementioned fungal treatments, respectively. In contrast, in the case of *S. oryzae*, the powder used in our tests was superior to the suspension. This supports with previous observations reported by Batta (2004) in bioassays with the same fungal strain against adults of this species. As in the case of *S. oryzae*, the conidial powder was much more effective than the suspension against *T. confusum* adults. Surprisingly, the application of the conidial powder after 7 d of exposure caused a 9-fold increase in *T. confusum* mortality in comparison with the corresponding mortality caused by the conidial suspension. Although longer exposures are required for fungi to infest their insect hosts, mortality was observed for all three species at short exposure intervals ($\leqslant$48 h) on wheat treated with *M. anisopliae* alone. This

fact could be attributed to physical effect due to massive fungal doses and early fungal penetration and growth as it has been reported by Pekrul and Grula (1979).

DE enhanced the insecticidal effect of the conidial powder at the highest dosage. In contrast, the liquid *M. anisopliae*/DE combination was not as effective against *T. confusum* as DE alone, suggesting that a detrimental effect takes place in this case. This is also evident for the other two species tested as well, for some of the fungal/DE combinations. Moore and Higgins (1997) noted that certain clays had a detrimental effect on conidial viability of *Metarhizium flavoviride* Gams and Rozsypal (Deuteromycotina: Hyphomycetes). Hence, given that DEs, like clays, are classified in the category of inert dusts, a similar detrimental effect on *M. anisopliae* conidia is likely to occur. However, viability is not a direct indication of fungal efficacy, since as Batta (2003, 2004) reported aged preparations of *M. anisopliae*, with reduced conidial viability, were equally effective as new ones. The strength of this detrimental effect is inversely related to the conidial concentration, which may also suggest that, the presence of the fungus may inhibit the DE activity. DE particles may partially lose their desiccant capacity due to a potential absorptive interaction with conidia. Moreover, this detrimental effect is more vigorously expressed, in the case of combined use of DE with aqueous conidia suspension given the increased moisture content of the treated grain. Increased moisture generally leads to a decreased DE efficacy (Arthur, 2000; Fields and Korunic, 2000; Vayias and Athanassiou, 2004) and that fact was also noticed in our experiments since the moisture content of the wheat sprayed with aqueous fungal suspension was higher than that of the wheat treated with conidial powder. Furthermore, the presence of DE particles on the insects' cuticle may reduce the conidial germination and/or attachment. Further experimental work is needed to clarify these hypotheses.

In light of our findings, the effect of the addition of DE on the fungal efficacy is determined by the conidial concentration. This may indicate the existence of a certain threshold of conidial concentration, which characterizes the DE–fungal interaction. Under this threshold the negative interaction is very strong and a detrimental effect on both substances may occur. Under conditions of high conidial containment (beyond the threshold) the fungal efficacy is not affected and might be enhanced by the presence of DE. In this case, *M. anisopliae* conidia may exploit better the activity of DE particles to certain epicuticular lipids that play an inhibitory role to fungus germination and growth. Lord (2001) reported a synergism between *B. bassianna* and the DE formulation "Protect-It" against adults of *R. dominica* and *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae). Furthermore, Akbar et al. (2004) revealed a synergistic effect of the same combination against larvae of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). In a recent study, Batta (2004), using the same strain of *M. anisopliae* as used in this current study found that the addition of several inert dust materials, such as charcoal or oven ash, increased the effectiveness of the fungus against *S. oryzae* adults. The basis of this effect has not been investigated in detail, but Akbar et al. (2004) reported that DE significantly increased the degree of the *B. bassiana* conidia attachment on the cuticle of *T. castaneum* larvae. It is likely that a potential complimentary effect on the insects' epicuticular lipids, is the basic characteristic of this effect (Moore et al., 2000).

Apart from parental mortality in the treated substrate, the avoidance of progeny production is essential for long-term stored grain protection. Both fungal preparations, at the highest dosage and in combination with DE, suppressed progeny production since very few *R. dominica* progeny were found. This can be considered as a direct consequence of the faster mortality that occurred. For the same reason, progeny production of *S. oryzae* in wheat treated with the aqueous fungal suspension was high, in comparison with DE alone. This species is considered as a DE-susceptible species (Fields and Korunic, 2000; Arthur, 2002). Previous studies by Athanassiou et al. (2003, 2005a) indicated that 0.5 g of SilicoSec®/kg of grain can, under certain circumstances, such as higher than 25 °C temperatures and longer than 7 d exposure intervals, provide 100% parental mortality and thus complete progeny suppression against *S. oryzae*. This was also noted in our study since mortality of all three species was increased with the increase of exposure on wheat treated with DE alone. Very few progeny of *T. confusum* were found in wheat treated with the high rate of both fungal preparations and no progeny production was recorded when DE was added at this rate. This may suggest that, at this rate, *M. anisopliae* is highly effective against *T. confusum* immatures, since, as a secondary pest, its larvae feed and develop in the external part of the seed; thus, the possibility of picking up conidia is increased. Also, Vayias and Athanassiou (2004) found that young larvae of *T. confusum* are very susceptible to SilicoSec®, which is in agreement with the results reported here.

One of the key elements of IPM in stored-products is the combination of several, reduced-risk, control methods, because storage pests are not always effectively controlled by the application of only one measure. Our results demonstrate that *M. anisopliae* is effective against *R. dominica*, *S. oryzae* and *T. confusum*, but its effectiveness is highly influenced by several factors such as the exposure interval, the target species, the dosage and, especially, the characteristics of the fungal preparation. In addition, according to Lord (2005), the presence of DE favors the insecticidal efficacy of *B. bassiana*. Combined use of DE with *M. anisoplae* could be of practical importance for pest control of stored products provided that the conidial concentration will be above a critical limit so a detrimental effect on both DE and fungal efficacy is not likely to occur. Although it is generally accepted that conidia formed on solid carriers are more stable than conidia in a liquid culture (Moore et al., 2000), the availability of a large

number of fungal isolates (Moore et al., 2000; Wakefield et al., 2002), makes the comparative evaluation of results obtained from different studies difficult. Practically, the selection of isolates based only on their virulence may not be the most important component, given that the addition of cheap materials, such as inert dusts, as fungal carriers may increase the effectiveness of a moderately effective isolate.

Acknowledgements

We thank M. Erb-Brinkmann (Biofa Gbmh) for providing the SilicoSec® quantity for experimentation and Dr. A. Markoglou (Agricultural University of Athens) for his assistance. This study was partially supported by the Ministry of Rural Development and Food (Directorate of Plant Produce Protection).

References

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265–267.

Aitken, A.D., 1975. Insect Travelers, I: Coleoptera. Technical Bulletin 31. H.M.S.O., London.

Akbar, W., Lord, J.C., Nechols, J.R., Howard, R.W., 2004. Diatomaceous earth increases the efficacy of *Beauveria bassiana* against *Tribolium castaneum* larvae and increases conidia attachment. J. Econ. Entomol. 97, 273–280.

Arthur, F.H., 2000. Toxicity of diatomaceous earth to red flour beetles and confused flour beetles (Coleoptera: Tenebrionidae): effects of temperature and relative humidity. J. Econ. Entomol. 93, 526–532.

Arthur, F.H., 2002. Survival of *Sitophilus oryzae* (L.) on wheat treated with diatomaceous earth: impact of biological and environmental parameters on product efficacy. J. Stored Prod. Res. 38, 305–313.

Athanassiou, C.G., Kavallieratos, N.G., Tsaganou, F.C., Vayias, B.J., Dimizas, C.B., Buchelos, C.Th., 2003. Effect of grain type on the insecticidal efficacy of SilicoSec® against *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). Crop Prot. 22, 1141–1147.

Athanassiou, C.G., Vayias, B.J., Dimizas, C.B., Kavalieratos, N.G., Papagregoriou, A.S., Buchelos, C.Th., 2005a. Insecticidal efficacy of diatomaceous earth against *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and *Tribolium confusum* Du Val (Coleoptera: Tenebrionidae) on stored wheat: influence of dosage, temperature and exposure interval. J. Stored Prod. Res. 41, 47–55.

Athanassiou, C.G., Kavallieratos, N.G., Economou, L.P., Dimizas, C.B., Vayias, B.J., Tomanović, S., Milutinović, M., 2005b. Persistence and efficacy of three diatomaceous earth formulations against *Sitophilus oryzae* (Coleoptera: Curculionidae) on wheat and barley. J. Econ. Entomol. 98, 1404–1412.

Batta, Y.A., 2003. Production and testing of novel formulations of the entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes). Crop Prot. 22, 415–422.

Batta, Y.A., 2004. Control of the rice weevil (*Sitophilus oryzae* L., Coleoptera: Curculionidae) with various formulations of *Metarhizium anisopliae*. Crop Prot. 23, 103–108.

Batta, Y.A., 2005. Control of the lesser grain borer *Rhyzopertha dominica* (F.), (Coleoptera: Bostrychidae) by treatments with residual formulations of *Metarhizium anisopliae*(Metschinkoff) Sorokin (Deuteromycotina: Hyphomycetes). J. Stored Prod. Res. 41, 221–229.

Cox, P.D., Wilking, D.R., 1996. The potential use of biological control of pests in stored grain. Research Review 36, Home-Grown Cereals Authority, London.

Dal-Bello, G., Padin, S., Lopez-Lastra, C., Fabrizio, M., 2001. Laboratory evaluation of chemical-biological control of the rice weevil (*Sitophilus oryzae* L.) in stored grains. J. Stored Prod. Res. 37, 77–81.

Ebeling, W., 1971. Sorptive dusts for pest control. Ann. Rev. Entomol. 16, 123–158.

Fields, P., Korunic, Z., 2000. The effect of grain moisture content and temperature on the efficacy of diatomaceous earths from different geographical locations against stored-product beetles. J. Stored Prod. Res. 36, 1–13.

Golob, P., 1997. Current status and future perspectives for inert dusts for control of stored product insects. J. Stored Prod. Res. 33, 69–79.

Greenspan, L., 1977. Humidity fixed points of binary saturated aqueous solutions. J. Res. Nat. Bur. Stand.—A. Phys. Chem. 81, 89–96.

Korunic, Z., 1998. Diatomaceous earths, a group of natural insecticides. J. Stored Prod. Res. 34, 87–97.

Lord, J.C., 2001. Desiccant dusts synergize the effect of *Beauveria bassiana* (Hyphomycetes: Moniliales) on stored-grain beetles. J. Econ Entomol. 94, 367–372.

Lord, J.C., 2005. Low humidity, moderate temperature and dessicant dust favor the efficacy of *Beauveria bassiana* (Hyphomycetes: Moniliales) for the lesser grain borer *Rhyzopertha dominica* (Coleoptera:Bruchidae). Biol. Control 34, 180–186.

Mewis, I., Ulrichs, Ch., 2001. Action of amorphous diatomaceous earth against different stages of the stored product pests *Tribolium confusum* (Coleoptera: Tenebrionidae), *Tenebrio molitor* (Coleoptera: Tenebrionidae), *Sitophilus granarius* (Coleoptera: Curculionidae) and *Plodia interpunctella* (Lepidoptera: Pyralidae). J. Stored Prod. Res. 37, 153–164.

Moore, D., Higgins, P.M., 1997. Viability of stored conidia of *Metarhizium flavoviride* Gams and Rozsypal, produced under different culture regimes and stored with clays. Biocontrol Sci. Technol. 7, 335–343.

Moore, D., Lord, J.C., Smith, S.M., 2000. Pathogens. In: Subramanyam, Bh., Hagstrum, D.W. (Eds.), Alternatives to Pesticides in Stored-Product IPM. Kluwer Academic Publishers, Dordreecht, pp. 193–227.

Padin, S.B., Dal-Bello, G., Fabrizio, M., 2002. Grain loss caused by *Tribolium castaneum*, *Sitophilus oryzae* and *Acanthoscelides obtectus* in stored durum wheat and beans treated with *Beauveria bassiana*. J. Stored Prod. Res. 38, 69–74.

Pekrul, S., Grula, E.A., 1979. Mode of action of the corn earworm (*Heliothis zea*) by *Bauveria bassiana* as revealed by scanning electron microscopy. J. Invertebr. Pathol. 34, 238–247.

Rice, W.C., Cogburn, R.R., 1999. Activity of the entomopathogenic fungus *Beauveria bassiana* (Deuteromycota: Hyphomycetes) against three coleopteran pests of stored grains. J. Econ. Entomol. 92, 691–694.

SAS Institute, 1995. SAS User's Guide: Statistics. SAS Institute, Gary, NC.

Sokal, R.R., Rohlf, F.J., 1995. Biometry, third ed. Freedman and Company, New York.

Stathers, T., 2002. Entomopathogenic fungi in grain storage—any lessons for Europe from elsewhere? In: Zdarkova, E., Lukas, J., Hubert, J. (Eds.), Proceedings of the 2nd meeting of WG 4 of COST Action 842, Prague, pp. 100–109.

Subramanyam, Bh., Roesli, R., 2000. Inert dusts. In: Subramanyam, Bh., Hagstrum, D.W. (Eds.), Alternatives to Pesticides in Stored-Product IPM. Kluwer Academic Publishers, Dordreecht, pp. 321–380.

Vayias, B.J., Athanassiou, C.G., 2004. Factors affecting efficacy of the diatomaceous earth formulation SilicoSec® against adults and larvae of the confused beetle *Tribolium confusum* Du Val (Coleoptera: Tenebrionidae). Crop Prot. 23, 565–573.

Wakefield, M., Cox, P.D., Wildey, K.B., Price, N.R., Moore, D., Bell, B.A., 2002. The use of entomopathogenic fungi for stored product insect and mite control—further progress in the "Mycopest project". In: Zdarkova, E., Lukas, J., Hubert, J. (Eds.), Proceedings of the 2nd meeting of WG 4 of COST Action 842, Prague, pp. 110–115.