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Effectiveness of *Metarhizium anisopliae* (Metschinkoff) Sorokin applied alone or in combination with diatomaceous earth against *Tribolium confusum* Du Val larvae: Influence of temperature, relative humidity and type of commodity

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Abstract

Laboratory experiments were carried out in order to assess the insecticidal effect of *Metarhizium anisopliae* (Metschinkoff) Sorokin (Deuteromycotina: Hyphomycetes) formulations against larvae of *Tribolium confusum* Du Val (Coleoptera: Tenebrionidae) on flour and wheat. *M. anisopliae* was applied at three dose rates, of 8×10^6, 8×10^8 and 8×10^{10} conidia/kg to either wheat or flour, respectively. Also the wheat/flour was treated with the diatomaceous earth (DE) formulation SilicoSec (Biofa, Germany), at two dose rates, 0.2 and 0.5 g/kg of wheat or flour either alone or in combination with *M. anisopliae* with each fungal rate. Mortality of *T. confusum* larvae was assessed 7 days after exposure to the treated substrate. The bioassays were conducted at three temperatures, 20, 25 and 30 °C, and two relative humidity (r.h.) levels, 55% and 75%. Larval mortality was notably varied among treatments, as well among temperature and humidity levels. For both fungus and DE, the increase of temperature increased their effectiveness. On the other hand, the increase of r.h. significantly reduced larval mortality for both *M. anisopliae* and SilicoSec. Both substances, either alone or in combination, were more effective on wheat than on flour. Also, the addition of 0.5 g of SilicoSec in the fungal preparation, especially at the highest dose rate, increased larval mortality, in comparison with the fungus alone or in combination with 0.2 g of SilicoSec. The results of the present work suggest that, under certain circumstances, the effectiveness of *M. anisopliae* against *T. confusum* larvae can be benefitted by the presence of DE.

Keywords: *Metarhizium anisopliae*; Diatomaceous earth; Synergism; *Tribolium confusum*; Stored wheat; Flour

1. Introduction

Stored-product insect control is currently based mainly on the use of two broad categories of insecticides: residual insecticides and fumigants. However, the development of resistance to these substances and the demands of consumers for residue-free food, has lead researchers to evaluate the use of alternative control methods, which do not leave residues on the product and are generally safe for the environment. Insect pathogens, such as entomopathogenic fungi, bacteria,

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viruses, protozoa and nematodes, offer many advantages, such as high efficacy and compatibility with other IPM methods, and thus, are considered to be among the most promising alternatives to chemical-based insect control (Moore et al., 2000).

A considerable proportion of the studies available on the use of pathogens in stored-products have utilized entomopathogenic fungi. These on naturally occurring organisms, which environmentally safe and have low mammalian toxicity (Cox and Wilkin, 1996). Furthermore, they have the potential to develop on the cadavers, thus reintroducing more inoculum into the system (Thomas et al., 1996, 1997). Hence, while long-term residual persistence is considered as a drawback in the case of conventional insecticides, it is a desirable characteristic for fungi (Moore et al., 2000). The most commonly examined fungal species in stored products is *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes). Several studies have demonstrated that *B. bassiana* is effective against several stored-product insect species in both laboratory and field tests (Rice and Cogburn, 1999; Moore et al., 2000; Meikle et al., 2001; Lord, 2001; Padin et al., 2002; Wakefield et al., 2002; Stathers, 2002; Akbar et al., 2004). In contrast, there are disproportionably few data for the use of *Metarhizium anisopliae* (Metschinkoff) Sorokin (Deuteromycotina: Hyphomycetes), despite the fact that there is evidence that *M. anisopliae* can be used with success in stored grain against several insect species (Dal-Bello et al., 2001; Batta, 2004, 2005).

The use of diatomaceous earth (DE) is another promising alternative to conventional pesticides. As in the case of fungi, DEs have a natural origin (they are composed of the fossils of diatoms) and low mammalian toxicity (Athanassiou et al., 2003, 2004). It is generally accepted that these materials act as desiccants on the insects' cuticle, and that insects exposed to DE particles die from water loss (Korunic, 1998; Subramanyam and Roesli, 2000; Mewis and Ulrichs, 2001). Lord (2001) and Akbar et al. (2004) found that DE increases the effectiveness of *B. bassiana* against stored-grain insect species, and their findings give promise for an integrated approach based on combinations of mycoinsecticides with DEs. However, there are many more aspects to be evaluated, for example, the influence of several biotic (insect life stage, etc.) or abiotic (temperature, moisture, etc.) factors on the potential synergism between these two substances.

The confused flour beetle, *Tribolium confusum* Du Val (Coleoptera: Tenebrionidae), is one of the most important pests of stored products (Aitken, 1975; Hill, 1990). It is a cosmopolitan species which can feed and develop in an extremely wide variety of stored products (Aitken, 1975). It is classified as a secondary pest, which means that this species can be developed easily in already damaged (mechanically damaged or infested) grains or processed grain products, especially wheat flour (Hill, 1990). However, it is also able to attack whole wheat grains (Daniels, 1956; Aitken, 1975). It can develop in a considerably wide temperature and moisture range, and is particularly tolerant to low humidities (Howe, 1960; Aitken, 1975). Like other species, it is now resistant to several conventional insecticides (Champ and Dyte, 1976; Zettler, 1991; Arthur and Zettler, 1992; Wool and Front, 2003). In the present work, we examined the potential of using *M. anisopliae* in combination with DE, against *T. confusum* larvae on wheat and flour. In addition, the influence of temperature and r.h. on the insecticidal effect of the fungal/DE formulations was also assessed.

2. Materials and methods

The *M. anisopliae* isolate used in the tests was strain Meta 1, obtained by Dr. Y. A. Batta [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, Via Israel]. The fungus was subcultured on plates with oat meal agar (O3506, Sigma, Germany) for mass production of the fungal conidia. During conidial production, plates were incubated at $20 \pm 1\,^{\circ}C$ and 16 h illumination per day. After 14 days of incubation, the fungal conidia were collected by scraping the conidial layers formed on the plate surface using a sterilized scalpel. The conidia were added to 100 ml sterile distilled water then stirred and filtered through muslin. A formulation containing fungal conidia and a dust carrier at 1:4 ratio (W/W) was prepared. The dust carriers that were obtained from local sources are wheat flour, oven ash (completely burned paper sheets), chalk powder (finely ground board chalks) and charcoal (finely ground coal pieces), as suggested by Batta (2004). Harvested conidia were thoroughly mixed with the carrier in screw-capped bottles. The concentration of fungal conidia in the conidial suspension was determined using a haemocytometer. Three formulations, corresponding to three concentrations of fungal conidia were prepared, containing 8×10^6, 8×10^8 and 8×10^{10} conidia/g of the formulation, respectively.

The *T. confusum* individuals were taken from a culture kept on wheat flour plus 5% brewers yeast (by weight) at $28 \pm 1\,^{\circ}C$ and $65 \pm 5\%$ r.h. and continuous darkness. This culture was kept at the Benaki Phytopathological Institute since 1994.

Untreated, clean, infestation-free soft wheat and flour were used for experimentation. The moisture content of the wheat, as determined by a Dickey–John moisture meter (Dickey–John Multigrain CAC II, Dickey–John Co, USA), ranged between 10.7% and 11.0%. In order to measure the moisture content of the flour, quantities of flour (500 g each) were placed in an oven at 130 °C for 90 min (Brabender III, Brabender Co, USA), and were reweighed at the end of the procedure. From this determination, the moisture content of the flour was shown to range between 13.3% and 13.6%. Samples of wheat or flour (10 g each) were treated with each of the three fungal dose rates, alone or in combination with two dose rates of the DE formulation SilicoSec (Biofa Gbmh, Germany). The DE rates used were 0.2 and 0.5 g/kg of product. An additional series of dishes was prepared, containing wheat or flour treated with the two DE dose rates alone. All samples were placed in glass-capped dishes, 9 cm in diameter and 2 cm in height. The fungal formulations were applied at the rate of 1 g/kg of product, corresponding to 8×10^{6}, 8×10^{8} and 8×10^{10} conidia/kg of wheat or flour. Thus, there were 11 combination treatments for each of the two products: the three fungal dose rates alone, the three fungal dose rates in combination with each of the two DE dose rates and the two DE rates alone. For each case (combination treatment and product) four dishes (replicates) were prepared. An additional series of dishes containing untreated wheat or flour served as a control. After the preparation of the dishes, 15 (third/fourth instar) *T. confusum* larvae were introduced into each dish. The dishes were placed in incubators set up at three temperatures, 20, 25 and 30 °C, and two relative humidities (r.h.), 55 and 75%. The required r.h. was maintained by using saturated salt solutions (sodium chloride and sodium bromide for 75% and 55% r.h., respectively), as recommended by Winston and Bates (1960). Larval mortality was assessed 7 days after exposure. All the experiments were repeated three times. Temperature and humidity during the experiments were monitored using HOBO data loggers (HOBO H8, Onset Computers, USA). Generally, temperature and humidity were stable during the entire experimental period.

Before the analysis all counts were arcsine transformed to normalize the variances and standardize the means. Mortality counts were corrected (on the transformed data) by using Abbott's (1925) formula; however, control mortality was generally low (usually $<5\%$). The data were analyzed separately for each treatment category (DE alone, fungus alone, 0.2 DE + fungus, 0.5 DE + fungus), by submitting the mortality counts to ANOVA's for dose rate, temperature and r.h. Means were separated using the Tukey–Kramer (HSD) test, at $P = 0.05$ (Sokal and Rohlf, 1995).

3. Results

3.1. SilicoSec alone

Generally, larval mortality was higher on wheat than on flour, regardless of the dose rate, the temperature and

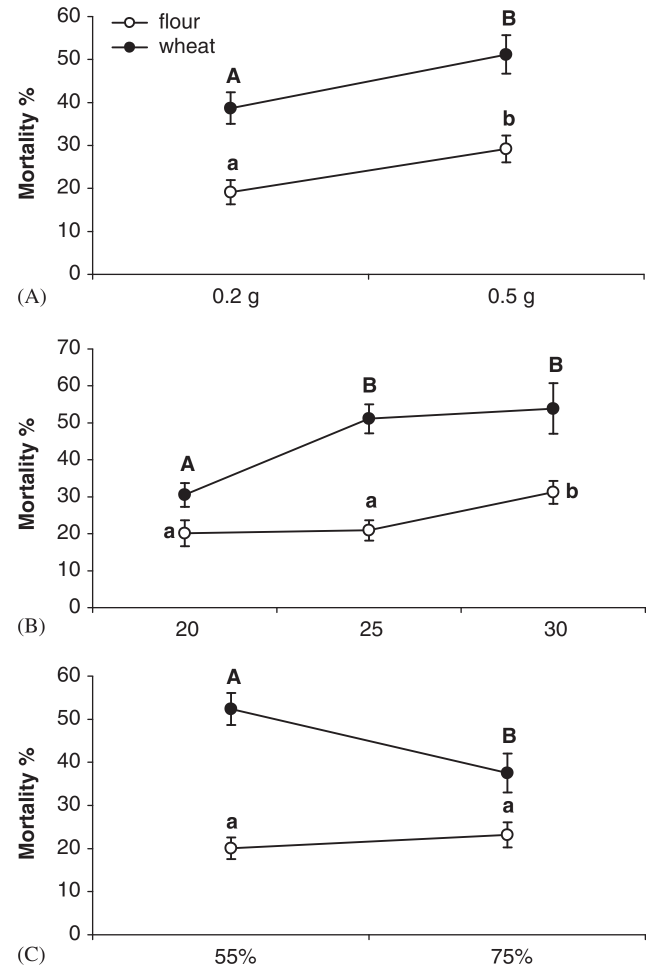

Fig. 1. Mean mortality (% ± SE) of *T. confusum* larvae exposed for 7 days on wheat or flour treated with (A) two dose rates of SilicoSec, (B) at three temperatures and (C) at two relative humidities (for each diagram, means within the same commodity followed by the same letter are not significantly different; Tukey–Kramer test at $P<0.05$).

the r.h. level (Fig. 1). The increase of SilicoSec dose rate significantly increased mortality, for both commodities tested (for wheat $F = 4.9$, $P = 0.03$, for flour $F = 8.2$, $P<0.01$, in both cases df = 1, 142) (Fig. 1A). However, even on wheat treated with 0.5 g of SilicoSec mortality did not exceed 51%. The increase of temperature significantly increased larval mortality (for wheat $F = 7.9$, $P<0.01$, for flour $F = 4.1$, $P = 0.02$, in both cases df = 2, 141), but dissimilar trends were noted among the two commodities (Fig. 1B). Hence, on wheat, no significant increase in mortality was noted at temperatures ⩾25 °C. In contrast, in the case of flour, significantly more larvae were dead at 30 °C, in comparison with the other two temperatures. Finally, the increase of r.h. significantly decreased SilicoSec efficacy, but only on wheat (for wheat $F = 7.1$, $P<0.01$, for flour $F = 2.2$, $P = 0.14$, in both cases df = 1, 142) (Fig. 1C).

3.2. *M. anisopliae* alone

Dose rate significantly affected larval mortality only in the case of flour (for wheat $F = 3.0$, $P = 0.06$, for flour $F = 5.9$, $P < 0.01$, in both cases df $= 2, 213$) (Fig. 2A). On the other hand, the increase of temperature was directly related with an increase of *M. anisopliae* efficacy, for both commodities examined (for wheat $F = 338.5$, for flour $F = 18.6$, in both cases df $= 2, 213$, $P < 0.01$) (Fig. 2B). Nevertheless, even at the highest dose rate, mortality was $< 55\%$. Also, for both commodities, *M. anisopliae* was significantly more effective at 55% than at 75% r.h. (for wheat $F = 19.9$, $P < 0.01$, for flour $F = 6.3$, $P = 0.01$, in both cases df $= 1, 214$) (Fig. 2C).

3.2.1. *M. anisopliae*+0.2 g of SilicoSec

Generally, when *M. anisopliae* was applied in combination with the lowest SilicoSec dose rate, mortality was not further increased in comparison with the application of *M. anisopliae* alone. Larval mortality was significantly affected by the fungal dose rate (for wheat $F = 5.8$, $P < 0.01$, for flour $F = 4.4$, $P = 0.01$, in both cases df $= 2, 213$) (Fig. 3A). Significantly less *T. confusum* larvae were dead on wheat treated with the lowest dose rate, in comparison with the other two fungal rates, while in the case of flour dose 8×10^8 was equally effective with the other two dose rates. As above, higher mortalities were noted at higher temperatures, (for wheat $F = 17.2$, for flour $F = 120.1$, in both cases df $= 2, 213$, $P < 0.01$) (Fig. 3B). On wheat, no further increase in mortality level was noted at temperatures $\geqslant 25\,^{\circ}$C, while on flour, significantly more

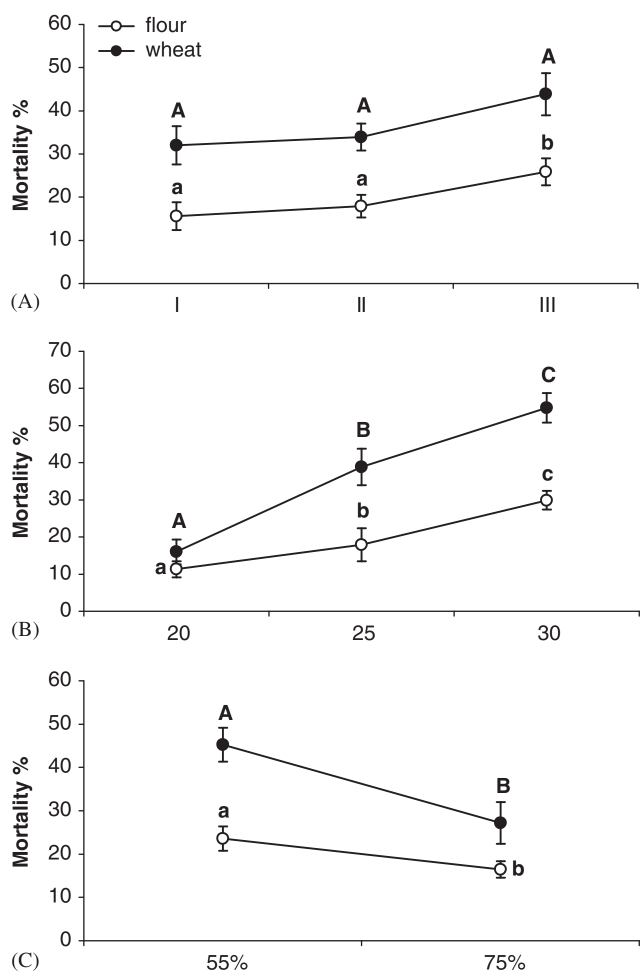

Fig. 2. Mean mortality (% ± SE) of *T. confusum* larvae exposed for 7 days on wheat or flour treated with (A) three dose rates of *M. anisopliae* (where $I = 8 \times 10^6$, $II = 8 \times 10^8$, $III = 8 \times 10^{10}$ conidia/kg), (B) at three temperatures and (C) at two relative humidities (for each diagram, means within the same commodity followed by the same letter are not significantly different; Tukey–Kramer test at $P < 0.05$).

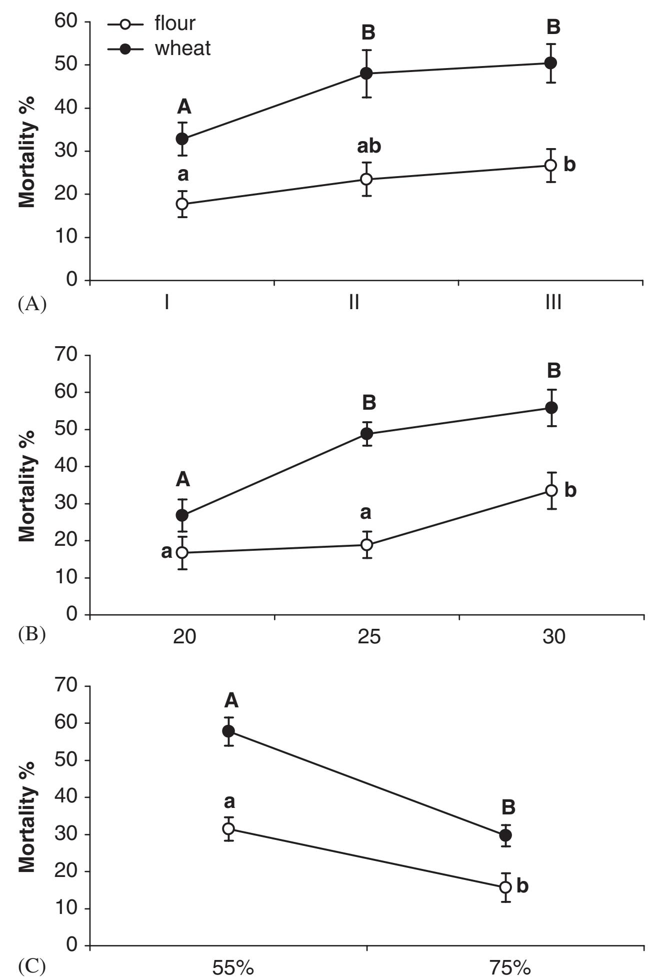

Fig. 3. Mean mortality (% ± SE) of *T. confusum* larvae exposed for 7 days on wheat or flour treated with (A) three dose rates of *M. anisopliae* (where $I = 8 \times 10^6$, $II = 8 \times 10^8$, $III = 8 \times 10^{10}$ conidia/kg) in combination with 0.2 g of SilicoSec/kg or wheat or flour, (B) at three temperatures and (C) at two relative humidities (for each diagram, means within the same commodity followed by the same letter are not significantly different; Tukey–Kramer test at $P < 0.05$).

larvae died at 30 °C than at the other two temperatures examined. Finally, the fungus was more effective at 55% than at 75% r.h. (for wheat $F = 45.8$, for flour $F = 39.9$, in both cases $df = 1, 214$, $P < 0.01$) (Fig. 3C).

3.2.2. *M. anisopliae+0.5 g of silicoSec*

The increase of SilicoSec dose rate in the fungal preparation increased mortality in comparison with the previous combination (Fig. 4). The increase of the fungal dose rate significantly affected larval mortality, especially in the case of wheat where mortality was directly related to fungal dose rate ($F = 6.4$, $df = 2, 213$, $P < 0.01$) (Fig. 4A). Hence, at the highest dose rate, on wheat, mortality reached 64%. In flour, significant differences were noted only between the lowest and the highest fungal rates ($F = 4.8$, $df = 2, 213$, $P = 0.01$). The increase of temperature also increased mortality for both commodities tested (for wheat $F = 29.1$, for flour $F = 10.2$, in both cases $df = 2, 213$, $P < 0.01$) (Fig. 4B). Thus, on wheat significantly more larvae were dead at 30 °C in comparison with the other two temperatures, while on flour, the fungus was equally effective at temperatures $\leqslant 25$ °C. Finally, as in the case of all the previously-mentioned cases, for both commodities, the fungal formulation was more effective at 55% than at 75% r.h. (for wheat $F = 5.7$, for flour $F = 18.8$, in both cases $df = 1, 214$, $P < 0.01$) (Fig. 4C).

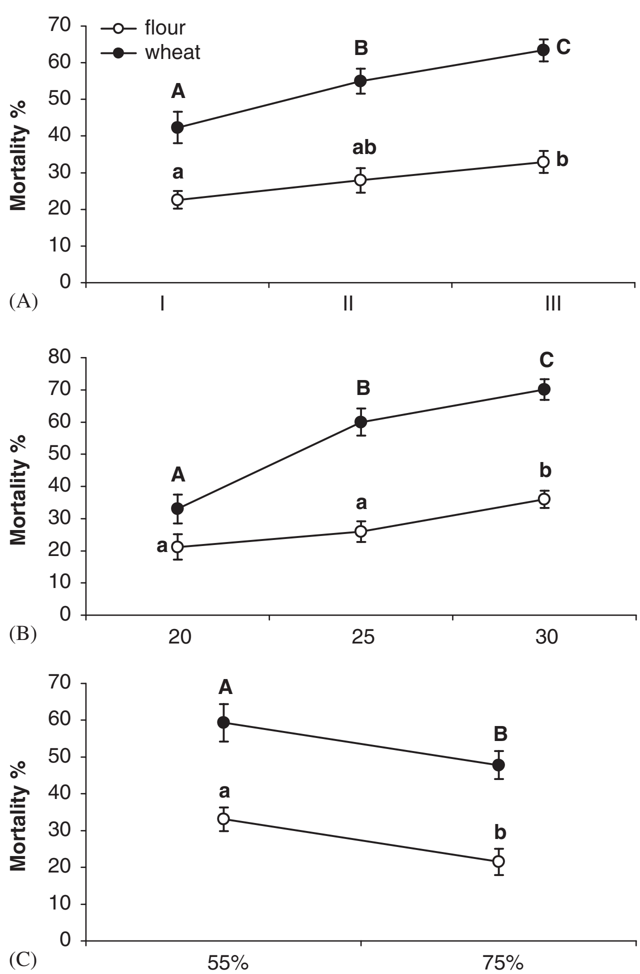

Fig. 4. Mean mortality (% ± SE) of *T. confusum* larvae exposed for 7 days on wheat or flour treated with (A) three dose rates of *M. anisopliae* (where $I = 8 \times 10^6$, $II = 8 \times 10^8$, $III = 8 \times 10^{10}$ conidia/kg) in combination with 0.5 g of SilicoSec/kg or wheat or flour, (B) at three temperatures and (C) at two relative humidities (for each diagram, means within the same commodity followed by the same letter are not significantly different; Tukey–Kramer test at $P < 0.05$).

4. Discussion

The results of the present study indicate that *M. anisopliae* and DE can be used successfully against *T. confusum* larvae, but their effectiveness is determined by several factors, such as the type of the commodity, the dose rate and the prevailing temperature and r.h. Also, our findings suggest that the presence of SilicoSec enhanced the insecticidal effect of *M. anisopliae* only under certain conditions.

From recent studies, it is known that DE synergises with *B. bassiana* against several stored-product beetle species (Lord, 2001; Akbar et al., 2004). Lord (2001) first reported this insecticidal effect using the DE formulation "Protect-It" against *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) and *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae). Similar results were also reported by Akbar et al. (2004) for *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). The present study is the first work on the potential use of *M. anisopliae* in combination with DE. Evidence that an enhanced effect is also likely to exist in this case was suggested by Batta (2004), who used *M. anisopliae* in dust carriers composed of several inert materials, such as charcoal and oven ash. He found that the addition of these materials increased the efficacy of the fungus against *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), and suggested that, since these materials provide a desiccant action, the incorporation of DE may be beneficial for the fungal formulation. Lord (2001) suggested that DEs may remove certain epicuticular lipids from the insects' bodies, enabling germinated fungal conidia to penetrate more easily into the insect haemoceal. In addition, Akbar et al. (2004) reported a significant increase in the degree of *B. bassiana* conidia attachment to the cuticle of *T. castaneum* larvae when the fungus was applied with DE. This finding can be a basic explanation for the synergism between *B. bassiana* and DE. However, Lord (2001), although finding a strong synergistic effect against *R. dominica*, found no significant increase in the degree of conidial attachment for this species.

Several inert materials, such as clays or silicas, have been used to improve the conidial viability of

Metarhizium flavoviride Gams and Rozsypal (Deuteromycotina: Hyphomycetes) (Moore et al., 1996; Moore and Higgins, 1997; Horaczek and Viernstein, 2004). Desiccant dusts are used in fungal formulations in order to dry the conidia to levels of 4–5% moisture content, which makes conidia durable and capable of long-term storage (Hedgecock et al., 1995; Moore and Higgins, 1997). From an industrial point of view, there are increasing needs to control the stability of entomopathogenic fungal formulations (Moore et al., 2000), but the influence of these additives on the insecticidal effect of the fungus has not been evaluated in detail. In our case, at some temperature/r.h. levels, the simultaneous presence of the fungus and DE gave lower levels of mortality than with the application of fungus or DE alone especially when the fungus was applied with the lowest SilicoSec dose rate. Moore and Higgins (1997) found that the presence of certain clays had a detrimental effect on the germination of conidia of *M. flavoviride*, and thus, in our case a negative influence in conidial viability might have occurred.

Larval mortality increased with the increase of the DE dose rate in the fungal formulations. For instance, on wheat, the mortality caused by the highest fungal dose rate with 0.5 g of SilicoSec was >70% at 30 °C, while the respective figure for same fungal rate alone was 54%. Interestingly, at the lowest fungal rates a small improvement or no improvement at all was recorded. This fact may suggest that DE benefits the fungal efficacy only when conidial concentration exceeds a certain "active threshold". Below this "threshold", a considerable amount of conidia may be damaged by the presence of DE, or DE particles may partially loose their desiccant capacity due to a potential absorptive interaction with conidia. On the other hand, when the conidial concentration is increased, this negative interaction is eliminated; this could be attributed to the fact that a sufficient amount of conidia remained "undamaged" to maintain a satisfactory insecticidal activity. We assume a specific amount of DE particles may interact with a specific quantity of conidia; hence, the conidial concentration has to be beyond this quantity for an effective combination formulation with sufficient conidial viability.

Based on our results, the type of commodity is determinative for the effectiveness of fungal or DE formulations. Therefore, consideration should be given to product type when considering applications of these substances. Both *M. anisopliae* and SilicoSec are more effective against *T. confusum* larvae on wheat than in flour. Most studies available for the effect of commodity type on results of treatment have been for DEs, while there are very few data available on entomopathogenic fungi. Generally, a specific DE is not equally effective at different commodities (Athanassiou et al., 2003, 2004, 2005; Athanassiou and Kavallieratos, 2005; Kavallieratos et al. 2005). For instance, in laboratory bioassays with SilicoSec against larvae of *T. confusum*, Vayias and Athanssiou (2004) found that larval survival was significantly higher on flour that on hard and soft wheat. As a secondary pest, *T. confusum* is fed and develops more easily on flour while in the case of sound grains, feeding and/or reproduction may be constrained (Aitken, 1975). As a result of feeding, water loss caused by DE is moderated and exposed individuals may partially recover (White and Loschiavo, 1989). Moreover, the presence of flour particles is likely to decrease the attachment of DE particles on the insects' cuticle, or remove them from their bodies. For the same reasons, flour may reduce the effectiveness of *M. anisopliae*. The impact of stored-product type on the effectiveness of *B. bassiana* has been confirmed by Rice and Cogburn (1999) and Padin et al. (2002). However, there are no relative data concerning *M. anisopliae*, and the influence of commodity in this case needs additional evaluation.

Temperature is one of the key elements for the effectiveness of DEs. It is well established that, with few exceptions, most stored-product beetle species are more vulnerable to DEs at higher temperatures (Arthur, 2000; Fields and Korunic, 2000; Subramanyam and Roesli, 2000; Vayias and Athanssiou, 2004; Athanassiou et al., 2004), and this is in agreement with our results. At higher temperatures, insects are more agile, and increased mobility may increase the contact with DE particles while water loss is more rapid at warmer conditions (Arthur, 2000; Fields and Korunic, 2000). Our results suggest that the increase of temperature increases the effectiveness of *M. anisopliae*, with or without the addition of SilicoSec. However, it is known that often an increase in temperature negatively affects and delays conidial germination (Moore et al., 1996; Moore and Higgins, 1997; Horaczek and Viernstein, 2004). Moore and Higgins (1997) found that conidia of *M. flavoviride* showed greater loss of viability when stored at 30 °C as compared to −10 °C. Also, Hedgecock et al. (1995) reported that temperature is a key factor for conidial longevity of *M. anisopliae*, and high temperatures cause catastrophic losses in conidial viability. However, there are no published studies on the insecticidal effect of *M. anisopliae* conidia at different temperatures, and additional experimental work should be carried out in this respect.

An increase of r.h. decreases the effectiveness of DEs, as has been documented by many reports (Arthur, 2000; Fields and Korunic, 2000; Subramanyam and Roelsi, 2000; Mewis and Ulrichs, 2001; Vayias and Athanassiou, 2004). Under moist conditions insects can moderate water loss (Mewis and Ulrichs, 2001), while DE particles may absorb moisture from the air (Stathers et al., 2004). Our results are in agreement with this fact. However, Akbar et al. (2004) did not find significant differences in the efficacy of the DE "Protect-It" against

T. castaneum between 55% and 75% r.h. Furthermore, the authors found no significant differences between these two humidities in the efficacy of *B. bassiana* against the same species. The available literature so far has given various and often contradictory results for the effect of moisture on the viability or the virulence of entomopathogenic fungi (Moore, 1973; Moore et al., 1996; Huafeng et al., 1998; Luz and Fargues, 1999; Akbar et al., 2004). Our results indicate clearly that *M. anisopliae* was more effective at 55% than at 75% r.h. High r.h. values may reduce the stability and persistence of *M. flavoviride* conidia (Hedgecock et al., 1995; Moore et al., 1996,2000; Moore and Higgins, 1997; Hong et al., 1998). Hence, moisture or humidity in a given commodity or storage facility should be seriously taken into account when an application of *M. anisopliae* is planned, since at moist conditions this application may be ineffective.

One of the fundamentals of an IPM-based strategy in stored-products is the combination of several, ecologically compatible control methods. *Metarhizium anisopliae* and DE are two very promising candidates for use in this strategy, and the present work provides evidence that the combination of these two substances can be used with success in stored-product commodities. Nevertheless, although some conclusions can be drawn, the performance observed in the present study is connected only with the specific fungal isolate and the DE formulation used, and thus, generalizations should be avoided.

Acknowledgments

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