OXFORD

Influence of Temperature and Relative Humidity on the Insecticidal Efficacy of *Metarhizium anisopliae* against Larvae of *Ephestia kuehniella* (Lepidoptera: Pyralidae) on Wheat

Christos G. Athanassiou,[1,2] Nickolas G. Kavallieratos,[3,4] Christos I. Rumbos,[1] and Demetrius C. Kontodimas[4]

[1]Laboratory of Entomology and Agricultural Zoology, Department of Agriculture Crop Production and Rural Environment, University of Thessaly, Phytokou str., 38446 Volos, Magnesia, Greece, [2]Corresponding author, e-mail: athanassiou@agr.uth.gr, [3]Laboratory of Agricultural Zoology and Entomology, Department of Crop Science, Agricultural University of Athens, 75 Iera Odos str., 11855 Athens, Attica, Greece, and [4]Laboratory of Agricultural Entomology, Department of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, 8 Stefanou Delta str., 14561 Kifissia, Attica, Greece

Abstract

A series of laboratory bioassays were conducted for the evaluation of the insecticidal efficacy of an isolate of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Hypocreales) against larvae of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), under various temperature–relative humidity (r.h.) conditions. The fungus was applied at four doses (0, 8×10^6, 8×10^8, and 8×10^{10} conidia ml^{-1}) on wheat and insect mortality was assessed after exposure of 1, 2, 7, and 14 d. Bioassays were conducted at three temperatures (20, 25, and 30 °C) and two r.h. levels (55 and 75%). Although complete control was not achieved in any case, the fungus provided a considerable level of insect control. Mortality of *E. kuehniella* larvae on wheat treated with *M. anisopliae* ranged between 41.1 and 93.3% after 14 d of exposure, whereas the respective mortality levels in control dishes never exceeded 28.3%. The increase of temperature resulted in most cases to higher efficacy, indicating that temperature is an important factor for the performance of the fungus. In contrast, in most cases r.h. did not significantly affect the efficacy of the fungus, at least for the humidity levels tested.

Key words: entomopathogenic fungi, *Ephestia kuehniella*, *Metarhizium anisopliae*, stored-product insect

Cereal production faces annually considerable losses due to post-harvest infestations (Reichmuth et al. 2007, Mason and McDonough 2012). Stored-product insect control is mainly accomplished by chemical means; however, in several cases conventional insecticides have lost their efficiency due to insecticide resistance development (Pereira et al. 2009, Nguyen et al. 2015). At the post-harvest stage of durable agricultural products, the methods that are available for pest control in the organic production are limited, and often not effective, compared to chemical control (i.e. application of contact insecticides and fumigants) (Grieshop et al. 2012). Therefore, there is a need for the evaluation of alternative agents for the management of stored-product insect pests, especially in the case of certain market durable commodities, such as organic food production and products that are used for sensitive groups, i.e. infants.

Biological control of stored-product insects with entomopathogenic fungi is a successful, sustainable alternative to chemical insecticides (Brower et al. 1996, Moore et al. 2000). Entomopathogenic fungi are natural enemies of insects, with low mammalian toxicity and limited environmental impact (Saik et al. 1990; Siegel and Shadduck 1990; Zimmermann 2007a,b). Fungal spores attach on the insect cuticle, germinate and mycelial hyphae penetrate into the host (Clarkson and Charnley 1996). After insect death, the inner mycelium grows out of the cadaver on the external part of the body of the dead host, sporulates and, theoretically, returns in this way more inoculum in the fungus-insect system (Goettel and Inglis 1997, Moore et al. 2000, Ortiz-Urquiza and Keyhani 2013).

Among the entomopathogenic fungi tested, *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Hypocreales) has been extensively evaluated against a wide spectrum of stored-product insect species (Dal Bello et al. 2001; Ekesi et al. 2001; Batta 2004, 2005; Kavallieratos et al. 2006, 2014). There are several reports on its effectiveness against major storage insects, such as the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) (Batta 2005, 2008; Athanassiou et al. 2008; Mahdneshin et al. 2009;

Shafighi et al. 2014), the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) (Dal Bello et al. 2001; Batta 2004, 2008; Kavallieratos et al. 2006, 2014; Athanassiou et al. 2008), the confused flour beetle, *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) (Kavallieratos et al. 2006, Michalaki et al. 2006), the groundnut bruchid, *Caryedon serratus* Olivier (Coleoptera: Bruchidae) (Ekesi et al. 2001), and the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) (Cherry et al. 2005). However, the efficacy of *M. anisopliae* varies among different studies. For instance, Kavallieratos et al. (2014) reported complete control of *S. oryzae* 14 d after application of a *M. anisopliae* conidial suspension on adults, in the presence or absence of food, at doses of 1.77×10^7 and 1.77×10^8 conidia ml^{-1}. In contrast, Dal Bello et al. (2001) reported low virulence of three *M. anisopliae* isolates against *S. oryzae* adults, with mortality levels ranging between 20 and 50% 1 month after exposure to 7×10^6 conidia ml^{-1}. Apparently, different fungal and insect strains are responsible for these variations. In this context, fungal strain is probably the most important factor for the performance of the entomopathogenic fungus.

The Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), is a cosmopolitan pest of agricultural stored products, particularly cereals, flour and other amylaceous commodities (Sedlacek et al. 1996, Tarlack et al. 2015) but also nuts and legumes (Locatelli et al. 2008), and its control is mandatory in mills and grain storage facilities (Trematerra and Gentile 2010, Belda et al. 2011). Athanassiou (2006) found that fifth-instar *E. kuehniella* larvae were tolerant to diatomaceous earths (DEs) when compared with first-instar ones. Recently, Pozidi-Metaxa and Athanassiou (2013) evaluated the insecticidal effect of four grain protectants (pirimiphos-methyl, chlorpyriphos-methyl, spinosad, and deltamethrin) against *E. kuehniella* larvae at different temperatures and reported that pirimiphos-methyl was the most effective among the insecticides tested. However, there is limited information on the insecticidal potential of entomopathogenic fungi against this stored-product moth species (Lord 2007a, Michalaki et al. 2007, Sabbour et al. 2012). For example, Michalaki et al. (2007) evaluated the insecticidal effect of the entomopathogenic fungus *Isaria fumosorosea* (Ifr) Wize (formerly *Paecilomyces fumosoroseus* (Wise) Brown and Smith) (Ascomycota: Hypocreales) against *E. kuehniella* larvae and reported their low susceptibility to this particular fungal isolate. In the only published study that tested the efficacy of *M. anisopliae* against *E. kuehniella* using nanoextractions of the fungus, Sabbour et al. (2012) reported increased susceptibility of the insect larvae to the fungal treatment.

Environmental conditions, e.g. temperature and humidity, have a strong impact on the virulence and success of entomopathogenic fungi against post-harvest insects. For instance, it is generally believed that entomopathogenic fungi are usually favored by high relative humidity conditions (Searle and Doberski 1984). As far as temperature is concerned, it is widely accepted that high temperatures affect negatively conidial viability and germination (Daoust and Roberts 1983, Moore et al. 1996, Horaczek and Viernstein 2004). Storage conditions, especially temperature and humidity, have a profound effect also on the quality of cereal grains and related commodities during storage (Sathya et al. 2008). In most cases, it has been proved that low humidity levels and temperatures can help to avoid spoilage of grains during storage and prolong storage time and grain viability (Volenik et al. 2006, Kaleta and Górnicki 2013). Therefore, it becomes evident that the successful implementation of entomopathogenic fungi for the control of insects in warehouses and storage facilities presupposes that storage

conditions will favor the performance of the fungus and simultaneously prevent the deterioration of stored grains. Few data are available on the effect of temperature and relative humidity (r.h.) on the performance of entomopathogenic fungi against stored-product insects. In this framework, the objective of the present study was to investigate the effect of temperature and r.h. on the insecticidal efficacy of *M. anisopliae* against *E. kuehniella*. For this reason, a *M. anisopliae* isolate was applied at three temperatures (20, 25, and 30 °C) and two r.h. levels (55 and 75%) against *E. kuehniella* larvae on wheat.

Materials and Methods

Test Insects and Commodity

Second or third instar larvae of *E. kuehniella* were used in the present study. Larvae were separated by instar according to their head capsule width (Harvey and Thompson 1995, Harvey and Vet 1997).

Ephestia kuehniella individuals were reared in 5 liter capacity glass jars containing 400 g of wheat flour, which were covered with muslin cloth fixed with a rubber band. Insect cultures were kept under constant conditions at 26 °C, 60 ± 5% r.h. and continuous darkness. Untreated, clean and infestation-free hard wheat, *Triticum durum* Desf. (var. Mexa), was used in the bioassays. The moisture content of the wheat samples was 11.2%, as determined by a Dickey-John moisture meter (mini GAC plus, Dickey-John Europe S.A.S., Colombes, France) at the initiation of the experiments.

Fungal Isolate and Conidial Suspensions

An isolate of *M. anisopliae* sensu lato from the culture collection of the Benaki Phytopathological Institute (Athens, Greece) was used in the bioassays. This isolate was first isolated from soil samples that were collected from the region of Marathon (Attika, Greece), using larvae of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), as bait (Zimmermann 1986). The fungus was initially grown on half-strength sabouraud dextrose agar (SDA) at 25 °C for 15 d in petri dishes. Then, the petri dishes were removed and stored at 4 °C. Before the experiments, the isolates were subcultured on SDA (ten petri dishes each) and placed in incubators at 25 °C for 12 d in continuous darkness (Kavallieratos et al. 2014). The conidia were harvested by scraping the surface of the petri dishes with a sterile scalpel. A sterile liquid solution of 0.1% Tween 80 was poured into the petri dishes (20 ml per plate) to carry away most of the conidia. The conidial suspensions were stirred using a magnetic stirrer (Heidolph MR 3001, Heidolph Instruments GmbH and Co. KG, Germany) and filtered twice using a sterile nylon membrane (45-μm pore size) using sterile glass beakers. The concentration of *M. anisopliae* conidia in the conidial suspension was determined by a haemocytometer (Precicolor, HBG, Giessen-Luetzellinden, Germany). Standard first concentration of *M. anisopliae* 8×10^{10} conidia per ml was prepared. From that conidial suspension, a series of 1/100 dilutions was prepared by diluting 0.5 ml of the preceding suspension in 49.5 ml of sterile 0.1% Tween 80 solution providing the final concentrations of 8×10^6 and 8×10^8 conidia ml^{-1}. Conidial germination of the inoculated conidia was observed microscopically (100x) after incubation for 24 h at 26 °C on SDA and exceeded 97% throughout the study.

Bioassays

One-kg lots of wheat were treated each with 1 ml of the suspension corresponding to the certain concentration (8×10^6, 8×10^8, or 8×10^{10} conidia ml^{-1}), as a thin layer on a tray. Conidial application

in the form of water suspension is commonly used in studies that evaluate the effectiveness of entomopathogenic fungi as grain protectants (Kavallieratos et al. 2014). Different trays were used for each spraying. An additional 1-kg lot of wheat was sprayed with a sterile aqueous solution of 0.1% Tween 80 and served as control. Spraying was carried out starting always from the control and proceeding from the lower to the higher concentration, using an AG-4 airbrush (Mecafer S.A., Valence, France) at an operating pressure of 1.6 bar. The airbrush was triple rinsed with sterile water and acetone between treatments. Then, the treated lots of wheat were transferred in glass jars and shaken manually to achieve even distribution of the conidial suspensions in the entire grain mass. Sterile plastic petri dishes (8 cm diameter by 1.5 cm high) were the experimental units for the bioassays. Each petri dish was filled with 15 g of the treated wheat with a scoop, using different scoop for each treatment. Finally, 15 *E. kuehniella* larvae were placed in each dish. The lid of the petri dishes had a 1.5 cm diameter hole in the middle, which was covered by gauze, to allow sufficient aeration inside the petri dish. The quantity of 15 g was weighed with a Precisa XB3200D compact balance (Alpha Analytical Instruments, Gerakas, Greece). Mortality of the exposed larvae was assessed after 1, 2, 7, and 14 d of exposure. Petri dishes were kept at the following six temperature × r.h. combinations in controlled chambers: 20 °C and 55% r.h., 20 °C and 75% r.h., 25 °C and 55% r.h., 25 °C and 75% r.h., 30 °C and 55% r.h. or 30 °C and 75% r.h. The humidity levels were achieved using saturated salt solutions as recommended by Greenspan (1977), whereas temperature and r.h. throughout each bioassay were monitored using a calibrated HOBO (Onset Computers, USA). These temperature and humidity levels were chosen in order to simulate favorable and unfavorable conditions for the development of insects and molds. Temperatures between 25 and 30 °C and r.h. levels between 70 and 80% are considered favorable for most grain storage insects (Hayma, 2003), whereas the minimum r.h. requirements of most common molds are in the 70–90% range (Brooker et al. 1992).

Experimental Design and Data Analysis

There were four dish replicates for each treatment, whereas each bioassay was repeated three times by preparing new lots of treated wheat each time (3 × 4 = 12 dishes for each treatment). Insect mortality was used as dependent variable in a generalized linear mixed effects model with exposure intervals as repeated measures and dose, temperature and r.h. as main effects using a negative binomial distribution and log link function (Stroup 2013). All associated interactions of the main effects were also incorporated in the analysis. Differences between mortality means for each exposure interval and temperature–r.h. combination were compared using Bonferroni *t*-tests at the 5% level (Sokal and Rohlf 1995). The SPSS version 24.0 software (SPSS Inc., Chicago, IL, USA) was used for all analyses.

Results

Mortality of *E. kuehniella* larvae was significantly affected by dose, temperature, r.h., and exposure interval (Table 1). Control mortality was generally low and ranged between 2.8 and 11.1, 3.9 and 12.8, 8.9 and 20.0, and 12.8 and 28.3% after 1, 2, 7, and 14 d of exposure, respectively, for all temperature–r.h. combinations tested (Tables 2–5).

After 1 d of exposure, mortality of *E. kuehniella* larvae on wheat treated with *M. anisopliae* was generally low at 20 and 25 °C for all doses and r.h. levels tested and hardly exceeded 21% (Table 2). The increase of temperature to 30 °C in most cases resulted in

Table 1. *F* statistics and *P* values from a generalized linear mixed effects model testing the effects of dose, temperature, r.h. and exposure interval, as well as their associated interactions, on the mortality levels of *Ephestia kuehniella* larvae exposed for 1, 2, 7, and 14 d (repeated measures) on *Metarhizium anisopliae*, applied at four dose rates (0, 8×10^6, 8×10^8, and 8×10^{10} conidia ml^{-1}) at three temperatures (20, 25, and 30 °C) and two r.h. levels (55 and 75%)

Source	df	*F*	*P*
Dose	3, 1056	88.8	<0.001*
Temperature	2, 1056	10.1	0.001*
r.h.	1, 1056	4.6	0.032*
Exposure	3, 1056	155.5	<0.001*
Dose × temperature	6, 1056	2.0	0.057
Dose × r.h.	3, 1056	2.5	0.059
Dose × exposure	9, 1056	32.0	<0.001*
Temperature × r.h.	2, 1056	1.5	0.213
Temperature × exposure	6, 1056	17.4	<0.001
r.h. × exposure	3, 1056	3.1	0.024*
Dose × temperature × r.h.	6, 1056	0.3	0.946
Dose × temperature × exposure	18, 1056	0.7	0.842
Dose × r.h. × exposure	9, 1056	1.2	0.316
Temperature × r.h. × exposure	6, 1056	4.3	<0.001
Dose × temperature × r.h. × exposure	18, 1056	0.6	0.913

*Indicates *P* < 0.05; GENLINMIXED Procedure.

significantly increased mortalities for *E. kuehniella* larvae for both r.h. levels. After application of the highest dose of the fungus, mortality reached 38.9 and 35.6% at the 55 and 75% r.h. level, respectively. In general, 1 d post-application mortality was in most cases not significantly affected by the application dose.

After 2 d of exposure mortality was further increased at all doses and r.h. levels tested (Table 3). For this exposure interval, when temperature was raised to 30 °C mortality reached 61.1 and 50.6% at the 55 and 75% r.h. level, respectively.

One week after application of *M. anisopliae* at 20 °C, the percentage of dead larvae ranged between 36.1 and 42.8% at the 55% and between 34.4 and 46.7% at the 75% r.h. level with no significant differences among doses (Table 4). For the same exposure interval, the highest mortality (78.3%) was recorded at 30 °C and 55% r.h. after application of the highest dose of the fungus.

The highest mortality levels were noted after 14 d of exposure, although control was not complete (100%) in any of the temperature–r.h. combinations and doses tested (Table 5). For instance, at 25 °C and 55% r.h. almost 94% of the exposed larvae died 2 weeks after treatment at the highest dose of the fungus. However, the increase of dose did not always correlate with an equivalent increase of mortality. Thus, at 20°C and 55% r.h., 25°C and 75% r.h., 30°C and 55% r.h. or 30°C and 75% r.h. the highest mortality levels were noted after application of 8×10^8 conidia ml^{-1}.

Discussion

Abiotic factors, such as temperature and r.h. can strongly affect the virulence and success of entomopathogenic fungi in controlling post-harvest insects. Temperature is a key element for their efficacy against storage insects. Conidial viability and germination generally decreases under high temperature conditions (Daoust and Roberts 1983, Moore et al. 2000, Horaczek and Viernstein 2004). Vassilakos et al. (2006) reported that *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) was more effective against

Table 2. Mean mortality ± SE of *Ephestia kuehniella* larvae exposed for 1 d on *M. anisopliae*, applied at three dose rates (8×10^6, 8×10^8, and 8×10^{10} conidia ml^{-1}) at three temperatures (20, 25, and 30 °C) and two r.h. levels (55 and 75%).

Temperature (°C)/r.h. (%)	Mortality (%)			
	Control	8×10^6	8×10^8	8×10^{10}
20 °C and 55%	3.3 ± 2.2 B	20.6 ± 2.5 Ab	12.8 ± 1.5 Abc	14.4 ± 2.1 Ab
20 °C and 75%	6.1 ± 2.8 B	13.3 ± 1.8 ABb	17.8 ± 1.7 Aab	18.9 ± 2.1 Ab
25 °C and 55%	2.8 ± 1.7 B	21.1 ± 2.1 Ab	8.3 ± 1.7 Bc	5.0 ± 0.9 Bc
25 °C and 75%	5.0 ± 2.3 B	17.2 ± 1.7 Ab	15.0 ± 2.3 Abc	16.1 ± 2.1 Ab
30 °C and 55%	11.1 ± 5.0 B	34.4 ± 2.0 Aa	26.7 ± 3.2 Aa	38.9 ± 2.7 Aa
30 °C and 75%	8.9 ± 4.5 C	20.0 ± 2.2 Bb	26.1 ± 1.9 ABa	35.6 ± 1.7 Aa

Within each row, means followed by the same uppercase letter do not differ significantly (in all cases df = 3, 47; Bonferroni *t*-test at *P* = 0.05). Within each column, means followed by the same lowercase letter do not differ significantly (in all cases df = 5, 71; Bonferroni *t*-test at *P* = 0.05). Where no letters exist, no significant differences were noted.

Table 3. Mean mortality ± SE of *Ephestia kuehniella* larvae exposed for 2 d on *M. anisopliae*, applied at three dose rates (8×10^6, 8×10^8, and 8×10^{10} conidia ml^{-1}) at three temperatures (20, 25, and 30 °C) and two r.h. levels (55 and 75%).

Temperature (°C)/r.h. (%)	Mortality (%)			
	Control	8×10^6	8×10^8	8×10^{10}
20 °C and 55%	9.4 ± 5.2 B	26.7 ± 2.3 Abc	27.8 ± 2.3 Ac	21.7 ± 3.4 ABbc
20 °C and 75%	7.2 ± 3.3 B	23.9 ± 2.7 Ac	27.8 ± 2.9 Ac	30.6 ± 4.0 Ab
25 °C and 55%	3.9 ± 1.9 C	31.1 ± 3.4 Abc	11.7 ± 2.2 BCd	13.9 ± 1.9 Bc
25 °C and 75%	10.0 ± 4.6 B	36.7 ± 3.4 Ab	33.9 ± 3.2 Abc	30.0 ± 3.2 Ab
30 °C and 55%	12.8 ± 5.3 B	50.6 ± 2.4 Aa	52.8 ± 4.0 Aa	61.1 ± 4.3 Aa
30 °C and 75%	11.7 ± 5.3 B	36.1 ± 3.3 Abc	46.1 ± 2.9 Aab	50.6 ± 2.8 Aa

Within each row, means followed by the same uppercase letter do not differ significantly (in all cases df = 3, 47; Bonferroni *t*-test at *P* = 0.05). Within each column, means followed by the same lowercase letter do not differ significantly (in all cases df = 5, 71; Bonferroni *t*-test at *P* = 0.05). Where no letters exist, no significant differences were noted.

Table 4. Mean mortality ± SE of *Ephestia kuehniella* larvae exposed for 7 d on *M. anisopliae*, applied at three dose rates (8×10^6, 8×10^8, and 8×10^{10} conidia ml^{-1}) at three temperatures (20, 25, and 30 °C) and two r.h. levels (55 and 75%).

Temp (°C)/r.h. (%)	Mortality (%)			
	Control	8×10^6	8×10^8	8×10^{10}
20 °C and 55%	12.2 ± 5.6 B	39.4 ± 3.4 Ab	42.8 ± 2.9 Ac	36.1 ± 3.0 Ad
20 °C and 75%	8.9 ± 4.4 B	34.4 ± 4.3 Ab	44.4 ± 3.9 Abc	46.7 ± 6.4 Abcd
25 °C and 55%	20.0 ± 8.9 B	69.4 ± 4.2 Aa	65.0 ± 5.9 Aa	57.2 ± 3.4 Abc
25 °C and 75%	13.3 ± 5.6 C	49.4 ± 4.8 ABb	62.2 ± 4.0 Aab	38.3 ± 4.0 Bcd
30 °C and 55%	13.9 ± 5.5 B	71.1 ± 3.0 Aa	71.1 ± 4.8 Aa	78.3 ± 5.0 Aa
30 °C and 75%	16.1 ± 7.1 C	47.2 ± 3.8 Bb	68.9 ± 3.8 Aa	64.4 ± 4.6 ABab

Within each row, means followed by the same uppercase letter do not differ significantly (in all cases df = 3, 47; Bonferroni t-test at *P* = 0.05). Within each column, means followed by the same lowercase letter do not differ significantly (in all cases df = 5, 71; Bonferroni *t*-test at *P* = 0.05). Where no letters exist, no significant differences were noted.

R. dominica and *S. oryzae* at 26 than at 30 °C. At the same time, the efficacy of *B. bassiana* against adults of the granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculonoidae), was decreased with the increase of temperature from 25 to 30 °C (Athanassiou and Steenberg 2007). In our study, the highest mortality levels of *E. kuehniella* larvae were recorded for most exposure intervals at 30 °C. Similarly, the effectiveness of *M. anisopliae* against *T. confusum* larvae was also increased with the increase of temperature (Michalaki et al. 2006). The reasons for the increase in efficacy of *M. anisopliae* against storage insects are poorly understood; however, the increased insect movement and metabolic activity at high temperatures (Fields and Korunić 2000) may be correlated with increased conidial attachment, and subsequently with higher fungal infection.

Regarding the effect of r.h. on the insecticidal efficacy of entomopathogenic fungi, it was believed until recently that entomopathogenic fungi need humid conditions to be effective, or that they are poorly effective at dry environments. Several reports supported this hypothesis showing the increased fungal efficacy against storage insects under humid conditions. For instance, Searle and Doberski (1984) reported reduced efficacy of *B. bassiana* against adults of the sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), at humidity levels lower than 90%, and identified humidity as the major factor determining whether spores will germinate and infect. Apparently, high humidity is favorable for fungal development, as fungi require a baseline of humid conditions to complete growth and spore production. This perception has probably influenced negatively the exploitation and commercialization

Table 5. Mean mortality ± SE of *Ephestia kuehniella* larvae exposed for 14 d on *M. anisopliae*, applied at three dose rates (8×10^6, 8×10^8, and 8×10^{10} conidia ml^{-1}) at three temperatures (20, 25, and 30 °C) and two r.h. levels (55 and 75%).

Temperature (°C)/r.h. (%)	Mortality (%)			
	Control	8×10^6	8×10^8	8×10^{10}
20 °C and 55%	23.9 ± 9.9 B	71.1 ± 3.6 Aabc	70.6 ± 3.5 Abc	59.4 ± 3.5 Ac
20 °C and 75%	12.8 ± 5.5 B	41.1 ± 5.4 Ad	59.4 ± 5.3 Ac	63.3 ± 7.5 Abc
25 °C and 55%	28.3 ± 11.4 B	88.3 ± 3.9 Aa	88.3 ± 0.9 Aa	93.9 ± 3.3 Aa
25 °C and 75%	18.3 ± 6.6 B	63.3 ± 5.3 Abc	85.0 ± 3.3 Aab	68.9 ± 7.4 Abc
30 °C and 55%	16.1 ± 5.6 B	80.0 ± 3.9 Aab	86.1 ± 5.0 Aab	85.6 ± 4.6 Aab
30 °C and 75%	19.4 ± 7.5 C	54.4 ± 4.8 Bcd	88.3 ± 3.7 Aa	78.3 ± 4.6 Aabc

Within each row, means followed by the same uppercase letter do not differ significantly (in all cases df = 3, 47; Bonferroni *t*-test at $P = 0.05$). Within each column, means followed by the same lowercase letter do not differ significantly (in all cases df = 5, 71; Bonferroni *t*-test at $P = 0.05$). Where no letters exist, no significant differences were noted.

of fungal formulations for insect control at the post-harvest stages of agricultural commodities, as high humidity in stored products is not desirable (Navarro et al. 2012). Nevertheless, recent reports relate the improved efficacy of entomopathogenic fungi with reduced r.h. levels (Lord 2005, 2007a,b; Michalaki et al. 2006; Athanassiou and Steenberg 2007). The insecticidal effect of *B. bassiana* was stronger at low r.h. levels against *R. dominica* (Lord 2005) and the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Lord 2007b). Desiccation increased the efficacy of *B. bassiana* against larvae of the Indian meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae), and adults of *S. oryzae* and the maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) (Lord 2007a). Similarly, the mortality of *S. granarius* in wheat treated with *B. bassiana* was higher at 55 than at 75% r.h. at 20 and 25 °C (Athanassiou and Steenberg 2007). At the same time, *M. anisopliae* was generally more effective at 55 than at 75% r.h. against *T. confusum* larvae (Michalaki et al. 2006). The reason for these results has not been fully understood yet. Lord (2005) speculated that desiccation stress may cause changes in the cuticular chemistry that affect the ability of conidia to attach, germinate and penetrate, or even alter insect behavior in a way that favors fungal infection. However, these findings have high practical value, as they support the hypothesis that entomopathogenic fungi can be effective against insects in stored-product environments, where usually dry conditions prevail. In the present study, in most cases no significant differences in fungal efficacy were noted between the two humidity levels tested, indicating that the infection of *E. kuehniella* larvae from *M. anisopliae* did not depend on humidity. Hence, the strain tested here can be effective against *E. kuehniella* larvae at ambient r.h. levels (around 50%), as the ones tested in this study. Other studies suggested that the infection process of post-harvest insects by entomopathogenic fungi can be independent by humidity (Rodrigues and Pratissoli 1990, Adane et al. 1996, Akbar et al. 2005). For example, Akbar et al. (2005) reported that the efficacy of *B. bassiana* against larvae of *T. castaneum* was similar at the two levels of r.h. tested, i.e. 55 and 75%. This is particularly important, as some fungal strains that are effective for insect control at dry conditions can be further evaluated and probably registered for their use in durable stored products, such as grains and legumes. The mechanisms on which some strains are more effective at dry conditions are poorly understood, and probably their high efficacy is related with increased water stress of the target insects. Lord (2005, 2007a) clearly indicated that fungal efficacy is higher when the fungus is combined with a desiccant, such as DEs. Consequently, increased efficacy is probably mostly related with increased insect stress, than with higher conidial germination and penetration.

Moreover, high r.h. values may reduce the stability and persistence of fungal conidia, as it has been shown for *Metarhizium flavoviride* Gams and Rozsypal (Deuteromycotina: Hyphomycetes) (Hedgecock et al. 1995, Moore et al. 2000).

To conclude, the certain *M. anisopliae* isolate tested in the present study provided a considerable level of *E. kuehniella* larvae control, with mortality levels up to 90% after 14 d of exposure, indicating the promising insecticidal potential of this fungal isolate under the common grain storage conditions. It is postulated that, given the slow-acting nature of most entomopathogenic fungi (Moore et al. 2000), mortality would have been higher at longer exposure intervals. Moreover, we identified temperature as an important factor for the efficacy of the fungus, and reported that the increase of temperature is related with higher efficacy levels. In contrast, r.h. did not significantly affect the efficacy of the fungus, at least for the humidity levels tested.

Funding

References Cited

Adane, K., D. Moore, and S. A. Archer. 1996. Preliminary studies on the use of *Beauveria bassiana* to control *Sitophilus zeamais* (Coleoptera: Curculionidae) in the laboratory. J. Stored Prod. Res. 32: 105–113.

Akbar, W., J. C. Lord, J. R. Nechlos, and T. M. Loughin. 2005. Efficacy of *Beauveria bassiana* for red flour beetle when applied with plant essential oils or mineral oil and organosilicon carriers. J. Econ. Entomol. 98: 683–688.

Athanassiou, C. G. 2006. Influence of instar and commodity on insecticidal effect of two diatomaceous earth formulations against larvae of *Ephestia kuehniella* (Lepidoptera: Pyralidae). J. Econ. Entomol. 99: 1905–1911.

Athanassiou, C. G., and T. Steenberg. 2007. Insecticidal effect of *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) in combination with three diatomaceous earth formulations against *Sitophilus granarius* (L.) (Coleoptera: Curculionidae). Biol. Control. 40: 411–416.

Athanassiou, C. G., N. G. Kavallieratos, B. J. Vayias, J. B. Tsakiri, N. H. Mikeli, C. M. Meletsis, and Z. Tomanović. 2008. Persistence and efficacy of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycotina: Hyphomycetes) and diatomaceous earth against *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) on wheat and maize. Crop Prot. 27: 1303–1311.

Batta, Y. A. 2004. Control of 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: Bostrichidae) by treatments with residual formulations of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycotina: Hyphomycetes). J. Stored Prod. Res. 41: 221–229.

Batta, Y. A. 2008. Control of main stored-grain insects with new formulations of entomopathogenic fungi in diatomaceous earth dusts. Int. J. Food Engin. 4: 9.

Belda, C., M. Ribes-Dasi, and J. Riudavets. 2011. Improving pest management in pet food mills using accurate monitoring and spatial analysis. J. Stored Prod. Res. 47: 385–392.

Brooker, D. B., and F. W. Bakker-Arkema, and C. W. Hall. 1992. Drying and storage of grains and oilseeds. Van Nostrand Reinhold, New York.

Brower, J. H., L. Smith, P. V. Vail, and P. W. Flinn. 1996. Biological control, pp. 223–286. *In* B. Subramanyam and D. W. Hagstrum (eds.), Integrated management of insects in stored products. Marcel Dekker, New York.

Cherry, A. J., P. Abalo, and K. Hell. 2005. A laboratory assessment of the potential of different strains of the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) to control *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) in stored cowpea. J. Stored Prod. Res. 41: 295–309.

Clarkson, J. M., and A. K. Charnley. 1996. New insights into the mechanisms of fungal pathogenesis in insects. Trends Microbiol. 4: 197–203.

Dal Bello, G., S. Padín, C. L. Lastra, and M. Fabrizio. 2001. Laboratory evaluation of chemical-biological control of the rice weevil (*Sitophilus oryzae* L.) in stored grains. J. Stored Prod. Res. 37: 77–84.

Daoust, R. A., and D. W. Roberts. 1983. Studies on prolonged storage of *Metarhizium anisopliae* conidia: effect of temperature and relative humidity on conidial viability and virulence against mosquitoes. J. Invertebr. Pathol. 41: 143–150.

Ekesi, S., E. A. Egwurube, A. D. Akpa, and I. Onu. 2001. Laboratory evaluation of the entomopathogenic fungus, *Metarhizium anisopliae* for the control of the groundnut bruchid, *Caryedon serratus* on groundnut. J. Stored Prod. Res. 37: 313–321.

Fields, P., and Z. Korunić. 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.

Goettel, M. S., and G. D. Inglis. 1997. Fungi: hyphomycetes, pp. 213–248. *In* L. A. Lacey (ed.), Manual of Techniques in Insect Pathology. Chapter V-3, Academic Press, San Diego.

Greenspan, L. 1977. Humidity fixed points of binary saturated aqueous solutions. J. Res. Natl. Bur. Stand. A. 81: 89–96.

Grieshop, M. J., D. T. Rogers, and F. H. Arthur. 2012. Organic approaches and regulations for stored product pest management, pp. 233–242. *In* D. W. Hagstrum, T. W. Phillips and G. Cuperus (eds.), Stored product protection. Kansas State University Kansas, USA.

Harvey, J. A., and D. J. Thompson. 1995. Developmental interactions between the solitary endoparasitoid *Venturia canescens* (Hymenoptera: Ichneumonidae) and two of its hosts *Plodia interpunctella* and *Corcyra cephalonica* (Lepidoptera: Pyralidae). Eur. J. Entomol. 92: 427–435.

Harvey, J. A., and L. E. M. Vet. 1997. *Venturia canescens* parasitizing *Galleria mellonella* and *Anagasta kuehniella*: differing suitability of two hosts with highly variable growth potential. Entomol. Exp. Appl. 84: 93–100.

Hayma, J. 2003. The storage of tropical agricultural products. 4th Edn. Agromisa Foundation, Wageningen, The Netherlands.

Hedgecock, S., D. Moore, P. M. Higgins, and C. Prior. 1995. Influence of moisture content on temperature tolerance and storage on *Metarhizium flavoviride* conidia in an oil formulation. Biocontrol Sci. Technol. 5: 371–377.

Horaczek, A., and H. Viernstein. 2004. Comparison of three commonly used drying technologies with respect to activity and longevity of aerial conidia of *Beauveria brongniartii* and *Metarhizium anisopliae*. Biol. Control. 31: 65–71.

Kaleta, A., and K. Górnicki. 2013. Criteria of determination of safe grain storage time – A review, pp. 294–318. *In* S. Grundas and A. Stepniewski (eds.), Advances in agrophysical research. InTech, Rijeka, Croatia.

Kavallieratos, N. G., C. G. Athanassiou, M. P. Michalaki, Y. A. Batta, H. A. Rigatos, F. G. Pashalidou, G. N. Balotis, Z. Tomanović, and B. J. Vayias. 2006. Effect of the combined use of *Metarhizium anisopliae* (Metschinkoff) Sorokin and diatomaceous earth for the control of three stored-product beetle species. Crop Prot. 25: 1087–1094.

Kavallieratos, N. G., C. G. Athanassiou, M. Aountala, and D. C. Kontodimas. 2014. Evaluation of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Isaria fumosorosea* for control of *Sitophilus oryzae*. J. Food Protect. 77: 87–93.

Locatelli, D. P., L. Limonta, and M. Stampini. 2008. Effect of particle size of soft wheat flour on the development of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). J. Stored Prod. Res. 44: 269–272.

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

Lord, J. C. 2007a. Desiccation increases the efficacy of *Beauveria bassiana* for stored-grain pest insect control. J. Stored Prod. Res. 43: 535–539.

Lord, J. C. 2007b. Enhanced efficacy of *Beauveria bassiana* for red flour beetle with reduced moisture. J. Econ. Entomol. 100: 1071–1074.

Mahdneshin, Z., M. H. Safaralizadah, and Y. Ghosta. 2009. Study on the efficacy of Iranian isolates of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metsch.) Sorokin against *Rhyzopertha dominica* F. (Coleoptera: Bostrychidae). J. Biol. Sci. 9: 170–174.

Mason, L. J., and M. McDonough. 2012. Biology, behavior, and ecology of stored grain and legume insects, pp. 7–20. *In* D. W. Hagstrum, T. W. Phillips and G. Cuperus (eds.), Stored product protection. Kansas State University Kansas, USA.

Michalaki, M.,, C. G. Athanassiou, N. G. Kavallieratos, Y. A. Batta, and G. N. Balotis. 2006. Effectiveness of *Metarhizium anisopliae* (Metschinkoff) Sorokin applied alone or in combination with diatomaceous earth against *Tribolium confusum* Jacquelin du Val: influence of temperature, relative humidity and type of commodity. Crop Prot. 25: 418–425.

Michalaki, M. P., C. G. Athanassiou, T. Steenberg, and C. Th. Buchelos. 2007. Effect of *Paecilomyces fumosoroseus* (Wise) Brown and Smith (Ascomycota: Hypocreales) alone or in combination with diatomaceous earth against *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). Biol. Control. 40: 280–286.

Moore, D., O. K. Douro-Kpindou, N. E. Jenkins, and C. J. Lomer. 1996. Effects of moisture content and temperature on storage of *Metarhizium flavoviride* conidia. Biocontrol Sci. Technol. 6: 51–61.

Moore, D., J. C. Lord, and S. M. Smith. 2000. Pathogens, pp. 193–227. *In* Bh. Subramanyam and D. W. Hagstrum (eds.), Alternatives to pesticides in stored product IPM. Kluwer Academic Publishers, Dordrecht.

Navarro, S., R. T. Noyes, M. Casada, and F. H. Arthur. 2012. Grain aeration, pp. 121–134. *In* D. W. Hagstrum, T. W. Phillips and G. Cuperus (eds.), Stored product protection. Kansas State University Kansas, USA.

Nguyen, T. T., P. J. Collins, and P. R. Ebert. 2015. Inheritance and characterization of strong resistance to phosphine in *Sitophilus oryzae* (L.). PLoS One. 10: e0124335.

Ortiz-Urquiza, A., and N. O. Keyhani. 2013. Action on the surface: entomopathogenic fungi versus the insect cuticle. Insects. 4: 357–374.

Pereira, C. J., E. J. G. Pereira, E. M. G. Cordeiro, T. M. C. Della Lucia, M. R. Tótola, and R. N. C. Guedes. 2009. Organophosphate resistance in the maize weevil *Sitophilus zeamais*: magnitude and behavior. Crop Prot. 28: 168–173.

Pozidi-Metaxa, E., and C. G. Athanassiou. 2013. Comparison of spinosad with three traditional grain protectants against *Prostephanus truncatus* (Horn) and *Ephestia kuehniella* (Zeller) at different temperatures. J. Pest Sci. 86: 203–210.

Reichmuth, C., M. Schöller, and C. Ulrichs. 2007. Stored product pests in grain: morphology – biology – damage – control. AgroConcept Verlagsgesellschaft, Bonn, Germany.

Rodrigues, C., and D. Pratissoli. 1990. Pathogenicity of *Beauveria brongniartii* (Sacc.) Petch. and *Metarhizium anisopliae* (Mols.) Sorok. and its affect on corn weevil and bean beetle. Ann. Entomol. Soc. Braz. 19: 302–306.

Sabbour, M. M., S. E.-S. Abd-El-Aziz, and M. A. Sherief. 2012. Efficacy of three entomopathogenic fungi alone or in combination with diatomaceous earth medications for the control of three pyralid moths in stored grains. J. Plant Prot. Res. 52: 359–363.

Saik, J. E., L. A. Lacey, and C. M. Lacey. 1990. Safety of microbial insecticides to vertebrates - domestic animals and wildlife, pp. 115–132. *In* M. Laird, L. A. Lacey and F. W. Davidson (eds.), Safety of microbial insecticides. CRC Press, Boca Raton, FL.

Sathya, G., D. S. Jayas, and N. D. G. White. 2008. Safe storage guidelines for rye. Can. Biosyst. Eng. 50: 3.1–3.8.

Searle, T., and J. Doberski. 1984. An investigation of the entomogenous fungus *Beauveria bassiana* (Bals.) Vuill. as a potential biological control agent for *Oryzaephilus surinamensis* (L.). J. Stored Prod. Res. 20: 17–23.

Sedlacek, J. D., P. A. Weston, and J. Barney. 1996. Lepidoptera and Psocoptera, pp. 41–70. *In* Bh. Subramanyam and D.W. Hagstrum (eds.), Integrated management of insects in stored products. Marcel Dekker, Inc., New York, NY.

Shafighi, Y., M. Ziaee, and Y. Ghosta. 2014. Diatomaceous earth used against insect pests, applied alone or in combination with *Metarhizium anisopliae* and *Beauveria bassiana*. J. Plant Prot. Res. 33: 81–97.

Siegel, J. P., and J. A. Shadduck. 1990. Safety of microbial insecticides to vertebrates-humans, pp. 101–113. *In* M. Laird, L. A. Lacey and E. W. Davidson (eds.), Safety of microbial insecticides. CRC Press, Boca Raton, FL.

Sokal, R. R., and F. J. Rohlf. 1995. Biometry. 3rd edn., Freeman W. H. and Company, New York.

Stroup, W. W. 2013. Generalized linear mixed models. CRC Press, Boca Raton, FL.

Tarlack, P., F. Mehrkhou, and M. Mousavi. 2015. Life history and fecundity rate of *Ephestia kuehniella* (Lepidoptera: Pyralidae) on different wheat flour varieties. Arch. Phytopathol. Plant Prot. 48: 95–103.

Trematerra, P., and P. Gentile. 2010. Five years of mass trapping of *Ephestia kuehniella* Zeller: a component of IPM in a flour mill. J. Appl. Entomol. 134: 149–156.

Vassilakos, T. N., C. G. Athanassiou, N. G. Kavallieratos, and B. J. Vayias. 2006. Infuence of temperature on the insecticidal effect of *Beauveria bassiana* in combination with diatomaceous earth against *Rhyzopertha dominica* and *Sitophilus oryzae* on stored wheat. Biol. Control. 38: 270–281.

Volenik, M., V. Rozman, I. Kalinović, A. Liška, and B. Šimić. 2006. Influence of relative humidity and temperature on changes in grain moisture in stored wheat and sunflower. *In* 9th International Working Conference on Stored Product Protection, Campinas, Brazil, pp. 24–29.

Zimmermann, G. 1986. The *Galeria* bait method for detection of entomopathogenic fungi in soil. J. Appl. Entomol. 102: 213–215.

Zimmermann, G. 2007a. Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. Biocontrol Sci. Techn. 17: 553–596.

Zimmermann, G. 2007b. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontrol Sci. Techn. 17: 879–920.