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

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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 × 10⁶, 8 × 10⁷, and 8 × 10⁸ 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; Battia 2004, 2005; Kavallieratos et al. 2006, 2014). There are several reports on its effectiveness against major storage insects, such as the lesser grain borer, *Rhyncopertha dominica* (F.) (Coleoptera: Bostrichidae) (Battia 2005, 2008; Athanassiou et al. 2008; Mahdneshin et al. 2009;
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* Jacquin du Val (Coleoptera: Tenebrionidae) (Kavallieratos et al. 2006, Michalaki et al. 2006), the groundnut bruchid, *Caryledon 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 $1.77 \times 10^7$ and $1.77 \times 10^8$ 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 $7 \times 10^5$ 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 (Sedlaczek 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-Metakon 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* (W.) 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 (Sathy 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 (Volensik et al. 2006, Kalera and Gornicki 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 (SAD) 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 SAD (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* $8 \times 10^{10}$ 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 conidial suspension in 49.5 ml of sterile 0.1% Tween 80 solution providing the final concentrations of $8 \times 10^6$ and $8 \times 10^8$ conidia ml$^{-1}$. Conidial germination of the inoculated conidia was observed microscopically (100x) after incubation for 24 h at 26 °C on SAD 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 ($8 \times 10^6$, $8 \times 10^8$, or $8 \times 10^{10}$ 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 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 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, as well as their associated interactions, on the mortality levels of *Ephesia 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 × 10^7, and 8 × 10^8 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 | 153.5 | <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.

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 and12.8, 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 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, Horacek and Vierneist 2004). Vassilakos et al. (2006) reported that * Beauveria bassiana* (Balsamo Vuillemin (Ascomycota: Hypocreales) was more effective against
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
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 Metarizium 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.

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