Aerobiologia
DOI 10.1007/s10453-012-9277-8

ORIGINAL PAPER

Study of the persistence and viability of *Metarhizium acridum* in Mexico's agricultural area

César Guerrero-Guerra · María del Roció Reyes-Montes · Conchita Toriello · Víctor Hernández-Velázquez · Ivonn Santiago-López · Lucia Mora-Palomino · María Elena Calderón-Segura · Silvia Docampo Fernández · Carmen Calderón-Ezquerro

Received: 28 June 2012 / Accepted: 25 September 2012

Abstract Locust is one of the most predominant agricultural pests in Mexico, followed by non-migratory grasshoppers. Their devastating effects cover numerous Mexican states, generating important economic losses. *Metarhizium acridum* (Hypocreales: Clavicipitaceae) has been employed in Mexico as a microbial agent for biological control for some time. However, questions related to the long-term persistence and viability of the fungus after it has been sprayed over crop fields remain to be elucidated. To resolve these questions, we performed a study in a crop field where an abundant population of the grasshopper *Sphenarium purpurascens* (Orthoptera: Pyrgomorphidae) is present. The fungus *M. acridum* was sprayed during August and October 2007. Its presence in the air, soil and vegetative cover was subsequently monitored using molecular methods and culture techniques. The results showed that the fungus was viable on the ground during the follow-up period (66 weeks). Its population fluctuated markedly, however, reaching four times its initial concentration and later decreasing to nearly half its initial concentration by the end of the study. The initial concentrations of the fungus in the air and in the vegetative cover decayed steadily (with some small, isolated recoveries) until its complete disappearance after 8 months.

Keywords Persistence · Biological control · *Metarhizium acridum* · *Sphenarium purpurascens*

C. Guerrero-Guerra · I. Santiago-López · M. E. Calderón-Segura · C. Calderón-Ezquerro (✉)
Centro de Ciencias de la Atmósfera, Universidad Nacional Autónoma de México (UNAM), Circuito Exterior, Ciudad Universitaria, C.P. 04510 México, D.F., Mexico
e-mail: mclce@atmosfera.unam.mx

C. Guerrero-Guerra
Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Av. Ciudad Universitaria 3000, C.P. 04360 Coyoacán, D.F., Mexico

María del R. Reyes-Montes · C. Toriello
Departamento de Micología y Parasitología Facultad de Medicina, UNAM, Circuito Exterior, Ciudad Universitaria, C.P. 04510 México, D.F., Mexico

V. Hernández-Velázquez
Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Cuernavaca, Mexico

L. Mora-Palomino
Departamento de Edafología, Instituto de Geología, UNAM, Circuito Exterior, Ciudad Universitaria, C.P. 04510 México, D.F., Mexico

S. D. Fernández
Departamento de Biología Vegetal, Facultad de Ciencias, Universidad de Málaga, Apdo. 59, 29080 Málaga, Spain

Published online: 06 October 2012

1 Introduction

Locusts and grasshoppers (Orthoptera) are common and highly unpredictable agricultural pests. These insects cause enormous losses to crops all over the world because when the climatic conditions are favourable for plant growth and egg eclosion, their populations can increase rapidly with devastating consequences. If appropriate control measures are not taken, outbreaks of these pests can proliferate without control (Wilps 1997).

Currently, the management and control of the desert locust and other harmful acridid rely on the application of synthetic pesticides, which have been shown to be toxic to the environment and human health (WHO 2004; PAN 2009).

The advent of chemical insecticides in the mid-twentieth century created the concept that insect pests could be all but eliminated from threatened crops. A succession of compounds has appeared since then. Initially, many were quite toxic and environmentally damaging. In recent years, however, new materials have appeared, which address human and environmental safety concerns caused by the earlier materials. In parallel, scientists have realised the inadvisability of using chemicals as the only strategy for pest control, and integrated pest management schemes have evolved to employ a variety of cultural, chemical and biological tools to manage (not eradicate) pest invasion to a point below an economic threshold (Jaronski 2010).

Hence, considerable effort has been directed toward the development of alternative approaches, such as biological control within an integrated pest management programme. Among the most effective biological control measures is the use of entomopathogenic fungi; *Metarhizium acridum* (Driver and Milner) J. F. Bisch., Rehner and Humber 2009 (Hypocreales: Clavicipitaceae) (formerly *Metarhizium anisopliae* var. *acridum*) is one of the most studied (Lomer et al. 2001).

A great deal of research has focused on assessing the virulence of fungal isolates and their feasibility as effective microbial agents (Lomer et al. 2001; Prior and Streett 1997; Milner and Hunter 2001). One result of these efforts in Africa has been the development of a commercial biopesticide called Green Muscle®, which is effective against the desert locust, *Schistocerca gregaria* (Forskål, 1775) (Orthoptera: Acrididae), the red locust, *Nomadacris septemfasciata* (Serville, 1883) (Orthoptera: Acrididae), and other African grasshopper species (Aston 2004; Kooyman et al. 2003). Further research in Australia with different fungal isolates has produced another commercial biopesticide, Green Guard®, which is effective against the Australian locust, *Chortoicetes terminifera* (Walker, 1870) (Orthoptera: Acrididae), the migratory locust (*Locusta migratoria* (Linnaeus, 1758) (Orthoptera: Acrididae) and other species (Zhang and Hunter 2005; Hunter 2005). The efficacy of *M. acridum* for controlling locust swarms has also been assessed elsewhere, such as in Brazil (Magalhaes et al. 2001) and Mexico (Hernández-Velázquez et al. 2000).

Most studies have focused on the efficiency of this fungus in controlling insect pests, and the usual method to determine the latter characteristic is to quantify the percentage of viable fungi in field samples that are capable of infecting a particular insect species (Kaaya et al. 1996; Vaenninen et al. 2000; Hu and St. Leger 2002; Ekesi et al. 2005).

Extrapolating these studies to different agricultural regions is complicated because these are dynamic ecosystems in which constant interchanges of matter and energy take place and which are strongly affected by human activity. For example, crop areas are highly perturbed during the harvest season, and entomopathogenic fungal communities in arable soils are different from those in less disturbed habitats (Steenberg 1995; Bidochka et al. 1998; Meyling and Eilenberg 2006).

The inconsistent performance of biological control agents is often associated with an incomplete understanding of the ecological constraints of the biological system in which they are placed. This is particularly true for entomopathogenic fungi. There is little or no knowledge of their biology outside of their insect host. However, these fungi are often inundatively introduced into the environment in the absence of their host in the hope that they will persist and infect their target once the host immigrates into the treated area (Bruck 2005).

However, questions related to the long-term persistence and viability of the fungus after it has been sprayed over crop fields remain to be elucidated.

Fungal persistence is important to consider for at least two reasons. First, it is desirable for the fungus to have a limited time span to minimise its possible harmful effects on non-target species. On the other hand, the fungus should persist for as long as possible to reduce the need for continuous spraying (Zimmermann 2007).

Several studies have shown that *M. anisopliae* can survive for days, months or even years in the field. However, this persistence depends largely on the environmental conditions of each area and the fungal isolate used (Fargues and Robert 1985; Vaenninen et al. 2000; Milner et al. 2003; Van der Valk 2007; Zimmermann 2007). Hence, the extrapolation of results to other regions is not straightforward.

Consequently, it is crucial to determine the persistence of *M. acridum* in the areas where it is regularly used. Precise knowledge of the amount of fungal spores that remain will enable managers to take appropriate measures in response to population increases or declines at any given time.

Molecular techniques based on PCR provide the necessary tools to undertake environmental sampling to monitor the entomopathogenic fungi (Castrillo et al. 2007).

In Mexico, *M. acridum* is commonly applied as a microbial agent to control the Central-American locust (*Schistocerca piceifrons piceifrons*) (SENASICA 2010) and has been proved effective in biological control of locusts, reaching up to 90 % mortality in field (Hernández-Velázquez et al. 2003; Barrientos-Lozano et al. 2005). To date, however, its persistence and viability after it has been sprayed over crop fields have not been determined. Therefore, this study was designed to determine the persistence and viability of *M. acridum* in the environment (soil, vegetation and air) of an agricultural area (an amaranth crop field) over a 66-week period after the fungus had been sprayed as a biological control for the grasshopper *Sphenarium purpurascens*. This insect can become a major agricultural pest in vast regions of Mexico.

2 Materials and methods

2.1 Study area

San Mateo Coatepec is a small community in the state of Puebla, Mexico. Its geographical coordinates are 18° 48′ 33.45″ N and 98° 39′ 10.82″ W. The town is mainly dedicated to agricultural activities, especially the growing of maize (*Zea mays*), beans (*Phaseolus vulgaris*), squash (*Cucurbita pepo*) and amaranth (*Amaranthus hypochondriacus*). It has a semi-warm subhumid climate with summer rains and classificated as ecoregion 14.4.1 (Balsas Depression with Low Tropical Deciduous Forest and Xerophytic Shrub) (Wiken et al. 2011). The town has no water sources (rivers or springs) in its vicinity. This site was chosen for a long-term study because since the 1990s, the region has been infested by grasshoppers (*S. purpurascens*). Previously, these insects had been exclusively controlled with chemical pesticides. The fungus *M. acridum* had not been previously applied as a biological control agent.

Climatic variables including rainfall; maximum, average and minimum temperatures; average and maximum wind speeds; relative humidity; potential evaporation; and maximum solar radiation were obtained from a meteorological station (located at 2.5 m above the ground) belonging to the national climatology net (Red Nacional de Estaciones Estatales Agroclimatológicas (RNEEA). http://clima.inifap.gob.mx/redclima/).

2.2 Fungal spraying

Monospore *M. acridum* culture EH-502/8 was used in this study. This culture was derived from the original MaPL40 strain, which has been successfully applied in field trials (Hernández-Velázquez et al. 2003) and registered in the World Database Center for Microorganisms, WDCM, as BMFM-UNAM 834 (C. Toriello, director). The fungus was produced by the State Plant Health Committee of Guanajuato using biphasic solid substrate fermentation. Whole rice was used for the solid phase and the viability of the conidia was greater than 95 %.

Experiments were conducted in a 1 ha crop field used for amaranth (*Amaranthus hypochondriacus*) cultivation. The fungus was sprayed during the early morning on 17 August and 9 October 2007 with a Micron Ulva + sprayer (Bromyard, UK) at a flow of 60 ml/min and walking speed of 1 m/s. The concentration was 4×10^{12} conidia/Ha, and an oil-based ultra-low-volume technique was employed to spread the fungus. Mineral oil used was citrolina (primaveral dormant spray). At the time of spraying, the amaranth plants exhibited an average height of 30 cm. Care was taken to distribute the fungal mixture uniformly over the plants.

2.3 Samples

Air, soil and vegetation samples (twenty-six duplicate samples) were collected in the study area throughout

the 1 year and four months (68 weeks) of the study to quantify the amount of viable *M. acridum* particles in each substrate. The crop area was divided into plots of equal size (400 cm^2 aprox.) (excluding the parts that people use to cross the field) and two were selected randomly for the sampling in each date. These selected areas were not considered for subsequent sampling. For air samples, these were collected always on the same location (see details below).

2.4 Soil sampling

On each sampling date, 250 g of soil was collected to a depth of 15 cm in the sprayed area (except the day of spraying, this day the samples were taken from the soil surface). The soil was collected from as near as possible to the plant roots without damaging them because the highest concentrations of the fungus occur in this area (Hu and St. Leger 2002). The samples were collected with sterile metallic spoons and put individually in sterile plastic bags for transfer to the laboratory. Each soil sample was stored at 4 °C until analysis (typically 1–2 days).

2.5 Plant sampling

On each sampling date, approximately 50 g of leaves was collected from the vegetation within the area where *M. acridum* had been applied. Leaves were taken from the crop and the vegetation nearby. Samples were cut with sterile scissors and put individually in sterile plastic bags for transfer to the laboratory and stored at 4 °C until analysis (typically 1–2 days).

2.6 CFU quantification

One gram of each soil or vegetation sample was resuspended in a 1 % Nonidet P40 (Roche®, Germany) solution, stirred in a vortex for 3 min and left to stand for 7 min. This mixture was then serially diluted to achieve dilutions of 10^{-2} and 10^{-3}, both dilutions were used for analysis, and this procedure was performed two times for each sampling date. Samples were seeded on Petri dishes containing a potato-dextrose-agar medium (PDA) (Bioxon®, Mexico) supplemented with 500 mg/l of chloramphenicol and incubated at 28 °C for 7 days. Developing colonies with general characteristics of the genus *Metarhizium* (colour, conidia size, conidia shape, etc.) was grouped and 2 colonies from each group were removed from the Petri dish to confirm their identity by PCR (see details below). *M. acridum* colonies were quantified to determine the number of CFU/g of soil or vegetation. The remainder of each soil sample was used for physicochemical analyses.

2.7 CFU quantification in air samples

To determine the viability of *M. acridum* (CFU's in air), a N6 Single-Stage Viable Andersen Cascade Impactor (Thermo-Andersen) was used. The sampler draws in air at a rate of 1 cubic foot per minute and impacts it on a Petri dish (90 mm in diameter). The Petri dish contains 30 ml of PDA medium supplemented with 500 mg/l of chloramphenicol. The sampler was placed at a height of 70 cm above ground level and was always in the same position next to the Hirst spore trap (see below). It was run for intervals of 5 and 10 min. The number of viable particles was derived from the formula CFU/m^3 of air = (CCD/t)/0.0283, where CCD is the colony count per dish, t is the sampling time in minutes, and 0.0283 is the equivalence factor between 1 ft^3 and 1 m^3. When more than 300 colonies per dish were present, the value was adjusted using the following equation: $P_r = N[1/\mathrm{N} + 1/N - 1 + 1/N - 2 + \ldots + 1/\mathrm{N} - (r + 1)]$, where P_r is the expected number of viable particles that are produced by a number 'r' of colonies in the dish, and N is the total number of holes in the sampler (400 in this case). Two colonies from each group were removed from the Petri dish to confirm their identity by PCR (see details below).

To assess the dynamics of *M. acridum* in the air in more detail, a Hirst spore trap (Burkard Manufacturing Co®., UK) was employed. This equipment draws in 10 l of air per minute and can operate 24 h a day during a whole year. The sampler incorporates a rotating drum that moves clockwise. Collected air particles impact on cellophane tape (Melinex Dupont®, USA) that is adhered over the surface of the drum and covered with a thin layer of a mixture (1:5) of Vaseline (Racel®, Mexico) and hexane (J. T. Baker®, USA). This tape was replaced every week on Thursday at 13:00 h. The tape was then cut into 7 pieces of 48 mm, each piece corresponding to 24 h of sampling. These pieces were placed in 1.5-ml microtubes for DNA extraction and detection. The methodology is described below. Thus,

the presence or absence of *M. acridum* DNA in the air was determined on each day of sampling. The equipment was removed from the study area on 13 August 2008.

2.8 DNA extraction

The rupture of spore walls and extraction of DNA initially followed an adaptation of the method of Williams et al. (2001) and later followed an adaptation of the method of Calderon et al. (2002). The colonies chosen for PCR analysis were placed in 2-ml microtubes that had been previously filled with 0.2 g of glass beads (400–455 μm in diameter, rinsed with hydrochloric acid) and 250 μl of 1 % Nonidet P40 (Roche®, Germany) solution was added. The microtubes were stirred using a FastPrep vortex (Thermo® MA, USA) three times for 40 s each at 6 m/s and cooled on ice for 2 min between each vortexing.

Subsequently, 50 μl of the resulting suspension was used for DNA purification following the method of Lee and Taylor (1990) as modified by Williams et al. (2001). Briefly, 100 μl of a phenol/chloroform (J. T. Baker, USA) mixture (1:1) was added to each microtube, and the mixture was stirred using a vortex for 10 s and centrifuged at 14,000 rpm for 15 min. The supernatant was transferred to another tube containing 60 μl of cold isopropanol (J. T. Baker®, USA), 4 μl of 6 M ammonium acetate (J. T. Baker®, USA) and 20 μg of glycogen (Roche®, Switzerland). The microtubes were stored at −20 °C for 1 h and centrifuged for 15 min at 14,000 rpm. The supernatant was discarded, and the pellet was rinsed with 100 μl ethanol (70 %) at −20 °C. The microtubes were centrifuged again, the supernatant was discarded, and the DNA pellet was air-dried and resuspended in 50 μl of Milli-Q sterile water (Millipore®, MA, USA). For the PCR assay, 5 μl of this suspension was used.

2.9 Detection of *M. acridum* DNA by nested PCR

Based on isolations of *M. acridum* collected in Mexico, two pairs of specific oligonucleotides were designed for *M. acridum* (Calderón-Ezquerro et al. 2012). The first pair consisted of the DNA primers OPA-04(461) 1F (5′ GGC-TGA-CCG-ACG-AGG-TTA-T 3′) and OPA-04(461) IR (5′ GAC-TGA-CGC-AAG-CTT-CAT-CC 3′), which amplify a 461-bp DNA region (Gene Bank, access number GU724978). The second pair consisted of the primers OPA-04(526) 2F (5′ GCC-GCA-AGT-TGG-ACT-ACG 3′) and OPA-04(526) 2R (5′ CAA-GCT-TCA-TCC-GGC-ACT-T 3′), which amplify a 293-bp DNA fragment.

The first PCR was performed in a 50-μl reaction mixture containing 0.125 mM dNTPs (all reagents were obtained from Fermentas®, Hanover, MD), 1.5 mM $MgCl_2$, 1 μM of each primer, 1.5 U Taq DNA polymerase and 5 μl of DNA. The cycling conditions consisted of 1 cycle at 94 °C for 3 min followed by 25 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min and a final step at 72 °C for 5 min. For the second (nested) PCR, the 50-μl reaction mixture contained 0.125 mM dNTPs, 1.5 mM $MgCl_2$, 1 μM of each primer, 1.5 U Taq DNA polymerase and 1 μl of the first reaction product. The cycling conditions consisted of 1 cycle at 94 °C for 3 min followed by 25 cycles at 94 °C for 1 min, 72 °C for 2 min and 72 °C for 1 min and a final step at 72 °C for 5 min. The following controls were performed: (1) a positive control consisting of DNA extracted from 10,000 conidia and (2) a negative control consisting of the PCR mixture without DNA.

Amplification products were electrophoresed through 2 % agarose in 0.5 % Tris–borate–EDTA buffer (45 mM Tris base, 45 mM boric acid and 1 mM EDTA). Electrophoresis was conducted at 80 V for 60 min. The 100 bp DNA Ladder (Invitrogen®) was used as a molecular size standard. The bands were visualised with a UV transilluminator after ethidium bromide staining (10 μg/ml). Images were captured using a Digi-Doc-It photodocumentation system (UVP®, USA).

2.10 Soil chemical analysis

The remainder of each soil sample was used to measure pH and interchangeable cations (K^+, Mg^{++}, Ca^{++} and Na^+), which were measured by ionic exchange chromatography. Values for available phosphorus were also recorded.

2.11 Statistical analysis

For each CFU sample, mean values and standard error were calculated and plotted. The results were correlated (Pearson correlation) with climatic variables (obtained with the RNEEA: rainfall; maximum, average and minimum temperatures; average and maximum wind speeds; relative humidity; potential evaporation; and

maximum solar radiation) to determine their influence on fungal populations. All analyses were carried out with Statistica® software version 8.

3 Results

Four analyses carried out in May and June 2007, before the application of the microbial agent on the amaranth crop field, showed no CFUs of *M. acridum* in the soil, vegetation and air samples. Data obtained from the samples collected during the period between 17 August 2007 and 3 December 2008 are shown in Figs. 1, 2 and 3.

From the fungal application (17 August 2007) to the end of the sampling period (3 December 2008), the number of *M. acridum* CFUs/g in the soil samples (identity confirmed by PCR) varied widely, showing a 400 % increase in soil collected on 7 March 2008 (162,600) compared to the day of application (31,000). However, the number of CFUs tended to decline until the last soil samples were collected on 3 December 2008 (Fig. 1).

The decline of the fungus was more abrupt in the plants than in the soil (Fig. 2). A decline from 122,500 CFU/g of vegetation to 0 CFU/g occurred in 1 week, with a brief recovery after 9 October 2007 when a second spraying took place. Subsequently, fungal concentrations remained low until 7 March 2008 when they rose to the same magnitude observed after the initial spraying. From May onward, no fungi were detected in the vegetative cover.

After the fungal application, airborne spores (1,120 spores/m^3 air) were detected on 24 August 2007 and decreased to zero a week later. These values fluctuated slightly until 7 March 2008; after that date, no *M. acridum* CFUs were detected in the air (Fig. 3).

3.1 Correlations with recorded climatic parameters

Table 1 shows the correlations between the recorded climatic variables and CFUs in the soil, air and vegetation. For the soil samples, significant correlations (Pearson′s correlations) were found between the number of CFUs and the following variables: maximum wind velocity, average wind velocity, potential evaporation and relative humidity. For the vegetation samples, no correlation was found between the number of CFUs and any of the recorded variables. For airborne fungi, the number of CFUs was correlated with rainfall and maximum solar radiation.

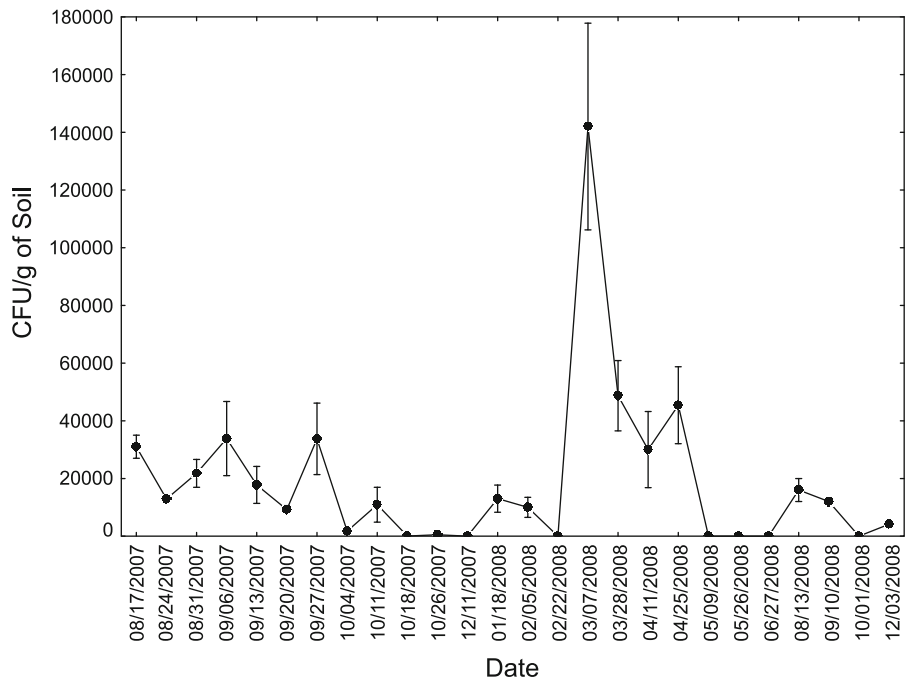

Fig. 1 CFU concentrations in the soil during sampling (*bars* represent SE)

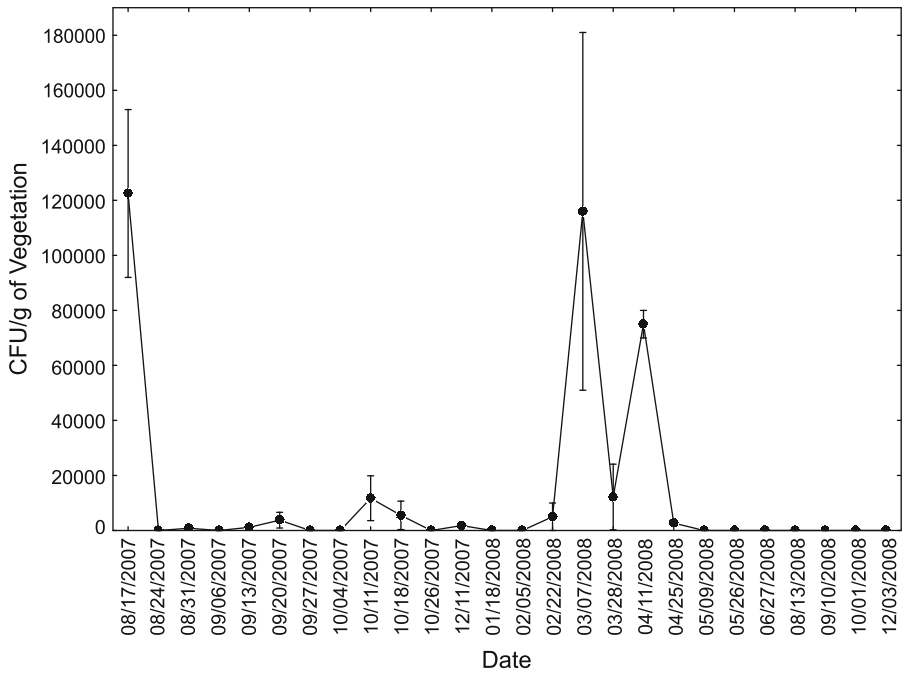

Fig. 2 CFU concentrations in the vegetative cover during sampling (*bars* represent SE)

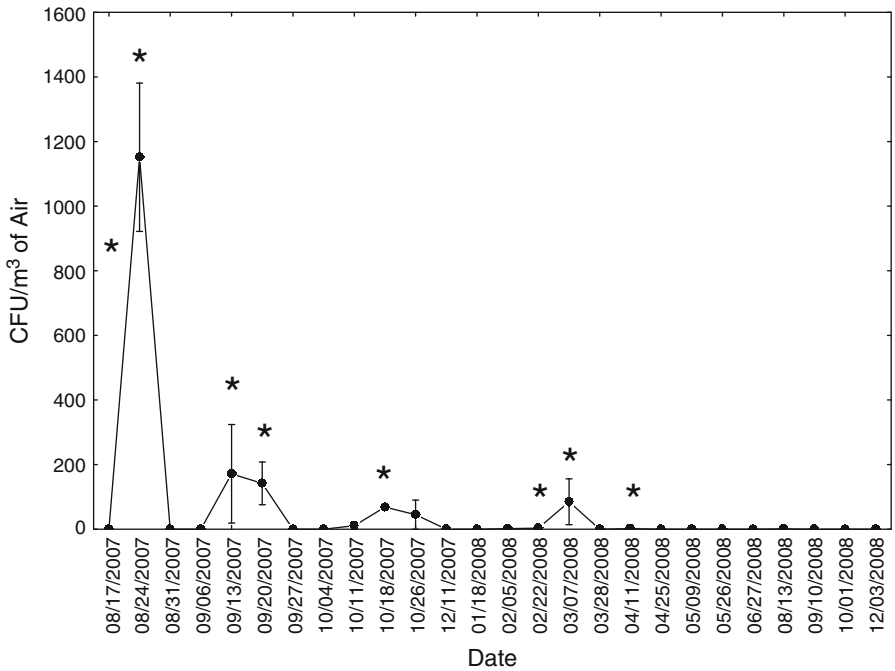

Fig. 3 CFU concentrations in the air during sampling (*bars* represent SE). *Asterisk* indicates DNA detection of *M. acridum* in airborne samples collected using Hirst spore trap on the same dates

Table 1 Pearson's correlation coefficients between the numbers of CFUs found in the soil, vegetative cover, and air and the recorded climatic parameters

	Rainfall	Temperature	Wind speed				Potential evaporation	Relative humidity	Maximum solar radiation
		Max	Min	Average	Max	Average			
CFU/g of soil	0.015	0.101	−0.067	0.131	0.424*	0.485*	0.466*	−0.400*	0.166
CFU/g of vegetative cover	0.375	0.154	0.057	0.193	0.317	0.364	0.353	−0.359	0.267
CFU/m^3 of air	0.447*	−0.179	0.170	−0.131	−0.203	−0.193	−0.178	0.269	0.433*

* $p < 0.05$

3.2 Correlations with soil physicochemical parameters

Table 2 shows the correlations between the numbers of CFUs and the chemical parameters analysed in soil samples from the study area. No correlation was found between the number of CFUs/g in the soil and the soil physicochemical parameters recorded.

3.3 Airborne conidia detection using Hirst spore traps

Hirst spore traps were used from 17 August 2007 to 13 August 2008. These traps allowed the variations in the persistence of airborne fungi to be observed in greater detail, as shown in Fig. 4. This graph shows the dates (a total of 32 days throughout the year) on which *M. acridum* DNA was detected from air-suspended conidia.

4 Discussion

Our results indicate that the fungus *M. acridum* remains viable and detectable for 1 year and 4 months in the soil and for approximately eight months in plant leaves and in the air, with negative periods between detections. Milner et al. (2003) assessed the persistence of commercial formulations of *M. anisopliae* and *M. anisopliae* var. *lepidiotum* sprayed on sugarcane cultivars in Australia and found that the fungi persisted in the soil for 3.5 years. This result is similar to those obtained by Vaenninen et al. (2000), who evaluated the persistence of *M. anisopliae* in Finnish crops (with snow during winter) and reported that soil samples were capable of infecting larvae of *Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae) up to 3 years after the fungal application. Moreover, other reports have asserted that *M. acridum* can remain viable on sterile sand for 8 months at 25 °C (Kaaya et al. 1996) and for 183 days in cultivated soil (Ekesi et al. 2005). Even it has been determined that *M. acridum* applications may effect some years later and have a significant effect on the desert locust (Bak et al. 2007).

The results of the present study are consistent with the time intervals given by these authors. During our study, the fungus was detected after 1 year and 4 months, so it probably has a longer viability.

Our results differ from those of the above-mentioned studies in that they observed continuous declines in *M. acridum* concentrations, whereas we observed periods in which the fungal population in the soil increased markedly, especially in March 2008. This increase was also reflected in the vegetative cover and to a lesser extent in the air (Figs. 1, 2, 3).

The increase in the number of CFU/g of soil may have been due to agricultural practices. Field observations during the period in which the fungal population increased showed that the soil had been fallowed to prepare it for the next sowing season. We hypothesise that this activity released conidia to the soil surface and even into the air, resulting in the

Table 2 Pearson's correlation coefficients between the numbers of *M. acridum* CFUs present in the soil and the recorded chemical parameters

	pH	Interchangeable cations				Available *p*
		Ca^{++}	Mg^{++}	K+	Na+	
CFU/g of soil	−0.114	0.150	0.207	−0.010	0.420	−0.122

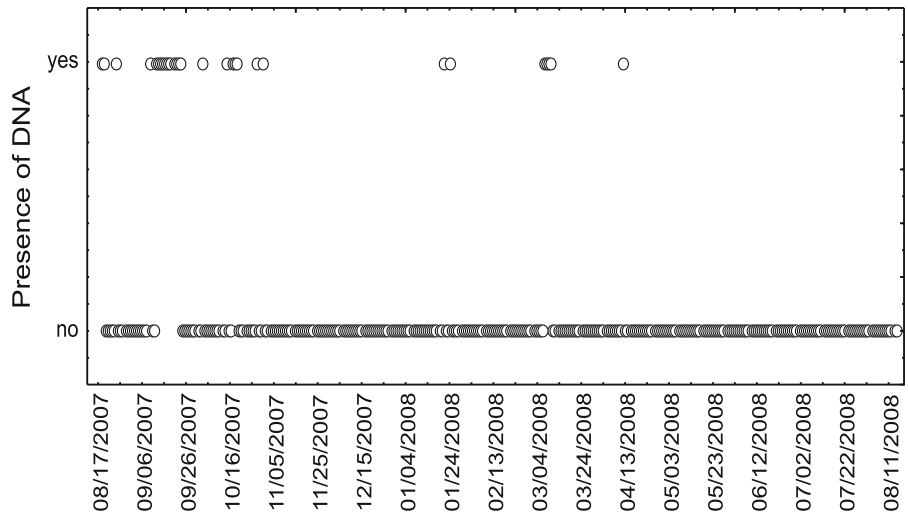

Fig. 4 DNA detection of *M. acridum* in airborne samples collected using Hirst spore trap during the period from August 2007 to August 2008

sudden increases detected in both the vegetative cover and the air. Further research concerning the persistence of the fungus in agricultural areas is needed to verify whether this behaviour is repeated.

Other studies have reported that *Metarhizium* is a common species in environments perturbed by humans. In Canada, for example, this fungus is more frequent in agricultural fields than in forests (Bidochka et al. 1998); in Denmark, *Metarhizium* is abundant in crop fields (Steenberg 1995). Jabbour and Barbercheck (2009) measured entomopathogenic fungi in crop fields and determined that *M. anisopliae* was more frequent in ploughed fields.

The large number of CFUs in the soil demonstrates the potential for *M. acridum* to accumulate. However, these CFUs were sampled at a depth of 15 cm, which is inaccessible to grasshoppers of the species *S. purpurascens*. These insects spend most of their life cycles on the aboveground vegetation. Additionally, the increase in CFUs during March occurred after the life cycle of the grasshoppers had ended. Moreover, the high concentrations of fungi would have a negligible effect on the oothecae of the grasshoppers, which are buried at an average depth of about 3 cm (Cueva del Castillo 2003). Therefore, high fungal concentrations in the soil would have almost no effect as a biological control for *S. purpurascens*.

The solar radiation causes the soil temperature can reach over 40 °C on the surface of the study area, so the viability of conidia at low depth is reduced, especially after harvest (during the month of October, approximately) when there are no plants to protect the soil, as a result, the concentrations of viable conidia are smaller than the depth of 15 cm. Probably, *M. acridum* concentrations at these depths may be high enough to infect the oothecas of *S. purpurascens*, but we do not evaluate this. However, perhaps other grasshoppers' species that spend more time on the ground are more susceptible to these high fungal concentrations, so this possible effect should be studied in more detail.

In the vegetative cover, that initial concentrations of the fungus were very high, up to five times those observed in the soil. However, these concentrations almost disappeared within one week. Ultraviolet solar radiation can cause conidium inactivation over a period of hours (Braga et al. 2001), and rain can reduce conidium viability within days (Inyang et al. 2000). August 2007 was particularly rainy in the study area, 334 mm of rain against 130 mm of rain of other years.

Small increases in fungal concentration detected during the sampling period may be due to conidia transported from the soil to the leaves.

Although little information is available on fungal persistence in the vegetative cover, the results of the few previous studies are in agreement with our findings. Moore et al. (1996) reported that the half-life of conidia in treated vegetation was 4.3 days. Inyang et al. (2000) simulated rainfall and showed a reduction in spore viability after 1 week. Hunter et al.

(2001) reported that *M. acridum* persisted for almost 7 days in the vegetation of agricultural areas in Australia. Finally, a review performed for Van der Valk (2007) for experiments conducted against the desert locust in Africa showed a half-life of conidia of 3.3 to 34 days in treated vegetation.

The abrupt decrease in *M. acridum* concentrations in the vegetative cover observed in this study shows that efficient pest management must employ frequent sprayings at one-week intervals to maintain high conidial concentrations in plants and ensure that insects become infected. Even more frequent sprayings may be required if rainfall is abundant.

Concerning fungal persistence in the air, no conidia were detected on the day of spraying using the viable particle sampler, but positive DNA detection was accomplished using the Hirst spore traps. The conidia of *M. acridum* were suspended in an oil base at the time of spraying, and our results show that the conidia may freed themselves from the oil after a period of hours and may have been carried by the wind from the soil and vegetative cover to the air. As a result, the Hirst spore traps could detect them on the same day. This observation highlights the need to employ continuous-type samplers to more accurately understand the atmospheric dynamics of biological particles in crop fields.

Finally, Zimmermann (2007) has asserted that *M. anisopliae* is not naturally found in the air, although it can be transported by external agents. The results obtained using the viable particle sampler as well as those obtained using the Hirst spore traps reveal that conidia sprayed on the fields can become suspended in the air, where they were detected on several occasions (on 32 days during the one-year period of sampling using the Hirst spore traps).

Several studies have focused on the effects of climatic variables, among which humidity, solar radiation and temperature are the most relevant (Rangel et al. 2004, 2005). In general, humidity must be high to induce the germination of conidia, but no studies have shown a long-term effect of this variable. In the present study, three weather variables associated with moisture were analysed: rainfall, relative humidity and potential evaporation. Interestingly, only rainfall was significantly correlated with the number of CFUs disseminated in the air.

McCartney (1991) has demonstrated that raindrops falling on a plant can cause spore release in two ways. First, the impact of water on a leaf can shake it and provide momentum to the spore. Second, the raindrops may create wind currents. In this manner, the release of conidia from the plant surface can increase the concentration of airborne conidia.

The number of CFUs in the soil was negatively correlated with relative humidity, probably because high humidity favours the growth of competitors and predators of *M. acridum*, thus reducing its population, as stated by Keller and Zimmermann (1989) and Hajek (1997). These authors have established that *Metarhizium* species are poor competitors for organic resources compared to opportunistic saprobic fungi. Another reason may be a reduction in oxygen levels (Jabbour and Barbercheck 2009). This explanation is reinforced by the correlation found with potential evaporation, which indicate water loss in an area; this variable was positively correlated with soil CFUs, unlike relative humidity. These data support the hypothesis that excessive humidity is detrimental for long-term fungal survival in the field.

Wind velocity was positively correlated with soil CFUs, probably because airflow removes conidia from the vegetation or from insects and deposits them in the soil. Nevertheless, the wind velocity necessary to remove *M. acridum* conidia from the vegetative cover has not yet been established. Experiments with *Aspergillus fumigatus, Cladosporium* spp. and *Penicillum* spp. (Pasanen et al. 1991) have found that wind speeds between 0.5 m/s and 1 m/s are required. In our study area, wind velocities vary from 1.08 m/s (average speed) to 6.6 m/s (maximum speed).

The number of airborne CFUs was also correlated with the maximum solar radiation. Unexpectedly, this correlation was positive, indicating an increase in CFUs depending on an increase in maximum solar radiation. Several studies, including those of Braga et al. (2001) and Rangel et al. (2004), have demonstrated that solar UV radiation can inactivate conidia in a matter of hours. One explanation for our results may be that solar radiation increases the temperature at the leaf surface, which in turn reduces the humidity around the leaf. As a consequence, the surface tension that binds conidia to the leaf decreases, causing their release, as suggested by Jones and Harrison (2004). However, further research under controlled climatic conditions is needed to corroborate this hypothesis.

More research is needed to determine whether the concentrations of *M. acridum* recorded in this study may be capable of causing harm to people exposed to the

fungus as farmers. Zimmermann' s biosegurity review (2007) describes cases of some people with allergic reactions to high concentrations of *M. acridum* and people with asthmatic symptoms exposed to *M. anisopliae* in sugar cane crops, but, as the author suggests, are rare cases. Currently, there are no published records of disease due to *M. acridum* in Mexico, but is prevalent to work in the crops without protection of any kind and is rare that farmers seek medical attention with the first symptoms of the disease, so the risk may be higher in case of the use of *M. acridum* spreading; therefore, more research is needed.

5 Conclusions

The results of this study show that the population of *M. acridum* fluctuates importantly through time and has the potential of accumulating in the soil given the proper conditions. With respect to the vegetative cover and air, conidia present in these areas decay rapidly so their effect as a microbial agent is minimised. Therefore, fungal spraying must be frequent (on a weekly basis) to achieve a better control of plagues.

Finally, it is necessary to determine whether the cumulative concentrations over time could be harmful to non-target species or people exposed.

Acknowledgments We want to express our gratitude to Posgrado de Ciencias Biológicas of the Universidad Nacional Autónoma de México (UNAM) for the support given to the student C. Guerrero-Guerra for the present study as part of his Doctoral thesis and to Consejo Nacional de Ciencia y Tecnología of Mexico (CONACYT) for the scholarship granted (203979). We also want to express our gratitude to M.Sc. Miguel Meneses and the engineers Gutiérrez López W. and García y Espinos M. for their technical assistance.

References

Aston, R. (2004). Medium scale field trials with the insect pathogenic fungus *Metarhizium anisopliae* ("Green Muscle") against the desert locust (*Schistocerca gregaria*) in Niger. *Draft project report OSRA/RAF/801/USA*. February 2004. Food and Agriculture Organization of the United Nations, Rome.

Bak, J., Fisker, E., & Kooyman, C. (2007). Evaluating the importance of residual effects from previous years' treatment on the efficiency of different strategies for control of Sahelian grasshoppers with *Metarhizium anisopliae* var. *acridum*. *Biocontrol Science and Technology, 2007*(17), 413–422.

Barrientos-Lozano, L., Hunter, D. M., Ávila-Valdéz, J., García-Salazar, P., & Horta-Vega, J. V. (2005). Control biológico de la langosta centroamericana *Schistocerca piceifrons piceifrons* walker (orthoptera: Acrididae) en el noreste de méxico. *Vedalia, 12*, 119–128.

Bidochka, M. J., Kasperski, J. E., & Wild, G. A. M. (1998). Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. *Canadian Journal of Botany, 76*, 1198–1204.

Braga, G. U. L., Flint, S. D., Miller, C. D., Anderson, A. J., & Roberts, D. W. (2001). Both solar UVA and UVB radiation impair conidial culturability and delay germination in the entomopathogenic fungus *Metarhizium anisopliae*. *Photochemistry and Photobiology, 74*, 734–739.

Bruck, D. (2005). Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: Implications for pest management. *Biological Control, 32*, 155–163.

Calderon, C., Ward, E., Freeman, J., Foster, J. S., & McCartney, H. A. (2002). Detection of airborne inoculum of *Leptosphaeria maculans* and *Pyrenopeziza brassicae* in oilseed rape crops by polymerase chain reaction (PCR) assays. *Plant Pathology, 51*, 303–310.

Calderón-Ezquerro, C., Santiago-López, I., Guerrero-Guerra, C., Reyes-Montes, M. R., Calderón-Segura, M. E., & Toriello, C. (2012). Molecular detection of the airborne entomopathogen fungus *Metarhizium acridum* using specific oligonucleotides. *Aerobiologia, 28*, 233–239.

Castrillo, L. A., Thomsen, L., Juneja, P., & Hajek, A. (2007). Detection and quantification of *Entomophaga maimaiga* resting spores in forest soil using real-time PCR. *Mycological Research, 111*, 324–331.

Cueva del Castillo, R. (2003). Body size and multiple copulations in a Neotropical grasshopper with an extraordinary mate-guarding duration. *Journal of Insect Behavior, 16*, 503–522.

Ekesi, S., Maniania, N., & Lux, S. (2005). Effect of soil temperature and moisture on survival and infectivity of *Metarhizium anisopliae* to four tephritid fruit fly puparia. *Journal of Invertebrate Pathology, 83*, 157–167.

Fargues, J., & Robert, P. H. (1985). Persistance des conidiospores des Hyphomycetes entomopathogenes *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metsch.) Sor., *Nomuraea rileyi* (F.) Samson et *Paecilomyces fumosoroseus* Wize dans le sol, en conditions controlees. *Agronomie, 5*, 73–80.

Hajek, A. E. (1997). Ecology of terrestrial fungal entomopathogens. *Advances in Microbial Ecology, 15*, 193–249.

Hernández-Velázquez, V. M., Berlanga-Padilla, A. M., & Barrientos-Lozano, L. (2000). Vegetable and mineral oil formulations of *Metarhizium anisopliae* var. *acridum* to control the Central American locust (*Schistocerca piceifrons piceifrons* Walker) (Orthoptera: Acrididae). *Journal of Orthoptera Research, 9*, 223–227.

Hernández-Velázquez, V. M., Hunter, D. M., & Barrientos-Lozano, L. (2003). Susceptibility of *Schistocerca piceifrons* (Orthoptera: Acrididae) to *Metarhizium anisopliae* var. *acridum* (Deuteromycotina: Hyphomycetes): Laboratory and Field Trials. *Journal of Orthoptera Research, 12*, 89–92.

Hu, G., & Leger, St. (2002). Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Applied and Environmental Microbiology, 68*, 6383–6387.

Hunter, D. M. (2005). Mycopesticides as part of integrated pest management of locusts and grasshoppers. *Journal of Orthoptera Research, 14*, 197–201.

Hunter, D. M., Milner, R. J., & Spurgin, P. A. (2001). Aerial treatment of the Australian plague locust, *Chortoicetes terminifera* (Orthoptera: Acrididae) with *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). *Bulletin of Entomological Research, 91*, 93–100.

Inyang, N., McCartney, A., Oyejola, B., Ibrahim, L., Pye, J., Archer, A., et al. (2000). Effect of formulation, application and rain on the persistence of the entomogenous fungus *Metarhizium anisopliae* on oilseed rape. *Mycological Research, 104*, 653–661.

Jabbour, R., & Barbercheck, M. E. (2009). Soil management effects on entomopathogenic fungi during the transition to organic agriculture in a feed grain rotation. *Biological Control, 51*, 435–443.

Jaronski, S. (2010). Ecological factors in the inundative use of fungal entomopathogens. *BioControl, 55*, 159–185.

Jones, A. M., & Harrison, R. M. (2004). The effects of meteorological factors on atmospheric bioaerosol concentrations—A review. *Science of the Total Environment, 326*, 151–180.

Kaaya, G., Mwangi, E., & Ouna, E. (1996). Prospects for biological control of livestock ticks, *Rhipicephalus appendiculatus* and *Amblyomma variegatum*, using the entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae*. *Journal of Invertebrate Pathology, 67*, 15–20.

Keller, S., & Zimmermann, G. (1989). Mycopathogens of soil insects. In N. Wilding, N. M. Collins, P. M. Hammond, & J. F. Webber (Eds.), *Insect-fungus interactions*. London: Academic Press.

Kooyman, C., Bahana, J., Godonou, I., Katheru, J. N., Magorna, R. N., Seyourn, E., Spurgin, P. (2003). Operational trial of Green Muscle against red locust nymphs in the Wenbere plains. *Tanzania Project report of projects* DFID R7818 and FAO TCP/URT/201/NOR.

Lee, S., & Taylor, J. (1990). Isolation of DNA from fungal mycelia and single spores. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols. A guide to methods and applications*. California: Academic Press.

Lomer, C. J., Bateman, R. P., Johnson, D. L., Langewald, J., & Thomas, M. (2001). Biological control of locusts and grasshoppers. *Annual Review of Entomology, 46*, 667–702.

Magalhaes, B. P., de Faria, M. R., Lecoq, M., Schmidt, F. G. V., Silva, J. B. T., Frazao, H. S., et al. (2001). The use of *Metarhizium anisopliae* var. *acridum* against the grasshopper *Rhammatocerus schistocercoides* in Brazil. *Journal of Orthoptera Research, 10*, 199–202.

McCartney, H. A. (1991). Airborne dissemination of plant pathogens. *Journal of Applied Bacteriology, 70*, 49S–59S.

Meyling, N., & Eilenberg, J. (2006). Occurrence and distribution of soil borne entomopathogenic fungi within a single organic agroecosystem. *Agriculture, Ecosystems & Environment, 113*, 336–341.

Milner, R. J., & Hunter, D. M. (2001). Recent developments in the use of fungi as biopesticides against locusts and grasshoppers in Australia. *Journal of Orthoptera Research, 10*, 271–276.

Milner, R. J., Samson, P., & Morton, R. (2003). Persistence of conidia of *Metarhizium anisopliae* in sugarcane fields: Effect of isolate and formulation on persistence over 3.5 years. *Biocontrol Science and Technology, 13*, 507–516.

Moore, D., Higgins, P. M., & Lomer, C. J. (1996). Effects of simulated and natural sunlight on the germination of conidia of *Metarhizium flavoviride* Gams and Rozsypal and interactions with temperature. *Biocontrol Science and Technology, 6*, 63–76.

PAN (Pesticide Action Network). (2009). *Pesticide database*. http://www.pesticideinfo.org. Cited June 2009.

Pasanen, A. L., Pasanen, P., Jantunen, M. J., & Kalliokoski, P. (1991). Significance of air humidity and air velocity for fungal spore release into the air. *Atmospheric Environment, 25*, 459–462.

Prior, C., & Streett, D. A. (1997). Strategies for the use of entomopathogens in the control of the desert locust and other acridoid pests. *Memoirs of the Entomological Society of Canada, 129*, 5–25.

Rangel, D. E. N., Braga, G. U. L., Anderson, A. J., & Roberts, D. W. (2005). Variability in conidial thermotolerance of *Metarhizium anisopliae* isolates from different geographic origins. *Journal of Invertebrate Pathology, 88*, 116–125.

Rangel, D. E. N., Braga, G. U. L., Flint, S. D., Anderson, A. J., & Roberts, D. W. (2004). Variations in UV-B tolerance and germination speed of *Metarhizium anisopliae* conidia produced on insects and artificial substrates. *Journal of Invertebrate Pathology, 87*, 77–83.

SENASICA. (2010). *Estrategia operativa de la campaña contra la langosta. Dirección General de Sanidad Vegetal*. DPF/CNRF, 2010. url: http://www.senasica.gob.mx/includes/asp/download.asp?IdDocumento=17723&idurl=25936.

Steenberg, T. (1995). Natural occurrence of *Beauveria bassiana* (Bals.) Vuill. with focus on infectivity to *Sitona* species and other insects in Lucerne. Ph.D. Thesis. The Royal Veterinary and Agricultural University, Denmark.

Vaenninen, I., Tyni-Juslin, J., & Hokkanen, H. (2000). Persistence of augmented *Metarhizium anisopliae* and *Beauveria bassiana* in Finnish agricultural soils. *BioControl, 45*, 201–222.

Van der Valk, H. (2007). Review of the efficacy of *Metarhizium anisopliae* var. *acridum* against the Desert Locust. Plant Production and Protection Division. Locusts and other migratory pests group. No. AGP/DL/TS/34. *Desert Locust Technical Series*. FAO.

WHO (World Health Organization). (2004). *The WHO recommended classification of pesticides by hazard and guidelines to classification*. Geneva: WHO.

Wiken, E. D., Jiménez Nava, F., & Griffith, G. (2011). *North American terrestrial ecoregions—Level III*. Montreal, Canada: Commission for Environmental Cooperation.

Williams, R. H., Ward, E., & McCartney, A. (2001). Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores. *Applied and Environmental Microbiology, 67*, 2453–2459.

Wilps, H. (1997). Ecology of *Schistocerca gregaria* (Forskal): Observations in West Africa from 1990 to 1994. In S. Krall,

R. Peveling, & D. Ba-Diallo (Eds.), *New strategies in locust control*. Basel: Birkhauser.

Zhang, L., & Hunter, D. M. (2005). Laboratory and field trials of Green Guard (*Metarhizium anisopliae* var. *acridum*) (Deuteromycotina: Hyphomycetes) against the oriental migratory locust (*Locusta migratoria manilensis*) (Orthoptera: Acrididae) in China. *Journal of Orthoptera Research, 14*, 27–30.

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology, 17*, 879–920.