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ORIGINAL PAPER

Molecular detection of the airborne entomopathogen fungus *Metarhizium acridum* using specific oligonucleotides

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Abstract We describe a technique to detect the presence of airborne conidia from the fungus *M. acridum* (formerly *Metarhizium anisopliae* var. *acridum*) (Hypocreales: Clavicipitaceae) with great accuracy. Airborne conidia were collected using Hirst-type spore traps. DNA extractions were optimized to achieve the best possible recovery. DNA was examined using polymerase chain reaction (PCR) with specific oligonucleotides to enable the detection of a single conidium. Experiments using a mini-wind tunnel were conducted to validate the method. Subsequently, this technique was applied to an agricultural region of Mexico, where *M. acridum* was sprayed to control the grasshopper, *Sphenarium purpurascens,* population (Orthoptera: Pyrgomorphidae). *M. acridum* conidia were detected 2 days after spraying in San Mateo Coatepec (Puebla, site of grasshopper study).

Keywords Molecular detection · Airborne conidia · PCR · *Metarhizium acridum* · Hypocreales · Clavicipitaceae

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1 Introduction

Locusts and grasshoppers are both common and unpredictable pests. They cause enormous losses to crops throughout the world. In weather conditions that favor egg eclosion and plant growth, these insect populations can increase rapidly to yield gregarious migratory populations (Lomer et al. 2001). Unless proper and definitive control measures are applied, these pests can reach devastating proportions (Wilps 1997).

Currently, synthetic pesticides used to control the desert locust and other harmful Orthoptera are toxic for the environment and human health. For this reason, there has been a growing interest in the development of biological-control options. Among the best choices for this purpose is the use of the entomopathogenic fungus, *M. acridum* (formerly *M. anisopliae* var. *acridum*) (Driver and Milner) J.F. Bischof., Rehner and Humber stat. nov.*;* (Lacey et al. 2001; Lomer et al. 2001).

Numerous studies have focused on the assessment of the virulence of various isolates of this fungus, as well as the feasibility of its use as a microbial agent (Hernández-Velázquez et al. 2003; Hunter et al. 2005). As a result, the commercial pesticide Green Muscle® has been developed, which has proven to be effective in controlling the desert locust (*Schistocerca gregaria*) and red locust (*Nomadacris septemfasciata*) and several species of African grasshoppers (Aston 2004; Kooyman et al. 2003). More recent research in Australia, using various fungal isolates, led to the

production of Green Guard®, another commercial pesticide effective against the Australian locust (*Chortoicetes terminifera*), the migratory locust (*Locusta migratoria*), and several more species (Hunter 2005).

Fungal efficacy in controlling locust populations has been demonstrated in several places such as Africa (Douthwaite et al. 2001; Lomer et al. 2001), Australia (Hunter 2005; Milner and Hunter 2001), Brazil (Magalhães et al. 2001), and Mexico (García-Gutiérrez et al. 2002; Hernandez-Velazquez et al. 2000). However, despite these studies, little is known regarding the basic ecological issues concerning fungi, mainly in agroecosystems (Meyling and Eilenberg 2007). To produce a more efficient biological-control agent, factors such as persistence, viability, and dispersion of the microbial agent after its application in the field, in addition to the effects of agricultural practices, are indispensable (Barbosa 1998).

A review by Meyling and Eilenberg (2007) reveals that there is lack of available information concerning these factors for the entomopathogenic fungus *M. acridum*. In addition, there is no information on the possible presence of indigenous *M. acridum* populations in agroecosystems prior to fungal application. A simple, molecular method using specific fungal DNA oligonucleotides would be very useful in environmental investigations of this fungus. Conventional identification and counting methods for fungi and other airborne microorganisms are dependent on light microscopy or adequate culture techniques, which are both cumbersome and time-consuming (Calderon et al. 1995; Williams et al. 2001). These difficulties have hindered routine sampling of biological particles.

Other methods have been developed to overcome these drawbacks. Among the new approaches to detect and identify diverse biological airborne particles are immunoassays and ELISA (Brasel et al. 2005; Kennedy et al. 1999; Kennedy and Wakeham 2008; Schmechel et al. 1996; Wakeham and Kennedy 2010). However, the application of these methods is limited by the difficulty in obtaining sufficiently specific antibodies.

Methods based on DNA detection are currently more commonly used. Polymerase chain reaction (PCR) is a fast and specific procedure that allows the detection of DNA target molecules in complex mixtures, thus offering an alternative method for fungal identification (Jurado et al. 2005). Several studies have used traditional PCR methods to identify fungal species (Buchheidt and Hummel 2005; Calderon et al. 2002a, b; Freeman et al. 2002; Mayoral et al. 2005; Taylor et al. 2005), whereas more recent studies have used real-time PCR (Hamal 2007; Keswani et al. 2005; Klingspor and Jalal 2006; Rogers et al. 2009).

Most of the PCR-based fungal detection methods are not readily adaptable for use in the field. Therefore, specialized techniques are required that allow adequate sampling such as DNA extraction and purification, avoiding genetic material loss, and the availability of specific oligonucleotides for specific fungal species, and a specific and sensitive PCR assay.

This study describes the development of a molecular detection technique specific for *M. acridum* airborne conidia. In addition, this technique was used to *detect M. acridum* in an amaranth crop in Puebla, Mexico, after a fungal application was used to control the grasshopper *S. purpurascens* (Orthoptera: Pyrgomorphidae).

2 Materials and methods

2.1 Area of study

San Mateo Coatepec is a small community that is part of the Atzizihuacán municipality located in the western part of the state of Puebla. Its geographical coordinates are 18° 48′ 33.45″ N and 98° 39′ 10.82″ W. It consists of about 2,000 inhabitants.

The town is mainly dedicated to agricultural activities, and it produces maize (*Zea mays*), beans (*Phaseolus vulgaris*), squash (*Cucurbita pepo*), and amaranth (*Amaranthus hypochondriacus*). It has a semi-warm, subhumid climate with summer rains. There are no sources of water (rivers or springs) within the vicinity of the town. The predominant soil type is cambisol (Ayuntamiento-Atzitzihuacan 2007).

Since the mid-1990s, this region has been infested with grasshoppers (*S. purpurascens*), which have been exclusively controlled using chemical pesticides. *M. acridum* has never been applied as a biological-control agent .

2.2 Spore source

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). Strain EH-502/8 was stored in a 10% glycerin solution in liquid nitrogen at −196°C. It was deposited in the Basic Mycology Laboratory culture collection in the Microbiology and Parasitology Department in the Faculty of Medicine at the Universidad Nacional Autónoma de México (UNAM) and registered in the World Database Center for Microorganisms, WDCM, as BMFM-UNAM 834 (C. Toriello, director). The original culture was isolated from locust (*Schistocerca piceifrons* ssp. *piceifrons*) from the Centro Nacional de Referencia de Control Biológico of Mexico (Dirección General de Sanidad Vegetal, Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación). The fungus was cultured in potato dextrose agar (PDA) media for 2–3 weeks at 28°C until spores formed.

2.3 Spore suspension for DNA extraction

A conidial suspension from PDA plates was obtained by adding 5 ml of 1% sterile Nonidet P40 onto the surface of the plate. The suspension was adjusted by conidial quantification in a Neubauer chamber. Subsequently, serial $\log_{10}$ dilutions were performed and used for DNA extractions.

2.4 Spore walls rupture

The methods used to break spore walls and extract DNA were described by Williams et al. (2001) and Calderón (2002a). A 250-µl spore suspension was placed into 2-ml microtubes containing 0.2 g of glass beads (400–455 µm in diameter that had been washed with hydrochloric acid). Microtubes were shaken in a Fast-Prep vortex (Thermo Savant NY, USA) three times for 40-s period at 6 m/s and placed on ice for 2 min between periods. The same method and conditions were used to DNA extraction from the conidia impacted on the Melinex tape used in the Hirst-type spore traps.

2.5 DNA extraction

DNA purification was carried out in 50 µl of spore suspension using the method described by Lee and Taylor (1990) and modified by Williams et al. (2001). Briefly, 100 µl of phenol/chloroform (1:1) were added to each microtube, vortexed 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, 4 µl of 6 M ammonium acetate, and 20 µg of glycogen (Roche, Switzerland). Microtubes were stored at -20°C for 1 h and centrifuged for 15 min at 14,488 × g. The supernatant was discarded, and the pellet was rinsed with 100 µl of ethanol (70%) that had been stored at –20°C. The samples were further centrifuged, the supernatant was discarded, and the DNA pellet was air-dried and resuspended in 50 µl of Milli-Q sterile water (Millipore, MA, USA). We used 5 µl of this suspension for each PCR assay.

2.6 Specific DNA detection of *M. acridum* by PCR

Based on isolates collected in Mexico, two pairs of specific oligonucleotides for *M. acridum* were designed. The first pair of oligonucleotides is 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 1317318). The second pair of oligonucleotides is OPA-04_{293} 2F (5′ GCC-GCA-AGT-TGG-ACT-ACG 3′) and OPA-04_{293} 2R (5′ CAA-GCT-TCA-TCC-GGC-ACT-T 3′), which amplify a 293-bp DNA region that is within the region amplified by of the first pair of oligonucleotides, that is, the primers are designed to work in conjunction with each other by using nested PCR. Amplicons were confirmed by sequencing (data not shown).

PCR conditions were 1.5 mM $MgCl_2$, 0.125 mM deoxyribonucleotide triphosphates, 50 pmol of both primers, 1.5 U Taq polymerase (Biogenica, Mexico), and 1 µl of the first primer-pair PCR product for the second probe-pair PCR. The following PCR controls were used: (1) the positive control consisted of DNA extracted from 10,000 conidia and (2) the negative control was the PCR mixture without DNA. The assay conditions for the first primer-pair PCR, OPA-04_{461}1, were 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 cycle at 72°C for 5 min. The assay conditions for the second probe-pair PCR, OPA-04_{293}2, were 1 cycle at 94°C for 3 min; followed by 25 cycles at 94°C for 1 min, 72°C for 2 min, 72°C for 1 min; and a final cycle at 72°C for 5 min.

2.7 Visualization of PCR products

All PCR products were visualized by gel electrophoresis in 2% agarose gels stained with ethidium bromide. The gel was run at 80 V in 0.5 × TBE buffer (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA). A 100-bp DNA standard ladder (Invitrogen, CA, USA) was used. Images were captured in a photodocumentation device Digi-Doc-It (UVP, CA, USA).

2.8 Spore traps

To collect airborne fungal conidia, Hirst-type spore traps were maintained continuously for 7 days (Burkard Manufacturing Co., Rickmansworth, UK). Details on how they function can be found in Lacey and West (2006) and Lacey and Venette (1995). Briefly, a sampler suctions 10 l of air per minute, 24 h a day, 365 days a year. This trap has an integrated rotating drum (spinning in clockwise direction) containing cellophane tape (Melinex, Rickmansworth, UK) covered with a thin layer of Vaseline/hexane (5:1) in which airborne conidia get trapped. The tape is subsequently withdrawn and cut into 48-mm pieces that correspond to 24-h samplings. The pieces are placed inside conic microtubes with glass beads, and DNA is extracted by the same method mentioned previously in spore walls rupture section. A review of the tape under a microscope showed that there is nothing attached to it after the breakup of the spores.

2.9 Mini-wind tunnel experiments

Prior to detecting airborne conidia in the field, we detected conidia using a mini-wind tunnel (90 cm long, 15 cm wide, and 15 cm high). Cotton wicks (impregnated with PDA media covered with *M. acridum* conidia) were placed inside the tunnel. The tunnel is connected to a chamber (35, 24, and 24 cm) that is coupled to a humidifier. An electric fan is located outside this device. The samples are placed in compartments found within the tunnel (Fig. 1).

A 4 m/s wind draft was applied so that conidia attached to the wicks would be released and captured by the Hirst-type spore traps. Subsequently, the adhesive tape was recovered, and DNA was extracted from the samples collected to confirm that *M. acridum* DNA could be detected.

Fig. 1 Mini-wind tunnel

2.10 Field experiments

During August 2007, Hirst-type spore traps were placed in amaranth fields in San Mateo Coatepec, Puebla. The fields chosen for this study are in an area plagued with the grasshoppers (*S. purpurascens)*.

In this area, an oil solution containing *M. acridum* conidia (strain EH-502/8, 1×10^{12} conidia/Ha) was sprayed (Hernandez-Velazquez et al. 2000). Traps were left for 1 week prior to and 1 week after spraying took place to determine the capacity to detect *M. acridum* conidia.

3 Results

3.1 PCR detection sensitivity using *M. acridum* spore DNA primers

Various conidia suspension densities were evaluated to obtain the lowest *M. acridum* detection limit using the different primers (Fig. 2). The lowest detection limit for the OPA-04_{461} 1F and 1R primers was 100 conidia, as shown in Fig. 2a.

Otherwise, the lowest detection limit for the OPA-04_{293} 2F and 2R primers was 1 spore, as shown in Fig. 2b.

3.2 Experiments using *M. acridum* inoculated cotton wicks in the mini-wind tunnel

Cellophane tape that contained fungal conidia, due to spore dispersion, were collected from day 1 to day 7.

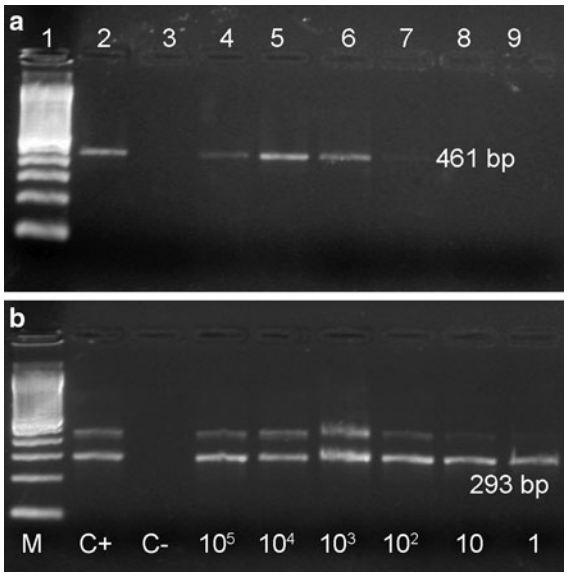

Fig. 2 PCR results showing the number of conidia detected by oligonucleotides OPA-04(526) 1F and 1R **a**, and OPA-04(526) 2F and 2R **b**. M: 100-bp marker, *C*+ positive control, *C*− negative control

No detection was possible using the OPA-04_{461} 1F and 1R primers. Detection using the OPA-04_{461} 1F and 1R primers was possible after 2 days collection on days 3 and 6.

3.3 Molecular detection of airborne *M. acridum* conidia collected with Hirst-type traps in crop fields

Once the detection limit of the specific primers to detect (*M. acridum)* fungal DNA was determined, samples were collected in San Mateo Coatepec, in August 2007, where it was being used to control grasshopper populations. *M. acridum* detection was possible on days 2 and 6 after fungal application.

4 Discussion

The entomopathogenic fungus, *M. acridum* has been widely used as a microbial agent to control acridids in Australia (Hunter et al. 1999; Hunter et al. 2001), Brazil (Magalhães et al. 2001), China (Lee et al. 2000), Africa (Lomer et al. 2001), and Mexico (Hernández-Velázquez et al. 2003). Currently, *M. acridum* is being developed for use as a biopesticide targeted against locusts and grasshoppers in China and Mexico.

However, the basic ecological characteristics of *M. acridum* are still unclear, and the use of molecular techniques would largely contribute to clarify many fundamental points, allowing a better understanding of its ecological niche.

Molecular detection to detect airborne fungi has previously been employed, mainly in phytopathogen detection (Calderon et al. 2002a, b; Freeman et al. 2002). This is the first study that detects airborne entomopathogenic fungi, specifically *M. acridum*. Previously, a molecular diagnostic test was developed to detect phytopathogenic fungi spores once the first propagules began to appear in agricultural areas susceptible to fungal infection, allowing convenient and timely palliative techniques (McCartney et al. 2003). In the present study, we were interested in this molecular technique because it could be used to establish conidia air propagation patterns, not yet studied for *M. acridum*, in addition to contributing to the knowledge of conidial dispersion and persistence (Meyling and Eilenberg 2007).

In this study, Hirst-type spore traps (Burkard) were chosen for the collection of samples because of the small quantities of airborne fungal spores that were present. This allowed the collection of sufficient airborne conidia for DNA extraction from the cellophane tape using a continuous flow of 10 l of air/min. The use of Fast-Prep equipment facilitates airborne fungal cell wall rupture. The extraction method proposed allowed the detection of a single conidium (Fig. 2).

The method employed in this study was successful in detecting *M. acridum* airborne conidia, although one must bear in mind that this technique is mainly qualitative; thus, the number of airborne conidia cannot be determined. However, this technique can be greatly improved using real-time PCR in conjunction with methods to test spore viability. In addition, this study may provide a basis for future research to evaluate the long-term persistence of spores in the environment.

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