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ORIGINAL PAPER

Discovery of a North American genetic variant of the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* pathogenic to grasshoppers

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Abstract A genetic variant of the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae*, isolated from a soil in Alberta, Canada, from a location with a history of severe grasshopper infestations, was evaluated for pathogenicity in bioassays of living grasshoppers. Mortality in treated individuals drawn from a laboratory colony was 99% (LT_{50} = 6.7 days, LT_{90} = 9.6 days) at 12 days post-inoculation compared to 100% (LT_{50} = 4.1 days, LT_{90} = 5.8 days) mortality at 8 days in insects exposed to a commercial isolate of *M. anisopliae* var. *acridum* (IMI 330189). Experimental infection of field-collected grasshoppers under laboratory conditions with the native isolate of *M. anisopliae* var. *anisopliae* resulted in 100% (LT_{50} = 4.4 days, LT_{90} = 5.4 days) mortality attained within 7 days compared to 100% (LT_{50} = 4.7 days, LT_{90} = 6.3 days) mortality in 9 days in insects treated with *M. anisopliae* var. *acridum*. Amplification of fungal genomic DNA from the indigenous isolate with primers for the specific detection of *M. anisopliae* var. *anisopliae* produced a product almost 300 bp larger than expected based on previously known isolates. This is the first demonstration of a highly virulent, indigenous non-chemical control agent of grasshoppers in North America.

Keywords Agroecosystem pest · Entomopathogenic fungus · Grasshoppers · Microbial control · *Metarhizium*

GenBank Accession Nos. DQ342236, DQ342237.

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Introduction

Recurring outbreaks of grasshoppers cause significant economic damage to crops, forages and range in the Canadian Prairies, and result in extensive pesticide application. Concern over the impact of chemicals on human health and the environment has been the driving force for investigations into the use of entomopathogenic microbes for control. Previously conducted field trials of indigenous agents against grasshoppers have yielded divergent results, mostly attributed to timing and environmental conditions. Applications of *Nosema locustae* (Johnson and Dolinski 1997; Johnson 1989) showed modest to negligible reductions in host densities, although reductions in activity (Johnson 1989) and feeding (Johnson and Pavlikova 1986) were demonstrated. Johnson et al. (1988) required high doses of *Verticillium lecanii* to significantly reduce insect populations; more realistic field application rates demonstrated ineffective control. Moderate (70%) mortality due to mycosis was obtained in grasshoppers confined in laboratory cages following treatment with a US isolate of *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes) and collection within two days of application (Johnson and Goettel 1993); however, no significant reduction of field populations was observed with later applications of two virulent strains of *B. bassiana* (Inglis et al. 1997).

Molecular monitoring has offered further improvements on the detection and differentiation of entomopathogens in infected grasshoppers. Cloned DNA probes were used in conjunction with the polymerase chain reaction (PCR) for the specific detection of *B. bassiana* in infected insects, although the probes failed to distinguish between *B. bassiana* strains (Hegedus and Khachatourians 1993, 1996). In the United States, an introduced Australian pathotype of *Entomophaga grylli* (Fresenius) Batko (Zygomycotina: Entomophthorales) was distinguished from native isolates in *E. grylli*-infected grasshoppers collected after field release of laboratory-inoculated insects (Bidochka et al. 1996).

The ability to distinguish introduced strains of *Metarhizium* spp. from native populations has been limited, and has precluded full evaluation of their fate in target hosts and non-targets. Hu and St. Leger (2002) were able to trace the release of a transformant of *M. anisopliae* tagged with a green fluorescent protein reporter gene. This method is not practical, in part because introduction of genetically modified organisms into the environment is a highly contentious issue. Entz et al. (2005) developed a molecular assay that would differentiate *M. anisopliae* var. *acridum* from endemic strains of *M. anisopliae* var. *anisopliae*, and was applicable for detection of fungal DNA in infected grasshoppers. In conjunction with investigation into the possibility of application of *Metarhizium* spp. for grasshopper control in Canada, a soil survey of locales in southern Alberta with known histories of severe grasshopper infestations was conducted and yielded isolates of *M. anisopliae* var. *anisopliae*. We report on one of those isolates, a naturally occurring genetic variant, that showed virulence comparable to a standard strain (IMI 330189) of *M. anisopliae* var. *acridum* to laboratory-reared and field-collected grasshopper nymphs, and demonstrate the ability of a molecular assay to distinguish the isolate from other native isolates, as well as detect fungal DNA in infected grasshoppers.

Materials and methods

Fungal isolates

Metarhizium anisopliae var. *anisopliae* isolates S54, 6W-2, and 11S-1 were isolated from soils in southern Alberta, Canada. Samples were collected from the upper 10 cm, in fallow agricultural fields. Isolates S54 and 6W-2 were obtained from soil extracts that were inoculated onto selective medium according to the method outlined by Rath et al. (1992). Briefly, moist soil equivalent to 20 g, oven-dried weight, was added to 200 ml sterile Ringer's solution (Oxoid, Nepean, Ontario, Canada) in a 500-ml Erlenmeyer flask. The suspension was shaken at 150 rpm for 30 min on an orbital shaker, then 0.1 ml of neat and 1:10 dilutions in sterile distilled water were spread-plated on mycological agar (Difco, Oakville, Ontario, Canada) containing 50 μg/ml chloramphenicol (Sigma-Aldrich, Oakville, Ontario, Canada) and 200 μg/ml cycloheximide (Sigma-Aldrich), and modified with 10 μg/ml dodine (Liu et al. 1993). Plates were then incubated in the dark at room temperature (ca. 20°C) for 20 days before examination for colonies of *Metarhizium* spp. Isolate 11S-1 was recovered from a cadaver of *Galleria mellonella* larvae used in a bait assay conducted at room temperature (ca. 20°C) for twenty-one days (Zimmermann 1986).

Sources, hosts, and geographical origins of additional fungal isolates used in this study are shown in Table 1. All fungal isolates were propagated and maintained on potato dextrose agar (PDA).

Inoculation of grasshoppers

Third- and fourth-instar nymphs of a laboratory colony of *Melanoplus sanguinipes* and third- and fourth-instar field-collected nymphs of *M. sanguinipes*, *M. bivittatus* Say, and *M. packardii* Scudder (Orthoptera: Acrididae) were collected, inoculated, and housed as previously described (Entz et al. 2005). Insects randomly selected from this group were placed individually in sterile 20 ml glass vials stoppered with a sterile polyurethane foam plug; species, sex and instar were recorded for each insect.

Table 1 List of isolates studied

Isolate code[a]	Name	Host	Country of origin
ARSEF 437	*M. anisopliae* var. *anisopliae*	*Teleogryllus commodus* (Orthoptera: Gryllidae)	Australia
ARSEF 727	*M. anisopliae* var. *anisopliae*	unidentified tettigonid (Orthoptera: Tettigoniidae)	Brazil
IMI 330189	*Metarhizium anisopliae* var. *acridum*	*Ornithacris cavroisi* (Orthoptera: Acrididae)	Niger
UAMH 421	*M. anisopliae* var. *anisopliae*	Unidentified insect larvae	USA
UAMH 4450	*M. anisopliae* var. *anisopliae*	Soil	Canada
6W-2	*M. anisopliae* var. *anisopliae*	Soil	Canada
11S-1	*M. anisopliae* var. *anisopliae*	Soil	Canada
S54	*M. anisopliae* var. *anisopliae*	Soil	Canada

[a] IMI = International Mycological Institute, Egham, UK

ARSEF = Agriculture Research Service Entomopathogenic Fungus Collection, US Department of Agriculture

UAMH = University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada

On the day of inoculation, conidia of *Metarhizium anisopliae* var. *acridum* isolate IMI 330189 or *M. anisopliae* var. *anisopliae* isolate S54 were harvested from PDA cultures (15–20 days of growth) and resuspended in sunflower oil. The concentrations of conidia were estimated with a hemocytometer and adjusted to 5×10^7 conidia/ml. Subsequently, 2-µl aliquots were pipetted onto lettuce-leaf wafers (0.7 cm diameter), resulting in a dose of approximately 10^5 spores per insect (*via* handling and feeding). Each grasshopper was confined with one lettuce-leaf wafer for 24 h. Control grasshoppers were confined with untreated lettuce-leaf wafers bearing only sunflower oil. After 24 h confinement, all surviving insects were removed and individually housed in 240-ml transparent plastic containers. Throughout the experiment, insects were contained in a temperature regime of 24°/16°C day/night with a corresponding 16/8 h light/dark photoperiod under ambient relative humidity (40–55%). The insects were observed and fed daily with fresh wheat leaves. Cadavers were removed daily with sterile forceps and processed further for confirmation of *Metarhizium* infection as described below.

Control groups consisted of 30 grasshoppers. *M. anisopliae* var. *acridum* isolate IMI 330189 was used to treat 144 grasshoppers from a laboratory colony and 128 field-collected insects. *M. anisopliae* var. *anisopliae* isolate S54 conidia were inoculated to 79 laboratory-reared grasshoppers and 131 field-collected nymphs.

Confirmation of *Metarhizium* infection in grasshoppers

M. anisopliae var. *acridum* infection in grasshopper nymphs treated with IMI 330189 spores was confirmed by PCR assay with specific primers Mac-ITS-F1 and Mac-ITS-R1 (Entz et al. 2005). Cadavers were kept frozen at –20°C prior to DNA extraction. A 25-µl aliquot of cadaver homogenate, prepared prior to DNA extraction, was removed and spread onto selective medium previously described. Plates were incubated at 25°C for a maximum of 20 days before examination for colonies of *Metarhizium* spp.

Mortality attributed to native isolate *M. anisopliae* var. *anisopliae* isolate S54 was recorded as incidence of mycosis with evidence of external sporulation characteristic of *Metarhizium*. Dead insects were removed daily and disinfected by immersion for 1 min in 70% ethanol followed by 1 min in sterile distilled water. Cadavers were then placed individually on moistened sterile filter paper in a 60 mm × 10 mm Petri dish, the dish sealed with Parafilm®, and incubated at ca. 20°C for a maximum of 21 days. PCR assays were used to confirm *M. anisopliae* var. *anisopliae* infection in infected cadavers. Conidia from the surface of infected cadavers were transferred with a sterile loop to 500 µl of potato dextrose broth (PDB) and incubated at in the dark at room temperature (ca. 20°C) for 3 to 4 days; 1 µl of culture was then used directly as template in nested PCR assays described below. Insects that did not display signs of external sporulation after 21 days were then frozen at –20°C until DNA extraction for PCR amplification.

Mortality data analysis

Because the resulting mortality data were not normally distributed, the Kruskal–Wallis test (Steel and Torrie 1980) was used to compare the distributions for the three grasshopper species for each *Metarhizium* isolate.

For all treated grasshopper experiments, mortality data from control insect groups were used in Abbott's (1925) formula to determine corrected mortality. Corrected daily mortality data were then fitted to a Weibull distribution and the LIFEREG procedure (SAS Institute 2005) used to estimate lethal times for mortality of 50% (LT_{50}) and 90% (LT_{90}) of treated insects with upper and lower 95% confidence limits (CL).

Fungal genomic and total grasshopper DNA extraction

Fungal genomic and total grasshopper DNA extractions were as described in Entz et al. (2005). Estimates of DNA quantities were obtained by electrophoresis in 0.9% TAE (40 mM Tris acetate, pH approx. 8.3, containing 1 mM EDTA) agarose gels containing 10 μg ml^{-1} ethidium bromide (Sambrook et al. 1989). Fifty ng of fungal genomic DNA and 100 ng of total grasshopper DNA were subjected to PCR amplifications.

PCR Amplification

DNA primers for the specific detection of *M. anisopliae* var. *anisopliae* were used to initially amplify the partial 3′ end of the large subunit ribosomal RNA and intergenic spacer (IGS) region followed by a secondary reaction with primers that amplified a 380 bp fragment within the IGS region specific to *M. anisopliae* var. *anisopliae*. Nested PCR assays were conducted with an initial reaction with primers Ma-28S4 (5′-CCTTGTTGTTACGATCTGCTGAGGG-3′) and Ma-IGS1 (5′-CGTCACTT GTATTGGCAC-3′) (Pantou et al. 2003). A second reaction was performed with a 1-μl aliquot from the initial amplification and primers Ma-IGSspF (5′- CTAC-CYGGGAGCCCAGGCAAG-3′) and Ma-IGSspR (5′- AAGCAGCCTACCC-TAAAGC-3′) (Pantou et al. 2003). Amplifications were performed in a total volume of 50 μl containing 20 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.4 μM of each primer, 25 μM of each dNTP (Invitrogen, Carlsbad, CA), 2.5 units *Taq* DNA polymerase (MBI Fermentas, Hanover, MD, USA) and template DNA. Negative controls for all amplifications consisted of sterile water in place of DNA. DNA amplifications were performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) programmed as follows: initial denaturation 5 min at 94°C; 30 cycles of the following steps: denaturation 1 min at 94°C, annealing 1 min at 54°C (Ma-28S4 and Ma-IGS1) or at 58°C (Ma-IGSspF and Ma-IGSspR), extension 2 min at 72°C; with a final extension 5 min at 72°C. PCR products were analyzed on 1.5% TAE agarose gels with a 100 bp DNA ladder (MBI Fermentas) included as a size marker.

Sequencing of the IGS Region of native *M. anisopliae* var. *anisopliae* isolate S54 and 6W-2

The PCR products that resulted from amplification of *M. anisopliae* var. *anisopliae* S54 and 6W-2 DNA with primers Ma-28S4 and Ma-IGS1 were cloned in vector pGEM®-T Easy using the pGEM® and pGEM®-T Easy Vector Systems cloning kit (Promega, Madison, WI, USA). Standard protocols were used for plasmid DNA isolation, buffers, and electrophoresis techniques (Sambrook et al. 1989). Sequences

were determined by the dideoxy chain termination method and deposited in GenBank (DQ342236, isolate 6W-2; DQ342237, isolate S54).

Results

M. anisopliae var. *anisopliae* PCR assays with fungal genomic DNA

Most isolates of *M. anisopliae* var. *anisopliae* in this study produced a 380 bp product in a nested PCR assay with primer combinations Ma-28S4/Ma-IGS1 and Ma-IGSspF/Ma-IGSspR (Fig. 1). Isolate ARSEF 437 produced a slightly smaller product of approximately 350 bp. Amplification of fungal genomic DNA from native isolate S54 resulted in a PCR product approximately 670 bp in size.

PCR products produced by amplification of the partial 3' end of the large subunit ribosomal RNA and IGS region with the Ma-28S4/Ma-IGS1 primers were cloned and sequenced for native isolates S54 and 6W-2. Isolate 6W-2 was identified as a group-B *M. anisopliae* var. *anisopliae* based on the presence of a 20 bp GT-rich insertion sequence found to be present in group-B strains (Pantou et al. 2003). Isolate S54 was identified as a group-B variant lacking the priming site for the Ma-IGSspF primer. The Ma-IGSspF primer false-primed upstream of the missing site at nucleotide positions 163–183, resulting in a PCR product 300 bp larger than expected. Colony morphologies (dark green conidia) on PDA for the group-B and variant group-B isolates were similar, as were size and shape of conidia.

Analysis of differential impact of *Metarhizium* infection on field-collected grasshopper species

The Kruskal–Wallis test indicated that the distributions for the three grasshopper species were not significantly different for either fungal isolate (IMI 330189:

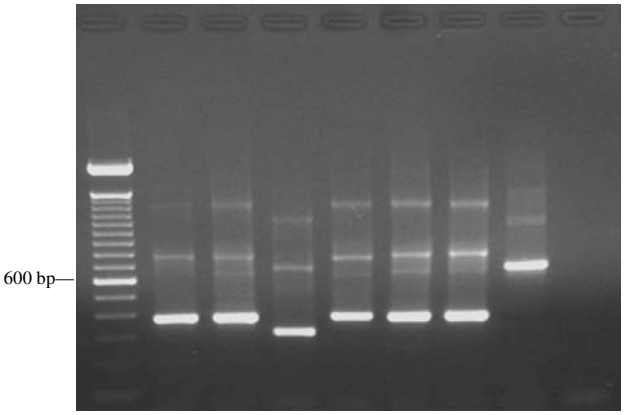

Fig. 1 Detection of *Metarhizium anisopliae* var. *anisopliae* fungal genomic DNA in nested PCR assays using Ma-28S4/Ma-IGS1 and Ma-IGSspF/Ma-IGSspR primers. Lane 1: 100 bp ladder Lane 2: *M. anisopliae* var. *anisopliae* UAMH 421 Lane 3: *M. anisopliae* var. *anisopliae* UAMH 4450 Lane 4: *M. anisopliae* var. *anisopliae* ARSEF 437 Lane 5: *M. anisopliae* var. *anisopliae* ARSEF 727 Lane 6: *M. anisopliae* var. *anisopliae* 6W-2 Lane 7: *M. anisopliae* var. *anisopliae* 11S-1 Lane 8: *M. anisopliae* var. *anisopliae* S54 Lane 9: Water

$P = 0.09$; S54: $P = 0.77$); mortality did not differ among the target species tested. Consequently, the data were further analyzed without respect to species for each treatment.

Infection of laboratory-reared and field-collected grasshopper nymphs with *M. anisopliae* var. *acridum* isolate IMI 330189

Complete mortality was observed by 8 days for laboratory colony nymphs versus 9 days for field-collected grasshoppers for treated insects exposed to *M. anisopliae* var. *acridum* isolate IMI 330189. The LT_{50} value for the IMI 330189-treated laboratory colony assay was 4.1 days versus 4.7 days for the IMI 330189-treated field-collected assay (Table 2). LT_{90} values were 5.8 and 6.3 days for the IMI 330189-treated laboratory and field-collected assays, respectively (Table 2). Daily corrected cumulative mortalities for laboratory-reared and field-collected grasshopper nymphs challenged with IMI 330189 are shown in Figs. 2 and 3, respectively.

The presence of *M. anisopliae* var. *acridum* DNA in IMI 330189-challenged insects was confirmed by successful amplification of a 420 bp DNA sequence from the total DNA extracted from 100% of treated laboratory colony and field-collected grasshopper nymphs in a PCR assay with the Mac-ITS-F1 and Mac-ITS-R1 primers (data not shown) and supported by isolation of a fungus with conidial morphology characteristic of *M. anisopliae* var. *acridum* on selective medium from 92.1% and 91.9% of treated laboratory colony and field-collected insects, respectively. No *Metarhizium* spp. were isolated from extracts of homogenized cadavers inoculated to selective medium from the control group, for either laboratory colony or field-collected nymphs. No amplified products were produced with the *M. anisopliae* var. *acridum*-specific primers in PCR assays of the control groups.

Infection of laboratory-reared and field-collected grasshopper nymphs with *M. anisopliae* var. *anisopliae* isolate S54

At 12 days post-inoculation, cumulative mortality was 97.9% in laboratory colony nymphs treated with *M. anisopliae* var. *anisopliae* isolate S54 (Fig. 2). Sporulation occurred in 91.5% of treated insects. LT_{50} was 6.7 days and LT_{90} was 9.6 days (Table 2). Nested PCR assays with primer combinations Ma-28S4/Ma-IGS1 and Ma-IGSspF/Ma-IGSspR produced amplified products 670 bp in size, corresponding to that expected for S54 DNA, in 97.2% of S54-treated laboratory colony nymphs, including all insects that exhibited sporulation and 4 out of 5 non-sporulating cadavers. No band sizes corresponding to those expected for *M. anisopliae* var. *anisopliae* with these primers were detected in untreated insects. The sole surviving insect in the treated group at the end of the experiment, 12 days post-inoculation, tested negative with the nested PCR assay.

Complete mortality (100% of experimental subjects) was observed 7 days post-inoculation in field-collected nymphs treated with isolate S54 (Fig. 3). The LT_{50} value for this bioassay was lower, 4.4 days compared to 6.7 days for infected laboratory colony nymphs (Table 2). The LT_{90} value was 5.4 days (Table 2). Nested PCR assays with *M. anisopliae* var. *anisopliae*-specific primers produced positive results for 97.6% of infected nymphs. *Metarhizium*-induced mycosis was confirmed by sporulation in 83.2% of infected insects one week after death.

Table 2 Lethal time for 50% (LT_{50}) and 90% (LT_{90}) population mortality with confidence limits ($1 - \alpha = 95\%$) of laboratory and field-collected grasshopper nymphs treated with conidia of two *Metarhizium* isolates. Numbers followed by the same letter in column are not significant at $\alpha = 0.05$

Insect Source/ Fungal Isolate	Number of insects (N)	LT_{50} (days)	LT_{50} Confidence limit Lower Upper	LT_{90} (days)	LT_{90} Confidence limit Lower Upper	Isolation of *Metarhizium* from cadavers (%)	Specific PCR positive (%)
Laboratory colony nymphs: IMI 330189	126	4.1	3.87–4.37[a]	5.8	5.50–6.15[d,e]	92.1	100
S54	71	6.7	6.20–7.29[b]	9.6	8.89–10.30[f]	91.5	97.2
Field-collected nymphs: IMI 330189	111	4.7	4.44–4.94[c]	6.3	5.96–6.57[e]	91.9	100
S54	125	4.4	4.29–4.60[a,c]	5.4	5.22–5.58[d]	83.2	97.6

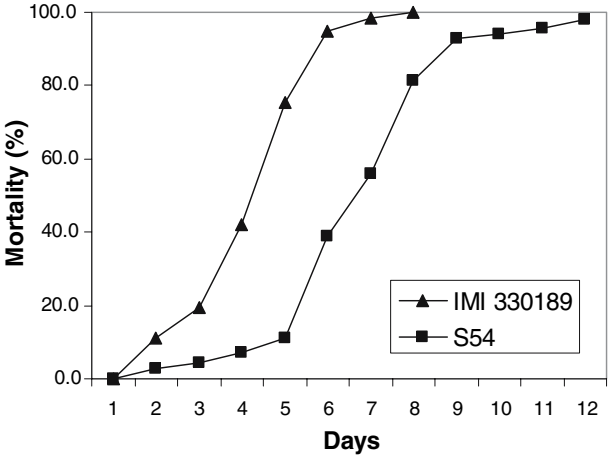

Fig. 2 Cumulative mortality of laboratory *Melanoplus sanguinipes* nymphs treated with *Metarhizium anisopliae* var. *acridum* IMI 330189 and *M. anisopliae* var. *anisopliae* S54

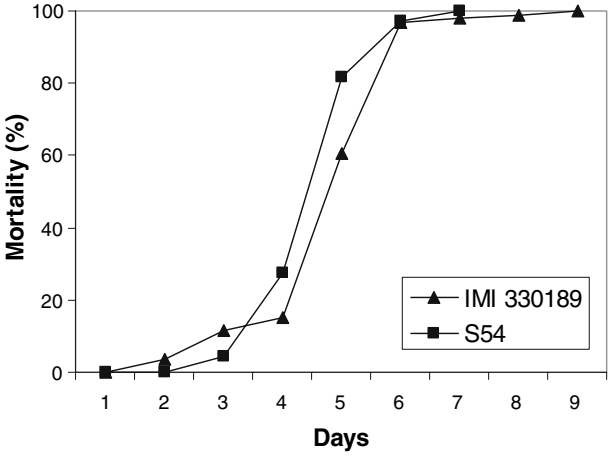

Fig. 3 Cumulative mortality of field-collected *Melanoplus sanguinipes*, *M. bivittatus*, and *M. packardii* nymphs treated with *Metarhizium anisopliae* var. *acridum* IMI 330189 and *M. anisopliae* var. *anisopliae* S54

The cumulative mortality curves for treated laboratory colony grasshoppers (Fig. 2) and treated field-collected nymphs (Fig. 3) approach the sigmoidal shape expected for populations of target insects treated with entomopathogenic fungi (Bateman et al., 1996).

Discussion

Prior to application of a biological control agent, a method is required that allows discrimination of the introduced organism from indigenous populations. The native isolate of *M. anisopliae* var. *anisopliae* evaluated in bioassays against North American grasshopper species in this study was selected on the basis of its distinctive genetic characteristics. The isolate, S54, was chosen because it could be distinguished from three other native isolates of *Metarhizium* spp. in a PCR assay. The three grasshopper species (*M. sanguinipes*, *M. bivittatus*, and *M. packardii*) used in assays

of field-collected grasshoppers were selected because they are the main agricultural pest species of grasshoppers in the Canadian Prairies.

The discovery of a soil isolate of *M. anisopliae* var. *anisopliae* that demonstrated comparable virulence with a commercialized *Metarhizium* isolate towards orthopteran species in southern Alberta is unexpected based on earlier studies that showed direct isolation from a target host was the most appropriate method for isolation of target-specific pathotypes (Shah 1994; Kooyman and Shah 1992). Furthermore, documentation of isolates of *M. anisopliae* var. *anisopliae* from orthopteran hosts is not common (Hernández-Crespo and Santiago-Álvarez 1997; Kooyman and Shah 1992; Prior 1992). However, other studies have demonstrated that *Metarhizium* isolates from non-orthopteran origins may have greater pathogenicity to acridids than isolates from Orthoptera (Bateman et al. 1996). In Madagascar, Welling et al. (1994) found a native virulent strain of *M. anisopliae* isolated from soil caused faster and higher mortality than an indigenous orthopteran isolate of *M. flavoviride* in bioassays of a laboratory strain of desert locusts, and concluded that soil-derived isolates may also be effective against certain target species and therefore should be included in routine bioassays.

In this study, the LT_{50} value of 4.1 days for laboratory-reared grasshoppers exposed to *M. anisopliae* var. *acridum* IMI 330189 fell within the range of reported values between 4 and 6 days in bioassays of laboratory stocks of acridids infected with *M. anisopliae* var. *acridum* in previous studies (Smits et al. 1999; Bateman et al. 1996; David Hunter, pers. comm.). The LT_{50} value of 4.7 days for field-collected nymphs treated with the same isolate also was comparable to results from previous tests with commercialized *Metarhizium* (Shah et al. 1998; D. Hunter, pers. comm.).

In contrast with results in bioassays of field-collected grasshoppers, in this study a southern Albertan isolate of *M. anisopliae* var. *anisopliae* showed significant slower mortality compared to an exotic isolate of *M. anisopliae* var. *acridum* in treated nymphs from a laboratory stock of *M. sanguinipes*. This may be a reflection of the genetic homogeneity of a laboratory culture of insects that has resulted from inbreeding within a closed genetic pool for over forty years. Over the initial twelve-year period during which the laboratory insect stock was established, Pickford and Randell (1969) noted no evidence of deleterious mutants, although noted that the population had been reduced to very small numbers on several occasions due to disease. The plot of cumulative mortality for laboratory-reared insects exposed to the exotic isolate IMI 330189 showed almost no initial lag in mortality as would be expected of a sigmoidal curve for a heterogeneous population treated with a pathogen, suggesting a narrow range of physiological response from the laboratory-reared insects. Similarly, since the native isolate of *M. anisopliae* var. *anisopliae* demonstrated a longer lag phase initially, this may be an indication that the individual laboratory stock nymphs possessed similar levels of resistance to the indigenous strain; however, this resistance was insufficient to prevent almost 100% mortality twelve days post-treatment. Large differences among host genotypes in insect populations in response to microbial pathogens have been previously well documented (Watanabe 1987). In this case, the results suggest that bioassays of native field-collected insects may better reflect the target response to indigenous fungal entomopathogens.

The Ma-IGSspF/Ma-IGSspR primers used in this study for the detection of *M. anisopliae* var. *anisopliae* were reported as species-specific in the amplification of a 380 bp product for this entomopathogen (Pantou et al. 2003). Contrary to the findings of that study, isolate ARSEF 437, obtained from an orthopteran host in

Australia, was observed to produce a slightly smaller product of approximately 350 bp (Fig. 1). More importantly, an isolate of *M. anisopliae* var. *anisopliae* found in southern Alberta produced a 670 bp product when amplified in nested PCR with the Ma-28S4/Ma-IGS1 and Ma-IGSspF/Ma-IGSspR primer combinations. Successful isolation of *M. anisopliae* var. *anisopliae* from soils in southern Alberta indicates that this species can survive and even possibly persist in semi-arid agroecosystems. The identification of an indigenous strain of *M. anisopliae* var. *anisopliae*, especially one with a unique built-in genetic fingerprint, that demonstrates high virulence towards native grasshopper pest species will ease some of the impediments to registration of a microbial control agent through the use of molecular methodology for monitoring and tracking the fate of the specific pest control product in the target population and in the environment.

Concerns over the safety and efficacy of exotic agents used to control native pests have led to the promotion of strategies for augmentation of native agents for biological control (Lockwood 1993a, b). The environmental impacts of a biological control agent native to the target area are expected to be reversible and more predictable than those that result from the introduction of an exotic agent (Howarth 2000). Further, regulatory agencies require data that address critical issues of toxicity and other effects on indicator non-target species. More work is required to determine host specificity of the native fungus, since there is no indication of the entomopathogen's host range, because it was isolated from soil; however, safety to ring-necked pheasants, *Phasianus colchicus* Linnaeus (Galliforma: Phasianidae), exposed to a closely related strain of *M. anisopliae* var. *acridum*, was previously demonstrated in a Canadian study (Johnson et al. 2002; Smits et al. 1999).

This study has identified an indigenous southern Albertan isolate of *M. anisopliae* var. *anisopliae* that shows high virulence to native grasshoppers, is readily propagated on culture media, and can be differentiated from other native isolates of *Metarhizium* spp. with a sensitive molecular assay. Further investigation is warranted because implementation of a native pathogen in a biological control program would help alleviate regulatory concerns about ecological consequences.

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