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Molecular methods and isolates of the entomopathogenic fungus Metarhizium anisolpliae for environmentally sustainable control of grasshoppers in Canada

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MOLECULAR METHODS AND ISOLATES OF THE ENTOMOPATHOGENIC
FUNGUS *Metarhizium anisopliae* FOR ENVIRONMENTALLY SUSTAINABLE
CONTROL OF GRASSHOPPERS IN CANADA

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Abstract

Metarhizium anisopliae var. *acridum*, a hyphomycetous fungus registered worldwide for grasshopper and locust control, is currently under consideration as a potential alternative to chemical insecticides for grasshopper control in Canada. Research in this thesis has contributed data required for the registration of biological control agents in Canada.

A diagnostic PCR assay was developed for the specific detection of *M. anisopliae* var. *acridum* DNA. The assay was highly sensitive and effective for the detection of fungal DNA in infected grasshoppers.

A survey of southern Alberta soils conducted in the spring of 2004 revealed the presence of *Metarhizium* spp. at low natural incidence. Two indigenous isolates demonstrated pathogenicity when bioassayed against laboratory-reared and field-collected grasshoppers. One of the isolates demonstrated virulence comparable to a commercial isolate.

An analysis of historical weather data revealed that summer weather in the Prairie provinces should not preclude the efficacy of *M. anisopliae* var. *acridum* under local conditions.

Preface

The following thesis is presented partially in manuscript format. The introduction and literature review are combined in a single chapter (Chapter 1) in traditional format. Chapters 2, 3, and 4 are presented as manuscripts. References for Chapters 2 to 4 are combined in a general references section as outlined in the table of contents. Chapter 5 outlines a retrospective prediction of *Metarhizium* efficacy under local conditions and is also written in traditional format. A general summary and conclusions are incorporated into Chapter 6.

Acknowledgements

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List of Abbreviations

ARSEF – (US Department of Agriculture) Agricultural Research Service
Entomopathogenic Fungus Collection

bp – base pair

CFU – colony forming unit

dNTP – deoxynucleotide

dpa – days post application

dpi – days post infection

EDTA – ethylenediaminetetraacetic acid

EPA – (US) Environmental Protection Agency

ha – hectare

IGS – intergenic spacer

IITA – International Institute of Tropical Agriculture

ITS – internal transcribed spacer

LD_{50} – lethal dose required for mortality of 50 % of population

LRC – Lethbridge Research Centre

LT_{50} – lethal time required for mortality of 50 % of population

LUBILOSA – Lutte Biologique Contre les Locustes et Sauteriaux

PCR – polymerase chain reaction

PDA – potato dextrose agar

PDB – potato dextrose broth

PMRA – Pest Management Regulatory Agency

RAPD – random amplified polymorphic DNA

rDNA – ribosomal deoxyribonucleic acid

RFLP – random fragment length polymorphism

RH – relative humidity

rRNA – ribosomal ribonucleic acid

SDA – Sabouraud-dextrose agar

SDS – sodium dodecyl sulphate

TAE – 40 mM Tris acetate, pH approx. 8.3, containing 1 mM EDTA

TE – 10 mM Tris-HCl containing 1 mM EDTA, pH 8

UAMH – University of Alberta Microfungus Collection and Herbarium

Chapter 1.   Introduction, Review of Literature, and Objectives

1.0   Introduction

Environmental and health concerns about the application of chemical insecticides to reduce large-scale insect pest infestations have led to renewed interest in the development of microbial agents for incorporation into integrated pest management strategies for the control of acridids. Grasshoppers and locusts are responsible for significant economic damage to the agriculture industry in grassland biomes of the world (Lomer *et al.*, 2001); over eighty-five species of grasshoppers exist in Alberta, and of these, four are considered to be serious agricultural pests (Calpas & Johnson, 2003). Microbial agents considered so far for the control of acridids include all major types including fungi, bacteria, viruses, nematodes, and protozoans.

Metarhizium anisopliae is a hyphomycetous fungus that is pathogenic to a wide range of insect orders. *M. anisopliae* var. *anisopliae* is cosmopolitan in nature; strains occur naturally in soil. Isolates with varying degrees of virulence have also been obtained from locusts and grasshoppers in Africa (Shah *et al.*, 1997), Australia (Prior, 1997), Madagascar (Delgado *et al.*, 1997a), and Spain (Hernández-Crespo & Santiago-Álvarez, 1997). The rarer, albeit more virulent, isolates of *M. anisopliae* var. *acridum* from acridoid hosts possess a pantropical distribution and have been recorded in Africa (Bateman *et al.*, 1996; Shah *et al.*, 1997), Australia (Milner & Hunter, 2001), Madagascar (Delgado *et al.*, 1997a), Brazil (Magalhães *et al.*, 1997), Mexico (Hernández-Velázquez *et al.*, 1997), and the Galapagos Islands (Prior, 1997).

Isolates of *M. anisopliae* var. *acridum* have been investigated and developed as biocontrol agents in Africa under the name of Green Muscle® (Douthwaite *et al.*, 2000)

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and as Green Guard® in Australia (Milner & Hunter, 2001) for control of acridids.

Registration has also been secured in Madagascar (Lomer *et al.*, 2001), and field trials

have been conducted in Brazil (Magalhães *et al.*, 2000b). Currently, there are no

Metarhizium-based products approved for grasshopper control in North America;

however, *M. anisopliae* var. *acridum* is under consideration as a potential alternative to

chemical insecticides for grasshopper control in Canada.

Prior to the introduction of a pathogen into a new environment, risks to non-target

organisms must be demonstrated as acceptable and the environmental fate of the

pathogen determined (Bidochka, 2001). Extensive studies have focused on the effects of

exposure on non-target organisms. Smits *et al.* (1999) found no significant pathological

or behavioural effects in ring-necked pheasant chicks (*Phasianus colchicus*) exposed to

M. anisopliae var. *acridum* in contaminated food sources. Field application of the

pathogen at recommended rates is not expected to pose any risks to fringe-toed lizards

(*Acanthodactylus dumerili*) based on pathological and behavioural examination of treated

lizards exposed to high challenge concentrations (Peveling & Demba, 2003). *M.*

anisopliae var. *acridum* was found non-pathogenic to non-target arthropods in the

families Coccinellidae, Tenebrionidae, Carabidae, Formicidae, and Ephydridae (Peveling

& Demba, 1997; Peveling *et al.*, 1999; Danfa & van der Valk, 1999) in addition to non-

target scavengers and locust predators (Arthurs *et al.*, 2003; Peveling & Demba, 1997).

Conversely, laboratory studies demonstrated susceptibility of parasitoids of acridids to

standard dose rates under simulated field conditions, although it was concluded that *M.*

anisopliae var. *acridum* posed a low risk due to a lack or low levels of infection (Stoltz *et*

al., 2002; Danfa & van der Valk, 1999). A simulated field dose formulated in oil resulted

in 11% mortality of exposed bees (*Apis mellifera*) under laboratory conditions; however,

application of a chemical pesticide at a dose that was just sub-lethal to locusts caused

100% mortality (Ball *et al.*, 1994). No adverse effects from exposure to doses almost one

hundred times greater than would be expected in field applications were recorded on

rainbow fish fry (*Melanotaenia duboulayi* Castelnau) or mayfly nymphs (*Ulmerophlebia*

sp.) housed in artificial water containers whereas significant mortality was observed in

cladocerans (*Ceriodaphnia dubia*) treated with high doses of *M. anisopliae* var. *acridum*.

When doses were lowered to rates comparable to realistic field operational conditions,

however, cladoceran mortality decreased to less than significant levels (Milner *et al.*,

2002). Further, additional samples of actual water sources in spray areas revealed low

levels of the entomopathogen; thus it was concluded that the level of conidia likely to be

present in affected aquatic ecosystems would be unlikely to pose any hazard to the

indigenous fauna.

In an analysis of published reports of the best-documented form of biological

control (of arthropods by arthropods), it has been estimated that only around ten percent

of attempts have been successful by definition of complete control of the target pest by

the established biocontrol agent where no other control methods were required or used

(Gurr & Wratten, 2000; Greathead & Greathead, 1992). The use of *M. anisopliae* var.

acridum as a biocontrol agent for acridids in Africa has been considered a qualified

success in terms of technical efficacy and public benefit (Gelernter & Lomer, 2000). In

addition to the studies on non-target organisms, a wealth of information generated by

global research (Lomer *et al.*, 2001) has been attained about the taxonomy, biology, and

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effect of environmental abiotic factors on the entomopathogen. A summary of results and knowledge available in the scientific literature follows later in this chapter.

Current methods of diagnosis of *M. anisopliae* var. *acridum* in infected insects depend upon development of fungal growth and sporulation in cadavers. Confirmatory tests include observation of morphological features on culture media, microscopic examination of spores and associated structures, and bioassay of target hosts. Difficulties in identification may be encountered due to observed variation of spore morphology within the same culture and between isolates (Milner *et al.*, 2003; Glare *et al.*, 1996). Further, Lomer *et al.* (2001) noted that *M. anisopliae* var. *acridum* cannot be distinguished from other *M. anisopliae* on the basis of spore size and shape.

Molecular probes have been developed for the specific detection of *M. anisopliae* var. *acridum*. Bidochka *et al.* (1994) used a random amplified polymorphic DNA (RAPD) fragment as a probe to differentiate acridid isolates of *M. flavoviride* (later renamed as *M. anisopliae* var. *acridum*) from acridid isolates of *M. anisopliae*. Inglis *et al.* (1999) applied telomeric fingerprinting to differentiate between *M. flavoviride* strains. Distinct telomeric fingerprints of acridoid *M. flavoviride* isolates were produced when genomic fungal DNA was hybridized with radiolabelled probe DNA prepared from a digest fragment of a plasmid carrying a repeated hexanucleotide telomere sequence. Application of both studies was limited due to a requirement for DNA extracted from axenic cultures.

Numerous studies on mycopathogens of insects and plants have targeted the internal transcribed spacer (ITS) region for development of assays for specific detection (Tymon *et al.*, 2004; Salazar *et al.*, 2000; Martínez-Culebras *et al.*, 2000; Mishra *et al.*,

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2000). The DNA coding for the ribosomal RNA gene complex (rDNA) consists of genes

encoding the 16S, 5.8S, and 28S fungal rRNA subunits that are separated by ITS

sequences (Freifelder, 1983). The region that separates the 16S from the 5.8S subunit is

referred to as ITS1; ITS2 separates the 5.8S from the 28S subunit. The section of DNA

that encompasses the 16S-ITS1-5.8S-ITS2-28S complex is defined as a repeat unit.

Multiple copies (up to 220), separated by nontranscribed spacer sequences, of the repeat

unit occur in the fungal ribosomal genome (Pipe *et al.*, 1995), a characteristic that makes

this region of DNA ideal as a target for molecular characterization. Other suitable

characteristics include high stability of rDNA as well as the combination of conserved

sequences in the 16S, 5.8S, and 28S subunits and divergence of sequences coding for the

ITS regions (White *et al.*, 1990). One of the objectives of this thesis was to analyze

sequence divergence within the ITS regions of the *Metarhizium* genome for the potential

for development of a molecular assay to differentiate an introduced strain of *M.*

anisopliae var. *acridum* from native populations of *M. anisopliae* and *M. flavoviride* in

environmental samples. Additional objectives are introduced in section 1.4.

1.1   *Metarhizium* taxonomy

1.1.1   Taxonomy based on morphological characterization

The genus *Metarhizium* consists of a number of species complexes that are found

worldwide and have been documented in more than two hundred species of seven orders

of insects (Veen, 1968), but there has been limited success in correlation of association

with either insect host or geographic origin. Early approaches to taxonomy relied upon

morphological characterization at the macro- and microscopic level. Metschnikoff

(1879) was the first to describe spores of *Entomophthora anisopliae*. In 1883, Sorokin

renamed the fungus *Metarhizium anisopliae*. *Metarhizium* is the current name for the

genus, although from 1880 to 1969 at least eight other names were proposed for the

genus alone (Tulloch, 1976). The nomenclature was further confounded with the

introduction of an incorrect spelling of *Metarrhizium* into numerous publications. Lack

of museum specimens of sufficient quality has lead to the removal, and later

reinstatement, of a second species *M. album* (Petch, 1931; Rombach *et al.*, 1987). *M.*

brunneum, first described by Petch (1935), was later determined to be *M. anisopliae* by

Roberts (1967) when irradiation of green spores of *M. anisopliae* produced brown

coloured mutants resembling *M. brunneum*. Insofar as no microscopic differences could

be found between *M. brunneum* and *M. anisopliae*, it was concluded that *M. brunneum*

was a naturally occurring mutant of *M. anisopliae*. In 1915, Johnston proposed the

classification of *M. anisopliae* into long- and short-spored forms. Gams and Rozsypal

(1973) described a third species *Metarrhizium flavoviride* isolated from insects and soil.

A short-spored form, *M. flavoviride* var. *minus*, was described by Rombach *et al.* (1986).

Other species have been proposed in China and Japan, but lack of material deposited in

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culture collections has led to difficulties for additional morphological and phylogenetic

analyses (Tzean *et al.*, 1993; Liang *et al.*, 1991; Shimazu, 1989; Guo *et al.* 1986).

1.1.2 Taxonomy based on molecular characterization

1.1.2.1 Random amplification of polymorphic DNA (RAPD)

Based on the numerous difficulties described previously, the taxonomic

classification of fungi has profited enormously from the possibilities offered by

molecular characterization, and this has been particularly true for *Metarhizium*. The

genetic diversity of isolates has been examined by RAPD analysis. RAPD uses a single

primer of arbitrary nucleotide sequence to amplify genomic sequences wherever the

primer finds regions of sufficient homology by polymerase chain reaction (PCR), thus no

prior knowledge of the genome to be analyzed is required. Fegan *et al.* (1993) found

considerable genetic diversity in Australian isolates that correlated weakly with

geographical location. The existence of high genetic diversity in Brazilian strains was

confirmed, but less variability was found in insect isolates in comparison with strains

from soil, suggesting that the fungus had developed a certain degree of host specificity

(Fungaro *et al.*, 1996; Tigano-Milani *et al.*, 1995). Bidochka *et al.* (1994) were unable to

demonstrate association with host or geographic origin in acridid isolates of *M.*

flavoviride, but did find evidence of high variability in acridid isolates of *M. anisopliae*.

In addition to confirmation of high DNA variability within isolates sampled throughout

the world, Cobb and Clarkson (1993) were able to differentiate *M. anisopliae* from *M.*

flavoviride, as well as establish a loose correlation of *M. anisopliae* with geographic

origin (Australia, the Caribbean and Asia) although there was no association with insect

host. Differentiation between isolates from different geographical locations and also

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between isolates from the same country was possible, but whereas isolates from

Australia, Brazil and the Philippines (*Oryctes rhinoceros*) were distinct, those from

Finland and *Nilaparvata lugens* in the Philippines showed similar profiles (Leal *et al.*,

1994).

1.1.2.2 Random fragment length polymorphism (RFLP)

Intraspecific variation in *Metarhizium* from various geographical locations and

insect hosts was also analyzed by RFLP. RFLP is a method that generates restriction

endonuclease digestion patterns of total or specific (e.g. mitochondrial) DNA. Analysis

of the derived patterns can assist with differentiation between species. Once again,

considerable DNA heterogeneity was demonstrated with this method. Pipe *et al.* (1995)

were able to group some *Metarhizium anisopliae* isolates with geographical origin, but

obtained poor differentiation of isolates from the same geographical region. Insufficient

numbers of isolates from the same geographic region but different insect hosts did not

allow investigation of any correlation with insect host. Mavridou and Typas (1998)

found that differences in intraspecific and interspecific variation were inadequate to allow

for any correlation with either insect host or geographic origin.

1.1.2.3 Amplified fragment length polymorphism (AFLP)

Using an alternative molecular analysis, Leal *et al.* (1997) performed

amplification and restriction endonuclease digestion of the major protease Pr1 gene to

characterize *Metarhizium* strains. AFLP is similar to RAPD, except that the PCR primers

in AFLP are complementary to specific adaptor sequences that have been ligated to the

ends of the digestion fragments. With this technique, the digest patterns of forty global

Metarhizium strains were clustered into four groups, with some of the groups correlated

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to geographic origin. Strains from the same country displayed more similarities than those from different countries. There appeared to be no correlation with host, except for some strains which had been isolated from orthopteran hosts.

1.1.2.4 Isoenzyme polymorphism analysis

Rakotonirainy *et al.* (1994) used electrophoretic banding patterns of seven enzymes to demonstrate the distinctiveness of *M. anisopliae* strains from New Zealand, suggesting a different genetic evolution of *Metarhizium* for these isolates, either through selective pressure by host or by environment. Banding patterns also permitted differentiation of isolates of *M. anisopliae* var. *majus* from additional strains of *M. anisopliae* var. *anisopliae*; however, there was no association with geographic region.

Bridge *et al.* (1997) applied a combination of isoenzyme analysis, RAPD PCR, and protease production to examine the relationships of thirty strains of *Metarhizium* from twenty-three countries. Isoenzyme analyses of catalase and propionyl-esterases gave distinct banding patterns with isolates from acridid or pyrgomorphid hosts. Principal Coordinate analysis of RAPD PCR results also showed isolates from acridid or pyrgomorphid hosts to be clearly separated as a distinct group. Further, in the same study, the strongest protease (elastase and chymoelastase) activity was demonstrated by acridid and pyrgomorphid isolates compared to those from other hosts, with one cercopid exception. They used their data to support the differentiation of *M. flavoviride* into 3 groups: 1) original isolates of *M. flavoviride* var. *flavoviride* from coleopteran hosts and soil in northern Europe, 2) isolates of *M. flavoviride* var. *minus* from homopteran hosts in Southeast Asia and 3) those isolates from acridoids in Africa (including Madagascar),

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Australia and the Galapagos. However, it should be noted that some other isolates of *Metarhizium*, not classified with the group 3 isolates, are pathogenic to acridoids.

1.1.2.5 Ribosomal RNA (rRNA) and DNA (rDNA) sequence analysis

In conjunction with their study on isoenzyme comparison, Rakotonirainy *et al.* (1994) used phylogenetic analyis of partial 28S rRNA sequences to confirm separation of *M. flavoviride* from *M. anisopliae*. The sequences clustered isolates into three groups: 1) *M. flavoviride*, 2) New Zealand strains of *M. anisopliae*, and 3) *M. anisopliae*. In the third cluster, there was a distinct division between strains of *M. anisopliae* var. *majus* and *M. anisopliae* var. *anisopliae*.

More recently, Driver *et al.* (2000) used rDNA sequences to assign representative isolates to ten separate clades. Acridoid isolates, most previously identified as *M. flavoviride* on the basis of conidial and phialide morphology, clustered as a distinct taxonomic group proposed as *M. anisopliae* var. *acridum*. Further, one of the proposed clades (*M. flavoviride* var. *novazealandicum*) incorporated a particular genotype of isolates from insects and soils in Australia and New Zealand. Table 1 outlines a simplified chronological history of the taxonomy of *Metarhizium*.

Although not all proposed clades have been universally accepted, it is generally agreed that there are three areas of consensus with regards to the taxonomy of *Metarhizium*: 1) *M. anisopliae* has a monophyletic evolution based on ITS and 28S rDNA sequence data, 2) *M. anisopliae* and *M. flavoviride* are related but can be distinguished with the use of molecular markers, and 3) genetically distinguishable subgroups exist within *M. anisopliae* and *M. flavoviride* (Bidochka & Small, 2005).

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Table 1-1. Chronological history of *Metarhizium* taxonomy

M. album	M. anisopliae	M. flavoviride	Reference
	Initially identified as *Entomophthora anisopliae*		Metschnikoff, 1879
	Renamed as *Metarhizium anisopliae*		Sorokin, 1883
	Recognition of long- and short-spored forms (forma *major* and forma *minor*)		Johnston, 1915
Recognition as authentic species			Petch, 1931 Rombach *et al.*, 1987
	M. brunneum identified as new species by Petch, later renamed *M. anisopliae* based on morphological comparison and exposure to gamma irradiation		Petch, 1935 Latch, 1965 Roberts, 1967
		Recognition of new species	Gams & Rozsypal, 1973
	Recognition of *M. anisopliae* (Metsch.) Sorok. var. *anisopliae* and *M. anisopliae* (Metsch.) Sorok. var. *majus* (Johnston) *comb. nov.*		Tulloch, 1976
		Recognition of new variety, *M. flavoviride* var. *minus*	Rombach *et al.*, 1986
	Recognition of genetic variability in this species but acknowledgement of homogeneity of *M. anisopliae* var. *majus* based on isozyme analysis		Riba *et al.*, 1986 St. Leger *et al.*, 1992

The hatched line represents the determination of taxonomy by morphological observation (above) and molecular characterization (below).

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Table 1-1 con't.

M. album	M. anisopliae	M. flavoviride	Reference
	Confirmation of high genetic variability by RAPD analysis		Fegan et al., 1993 Bidochka et al., 1994 Fungaro et al., 1996
	Recognition of subgroups, loosely correlated with geographical origin, by RAPD analysis and restriction endonuclease digestion of a protease gene		Cobb & Clarkson, 1993 Leal et al., 1994 Leal et al., 1997
		Low intraspecific variability in isolates virulent to acridids	Cobb & Clarkson, 1993 Bidochka et al., 1994
		API ZYM, isoenzyme, and RFLP banding patterns permit differentiation of M. flavoviride from M. anisopliae	Bridge et al., 1993
	Recognition of two subgroups with no correlation to geographic origin or insect host		Curran et al., 1994
Differentiation of M. album, M. anisopliae var. anisopliae, M. anisopliae var. majus, and M. flavoviride by analysis of rDNA sequences, rRNA sequences and isozyme profiles, and RFLPs			Curran et al., 1994 Rakotonirainy et al., 1994 Pipe et al., 1995 Mavridou & Typas, 1998
		Recognition of acridid isolates as a single, distinctive genotype with a pantropical distribution	Bridge et al., 1997
Recognition as a single distinct clade	Recognition of four distinct clades: M. anisopliae var. anisopliae, M. anisopliae var. majus, M. anisopliae var. lepidiotum var. nov., M. anisopliae var. acridum var. nov.	Recognition of five distinct clades: M. flavoviride var. flavoviride, M. flavoviride Type E, M. flavoviride var. minus, M. flavoviride var. novazealandicum var. nov.	Driver et al., 2000

Table 1-1 con't.

M. album	*M. anisopliae*	*M. flavoviride*	Reference
	Recognition of three subgroups of *M. anisopliae* var. *anisopliae* by IGS sequence analysis		Pantou *et al*, 2003
Proposal that all three currently recognized species be reduced to a single species *M. anisopliae*, further defined as varieties, genetic groups, cryptic species as additional phylogenetic and phylogeographic data are obtained.			Milner *et al.*, 1994 Bidochka & Small, 2005

Further consensus and correlation of molecular, morphological and ecological data will require examination of additional isolates.

1.2 The fungal infection process

The infectious propagule of *Metarhizium* is the uninuclear conidium, a fungal mitospore formed externally on a conidiogenous cell (Humber, 1997). Pathogenesis involves the following stages: recognition and spore attachment to host, germination and penetration of integument, growth and proliferation within host, and re-emergence from host followed by conidial production (Zacharuk, 1973).

1.2.1 Recognition and spore attachment to host

The process by which a spore recognizes an appropriate host has not yet been fully elucidated. Butt *et al.* (unpublished results in Butt, 2002) have observed a complex signalling apparatus (G-proteins, receptors, kinases, and secondary messengers) in some entomogenous fungi. Wang and St. Leger (2005) identified fungal transcriptional patterns induced by recognition of host-specific topography and chemical components displayed or released by the host. Attachment of the conidium to host cuticle is mediated through non-specific hydrophobic interaction between conidial rodlets and the waxy surface of the insect cuticle (St. Leger, 1993; Boucias *et al.*, 1988).

1.2.2 Germination and penetration of integument

Before production of a germ tube can be initiated, the spore must overcome fungistatic or toxic compounds present in the insect cuticle (Samuels & Reynolds, 2000; Sosa-Gómez *et al.*, 1997; St. Leger, 1991). Successful germination is also dependent upon appropriate humidity, available nutrients and surface topography (Dillon & Charnley, 1990; St. Leger *et al.*, 1994, 1991). Ibrahim *et al.* (1999) determined that

M. anisopliae conidia require water activity > 0.98 (=98% RH) for germination, irrespective of aqueous or oil-based formulations. Conversely, Bateman *et al.* (1993) demonstrated infectivity of desert locusts (*Schistocerca gregaria*) by an oil formulation of *M. flavoviride* at 35% RH, suggesting that the microclimate humidity of the insect cuticle was more of a determinant than ambient relative humidity for germination. Wang and St. Leger (2005) observed that *M. anisopliae* var. *acridum* conidia germinated on *S. gregaria*, however, germination on beetles or hemipteran bugs was either repressed or occurred with low levels of differentiation.

Penetration through the cuticle is accomplished by production of an appressorium, a specialized structure at the apex of the germ tube (St. Leger *et al.*, 1989; Zacharuk, 1970a). Formation of the appressorium is influenced by surface topography, with preference for hard, smooth surfaces (St. Leger *et al.*, 1991; St. Leger *et al.*, 1989). Penetration pegs produced by the appressorium enter the cuticle, usually at intersegmental folds, with the aid of mechanical pressure and cuticle-degrading enzymes including proteases, chitinases, lipases, esterases and phosphatases (Freimoser *et al.*, 2003; Gillespie *et al.*, 1998; St. Leger *et al.* 1996; Bidochka & Khachatourians, 1994; Zacharuk, 1970b).

1.2.3   Growth and proliferation within host

Once the fungus has entered the haemocoel, colonization is dependent upon the ability of the fungus to overcome a combination of cellular and humoral responses that comprise the host immune system (Gillespie *et al.*, 2000). Fungal cytotoxic compounds are produced by blastospores, free-floating yeast-like cells produced as hyphae bud within the haemocoel (Zacharuk, 1971). The production of secondary fungal metabolites,

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primarily destruxins A, B and E, by isolates of *M. flavoviride* has been demonstrated *in vitro* (Amiri-Besheli *et al.*, 2000). In contrast, Kershaw *et al.* (1999) could only detect destruxins in early adults of *S. gregaria* that were infected in a bioassay with an Australian isolate of *M. anisopliae* var. *acridum*; destruxins were not detected with isolates from Africa or the Galapagos Islands. Destruxins have been shown to possess immunomodulatory ability (Vilcinskas *et al.*, 1997; Vey *et al.*, 1995; Huxham *et al.*, 1989) as well as disrupt normal cell metabolism (James *et al.*, 1993). Expression of genes involved in stress response, detoxification and transmembrane transport in *M. anisopliae* var. *acridum* (Freimoser *et al.*, 2003) is likely induced by the host humoral response.

Subsequent to successful suppression of the host immune response, colonization of the haemocoel is completed and the insect succumbs. The reasons for host mortality are not yet fully understood, but may be due to a combination of mechanical damage to internal organs, nutrient depletion and/or toxicosis (Gillespie & Claydon, 1989).

1.2.4   Re-emergence from the host and conidiation

Under favourable environmental conditions, mycelial growth resumes and hyphae emerge soon after host death to colonize the cadaver surface. Hyphal differentiation into conidiogenous cells occurs and concomitant sporulation completes the infection process. At this point, abiotic factors are the main determinants in persistence of the infectious propagule in the environment.

1.3   Abiotic factors affecting persistence of *M. anisopliae* var. *acridum*

The growth and survival of fungi are determined primarily by abiotic factors including temperature, solar radiation and humidity. Other meteorological factors such

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as rainfall and wind contribute to the efficacy and dispersal of the entomopathogen in the

field, but the impact of weather on the effectiveness of *M. anisopliae* var. *acridum* has

not yet been widely studied. It should be noted that most data on abiotic factors have

been acquired from laboratory results, thus, generalization of pathogen survival observed

under controlled conditions must be verified or refuted by field experimentation (Ignoffo,

1992). Further, environmental parameters interact in their impact on entomopathogens

(Inglis *et al.*, 2001).

1.3.1 Temperature

Temperature acts not only on the germination of fungal spores and hyphal

development, but also on the speed and quantity of sporulation (Benz, 1987). Ambient

temperatures in most agroecosystems range from about 10 to 40 °C during the growing

season (Ignoffo, 1992). Many studies have determined that *M. anisopliae* var. *acridum*

would not be adversely affected by most temperatures within this range. The optimum

temperature for growth for *M. anisopliae* var. *acridum* has been shown to be around 28 to

30 °C, with some degree of growth noted over a range from 15 to 35 °C, although an

isolate from Madagascar has demonstrated the ability to grow at 38 °C (Welling *et al.*,

1994). Thomas and Jenkins (1997) observed optimal temperatures of *ca.* 30 °C for Green

Muscle® and another isolate of *M. anisopliae* var. *acridum*. However, the optimal

temperatures for hyphal extension and conidial yield on Sabouraud-dextrose agar (SDA)

were 27 °C and 25 °C, respectively, for Green Muscle® and 25.5 °C and 24 °C,

respectively, for isolate I91-609. It was noted that conidial production rose steeply with

increasing temperatures towards the optimum followed by a more gradual decline as

temperatures increased above the optimum. They also determined that the optimal

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temperature for infection of *Zonocerus variegatus* under controlled environmental conditions was *ca*. 30 °C for both isolates. In a study of vegetative growth on a semi-synthetic medium, Ouedraogo *et al*. (1997) demonstrated growth of several *M. flavoviride* isolates over a range of 8 to 35 °C. No isolates grew at 37 °C. Most isolates had optimal growth at 28 to 30 °C, but a few fell in the range of 25 to 28 °C. An optimal temperature of 30 °C for conidial germination was shown by Fargues *et al*. (1997); however, while Green Guard® still exhibited 98 % germination after 48 hours at 37 °C, Green Muscle® displayed only 11 % germination for the same temperature and duration. No germination was observed at 40 °C. In a comparison of isolates from Australia and Mexico, Milner *et al*. (2003) showed that all had optimal growth at 28 °C on SDA but found significant differences in growth rates, with the most rapid rate displayed by one of the Mexican isolates. All isolates showed little or no growth at 36 °C and slow growth at 16 °C. High mortality in wingless grasshopper, *Phaulacridium vittatum*, from varying doses was demonstrated in a laboratory bioassay over a range of temperatures from 20 to 35 °C, but sporulation occurred only at high doses at 20 °C, as well as at all doses at 25 °C and 30 °C. At 35 °C, one of the Mexican isolates was the only one to produce substantial sporulation. Arthurs and Thomas (2001a) found similar results with maximum conidiogenesis in cadavers in contact with a damp surface at 25 °C. Little sporulation occurred at 15 °C or 40 °C.

Studies have also focused on the thermal tolerance of *M. anisopliae* var. *acridum* conidia in oil formulations. McClatchie *et al*. (1994) observed a slight decrease in germination in oil-formulated conidia exposed to 60 °C for five hours. Even after five hours at 80 °C, 10 % of the exposed conidia were still capable of germination. A longer

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storage time at a lower temperature (six weeks at 55 °C) resulted in 20 % survival of dried conidia. Morley-Davies *et al.* (1995) demonstrated germination rates greater than 75 % after ninety days storage at temperatures ranging from -10 to 40 °C for Green Muscle[®] for both oil and dry powder formulations. Dry formulations generally outperformed oil formulations at all temperatures tested. Rangel *et al.* (2005) determined the conidial thermotolerance for Green Guard[®]. Germination of a conidial suspension in an aqueous solution was 90 % after twelve hours exposure to 45 °C. The median lethal dose (LD_{50}) was calculated as 49.4 °C for two hours.

Additional studies have investigated the effects of fluctuating temperature regimes on virulence in bioassays of acridids. Inglis *et al.* (1999) found a mean lethal time of 4.8 days for grasshopper nymphs infected with a Brazilian isolate of *M. flavoviride* under a regime of 20/30 °C (12 h:12 h) compared to 5.4 days for those incubated at a constant 25 °C. Both regimes produced *ca.* 80 % external colonization of cadavers compared to 24 % external colonization for infected nymphs that had been incubated under a regime of 10/40 °C (12 h:12 h). Welling *et al.* (1994) determined a median lethal time of 8.55 days for locust nymphs inoculated with an isolate of *M. flavoviride* from Madagascar and incubated at 30/25 °C (8 h:16 h) versus 6.83 days for nymphs exposed to 36/25 °C (8 h:16 h).

1.3.2   Solar radiation

Ignoffo (1992) declared natural sunlight to be the most destructive environmental factor affecting the persistence of entomopathogens. It has been determined that wavelengths in the 285 to 315 nm range (UV-B) are the most damaging to fungi. Morley-Davies *et al.* (1995) exposed oil formulations of *M. flavoviride* conidia to

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simulated sunlight for up to twenty-four hours at 40 °C to approximate conditions that

would exist at the equator. The impact on conidial germination after 24 hours exposure

was severe, with germination reduced to 28.6 % from 82.2 % for unexposed Green

Muscle® conidia and a decline from 69.4 % to 31 % for Green Guard®. After only eight

hours exposure, germination for Green Muscle® declined to 39.4 %. Fargues *et al.*

(1996) found isolates of *M. flavoviride* to be the most resistant, followed by *Beauveria*

bassiana, *M. anisopliae* and *Isaria* (formerly *Paecilomyces*) *fumosorosea*, to irradiation

by artificial sunlight. Green Muscle® was reduced to 5.2 % surviving colony forming

units (CFU) relative to a non-irradiated control, whereas Green Guard® declined to 11.1

% surviving CFU following eight hours of irradiation. As with the study by Morley-

Davies *et al.* (1995), Fargues *et al.* (1996) observed that Green Guard® conidia were

more resistant to simulated radiation than Green Muscle®, thus demonstrating

intravarietal differences. Braga *et al.* (2001a) compared the effects of full-spectrum

sunlight and UV-A radiation (320-400 nm) exposure on Green Guard® conidia. The

exposures were performed in Utah under naturally occurring sunlight and different filter

combinations. Radiation was recorded as irradiance measurements. Relative to full-

spectrum sunlight, conidia showed less decline in mean relative percent culturability (*ca.*

90 %) from four hours exposure to UV-A radiation compared to 70-80 %. Measurements

of mean relative percent germination over a period of 48 hours after four hours exposure

to either full-spectrum sunlight or UV-A radiation revealed no significant difference from

unexposed controls; however, there was a significant delay in germination when

measured over 24 hours for both spectra. Moore *et al.* (1996) found that temperature

influenced germination decay curves for oil formulations of Green Muscle® conidia

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exposed to natural solar irradiation in Niger. Minimal loss of germination was experienced at 5 °C and 10 °C during the first two hours of exposure, followed by a decline in the subsequent two hours. Conversely, germination was reduced by half at temperatures of 40 °C and above, with less than one hour exposure.

Some studies have looked at formulation composition as a source of stability enhancement in a natural setting. Moore et al. (1993) discovered that oil formulations of *M. flavoviride* showed 36.5 % germination over a 24-hour period after one hour exposure to UV from a solar simulator compared with 4.7 % germination of conidia in water. Addition of a sunscreen (oxybenzone) to an oil-based formulation resulted in 81.9 % conidial germination after three hours exposure compared with 28.1 % in a formulation without sunscreen. In contrast, Shah et al. (1998a) found no significant differences between field treatments of oil formulations of *M. flavoviride* conidia that contained oxybenzone and those without. Hunt et al. (1994) could not corroborate the findings of Moore et al. (1993) in an analysis of the protective effects offered by several sunscreens. None of the sunscreens tested, including oxybenzone, offered significant protection for oil-formulated conidia after five hours exposure to UV from a solar simulator.

1.3.3 Humidity

Free water is a critical element required for fungal spore germination and sporulation on mycosed cadavers. Whereas water availability in the microclimate of the host cuticle is more of a determinant for conidial germination than ambient relative humidity, conidiogenesis on cadavers is dependent on high moisture in the surrounding environment. Ibrahim et al. (1999) determined that conidia of *M. anisopliae* required water activity > 0.98 (= 98 % RH) for germination. The type of formulation (oil or

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aqueous) did not influence this requirement. They also demonstrated ability of water to diffuse from the haemocoel through a locust cuticle through to the surface, presumably via pore channels. Magalhães *et al.* (2000a) observed that *M. anisopliae* var. *acridum* sporulated internally within the grasshopper *Rhammatocerus schistocercoides* under ambient humidities of 53 % and 75 %; however, there was no external sporulation for either condition. Arthurs and Thomas (2001a) found conidial yield to be closely related to the water content of locust cadavers. In a simulation of overcast/wet weather conditions at field sites in the Sahel and South Africa, *S. gregaria* cadavers were exposed to a fluctuating regime of 25 °C/100 % RH and 40 °C/ 80 % RH (12 h: 12 h). Sporulation under this regime was less than that yielded by cadavers held at 25 °C/100 % RH and 40 °C/100 % RH, evidence that conidial yield was reduced by periodic exposure to low humidity. Fargues *et al.* (1997) found that a range of 13 to 100 % ambient humidity had no effect on cumulative mortality, cumulative mycosis or median lethal time in locusts treated with *M. flavoviride*. An estimated LT_{50} of five days was established by Bateman *et al.* (1993) for locusts inoculated with an oil formulation of *M. flavoviride* and incubated at 35 % RH. Death due to mycosis was confirmed by incubation of cadavers under high humidity to permit fungal outgrowth.

1.4   Objectives

As part of the initial studies necessary for the implementation of a biological control plan, the research in this thesis involved (i) the development of a molecular assay for the specific detection of *M. anisopliae* var. *acridum* and analysis of the efficacy of the assay for detection of the fungus in infected grasshoppers and spiked soil samples (Chapter 2), (ii) a survey of southern Alberta soils to determine natural incidence of

Metarhizium (Chapter 3), (iii) bioassay of grasshoppers with native isolates of

Metarhizium to determine potential virulence (Chapter 4), and (iv) evaluation of

historical weather and climate data in the Prairie provinces to estimate *M. anisopliae* var.

acridum efficacy in a temperate regime (Chapter 5). The research described within will

provide information for the determination of an integrated pest management strategy

applicable to current local conditions, and under a range of weather scenarios.

Chapter 2.   Development and Validation of a PCR Assay for the Specific Detection of *M. anisopliae* var. *acridum* *

2.0   Introduction

Comparative studies of nucleotide sequences of rRNA genes have provided significant data for analysis of phylogenetics and taxonomy (White *et al.*, 1990). Ribosomal DNA is present in multiple copies in the fungal genome and is thereby a preferred choice over single-copy genes for PCR amplification. White *et al.* (1990) introduced the use of PCR methods for amplification of the ITS region in nuclear rDNA of the fungal genome. The ITS sequences of the rDNA region are an ideal target for the development of species-specific primers because they evolve relatively rapidly and are highly variable in length and nucleotide content between closely related species and sometimes within a species as has been demonstrated for the genus *Metarhizium*. Driver *et al.* (2000) showed 14 to 18 % nucleotide divergence for the ITS region between the morphologically defined species of *M. album*, *M. anisopliae* and *M. flavoviride*, and up to 5 % divergence between recognized varieties within a species.

Sequence data from the distinct ITS rDNA regions for *M. anisopliae* var. *acridum* were analyzed for primer development for a PCR assay capable of specific detection of this entomopathogen. The objective was to develop a pair of primers that could differentiate *M. anisopliae* var. *acridum* from native isolates of *M. anisopliae* var. *anisopliae* and *M. flavoviride*. The assay was also required to detect specific *Metarhizium* DNA from a soil matrix and from infected grasshoppers.

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2.1   Materials and Methods

2.1.1   Fungal isolates and cultivation

The fungal isolates used in this study are listed in Table 2-1. All were propagated and maintained on potato dextrose agar (PDA). *M. anisopliae* var. *acridum* (IMI 330189; commercialized as Green Muscle® by the Lutte Biologique Contre les Locustes et Sauteriaux [LUBILOSA] programme) was obtained from the International Institute of Tropical Agriculture (IITA, Benin). *M. anisopliae* var. *anisopliae* isolates 421 and 4450 and other fungi coded as UAMH were obtained from the University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada. *Metarhizium* isolates coded as ARSEF were obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA. Those coded as LRC and *Isaria* (formerly *Paecilomyces*) *fumosorosea* (PFR-97) were obtained from the Lethbridge Research Centre (LRC), Canada. *Metarhizium anisopliae* var. *acridum* SP9 and *Beauveria bassiana* (GHA 726) were previously obtained from Mycotech Corporation, Butte, MT, USA. *M. anisopliae* var. *acridum* FI-985 (commercialized as Green Guard®) was procured from Bio-Care Technology Pty Ltd., Somersby, Australia.

2.1.2   Fungal DNA isolation

The procedure of Cenis (1992) was used for fungal DNA extraction. Briefly, hyphae were used to inoculate 500 µl of potato dextrose broth in a 1.5 ml Eppendorf tube. Following 3-5 days incubation at 25 °C, the mycelial mat was pelleted by centrifugation for 5 min at 16 000 X g, washed with 500 µl 10 mM Tris-HCl, 1 mM EDTA, pH 8 (TE), and pelleted again. The TE was decanted and 300 µl of 200 mM Tris-

Table 2-1. List of isolates studied

Isolate code[a]	Name[b]	Host	Country of origin
Metarhizium spp.:			
IMI 330189	*Metarhizium anisopliae* var. *acridum*	*Ornithacris cavroisi* (Orthoptera: Acrididae)	Niger
SP9	*M. anisopliae* var. *acridum*	*Locusta migratoria capito* (Orthoptera: Acrididae)	Madagascar
FI 985	*M. anisopliae* var. *acridum*	*Austracris guttulosa* (Orthoptera: Acrididae)	Australia
ARSEF 3391	*M. anisopliae* var. *acridum*	*Zoonocerus elegans* (Orthoptera: Pyrgomorphidae)	Tanzania
ARSEF 6421	*M. anisopliae* var. *acridum*	*Kraussaria angulifera* (Orthoptera: Acrididae)	Senegal
ARSEF 437	*M. anisopliae* var. *anisopliae*	*Teleogryllus commodus* (Orthoptera: Gryllidae)	Australia
ARSEF 727	*M. anisopliae* var. *anisopliae*	Unidentified tettigonid (Orthoptera: Tettigoniidae)	Brazil

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Table 2-1 con't.

Isolate code[a]	Name[b]	Host	Country of origin
Metarhizium spp.:			
UAMH 421	*M. anisopliae* var. *anisopliae*	Unidentified insect larvae	USA
UAMH 4450	*M. anisopliae* var. *anisopliae*	Soil	Canada
S54	*M. anisopliae* var. *anisopliae*	Soil	Canada
6W-2	*M. anisopliae* var. *anisopliae*	Soil	Canada
11S-1	*M. anisopliae* var. *anisopliae*	*Galleria mellonella* (Lepidoptera: Pyralidae)	Canada
ARSEF 1184	*M. flavoviride* Gams & Rozsypal	*Otiorhynchus sulcatus* (Coleoptera: Curculionidae)	France
ARSEF 2023	*M. flavoviride* var. *minus*	Unidentified acridid (Orthoptera: Acrididae)	Galapagos Islands

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Table 2-1. con't.

Isolate code[a]	Name[b]	Host	Country of origin
Other isolates:			
GHA 726	*Beauveria bassiana*	*Melanoplus sanguinipes* (Orthoptera: Acrididae)	USA
UAMH 4756	*Colletotrichum gloeosporioides* (telomorph *Glomerella cingulata*)	*Laeliocattleya* sp.	Canada
UAMH 1656	*Emericella nidulans*	Feed	Canada
LRC 2111	*Fusarium oxysporum*	Soil	Canada
LRC 2087	*Clonostachys rosea* f. *catenulata*	Soil	Canada
UAMH 772	*Hydropisphaera peziza*	Soil	Canada
UAMH 2876	*Isaria farinosa*	Soil	Canada
PFR 97	*I. fumosorosea*	*Phenacoccus solani* (Homoptera: Pseudococcidae)	USA
LRC 2176	*Penicillium bilaii*	Soil	Canada

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Table 2-1. con't.

Isolate code[a]	Name[b]	Host	Country of origin
Other isolates:			
LRC 2391	*Rhizopus* sp.	Soil	Canada
LRC 2524	*Trichoderma reesei*	Soil	Canada
LRC race 1	*Verticillium albo-atrum*	*Solanum tuberosum*	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
LRC = Lethbridge Research Centre, Lethbridge, Alberta, Canada

[b]name as received

HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS extraction buffer added. The mycelial mat was hand-ground for 1-2 minutes with a conical microtube pestle. Following homogenization, 150 μl of 3 M sodium acetate, pH 5.2, was added. The suspension was briefly vortexed and placed at -20 °C for 10 min. The microtube was then centrifuged as previously described, and the supernatant transferred to a new tube. An equal volume of isopropanol was added and after incubation at room temperature for approximately 10 min, the precipitated DNA was pelleted by centrifugation. The supernatant was removed, and the pellet washed with 70% ethanol. After another centrifugation and removal of the supernatant, the pellet was dried before being resuspended in 50 μl of TE and stored at -20 °C. 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). PCR amplifications with general fungal primers TW81 and AB28 (Curran *et al.*, 1994) and *M. anisopliae* var. *acridum*-specific Mac-ITS-spF and Mac-ITS-spR primers were performed on 50 ng DNA.

2.1.3   Production of a positive DNA control for *M. anisopliae* var. *acridum*

A positive control was generated by cloning the PCR product resulting from amplification of *M. anisopliae* var. *acridum* IMI 330189 DNA with primers TW81 and AB28 in vector pGEM®-T Easy using the pGEM® and pGEM®-T Easy Vector Systems cloning kit (Promega, Madison, WI). Standard protocols were used for plasmid DNA isolation, buffers, and electrophoresis techniques (Sambrook *et al.*, 1989). Correct nucleotide sequence of the cloned product was confirmed by sequencing (University

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Core DNA and Protein Services, University of Calgary) and comparison to the published

sequence for *M. anisopliae* var. *acridum* (AF137062; Driver *et al.*, 2000).

2.1.4 Construction of a simulated soil DNA pool spiked with *M. anisopliae* var. *acridum* DNA

A simulated soil DNA pool was prepared with fungal DNA (section 2.1.2), with

the exception of that from *M. anisopliae* var. *acridum* and *M. flavoviride* var. *minus*, at a

final concentration of 100 ng/µl. The pool consisted of equal proportions of *Metarhizium*

spp. DNA versus non-*Metarhizium* spp. DNA. The pool was spiked by addition of 100

ng *M. anisopliae* var. *acridum* DNA (concentration of 100 ng/µl) to 900 ng soil DNA

pool. Four 10-fold dilutions were made of the spiked DNA pool using the simulated soil

DNA pool as diluent, representing final concentrations of 1 ng, 100 pg, 10 pg, and 1 pg

per µl *M. anisopliae* var. *acridum* DNA. PCR amplifications using the Mac-ITS-spF and

Mac-ITS-spR primers were performed with 1 µl of each spiked sample.

2.1.5 Inoculation of soil with *M. anisopliae* var. *acridum* conidia

Spores of *Metarhizium anisopliae* var. *acridum* were applied at various

concentrations to a local southern Alberta soil (clay-loam). Prior to inoculation, the soil

was examined for *Metarhizium* spp. as per the method of Rath *et al.* (1992). Moist soil

equivalent to 20 g oven-dried weight of the soil sample was added to 200 ml of sterile

Ringer's solution (Oxoid, Ogdensburg, NY), the suspension shaken on an orbital shaker

at 150 rpm for 30 min at room temperature, and then spread-plated as 0.1 ml of neat or

10^{-1} dilutions in Ringer's solution onto a 100 x 15 mm Petri dish containing selective

media consisting of 3.5% mycological agar (Difco, Franklin Lakes, NJ) with 10 µg/ml

dodine (Cyprex 65-W, American Cyanamid, Wayne, NJ), 50 µg/ml chloramphenicol

(Sigma-Aldrich, St. Louis, MO), and 200 µg/ml cycloheximide (Sigma-Aldrich) (Liu *et*

al., 1993). Each dilution was plated as 5 replicates. Plates were incubated at 25 °C for 15 days before examination for colonies of *Metarhizium* spp. Also prior to inoculation, DNA was extracted from 0.25 g of the soil using the Ultra Clean Soil DNA kit (MoBio, Carlsbad, CA). Following extraction, the DNA was then subjected to PCR amplification with the general fungal TW81 and AB28 primers to confirm successful DNA extraction, and amplification with a set of primers (Mac-ITS-spF and Mac-ITS-spR) designed for the specific detection of *M. anisopliae* var. *acridum* DNA.

Spores of *M. anisopliae* var. *acridum* (IMI 330189) were scraped from a PDA plate and resuspended in 0.05% Tween 20. Spore concentration was estimated with a hemocytometer and concentrations adjusted to 10^2, 10^3, 10^4, and 10^5 spores, each in 200 μl of 0.05% Tween 20. The spore suspensions were each added to 0.25 g of soil, followed immediately by soil DNA extraction using the MoBio Ultra Clean Soil DNA kit. Extracted DNA (1 μl) was subsequently subjected to PCR amplification with the Mac-ITS-spF and Mac-ITS-spR primers.

2.1.6 Inoculation of grasshoppers

Nymphs (third and fourth instar) of a non-diapausing strain of *Melanoplus sanguinipes* (Pickford & Randell, 1969) were collected at random from a laboratory colony at the LRC and placed individually in sterile 20 ml glass vials stoppered with a sterile polyurethane foam plug. The experiment involved a total of 152 insects (26 in the control group, 126 in the treatment group) with approximately equal proportions of males and females in each group. On the day of inoculation, conidia of *M. anisopliae* var. *acridum* were harvested from PDA cultures (15-20 days of growth) and resuspended in sunflower oil (Safflo, Concord, ON). Formulation of the inoculum has been previously

described by Johnson *et al.* (2002). Briefly, the concentration of conidia was estimated with a hemocytometer and adjusted to 5 X 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 wafer for 24 h. Control grasshoppers were confined with wafers containing only sunflower oil. After 24 h confinement, all grasshoppers were removed and individually housed in 240-ml transparent plastic containers. Nymphs that did not survive the 24 h confinement were discarded (mortality was attributed to handling). Throughout the experiment, insects were exposed to a temperature regime of 24 °C/16 °C day/night with a corresponding 16/8 h light/dark photoperiod under ambient relative humidity (40-55%). Nymphs were observed and fed daily with fresh wheat leaves. Cadavers were removed daily with sterile forceps and stored in sterile 1.5 ml Eppendorf vials at -20 °C prior to DNA extraction. All treated grasshoppers were dead by day 8; all remaining control grasshoppers were then killed at -20 °C. Viability of conidia was determined by microscopic examination of germination following 48 h incubation at 25 °C of 2 X 10-µl replicate aliquots of the inoculum onto PDA blocks on a microscope slide.

2.1.7   Grasshopper DNA isolation

The method of Hegedus and Khachatourians (1993) was modified for the extraction of DNA from infected and noninfected grasshoppers. Individual nymphs were homogenized in 500 µl of TE with a sterile microtube pestle for 2-3 minutes accompanied by vigorous vortexing. A 25-µl aliquot of the homogenate was removed and spread on a 60 X 15 mm Petri dish containing selective media for *Metarhizium* spp.

as described above. Inoculated agar plates were incubated at 25 °C for confirmation of

presence/absence of *M. anisopliae* var. *acridum* colonies (maximum of 20 days). The

remaining solution was extracted with an equal volume of phenol:chloroform (1:1, v/v)

followed by a 10 min centrifugation at 16 000 x g. The upper aqueous phase was

removed and extracted once more with chloroform:isoamyl alcohol (24:1, v/v), followed

by addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and one volume of isopropanol

to the aqueous phase. Following incubation at room temperature (*ca.* 20 °C) for 10 min,

the mixture was centrifuged, and the supernatant removed. The pellet was washed with 1

ml of ice-cold 70% ethanol, centrifuged, and was dried briefly. The DNA was then

resuspended in 500 µl of TE containing 2 µl RNase A (Sigma-Aldrich) and stored at -20

°C. Quantitation of DNA was determined with use of a spectrophotometer (Pharmacia

Biotech, Piscataway, NJ) and 100 ng was later subjected to PCR amplification.

2.1.8 PCR amplification

General fungal primers TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28

(5'-ATATGCTTAAGTTCAGCGGGT-3') (Curran *et al.*, 1994) were used to amplify the

region of the ribosomal repeat from the 3' end of the 16S rDNA to the 5' end of the 28S

rDNA flanking the ITS1, the 5.8S rDNA, and ITS2 sequences, from total fungal DNA.

PCR amplifications were performed in a total volume of 50 µl containing 10 mM Tris,

pH 8.4, 50 mM KCl, 1.5 mM MgCl$_2$, 0.05% Tween 20, 0.05% NP40, 0.4 µM of each

primer, 25 µM of each dNTP (Invitrogen, Carlsbad, CA), 2.5 units *Taq* DNA polymerase

(MBI Fermentas, Hanover, MD) and template DNA. Negative controls contained sterile

water in place of DNA. DNA amplification was performed in a GeneAmp® PCR System

9700 (Applied Biosystems, Foster City, CA) programmed as follows: initial denaturation

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5 min at 94 °C; 30 cycles of: denaturation 1 min at 94 °C, annealing 1 min 30 sec at 55 °C, extension 2 min at 72 °C; with a final extension 5 min at 72 °C. PCR products were analyzed on a 1.5% TAE agarose gel in with a 100 bp DNA ladder (MBI Fermentas) included as a size marker.

Primers Mac-ITS-spF (5'-CTGTCACTGTTGCTTCGGCGGTAC-3') and Mac-ITS-spR (5'-CCCGTTGCGAGTGAGTTACTACTGC-3') were designed based on the ITS1 and ITS2 regions of the rDNA sequence data for *M. anisopliae* var. *acridum* (clade 7, Driver *et al.*, 2000). Total fungal and soil DNA and grasshopper DNA from infected and noninfected insects were used in PCR assays with this primer combination. 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, 2.5 units *Taq* DNA polymerase and template DNA. As previously noted, negative controls contained sterile water in place of DNA. DNA amplification was also performed in a GeneAmp® PCR System 9700 programmed as follows: initial denaturation 5 min at 94 °C; 30 cycles of: denaturation 1 min at 94 °C, combined annealing and extension 3 min at 72 °C; with a final extension 5 min at 72 °C. PCR products were analyzed as previously mentioned.

Nested PCR amplifications were carried out on grasshopper DNA from infected insects that initially produced weak products in a single amplification with the Mac-ITS-spF and Mac-ITS-spR primers. DNA from infected grasshoppers was amplified in an initial reaction with the TW81 and AB28 primers using conditions previously described. A second amplification was then performed with a 1-µl aliquot from the initial reaction

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and the Mac-ITS-spF/Mac-ITS-spR primer combination using conditions described above.

2.2 Results

As expected, use of the Mac-ITS-spF and Mac-ITS-spR primers in a PCR assay successfully amplified a 420 bp DNA sequence from the total genomic DNA extracted from *M. anisopliae* var. *acridum* (Figure 2-1). *M. anisopliae* var. *minus* also produced a 420 bp amplification product. Isolates of *M. anisopliae* var. *anisopliae* and *M. flavoviride* Gams & Rozsypal produced no amplified product, nor did isolates of *B. bassiana, I. fumosorosea, I. farinosa, V. albo-atrum, C. gloeosporioides, E. nidulans, T. reesei, F. oxysporum, C. rosea* f. *catenulata, P. bilaii, H. peziza*, or an isolate of *Rhizopus* sp. (data not shown). In contrast, the TW81 and AB28 primers produced a varying range (most around 500-600 bp) of amplified products in all isolates tested, thus confirming successful extraction of PCR-quality DNA from all fungal species (data not shown).

The sensitivity of the *M. anisopliae* var. *acridum*-specific PCR assay was determined for genomic fungal DNA extracted from an axenic culture of *M. anisopliae* var. *acridum*. The assay was sensitive enough to detect approximately 1 pg of genomic DNA (Fig. 2-2).

The *M. anisopliae* var. *acridium*-specific PCR assay successfully detected *M. anisopliae* var. *acridum* DNA in the presence of a simulated soil DNA pool. A detection limit of 10 pg was observed, representing 0.001% of total DNA in the sample.

M. anisopliae var. *acridum* spores were detected at a concentration of 10^4 spores per 0.25 g of soil. Use of general fungal TW81 and AB28 primers in conjunction with specific Mac-ITS-spF and Mac-ITS-spR primers in a nested PCR assay increased the
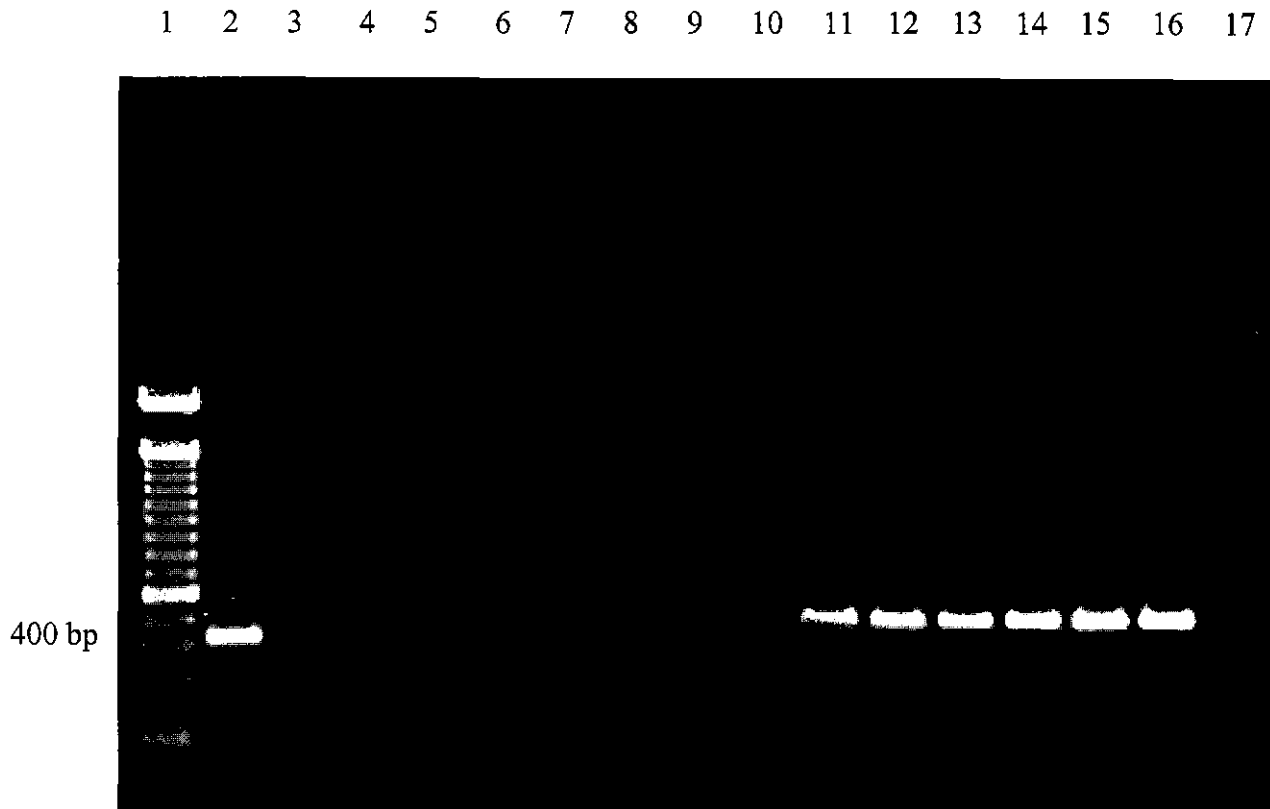
Fig. 2-1. Specificity determination of the PCR assay using the Mac-ITS-spF and
Mac-ITS-spR primers and genomic DNA from various fungal isolates

Lane 1: 100 bp ladder
Lane 2: Positive control (cloned *M. anisopliae* var. *acridum*)
Lane 3: *M. anisopliae* var. *anisopliae* UAMH 421
Lane 4: *M. anisopliae* var. *anisopliae* UAMH 4450
Lane 5: *M. anisopliae* var. *anisopliae* S54
Lane 6: *M. anisopliae* var. *anisopliae* 6W-2
Lane 7: *M. anisopliae* var. *anisopliae* 11S-1
Lane 8: *M. anisopliae* var. *anisopliae* ARSEF 437
Lane 9: *M. anisopliae* var. *anisopliae* ARSEF 727
Lane 10: *M. flavoviride* Gams & Rozsypal ARSEF 1184
Lane 11: *M. flavoviride* var. *minus* ARSEF 2023
Lane 12: *M. anisopliae* var. *acridum* IMI 330189
Lane 13: *M. anisopliae* var. *acridum* SP9
Lane 14: *M. anisopliae* var. *acridum* FI 985
Lane 15: *M. anisopliae* var. *acridum* ARSEF 3391
Lane 16: *M. anisopliae* var. *acridum* ARSEF 6421
Lane 17: Water
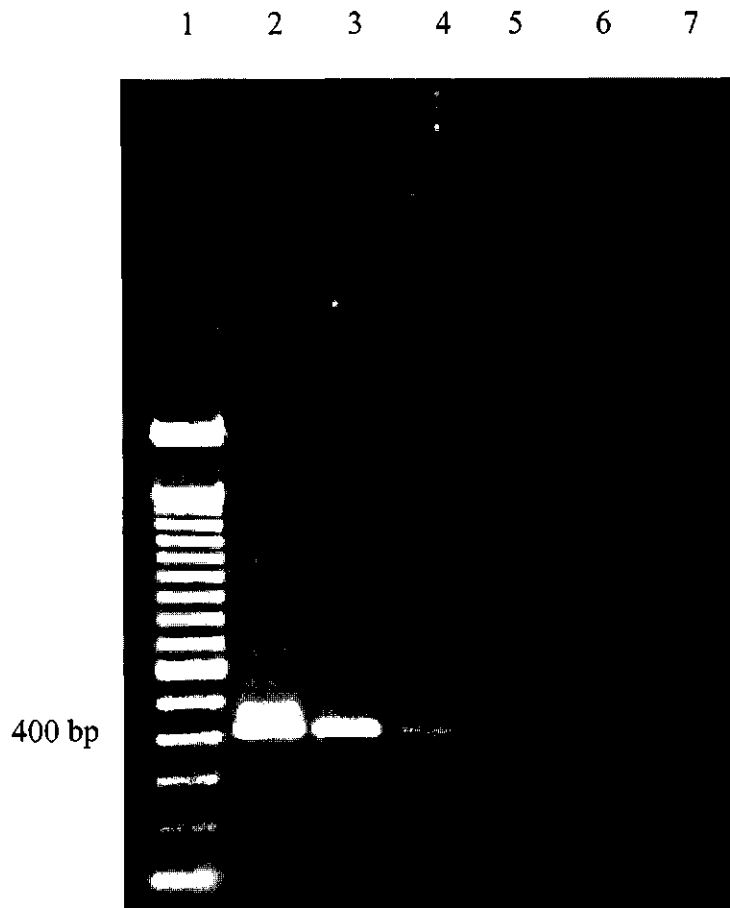
Fig. 2-2. Sensitivity determination of the PCR assay using the Mac-ITS-spF and
Mac-ITS-spR primers and genomic DNA from *M. anisopliae* var. *acridum*

Lane 1: 100 bp ladder
Lane 2: 1 ng
Lane 3: 100 pg
Lane 4: 10 pg
Lane 5: 1 pg
Lane 6: 100 fg
Lane 7: Water

detection limits to 10^2 spores per 0.25 g of soil. *M. anisopliae* var. *acridum* DNA was not detected and no *Metarhizium* spp. were isolated from the soil prior to inoculation.

The specific assay also successfully detected *M. anisopliae* var. *acridum* DNA in each of the 126 infected grasshoppers. Counts of viable conidia in the inoculum revealed a germination rate of >90% at 48 h after incubation at 25 °C. Ecdysis was either completed or initiated by 65 of the treated nymphs prior to death; however, this did not inhibit detection of fungal DNA. Only 28 of the treated cadavers displayed the reddish discolouration of the cuticle associated with infection by *M. anisopliae* var. *acridum*. Fungal colonies with *M. anisopliae* var. *acridum* morphological features, namely dark green conidia, were observed on 116 agar plates for the treated group. No growth was observed on nine plates, and for another plate, overgrowth by *Rhizopus* sp. interfered with examination for colonies of *M. anisopliae* var. *acridum*.

No amplified products were observed with PCR assay of the control group, and no colonies of *M. anisopliae* var. *acridum* were isolated from any of the agar plates for the control nymphs. Figure 2-3 shows the var. *acridum*-specific PCR amplification results for a representative group of infected and noninfected nymphs.

2.3 Discussion

Primers designed from the ITS nucleotide sequences for *M. anisopliae* var. *acridum* were used successfully in a PCR-based assay for amplification of a 420 bp sequence with genomic DNA extracted from *M. anisopliae* var. *acridum*. A 420 bp product observed after amplification of *M. flavoviride* var. *minus* DNA was also expected as this species has been recognized as *M. anisopliae* var. *acridum* by Driver *et al.* (2000). The ability to produce an amplified product specific to *M.* anisopliae var. *acridum*

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400 bp
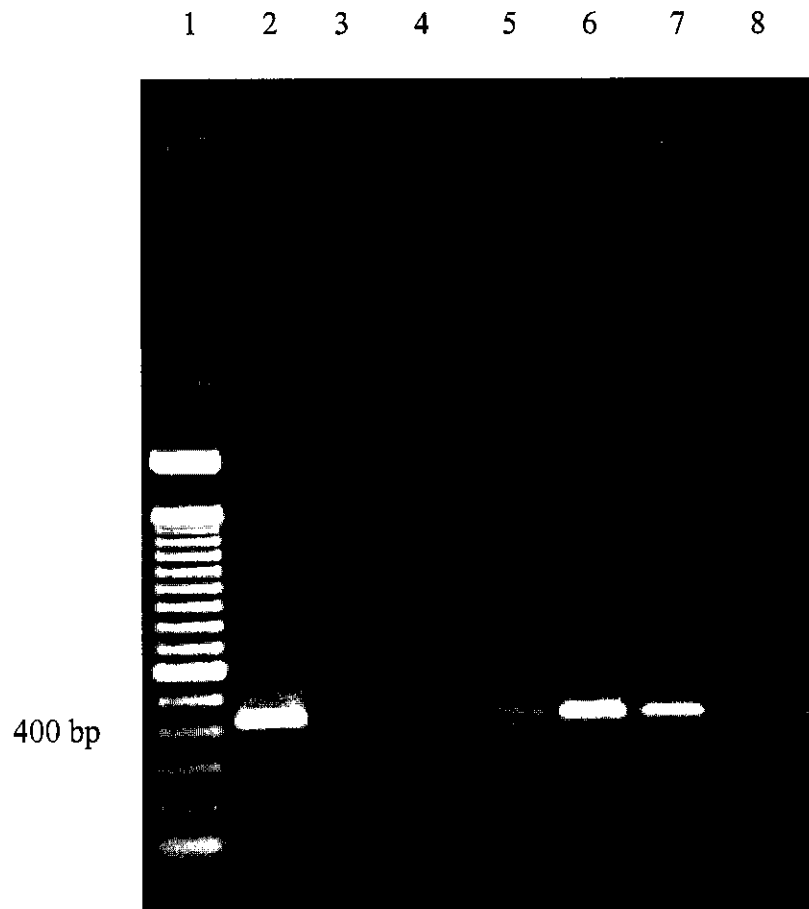
Fig. 2-3. Detection of *M. anisopliae* var. *acridum* DNA in infected grasshoppers using PCR primers Mac-ITS-spF and Mac-ITS-spR

Lane 1: 100 bp ladder
Lane 2: *M. anisopliae* var. *acridum* (positive control)
Lane 3: DNA from uninfected grasshopper
Lane 4: DNA from uninfected grasshopper
Lane 5: DNA from grasshopper infected with *M. anisopliae* var. *acridum* (4 dpi[c])
Lane 6: DNA from grasshopper infected with *M. anisopliae* var. *acridum* (5 dpi)
Lane 7: DNA from grasshopper infected with *M. anisopliae* var. *acridum* (6 dpi)
Lane 8: Water

[c]days post-infection

supports the concept of divergence between taxa and also corroborates the hypothesis of divergent evolutionary lines within the genus *Metarhizium* (Driver *et al.*, 2000). Although representatives from only two other clades of *Metarhizium* were evaluated, the high sequence variability of *M. anisopliae* var. *acridum* in comparison with other clades combined with the highly stringent composition of the synthesized sequences support the specificity of the Mac-ITS-spF and Mac-ITS-spR primers.

The fungal genera other than *Metarhizium* analyzed in this study encompassed a range of entomopathogenic, phytopathogenic, mycopathogenic, and soil saprophytic organisms. Several of the genera have previously been isolated from southern Alberta soils (Inglis *et al.*, 1998). *V. albo-atrum* and *C. gloeosporioides* are phytopathogens (Domsch *et al.*, 1980; Evans *et al.*, 2001). Others, such as *Isaria* (syn. *Paecilomyces* p.p) are entomopathogenic (Inglis *et al.*, 2001). One of these other entomopathogens, *B. bassiana,* was selected because it is an acridid pathogen (Johnson & Goettel, 1993). *Gliocladium* spp., *Trichoderma* spp., and *Fusarium* spp. have been identified as pathogens of fungi (Vey *et al.*, 2001). A pending survey of southern Alberta soils and insects necessitated analysis of these genera with the *M. anisopliae* var. *acridum*-specific primers to determine specificity of the PCR assay. Further, demonstration of successful amplification of *M. anisopliae* var. *acridum* DNA in the presence of other DNA, particularly from soil, was essential and has been demonstrated in this work.

Extraction of PCR-amplifiable DNA from insects is often difficult due to the number of PCR inhibitors in the form of tannic acids, quinones, polyphenols and chelators coisolated from the insect cuticle (Hackman, 1974). Some weak amplification products were experienced with the var. *acridum*-specific primers in a single

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amplification from infected grasshopper DNA but these products were subsequently more strongly amplified with a nested PCR assay that employed the TW81/AB28 primers for the first amplification and the Mac-ITS-spF/Mac-ITS-spR primers for the second amplification. Inhibitory compounds were diluted to a negligible amount when 1 μl of the first amplification reaction was used as template for the second amplification. Molting did not interfere with the ability of the assay to detect *M. anisopliae* var. *acridum* DNA in infected grasshoppers that underwent ecdysis. This supports a previous observation by Milner and Prior (1994) that ecdysis did not interfere in the infection of the Australian plague locust (*C. terminifera*) with *M. anisopliae* var. *acridum*.

Studies have demonstrated that, depending on the dose, the majority of laboratory bioassay mortality in acridids infected with *M. anisopliae* var. *acridum* occurs between 4-6 d post-infection (Delgado *et al.*, 1997b; Lomer *et al.*, 1997b; Magalhães *et al.*, 1997; Milner, 1997). In this study, the *M. anisopliae* var. *acridum*-specific PCR assay amplified sequences from DNA extracted from treated nymphs that died 1-3 d post-inoculation. Presumably, the majority of nymphs at this stage died from complications due to contact with the sunflower oil component of the inoculum rather than from active fungal infection. The *M. anisopliae* var. *acridum*-specific diagnostic PCR assay is qualitative and not designed to determine activity levels of the target organism. However, confirmation of *M. anisopliae* var. *acridum* colony growth for 92.1% of the treated grasshoppers indicates that the presence of viable spores can be detected early post-infection. Moreover, the intensity of amplification products increased with DNA from cadavers from the later days of the experiment, thus suggesting a progressive increase in fungal mass in the infected host.

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Surveys for natural incidence of *M. anisopliae* var. *acridum* have indicated that these levels are generally very low. In northern Benin, Shah *et al.* (1998b) found levels of 0.3-1.7% and 1.2-3.2% at different sites, respectively. Also in Benin, Douro-Kpindou *et al.* (1995) detected fungal incidence at 15% in field trial plots before application of a formulation of *M. anisopliae* var. *acridum* for biocontrol of *Zonocerus variegatus*. The ability of our assay to detect levels of *M. anisopliae* var. *acridum* DNA as low as 0.001% of total DNA present demonstrates its suitability for detection of this fungus at low incidence.

Laboratory and field tests indicate differential impacts of weather affect the operation and efficacy of entomopathogenic fungi (Inglis *et al.*, 1997). Further, spring temperature, overwintering conditions, and moisture strongly affect the target insect. Insect body temperature can be calculated (Lactin & Johnson, 1998) and is largely a result of immediate weather factors; however, the probable impact of weather on the effectiveness of *M. anisopliae* var. *acridum* is largely unknown. Improved knowledge of the biology and ecology of this fungus in a natural setting is a prerequisite for the development of an effective long-lasting pest management strategy for the biological control of acridids. This study offers a reliable, specific, and sensitive diagnostic PCR assay that can be performed on a number of templates including those with non-target DNA. This molecular method can be used to investigate the geographical extent of *Metarhizium* spp. in soils and native insects, to compare this distribution to possible future distributions under changing weather and climate, and to assess the opportunities for including *Metarhizium* spp. in integrated grasshopper management plans.

Chapter 3.   Determination of Natural Incidence of *Metarhizium* in Southern Alberta Soils

3.0   Introduction

Documentation of the natural incidence of entomopathogenic fungi in the environment is crucial to the successful application of biocontrol agents. Surveys yield valuable knowledge on the distribution and quantification of naturally occurring genotypes, as well as the suitability of the target area for persistence of the fungus. Further, the best source for potentially effective biocontrol agents is the site of natural interaction between a pathogen and its host (Hofstein & Chapple, 1999).

Detection of entomopathogens may be direct through observation of endemic levels of disease in target insect populations or indirect by culture on selective media, use of insect larvae in bait assays, or application of molecular techniques such as PCR. Direct isolation of a pathogen from a diseased target insect is more likely to yield a virulent pathotype than indirect isolation from soil; however, the incidence of *M. anisopliae* var. *acridum* infection in Orthoptera is very rare. In the last twenty years, only two isolates have been found on acridid hosts in Australia (Milner *et al.* 2003) and one hundred and twenty one isolates of *M. flavoviride* were collected from a survey of West Africa and Madagascar that involved tens of thousands of grasshoppers and locusts maintained in cages (Shah *et al.*, 1997). Various degrees of virulence were determined in the African isolates (Bateman *et al.*, 1996), possibly a consequence of increased levels in susceptibility that resulted from stressed insects in high population densities under controlled conditions at the time of isolation.

Dodine has successfully been used as a selective agent for the isolation of *Metarhizium* spp. from soil (Liu *et al.*, 1993). Milner and Lutton (1976) determined that

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mycological agar with 1% chloramphenicol and 0.5% cycloheximide permitted detection

levels down to 10^3 conidia of *M. anisopliae* per gram of soil. *Metarhizium anisopliae* has

also been successfully isolated from Canadian soils by investigation of laboratory

cultures of dead elaterids (Zacharuk & Tinline, 1968) and by bait assays of waxworm

larvae (Bidochka *et al.*, 1998). Recently, DNA-based PCR has been used to target the

highly variable ITS region for identification of various fungal pathogens from soils

(Lochman *et al.*; Ippilito *et al.*, 2002; Cumagun *et al.*, 2000; Bell *et al.*, 1999).

The objective of this study was to determine the natural incidence of *Metarhizium*

spp. in southern Alberta soils by indirect isolation on selective media and bait assay with

waxworm larvae, and by identification with nested PCR for three varieties of

Metarhizium.

3.1   Materials and Methods

3.1.1   Collection of soil samples

Soil samples were collected in May 2004 from twenty geographical locations in

southern Alberta (Fig. 3-1). Sites were chosen on the basis of highest grasshopper

densities as determined from the previous year's data collected by agricultural field

surveyors. All sites included fields that had been under cultivation of various types of

cereal crops and the adjoining uncultivated ditches. At each site, five replicates were

taken from the cultivated field and five taken from the adjacent ditch. Soil samples were

taken with a cylindrical soil corer with an internal diameter of 12 cm and from a soil

depth of 10 to 15 cm. Samples were double-bagged and stored at 4 °C until they were

used for fungal isolation.
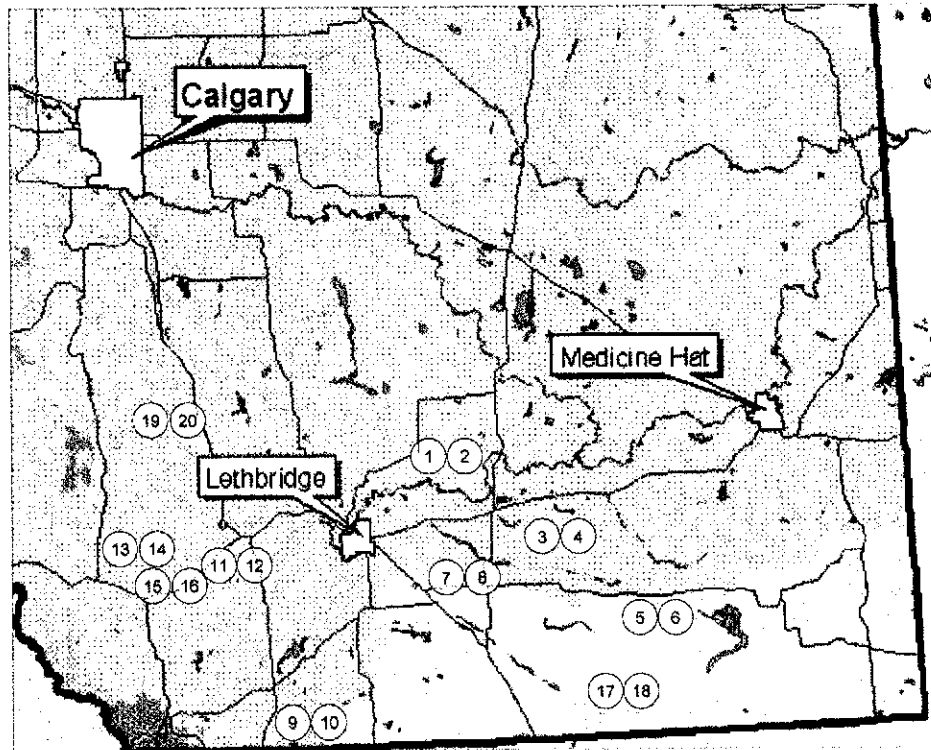
Fig. 3-1. Geographic locations in southern Alberta for soil survey of *Metarhizium* spp.
Map courtesy of Southern Alberta Sustainable Strategy (SASS).

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3.1.2 Determination of soil moisture

The mean soil moisture content of each sample was calculated after drying three separate 20 to 25 g quantities of the soil in an oven at 105 °C for 16 to 20 h. The sampling spoon was immersed in 70 % ethanol, rinsed in running distilled water, and dried between samples to eliminated sample-to-sample contamination.

3.1.3 Isolation of soil fungi on selective medium

Inoculation of soil extracts onto selective medium was performed according to the method outlined by Rath *et al.* (1992). A soil corer (2.5 cm diameter, 20 cm length), decontaminated as per section 3.1.2, was used to remove a subsample of each soil sample. The subsample was thoroughly mixed in a new clean plastic bag, and then moist soil equivalent to 20 g oven-dried weight was added to 200 ml sterile Ringer's solution (Oxoid) in a 500 ml Erlenmyer 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) containing 50 µg/ml chloramphenicol and 200 µg/ml cycloheximide (Veen & Ferron, 1968), and modified with 10 µg/ml dodine. Five replicate plates were inoculated for each dilution. Plates were sealed with Parafilm® and incubated in the dark at room temperature (*ca.* 20 °C) for 20 days before examination. *M. anisopliae* var. *anisopliae* isolate UAMH 421 was inoculated as a positive control onto one plate from each batch of medium produced.

3.1.4 Isolation of soil fungi with waxworm larvae

The *Galleria* bait method described by Zimmermann (1986) was used for isolation of soil fungi. Soil samples were aseptically transferred individually to sterile 115-ml containers with perforated screw-capped lids. Each sample was moistened with 5

ml of sterile distilled water, after which four waxworm (*Galleria mellonella*) larvae were added with sterile forceps to each container. The assay was conducted at room temperature (*ca.* 20 °C) for twenty-one days. Dead larvae were removed every seven days; larvae not showing any signs of fungal outgrowth were disinfected by immersion for one minute in 70 % ethanol followed by one minute in sterile distilled water. Cadavers were then placed individually on moistened sterile filter paper in a 60 mm x 10 mm Petri dish, the dish sealed with Parafilm®, and incubated at *ca.* 20 °C for a maximum of twenty-one days. Conidia from fungal outgrowth on dead larvae were transferred with a sterile loop to selective medium described in section 3.1.3. Uninoculated waxworm larvae were maintained on a wheat germ/oatmeal/honey mash shipped by the supplier (Massasauga Imports, Acton, ON). Two sets of control larvae (ten larvae per isolate) were inoculated with *Metarhizium anisopliae* isolates UAMH 421 and S54 by rolling individual larva onto actively growing cultures.

3.1.5   Identification of *Metarhizium* spp. in soil by PCR

3.1.5.1 Extraction of soil DNA

Soil DNA was extracted from 0.25 g quantities for four replicates of each soil sample as per the manufacturer's instructions for the Ultra Clean Soil DNA kit (MoBio). PCR primers TW81 and AB28 (Curran *et al.*, 1984) were used to confirm successful DNA extraction as previously described (section 2.1.8). Extracted DNA (1 µl) was subsequently subjected to PCR amplification.

3.1.5.2 Production of a positive DNA control for *M. flavoviride* var. *flavoviride*

A positive control for *M. flavoviride* Gams & Rozsypal ARSEF 1184 was constructed as previously described (section 2.1.3). Correct nucleotide sequence of the

cloned product was confirmed by sequencing and comparison to the published sequence

for *M. flavoviride* var. *flavoviride* (AF138267, clade 4, Driver *et al.*, 2000).

3.1.5.3 Design of PCR primers for the specific detection of *M. flavoviride* var.
flavoviride

Primers Mf-ITS-spF and Mf-ITS-spR were designed based on the ITS1 and ITS2

regions of the rDNA sequence data for *M. flavoviride* var. *flavoviride* (clade 4, Driver *et*

al., 2000).

3.1.5.4 PCR amplification of soil DNA

Each soil DNA extract was subjected to three different nested PCR. Assays for

specific detection of *M. anisopliae* var. *acridum* and *M. flavoviride* were conducted in

conjunction with a primary assay with general fungal primers TW81 and AB28. PCR

amplifications were described previously in section 2.1.8. Conditions for the *M.*

flavoviride-specific assay were identical to those used for the *M. anisopliae* var. *acridum*

assay.

Nested PCR assays that targeted the variable intergenic spacer (IGS) region

between the 16S and 28S rRNA subunits for the specific detection of *M. anisopliae* var.

anisopliae were conducted with an initial reaction with primers Ma-28S4 and Ma-IGS1

(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, 2.5 units *Taq* DNA polymerase and template DNA.

DNA amplification was performed in a GeneAmp® PCR System 9700 programmed as

follows: initial denaturation 5 min at 94 °C; 30 cycles of: denaturation 1 min at 94 °C,

annealing 1 min at 54 °C, extension 2 min at 72 °C; with a final extension 5 min at 72 °C.

PCR products were analyzed on 1 % TAE agarose gels with a 100 bp DNA ladder

included as a size marker. A second reaction was performed with a 1-µl aliquot from the initial amplification and primers Ma-IGSspF and Ma-IGSspR (Pantou *et al.*, 2003). Reaction mixtures were as described above. DNA amplification was performed in a GeneAmp® PCR System 9700 programmed as follows: initial denaturation 5 min at 94 °C; 30 cycles of: denaturation 1 min at 94 °C, annealing 1 min at 58 °C, extension 2 min at 72 °C; with a final extension 5 min at 72 °C. PCR products were analyzed as previously mentioned (section 2.1.8). Negative controls for all amplifications consisted of sterile water in place of DNA. Genomic DNA (50 ng) from *M. anisopliae* var. *anisopliae* isolate UAMH 421 was used as a positive control for the *M. anisopliae* var. *anisopliae* specific assays. In a preliminary study on specificity, nested PCR assays with the Ma-28S4/Ma-IGS1 and Ma-IGSspF/Ma-IGSspR primer combinations were conducted on genomic DNA from the fungal isolates listed in section 2.1.1. Table 3-1 contains a list of all primers used in this study and their sequences.

3.2   Results

3.2.1   Isolation on selective medium

M. anisopliae var. *anisopliae* was isolated from three of the twenty (15 %) geographical locations sampled in southern Alberta. Two sites had a single colony forming unit (CFU) and the third site had one replicate sample with one CFU on one plate and another replicate with two colonies on another plate. PCR testing confirmed the identification of all isolates as *M. anisopliae* var. *anisopliae*. 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 all isolates. One isolate from a wheat field, 6W-2, was identified as a group-B

Table 3-1. List of primers used for PCR amplification in this study

Primer	Sequence (5'-3')	Source
TW81	GTTTCCGTAGGTGAACCTGC	Curran *et al.* (1994)
AB28	ATATGCTTAAGTTCAGCGGGT	Curran *et al.* (1994)
Mac-ITS-spF	CTGTCACTGTTGCTTCGGCGGTAC	Entz *et al.* (2005)
Mac-ITS-spR	CCCGTTGCGAGTGAGTTACTACTGC	Entz *et al.* (2005)
Mf-ITS-spF	TGTCTACCGTTGCTTCGGCGGGTTC	This study
Mf-ITS-spR	TACGGCAGCAGCAGGGCACCAGTT	This study
Ma-28S4	CCTTGTTGTTACGATCTGCTGAGGG	Pantou *et al.* (2003)
Ma-IGS1	CGTCACTTGTATTGGCAC	Pantou *et al.* (2003)
Ma-IGSspF	CTACCYGGGAGCCCAGGCAAG	Pantou *et al.* (2003)
Ma-IGSspR	AAGCAGCCTACCCTAAAGC	Pantou *et al.* (2003)

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). The other two sites

(a wheat field and the adjoining brome ditch) yielded a strain (S54) that 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, resulting in a PCR product 300 bp

larger than expected (Fig. 3-2). Colony morphologies (dark green conidia) for the group-

B and variant group-B isolates were similar. Nucleotide sequences for the cloned PCR

fragments amplified by the Ma-28S4/Ma-IGS1 primers for these isolates can be found in

Appendices 1 and 2. Mean soil moisture content for the soil samples taken was quite

variable, ranging from 4.39 to 22.70 %. No isolates of *M. anisopliae* var. *acridum* or *M.*

flavoviride grew on the selective medium. Each batch of medium produced supported the

growth of *M. anisopliae* var. *anisopliae* UAMH 421.

3.2.2   Isolation on *Galleria* bait assay

Two *Metarhizium* isolates were recovered from only two infected *Galleria* larvae.

Both isolates came from two sites that differed from the sites positive for *M. anisopliae*

var. *anisopliae* isolation on selective medium. One isolate (11S-1) recovered from a

brome ditch next to a wheat field resembled the selective medium isolates in colony

morphology (Fig. 3-3a) and was confirmed as a non-variant (in terms of expected band

size) *M. anisopliae* var. *anisopliae* by PCR. The other isolate (20W-5), despite

exhibition of similar morphology on *Galleria* to the first isolate, showed a distinct

morphology characterized by white mycelial growth with very sparse green conidiation

(Fig. 3-3b) after six weeks incubation at *ca.* 20 °C on various media, including PDA,

SDA + 1 % yeast extract, Czapek-Dox agar, and the selective medium previously

52
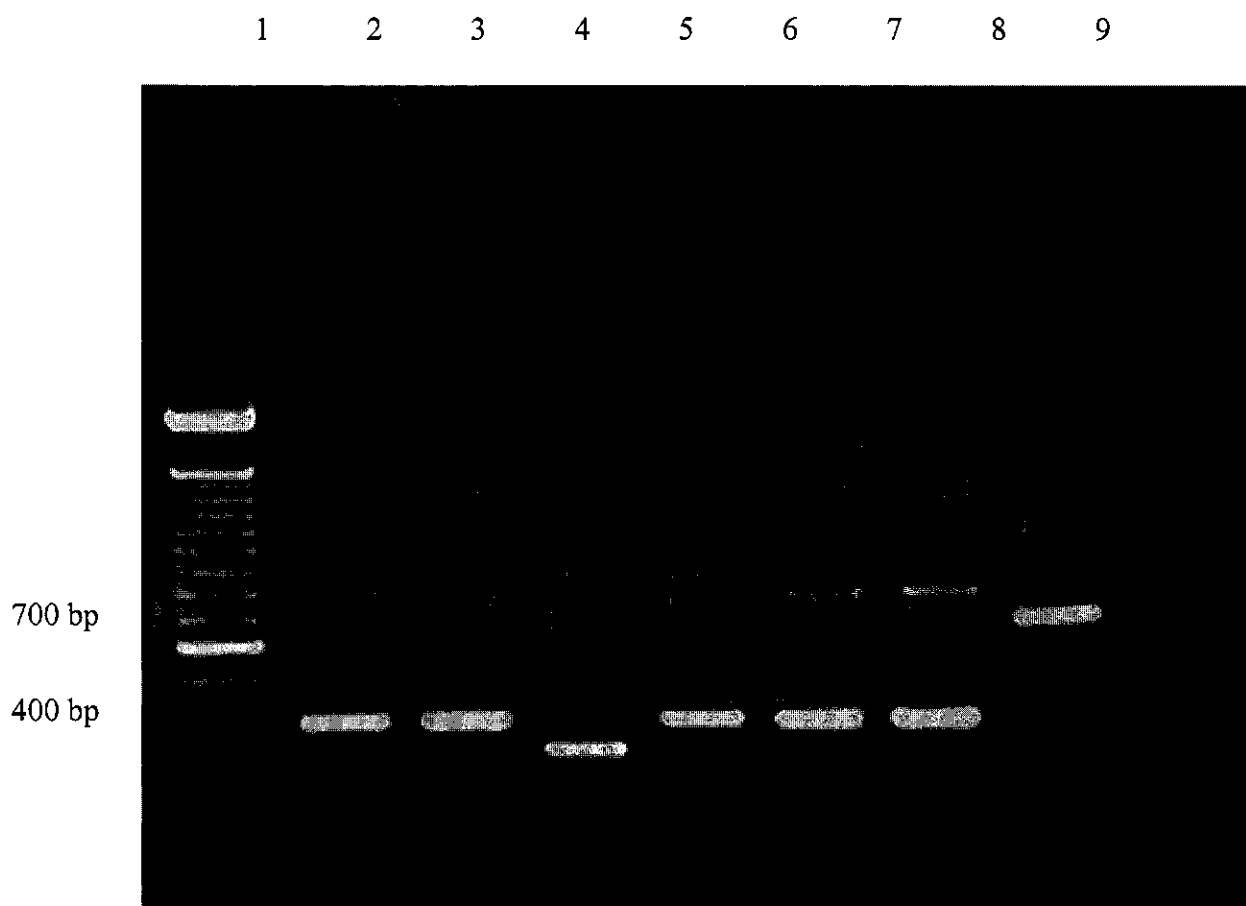
Fig. 3-2. Detection of *M. anisopliae* var. *anisopliae* DNA in nested PCR assays using Ma-28S4/Ma-IGS1 and Ma-IGSspF/Ma-IGSspR primers and genomic DNA from various *M. anisopliae* var. *anisopliae* isolates

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

53
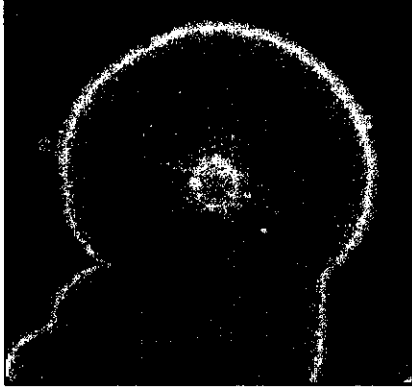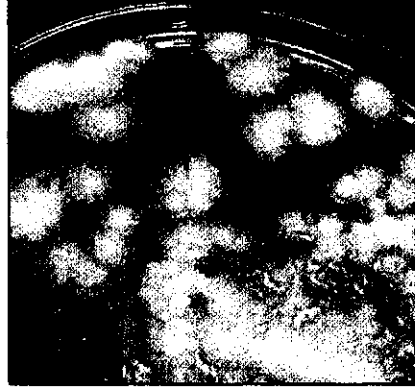
(a) isolate 11S-1   (b) isolate 20W-5

Fig. 3-2. Two types of colony morphologies of *Metarhizium* isolated by *Galleria* bait method from southern Alberta soils. Additional isolates obtained from direct plating onto selective media had morphologies similar to (a).

mentioned. Further, amplification of genomic DNA from isolate 20W-5 produced a band

approximately 250 bp in size in a PCR assay with general fungal primers TW81 and

AB28 (Fig. 3-4), but no product was produced in assays with *M. anisopliae* var.

anisopliae-specific primers Ma-IGSspF and Ma-IGSspR. The isolate was tentatively

identified as *M. anisopliae* on the basis of microscopic examination of spores and

resemblance in colony morphology to *M. anisopliae* var. *anisopliae* ARSEF 727, an

isolate from a tettigonid host in Brazil. Further DNA and morphological characterization

for this isolate is pending.

No mortality in uninoculated control larvae was attributed to fungal infection. All

larvae infected with *M. anisopliae* var. *anisopliae* UAMH 421 showed fungal outgrowth

typical of *Metarhizium* (green conidiation). All larvae inoculated with *M. anisopliae* var.

anisopliae S54 died but no fungal mycosis attributable to *Metarhizium* was observed on

any of the cadavers. No *Metarhizium* sp. were isolated from S54-inoculated cadavers.

3.2.3 PCR assays of soil samples with general fungal primers TW81 and AB28

All soil DNA extracts produced amplification products of varying sizes with

primers TW81 and AB28 (data not shown), thus indicating the presence of PCR-quality

DNA in the soil extracts produced by the MoBio Ultra Clean Soil DNA kit.

3.2.4 *M. flavoviride* var. *flavoviride* PCR assays

3.2.4.1 Specificity

A PCR assay with Mf-ITS-spF and Mf-ITS-spR primers successfully amplified a

340 bp DNA sequence from genomic DNA extracted from *M. flavoviride* var. *flavoviride*

(data not shown). No amplified product was produced by isolates of *M. anisopliae* var.

anisopliae, M. anisopliae var. *acridum, M. flavoviride* var. *minus, B. bassiana,*

55
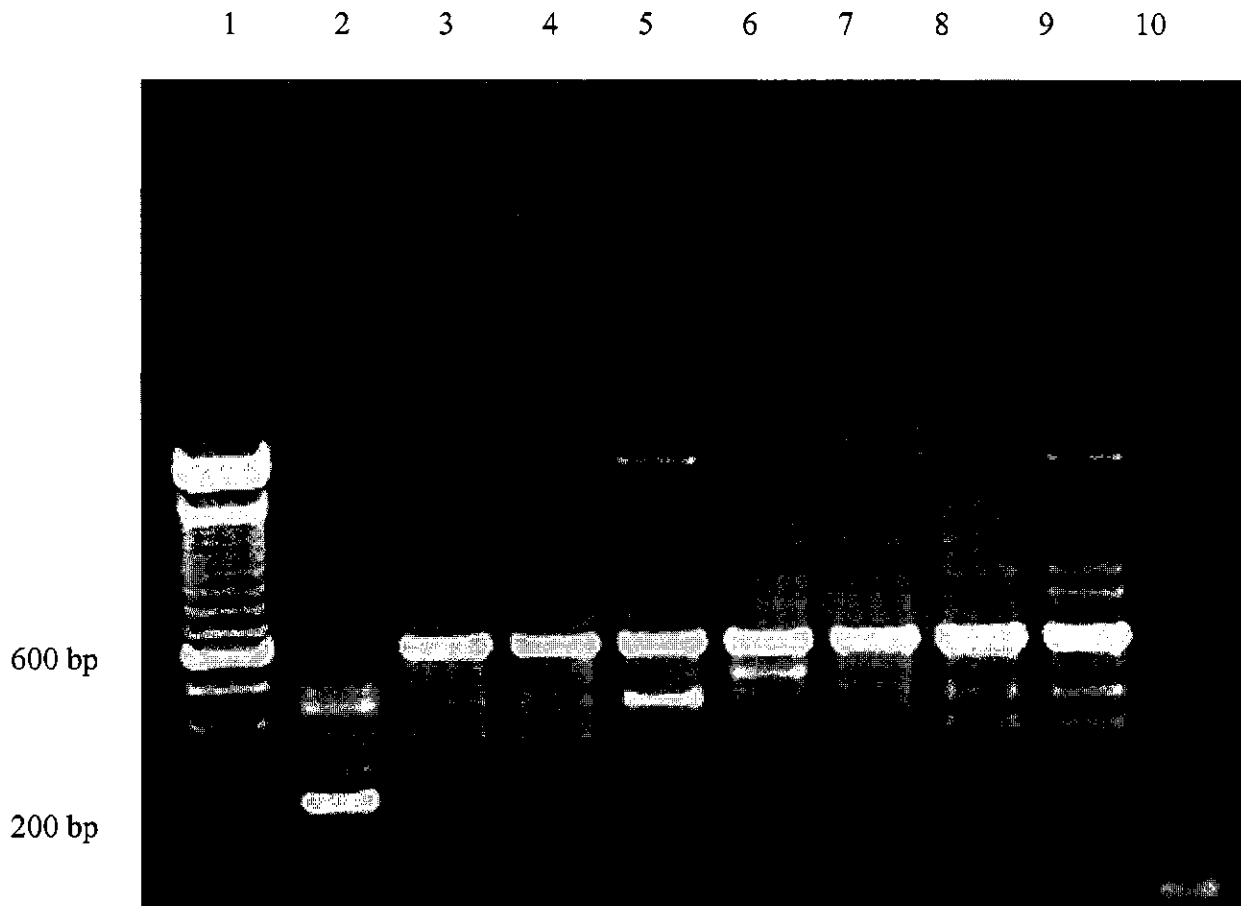
Fig. 3-4. Amplification products from genomic DNA of *M. anisopliae* isolate 20W-5 and other various *M. anisopliae* var. *anisopliae* in a PCR assay using general fungal primers TW81 and AB28

Lane 1: 100 bp ladder
Lane 2: *M. anisopliae* 20W-5
Lane 3: *M. anisopliae* var. *anisopliae* UAMH 421
Lane 4: *M. anisopliae* var. *anisopliae* UAMH 4450
Lane 5: *M. anisopliae* var. *anisopliae* ARSEF 437
Lane 6: *M. anisopliae* var. *anisopliae* ARSEF 727
Lane 7: *M. anisopliae* var. *anisopliae* 6W-2
Lane 8: *M. anisopliae* var. *anisopliae* 11S-1
Lane 9: *M. anisopliae* var. *anisopliae* S54
Lane 10: Water

I. fumosorosea, I. farinosa, V. albo-atrum, C. gloeosporioides, E. nidulans, T. reesei, F. oxysporum, C. rosea f. *catenulata, P. bilaii, H. peziza,* and an isolate of *Rhizopus* sp. (data not shown).

3.2.4.2 PCR assays of soil samples with *M. flavoviride* var. *flavoviride*-specific primers

With one exception, no products were produced in nested PCR assays with TW81 and AB28 primers in intial reactions and *M. flavoviride* var. *flavoviride*-specific primers in secondary amplifications (data not shown). One site produced a weak 340 bp band in a nested PCR with primer combinations TW81/AB28 and Mf-ITS-spF/Mf-ITS-spR (data not shown), but could not be more strongly amplified in a tertiary amplification of the nested reaction with the *M. flavoviride*-specific primers.

3.2.5 PCR assays of soil samples with *M. anisopliae* var. *acridum*-specific primers

No products were produced in nested PCR assays with TW81 and AB28 primers in intial reactions and *M. anisopliae* var. *acridum*-specific primers in secondary amplifications (data not shown).

3.2.6 *M. anisopliae* var. *anisopliae* PCR assays

3.2.6.1 Specificity

Most isolates of *M. anisopliae* var. *anisopliae* produced a 380 bp product in a nested PCR assay with primer combinations Ma-28S4/Ma-IGS1 and Ma-IGSspF/Ma-IGSspR (Fig. 3-2). Most other isolates from section 2.1.1 produced either no product or weak amplification products of varying sizes, some of which were around 400 bp in size (data not shown). *Trichoderma reesei* produced a strongly amplified product of approximately 500 bp (data not shown).

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3.2.6.2 PCR assays of soil samples with *M. anisopliae* var. *anisopliae*-specific primers

Eleven of the twenty sites produced PCR products in a nested assay with the Ma-28S4/Ma-IGS1 (outer) and Ma-IGSspF/MaIGSspR (inner) primer combinations (data not shown). Ten of these sites produced a 380 bp product comparable to the expected size for *M. anisopliae* var. *anisopliae*. Two of the ten sites corresponded to those from which *M. anisopliae* var. *anisopliae* isolates 6W-2 and S54 were recovered on selective medium (section 3.2.1). Another site was the same as the one from which the non-variant *M. anisopliae* var. *anisopliae* (11S-1) was isolated from a *Galleria* cadaver (section 3.2.2). One site produced a strongly amplified product approximately 500 bp in size. No sites produced a product corresponding to the size (approximately 700 bp) yielded by the variant group-B isolate from above (section 3.2.1).

3.3   Discussion

Results from the isolation of *M. anisopliae* var. *anisopliae* on selective medium and by *Galleria* bait assay indicate that this species is present at very low densities ($< 10^2$ CFU per g of soil) and occurrence (25%) in southern Alberta. The global abundance and distribution of *M. anisopliae* is wide ranging. Six of 163 soil (3 %) samples yielded isolates of *M. anisopliae* in subantarctic Macquarie Island (Roddam & Rath, 1997), while 96 % of investigated fields in Switzerland were positive for *M. anisopliae* isolation with either selective medium and/or *Galleria* bait method (Keller *et al.*, 2003), albeit at densities $< 10^4$ CFU per g of soil. Klingen (2000) detected *M. anisopliae* in 10 % of locations sampled in Norway, but Kleespies *et al.* (1989) recorded the presence of *M. anisopliae* in 42 % of samples tested in Germany. Hokkanen and Zimmermann (1986) isolated the entomopathogen in 16.7 % of Finnish agroforestry soil with *Galleria* larvae;

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ten years later, Vänninen (1996) recorded 15.6 % incidence in natural and agricultural

soils in Finland that resulted from bait assays of *Tenebrio molitor*, *Tribolium castaneum*,

Acanthocinus aedilis, and *G. mellonella*. In Tasmania, Rath *et al.* (1992) found *M.*

anisopliae in 28 % of pasture soil samples but at low abundances ($\leq 5 \times 10^4$ CFU per g of

soil), and Milner *et al.* (1998) isolated *M. anisopliae* on selective medium from 14 % of

Australian termite nests and feeding sites at levels close to 10^3 conidia per g of material.

In contrast, in the Near East, Shah *et al.* (1997) obtained no isolates from 243 soils

screened on dodine oatmeal agar in Oman; however, since numerous variables, including

incubation temperature, humidity, composition of medium, and inoculum, influence the

degree of successful isolation on synthetic medium, it is difficult to form any conclusions

about the lack of success. Moreover, the cosmopolitan distribution of *M. anisopliae* was

confirmed when Bidochka *et al.* (1998) found the fungus in 66.7 % of sites in Ontario

baited by *Galleria*. They also recognized an association with agricultural, cultivated

habitats.

In general, the choice of method of isolation of fungi from soils has inherent

limitations. Successful isolation and characterization are dependent upon the ability of

the fungus to grow and sporulate on the medium of choice. It has been assumed that

Metarhizium spp., although usually associated with a specific host, are readily capable of

saprophytic growth as demonstrated by nonfastidious growth on various culture media.

However, based on their experience, Milner *et al.* (1998) noted that *M. anisopliae* rarely,

if ever, grows saprotrophically in soil. Further, isolation directly from soil fails to

indicate if the recovered propagule was in the form of conidia, resting spores, mycelia

surviving on fragments of host tissue or mycelia living on non-insect substrates (Prior,

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1992). The selection of insect host for larval bait method will influence the degree of

success in isolation of a host-specific entomopathogen. Assays that employ *Galleria* as

the target require that the fungal genotype possesses the capability to recognize

Lepidoptera receptors in a suitable host. This capability was not evident in the variant

genotype of *M. anisopliae* var. *anisopliae* isolated in this study that grew well on

selective medium but failed to grow or sporulate on *Galleria* larvae. Coleoptera larvae

(*T. molitor*, *T. castaneum and A. aedilis*) have also been chosen for bait assays since

beetles have been documented as the most common host for *Metarhizium* (Zimmermann,

1993).

Climate conditions have also been correlated to distribution of *M. anisopliae*.

Rath *et al.* (1992) observed an increase in the proportion of soils that yielded *M.*

anisopliae as average annual rainfall increased from 450 to 1250 mm. In undisturbed

pastures, Milner (1992) found densities exceeding 10^6 spores per g of soil in wet climatic

zones. In contrast, densities were less than 2×10^3 in frequently cultivated fields in hot

dry climates. Further, Milner *et al.* (1998) collected most of their *M. anisopliae* isolates

from termite material located in the cooler wetter parts of southeast Australia. Low

densities of *M. anisopliae* in southern Alberta may be attributable to precipitation

amounts approximately two-thirds of average levels and an annual mean temperature

more than half a degree higher than the normal mean during the twelve months prior to

sampling.

The fact that *M. anisopliae* var. *acridum* and *M. flavoviride* were not detected is

in accord with the absence of documented reports of these varieties in North America.

There have been no documented records of *M. anisopliae* var. *acridum* isolated in

temperate regions and, as previously mentioned; the occurrence in tropical regions is rare. Only seven isolates of *M. anisopliae* var. *acridum* are listed in a collection of more than seven hundred cultures of *Metarhizium* (Humber, 2004); all originate from tropical regimes. Although *M. flavoviride* has been obtained from both tropical and temperate regions, no naturally occurring isolates from North America have been entered into the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) based in New York. Moreover, the geographical distribution for *M. flavoviride* var. *minus* has thus far been mostly restricted to the Philippines and the Solomon Islands, with a few isolates recorded from Brazil, Australia and Benin (Humber, 2004).

The successful isolation of *M. anisopliae* var. *anisopliae* isolates from southern Albertan soils confirms their cosmopolitan distribution. Further, the isolation of distinct genetic (isolate S54) and morphological (isolate 20W-5) variants supports the high genetic diversity within this clade (Driver *et al.*, 2000).

Soil samples from one-half of the sites produced PCR products comparable to the expected size with DNA primers for the specific detection of *M. anisopliae* var. *anisopliae*. Isolates of *M. anisopliae* var. *anisopliae* were recovered by direct isolation on selective medium or by waxworm bait assay from three of these 10 sites in support of the PCR results. All isolates were recovered at low incidence, a factor that may explain the lack of isolates recovered from additional PCR-positive sites. Further, the contributions of low levels of soil moisture in the samples (4.39 to 22.70 %), as well as a history of severe drought in southern Alberta over the summers of 2001 – 2003, to low recovery of fungal isolates are unknown.

The Ma-IGSspF/Ma-IGSspR primers used in this study for the detection of *M. anisopliae* var. *anisopliae* DNA 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. 3-2). 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/MaIGSspR primer combinations. Further, it is not known if the 500 bp product obtained from one soil sample is the result of a *Trichoderma* sequence or another *M. anisopliae* genetic polymorphism. Sequence analysis of amplification products could be used for verification, but is not practical for screening purposes. No *M. anisopliae* var. *acridum* were detected by nested PCR in this study, but whether this is a result of lack of this species in a temperate region or inability to detect low levels of target due to inherent low densities or purification losses is unknown. It was also not possible to verify detection of *M. flavoviride* by PCR in one sample due to inability to further amplify the product for additional analysis. The inability to more strongly amplify the weak 340 bp band in a tertiary amplification of the nested PCR reaction with the *M. flavoviride*-specific primers could be attributed to insufficient template DNA that could not be further increased without an accompanying increase in inhibitory products present in the nested reaction mixture, or the weak amplification product may have been an artefact inherent in performance of PCR assays.

Another southern Albertan *M. anisopliae* isolate (20W-5) failed to produce any product in nested PCR assays with the Ma-28S4/Ma-IGS1 and Ma-IGSspF/MaIGSspR

primer combinations. This isolate also produced a much smaller than expected band size of approximately 250 bp in a PCR with the general fungal primers TW81 and AB28 (Fig. 3-4). Driver and Milner (1998) found products that ranged in size from 506 to 573 bp when they sequenced the ITS1-5.8S-ITS2 region for a number of species/varieties of *Metarhizium*. However, Jackson (unpublished data in Glare *et al.*, 1998) identified a *M. anisopliae* isolate from Papua New Guinea that had an ITS1-5.8S-ITS2 region of approximately 300 bp and which displayed only 40-50% homology to other *Metarhizium* isolates. It is therefore apparent that use of sequence information from the rDNA region for separation of *Metarhizium* isolates into various clades may be confounded by some inconsistencies.

Soil samples are problematic for PCR assays due to the presence of inhibitory compounds such as humic and fulvic acids that inhibit *Taq* DNA polymerase through chelation of Mg^{2+} ions (Tebbe & Vahjen, 1993; Tsai & Olson, 1991). The most crucial limitation to PCR is nucleic acid quality. Extensive purification is required, but each purification step can result in 50 to 90 % loss of DNA. The loss of starting material, however, is offset by the sensitivity of PCR assays in the ability to detect very minute amounts of target. However, copy fidelity, formation of chimeric products (artifacts), sequencing errors, and variable hybridization efficiencies from a mixed pool of templates all contribute to PCR bias (von Wintzingerode *et al.*, 1997). Although PCR has been successfully used to detect soil-based fungal pathogens (Lochman *et al.*, 2004; Ippilito *et al.*, 2002; Cumagun *et al.*, 2000; Bell *et al.*, 1999), in this study inconsistencies were observed with the results of the PCR assays. In this case, the primers would be more

suitable in confirmation of identification of *Metarhizium* isolates, rather than direct identification within an environmental matrix.

Nonetheless, successful isolation of isolates of *M. anisopliae* var. *anisopliae* from soils in southern Alberta indicates that this species can survive and even possibly persist in agroecosystems under semi-arid conditions and thereby offers promise as recourse for grasshopper management. Moreover, employment of molecular methodology will permit differentiation of an applied strain of *M. anisopliae* var. *acridum* from native southern Albertan strains of *M. anisopliae* var. *anisopliae* during the phase of environmental monitoring.

Chapter 4. **Evaluation of Indigenous Isolates of *Metarhizium anisopliae* for Pathogenicity towards Grasshoppers in Southern Alberta**

4.0 Introduction

By definition, classical biological control has been defined as the intentional introduction of an exotic, sometimes co-evolved, biological control agent for permanent establishment and long-term pest control (Eilenberg *et al.*, 2001). Steps for this process include determination of a target, acquisition of natural enemies, selection of an environmentally safer natural enemy for release, establishment of the natural enemy in a suitable habitat, management of the project through education and integration of cooperative parties, and assessment of the final outcome (Van Driesche & Hoddle, 2000). Although the general consensus is that the pest's area of origin is the best location for finding natural controls, in the case of North American grasshopper species, there is much disagreement on determination of a pest's original home range. Carruthers and Onsager (1993) have argued that grasshoppers have not evolved in North America and therefore should not be considered native, thus supporting their claim that use of exotic agents for control of nonindigenous pests is merely a reunion of current pests with former natural enemies. Conversely, Lockwood (1993a,b) contended that orthoptera have indeed speciated over 300 million years on the North American land mass known as Laurasia (Rehn, 1954) and, therefore, should be considered native. This argument was used to emphasize the significant differences that potentially exist for undesirable ecological consequences expected from introduction of an exotic species for control of a native target versus use of a native species to control a native pest.

In North America, indigenous and exotic introductions of fungal biocontrol agents have been attempted for the control of grasshoppers, albeit with qualified success. In

Canada, 70 % mortality due to mycosis was obtained in grasshoppers confined in laboratory cages following treatment with a US isolate of *Beauveria bassiana* and collection within two days of application (Johnson & Goettel, 1993). However, significant and declining levels of infection were observed in insects collected after this time up to 19 days post-application. In the United States, an introduced Australian pathotype of *Entomophaga grylli* was identified in up to 23 % of *E. grylli*-infected grasshoppers one year after fungal introduction (Bidochka *et al.*, 1996). This figure declined to less than 2 % two years following introduction and no infection attributable to the exotic pathotype could be confirmed three years post-introduction. Currently, there has been no documentation in the literature on any introductions of *Metarhizium* (exotic or indigenous) for control of North American orthoptera.

This study shows the results of pathogenicity screening of two southern Albertan isolates of *Metarhizium anisopliae* var. *anisopliae* in comparison to a standard strain (IMI 330189) of *M. anisopliae* var. *acridum* in nymphs of a laboratory colony of *Melanoplus sanguinipes* and field-collected grasshoppers following application of spores in bioassays.

4.1   Materials and Methods

4.1.1   Fungal isolates and cultivation

Metarhizium anisopliae var. *anisopliae* fungal isolates S54 (genetic group-B variant) and 20W-5 (distinctive colony morphology from other native isolates), isolated from southern Alberta soils, were propagated and maintained on PDA. *M. anisopliae* var. *acridum* IMI 330189 was obtained and propagated as previously mentioned (section 2.1.1).

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4.1.2 Inoculation of grasshoppers

Third and fourth instar nymphs of a laboratory colony of *Melanoplus sanguinipes* were randomly collected, inoculated, and housed as previously described (section 2.1.6). Third and fourth instar field-collected nymphs of *M. sanguinipes*, *M. bivittatus*, *M. packardii*, and *Camnula pellucida* were also randomly collected, inoculated, and housed as previously described (section 2.1.6).

All three *Metarhizium* isolates listed in section 4.1.1 were used to treat nymphs of a laboratory colony of *M. sanguinipes*, but only isolates S54 and IMI 330189 were evaluated in assays with field-collected nymphs. Due to sparse sporulation on solid culture media, isolate 20W-5 was not tested on field-collected nymphs.

4.1.3 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 (section 2.1.8). Cadavers were frozen as previously described (section 2.1.6) prior to DNA extraction as per section 2.1.7. In order to minimize environmental contamination with an exotic isolate of *Metarhizium*, external sporulation was not allowed on IMI 330189-infected cadavers.

Mortality attributed to native isolates 20W-5 and 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 one minute in 70 % ethanol followed by one minute in sterile distilled water. Cadavers were then placed individually on moistened sterile filter paper in a 60 mm x 10 mm Petri dish, the dish sealed with Parafilm®, and incubated at *ca.* 20 °C for a maximum of twenty-one days. In the case of

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S54, *M. anisopliae* var. *anisopliae* infection in infected cadavers was also confirmed by PCR. Conidia from the surface of infected cadavers were transferred with a sterile loop to 500 μl of potato dextrose broth (PDB) and incubated 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 with outer primers Ma-28S4 and Ma-IGS1 followed by a second reaction with inner primers Ma-IGSspF and Ma-IGSspR as per section 3.1.5.3. Insects that did not display signs of external sporulation after 21 days were then frozen at -20 °C until DNA extraction as per section 2.1.7.

4.1.4   Data analysis

For all treated grasshopper experiments, mortality data from control insect groups were used in Abbott's (1925) formula to correct total insect 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 % of treated insects (LT$_{50}$) with upper and lower 95 % confidence limits (CL).

4.2   Results

4.2.1   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 4-1). Daily corrected cumulative mortalities for laboratory-reared and field-collected grasshopper nymphs challenged with IMI 330189 are shown in Figs. 4-1 and

Table 4-1. Lethal time for 50 % population mortality (LT_{50}) with confidence limits
$(1 - \alpha = 95\ \%)$ of laboratory and field-collected grasshopper nymphs treated with conidia
of three *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)	Confidence limit Lower Upper	Mycosis attributed to *Metarhizium* (%)	Specific PCR positive (%)
Laboratory colony nymphs:					
IMI 330189	126	4.1	$3.87 - 4.37^{a}$	N/A	100
20W-5	77	6.3	$5.92 - 6.68^{b}$	79.2	N/A
S54	71	6.7	$6.20 - 7.29^{b}$	91.5	97.2
Field-collected nymphs:					
IMI 330189	112	4.7	$4.43 - 4.93^{c}$	N/A	100
S54	125	4.4	$4.28 - 4.60^{a,c}$	83.2	97.6

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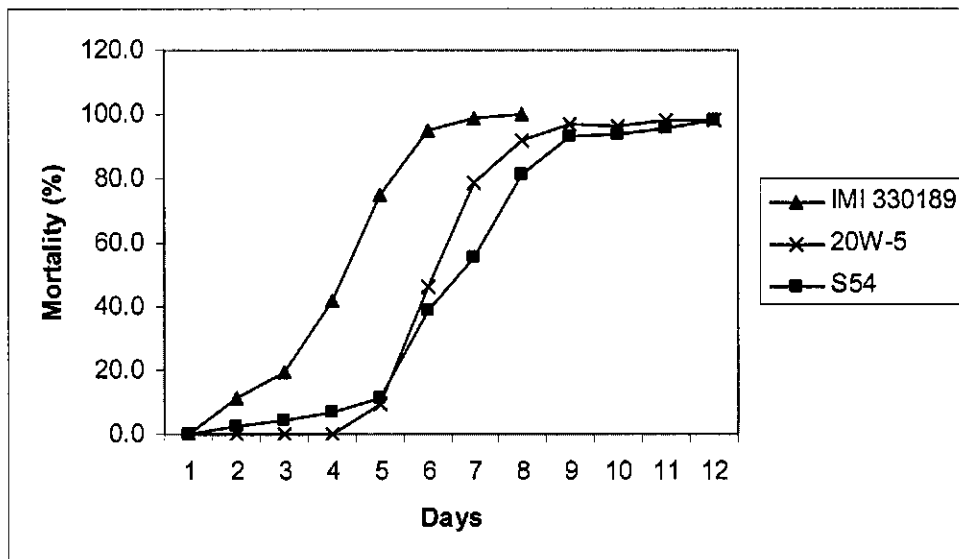
Fig. 4-1. Cumulative mortality of laboratory grasshopper nymphs treated with three isolates of *Metarhizium* under laboratory conditions of 24 °C/16 °C day/night with a corresponding 16/8 h light/dark photoperiod under ambient relative humidity (40-55 %).

4-2, 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 *M. anisopliae* var. *acridum* from 92.1 % and 92.0 % of treated laboratory colony and field-collected insects, respectively. No *Metarhizium* spp. were isolated 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 (data not shown).

As observed earlier, molting (51.6 % in the treated colony, 16.1 % in the treated field-collected group) did not inhibit detection of fungal DNA. Frequency of ecdysis was lower at 34.6 % eight days post-treatment in untreated laboratory colony nymphs; the converse was true for field-collected insects where molts were observed in 52.2 % of untreated grasshoppers 9 days post-treatment.

4.2.2 Infection of laboratory-reared grasshopper nymphs with *M. anisopliae* isolate 20W-5

Cumulative mortality in laboratory colony nymphs treated with spores of *M. anisopliae* isolate 20W-5 was 98.7 % twelve days post-inoculation. Sporulation was observed in 79.2 % of treated insects and occurred within 2 weeks after death (Fig. 4-3a). Molting occurred in 9.1 % of infected nymphs and LT_{50} was 6.3 days for insects exposed to isolate 20W-5 (Table 4-1). Daily corrected cumulative mortality for treated nymphs is shown in Fig. 4-1.

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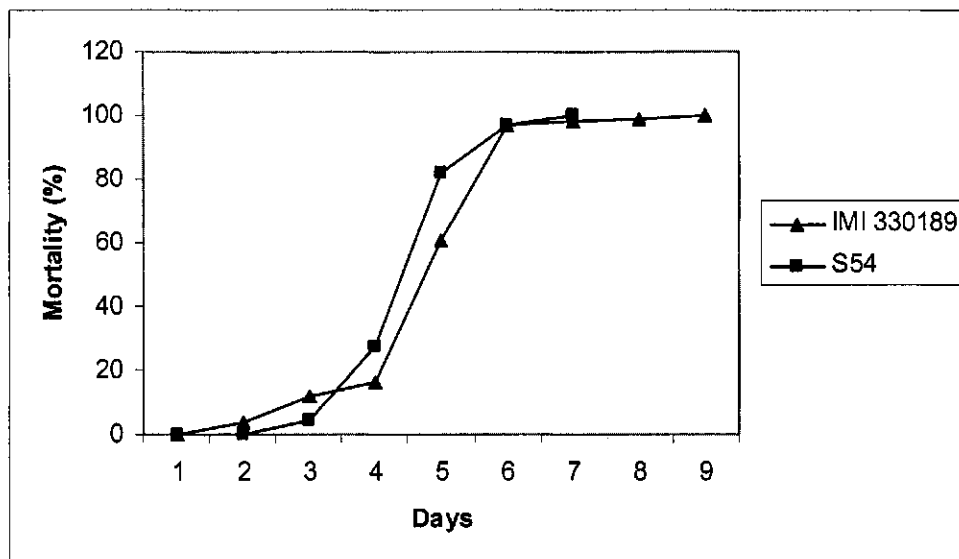
Fig. 4-2. Cumulative mortality of field-collected grasshopper nymphs treated with two isolates of *Metarhizium* under laboratory conditions of 24 °C/16 °C day/night with a corresponding 16/8 h light/dark photoperiod under ambient relative humidity (40-55 %).
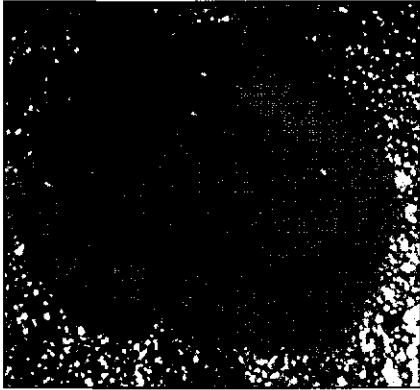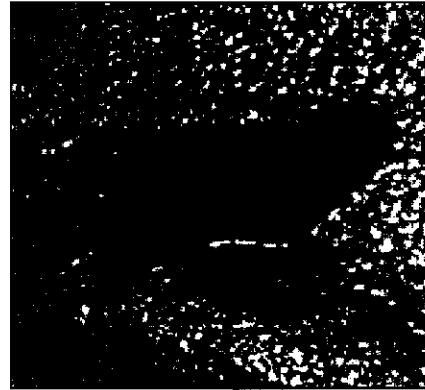
(a)   (b)

Fig. 4-3. Sporulation of southern Albertan soil isolates of *Metarhizium* on *Melanoplus sanguinipes* nymphs from a laboratory colony.
(a) isolate 20W-5   (b) isolate S54

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4.2.3 Infection of laboratory-reared and field-collected grasshopper nymphs with

***M. anisopliae* var. *anisopliae* isolate S54**

Similar final mortality was observed in laboratory colony nymphs treated with *M. anisopliae* var. *anisopliae* isolate S54. At 12 days post-inoculation, cumulative mortality was 98.6 % (Fig. 4-1). Conidial appearance on infected cadavers (Fig. 4-3b) was very similar to published photos of *M. anisopliae* var. *acridum* sporulation on infected locusts (Fig. 4-4). Sporulation occurred in 91.5 % of S54-treated insects and LT_{50} was 6.7 days (Table 4-1). Rate of sporulation on S54 cadavers was more rapid compared to insects challenged with isolate 20W-5, with 100 % conidiation completed within one week following death. Slightly less than 10 % of S54-infected insects molted in this bioassay.

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. No band sizes corresponding to those expected for *M. anisopliae* var. *anisopliae* with these primers were detected in untreated insects. The sole surviving insect 12 days post-inoculation of the treated group also tested negative with the nested PCR assay.

Complete mortality was obtained 7 days post-inoculation in field-collected nymphs treated with isolate S54 (Fig. 4-2). The LT_{50} value for this bioassay was lower at 4.4 days compared to 6.7 days for infected laboratory colony nymphs (Table 4-1). 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

74
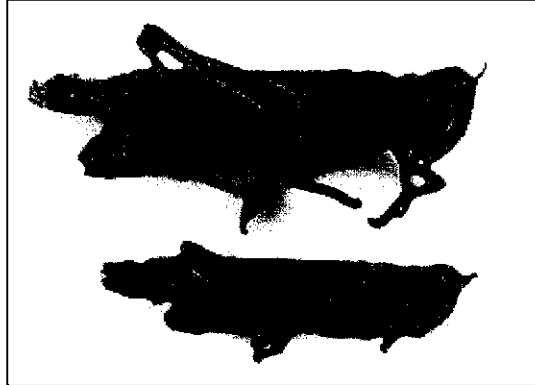
Fig. 4-4. Plague locusts infected with Green Guard® (*M. anisopliae* var. *acridum*)
Photo courtesy of the Commonwealth Scientific and Industrial Research Organisation
(CSIRO), Australia

sporulation in 83.2 % of infected insects one week after death. As with S54-infected laboratory colony grasshoppers, the incidence of ecdysis was low at 12.8 %.

In tandem with an observation of increased lethargy, there was also a noticeable reduction in food consumption by all insects in all *Metarhizium*-treated groups, with the sole exception of the surviving S54-treated laboratory colony nymph, versus their untreated counterparts although these observations were not quantified. As previously demonstrated, ecdysis did not interfere with development of mycosis. No mortality in control groups was attributable to *Metarhizium*-induced mycosis. Final cumulative mortalities in control groups were 46.2 % and 34.5 % for laboratory colony and 17.4 % for field-collected grasshopper nymphs.

The cumulative mortality curves for treated laboratory colony grasshoppers (Fig. 4-1) and treated field-collected nymphs (Fig. 4-2) approach the sigmoidal shape expected for populations of target insects treated with entomopathogenic fungi (Bateman *et al.*, 1996).

4.3 Discussion

Prior to application of a biological control agent, a method is required that allows discrimination of the introduced organism from indigenous populations. The two native isolates of *M. anisopliae* evaluated in bioassays against *M. sanguinipes* in this study were selected on the basis of their distinctive genetic characteristics. One isolate, S54, was chosen because it could be distinguished from other native isolates of *Metarhizium* in a PCR assay. The other isolate (20W-5) possessed unique morphological characteristics that would permit visual differentiation of this fungal isolate from others. The four grasshopper species (*M. sanguinipes, M. bivittatus, M. packardii*, and *C. pellucida*) used

in assays of field-collected grasshoppers were selected as they represent the main cereal pest species in Alberta.

The discovery of two soil isolates of *Metarhizium* that demonstrated comparable virulence with a commercialized isolate towards orthopteran species in southern Alberta is contrary to earlier studies that showed direct isolation from a target host was the most appropriate method for isolation of target-specific pathotypes. Kooyman and Shah (1992) collected one hundred and twenty-eight isolates of *Metarhizium* directly from orthopteran hosts, but failed to find isolates in two hundred and forty-three soils screened with dodine oatmeal agar (Shah, 1994) in a survey conducted in Africa and the Near East. Further, documentation of isolates of *M. anisopliae* var. *anisopliae* from orthopteran hosts is not common. In Spain, *M. anisopliae* (Metschnikoff) was isolated from four individuals out of three hundred and seventeen Moroccan locusts but degree of virulence was not determined (Hernández-Crespo & Santiago-Álvarez, 1997). Of the one hundred and twenty-eight isolates of *Metarhizium* identified by Kooyman and Shah (1992), only seven were classified as *M. anisopliae*. The remainder were *M. flavoviride*, some to be later renamed as *M. anisopliae* var. *acridum*. Prior (1992) noted that as of 1992, no examples had been found of non-orthopteran isolates of *Metarhizium* spp. with high virulence to the desert locust *S. gregaria*.

However, other studies have demonstrated the value of *Metarhizium* isolates that have non-orthopteran origins for pathogenicity to acridids. Bateman *et al.* (1996) recorded nine non-orthopteran isolates of *Metarhizium* spp., one of which originated from a coleopteran host in the US, which showed virulence to *S. gregaria* that was comparable to a highly virulent standard isolate originally isolated from an acridid in Niger. Further,

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four of the nine isolates originated from tropical soils. As well, 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. Their conclusion was 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; Inglis *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 works (Shah *et al.*, 1998a; D. Hunter, pers. comm.).

In contrast with previous results from bioassays of field-collected grasshoppers, in this study southern Albertan isolates of *M. anisopliae* induced a significantly 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 admitted 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 contrary to what 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 both native

isolates of *M. 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 two indigenous strains; however, this resistance was insufficient to

prevent almost 100 % mortality twelve days post-treatment. The degree of variability in

susceptibility of the target population could be confirmed by treatment with a varying

range of spore concentrations and use of probit transformation to convert the sigmoidal

curve resulting from a plot of mortality versus dose into a straight line. A slope with a

value of less than one indicates a greater variation in host susceptibility in terms of

concentration lethal to 50 % of the population. However, it should be noted that slope is

also a function of variability in actual dose administered (Meynell, 1957) in that

application of a particular dose is not necessarily an indication of the number of spores

that actually result in infection. Nonetheless, it has been well documented that there can

be large differences among host genotypes in insect populations in response to microbial

pathogens (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.

Regardless of the type and duration of physiological response shown by treated

insects in assays of laboratory stock and field-collected nymphs, reduction in feeding was

observed in all treated groups. These results are supported by a previous study that

quantified reduced feeding in variegated grasshoppers infected with *M. flavoviride* (Thomas *et al.*, 1997).

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). Hokkanen and Pimentel (1984) calculated a 75 % higher rate of success for biological control programs that employed new parasite-host associations over those that were based on long-evolved associations applied in classical biological control. Further, Lockwood (1993a,b) has pointed out that native biological agents can and do play a major role in the control of grasshopper population dynamics and that indigenous evolutionary and ecological processes would provide constraints on augmented native organisms, thus any potential effects would more likely be predictable, localized, and temporary. 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* with a simple molecular assay. Much more work is required to determine host specificity of this fungus, since there is no indication of the entomopathogen's host range because it was isolated from soil, but failure to sporulate on *Galleria* larvae suggests that the host range may not include lepidopterans. Specific non-target and efficacy trials should be done as the scale on control is expanded to commercial levels because implementation of a native pathogen in a biological control program would help alleviate regulatory concerns about ecological consequences.

Chapter 5.   Prediction of *Metarhizium anisopliae* var. *acridum* Efficacy for Grasshopper Biocontrol in a Temperate Region Based on Historical Weather and Climate Data

5.0   Introduction

Biocontrol of grasshoppers and locusts with an entomopathogenic fungal agent is typically constrained primarily by environmental temperature (Blanford & Thomas, 2000; Carruthers *et al.*, 1992). A key factor mediating pathogen development is the ability of target acridids to regulate their body temperature to a preferred set point (thermoregulation) independent of daytime ambient temperatures through interaction of environmental, physical, and behavioural factors (Lactin & Johnson, 1998; Chappell & Whitman, 1990; Kemp, 1986). Further, these insects have the capacity to increase the preferred set point in response to infection by *M. anisopliae* var. *acridum* (Ouedraogo *et al.*, 2003; Elliot *et al.*, 2002; Gardner & Thomas, 2002; Blanford & Thomas, 1999a,b; Blanford *et al.*, 1998).

In conjunction with temperature, humidity has been shown to play a more pivotal role in horizontal transmission of the entomopathogen through sporulation on infected cadavers and persistence in the environment (Arthurs & Thomas, 2001a, 1999; Arthurs *et al.*, 2001; Thomas *et al.*, 1997) than in germination on the insect cuticle (Bateman *et al.*, 1993). The impact of solar radiation on pathogen persistence in the environment has been acknowledged (Braga *et al.*, 2001a; Shah *et al.*, 1998a; Moore *et al.*, 1996), but confounded by conflicting results and difficulties in quantification of conditions in a natural setting. Intravarietal differences between isolates in tolerances to temperature, humidity and solar radiation also contribute to the uncertainty but also confer persistence in the environment to entomopathogens.

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In a few instances, weather conditions have been recorded and efficacy of

Metarhizium anisopliae var. *acridum* estimated in tropical climates. These observations

will be used to estimate *M. anisopliae* var. *acridum* efficacy in the temperate Prairie

provinces of Canada based on historical weather and climate data as well as observations

of native grasshopper thermoregulatory behaviour.

5.1   Thermoregulation

Several studies have shown that, given conditions optimal for thermoregulation,

grasshoppers and locusts aspire to reach a preferred body temperature of 38-42 °C under

laboratory (Blanford & Thomas, 1999a,b; Lactin & Johnson, 1995, 1996a) and field

(Blanford & Thomas, 2000; Blanford *et al.*, 1998; Carruthers *et al.*, 1992; Kemp, 1986)

conditions. The adaptive advantages for this behaviour include optimum temperatures

for rates of feeding and development (Lactin & Johnson, 1995, 1996a). Depending on

ambient temperature and availability of solar radiation, body temperatures will increase

rapidly from a few degrees below ambient up to preferred body temperatures, followed

by a decline as ambient temperatures decrease (Fig. 5-1). Equilibrium at preferred body

temperatures is maintained by adoption of heat aversion strategies through microhabitat

selection and postural adjustment (Blanford *et al.*, 1998), as well as behaviour such as

hyperventilation to promote reduction of body temperature by evaporative cooling

(Carruthers *et al.*, 1992; Chappel & Whitman, 1990). Figure 5-2 demonstrates the

nonlinear relationship between body and ambient temperatures.

In contrast, Lactin and Johnson (1998) noted a linear relationship between

ambient air temperature at 1 meter and mean body temperature of rangeland grasshoppers

distributed and oriented randomly within the vegetation canopy in southern Alberta.

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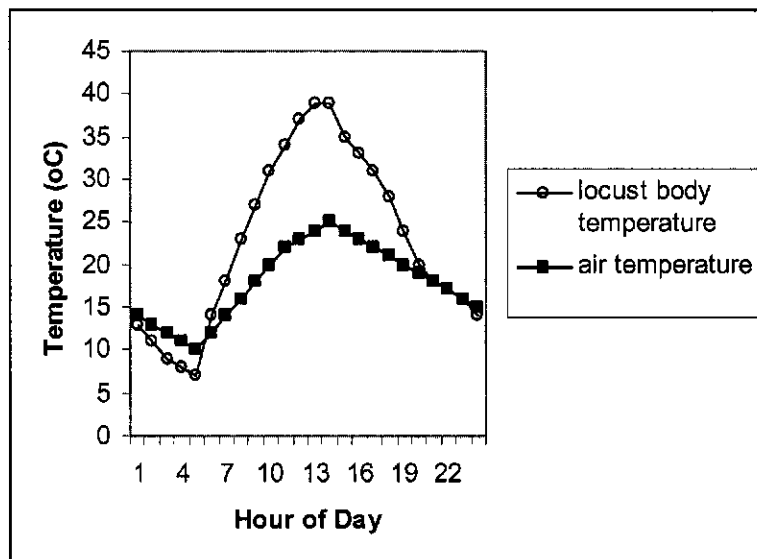
Fig. 5-1. Examples of an hourly pattern of thermoregulation for Australian plague locusts
(data courtesy of D. Hunter)
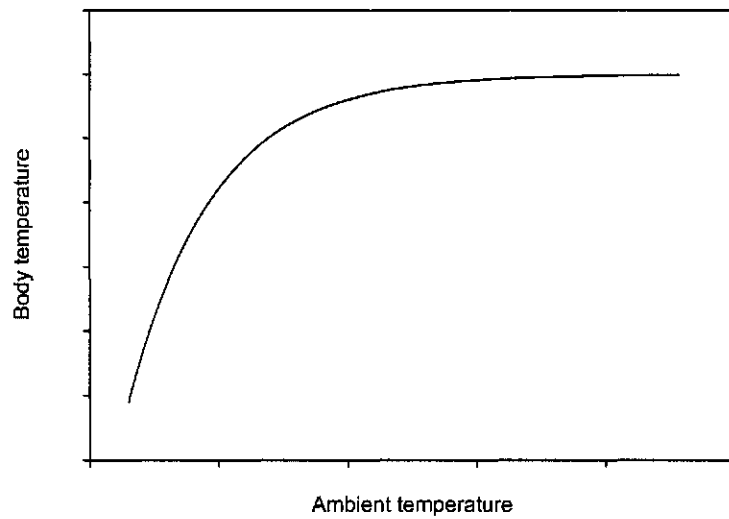
Fig. 5-2. Distribution of body temperature against ambient temperature. The curve
estimates best fit nonlinear regression describing the pattern of active thermoregulation
(modified from Blanford & Thomas, 2000).

They observed that within a range of ambient temperatures between 15-28 °C, body

temperatures were usually three to four degrees higher than ambient. Grasshopper data

from Australia are consistent with the Lactin and Johnson observations and suggest that

grasshoppers do not increase their body temperatures as much as locusts (D. Hunter, pers.

comm.).

Optimal *in vivo* development rates for *M. anisopliae* var. *acridum* occur at 28-30

°C (Arthurs & Thomas, 2001b; Fargues *et al.*, 1997; Thomas & Jenkins, 1997). Pathogen

growth and mortality still occur over a range of 15-35 °C, but the period of latent

infection can be significantly longer (Arthurs & Thomas, 2001b, 1999; Fargues *et al.*,

1997; Thomas & Jenkins, 1997). Maintenance of a set body temperature of 38-42 °C has

been shown to not alter the final outcome of infection but does influence the rate of

mortality since duration at temperatures sub-optimal for pathogen growth is constrained

by environmental factors such as ambient temperature and solar radiation (Arthurs &

Thomas, 1999). Blanford *et al.* (1998) noted that in tropical regimes, *in vivo* fungal

development can be checked for a maximum of 7-8 hours per day and most growth will

occur during cooler periods in the evening.

5.1.1 Behavioural fever

Some acridids have shown the ability to raise the preferred body temperature set

point a few degrees approaching 44 °C, resulting in possible suppression of infection

(Ouedraogo *et al.*, 2003; Elliot *et al.*, 2002; Gardner & Thomas, 2002; Blanford &

Thomas, 1999a,b; Blanford *et al.*, 1998). This response, known as behavioural fever, is

believed to assist the host immune system in suppression of pathogen development and

although therapeutic benefits have been recognized, the underlying mechanisms for this

host-mediated adaption are unclear (Gardner & Thomas, 2002). Further, the costs

associated with this behaviour include impacts on feeding efficiency, host development,

and susceptibility to predation (Gardner & Thomas, 2002). Negative consequences on

the pathogen are temporary and, as with regular thermoregulation, outcome of infection

will probably not be affected (Ouedraogo *et al.*, 2002; Blanford & Thomas, 1999a,b;

Blanford *et al.*, 1998). However, it has been speculated that low numbers of pathogen

may be eliminated (Elliot *et al.*, 2002) and that variability in behavioural fever effects

among host species may contribute to the variation in efficacy experienced with

application of biocontrol agents (Blanford *et al.*, 1998).

5.2   Efficacy of *M. anisopliae* var. *acridum* under tropical regimes

As previously mentioned, a common observation of efficacy of an applied

pathogen is variable performance and is likely due to spatial and temporal variations in

biotic and abiotic factors (Blanford *et al.*, 1998). In Brazil, Magalhães *et al.* (2000b)

estimated 88 % mortality of grasshopper nymphal bands 14 days post application (dpa) (2

x 10^{13} conidia per hectare [ha]) with a native isolate of *M. anisopliae* var. *acridum* in

field trials at the beginning of the rainy season with maximum temperatures ranging from

28-31 °C and minimum temperatures from 18-22 °C. Approximately 250 mm of

precipitation accumulated during this time from nine events of rainfall. In Mauritania,

Langewald *et al.* (1997) achieved mortality rates of 99 % in caged samples of treated

desert locusts (5 x 10^{12} conidia per ha) in 15 days and estimated 70 % reduction in

population numbers between 2 and 8 days post application of a treated hopper band in a

semi-desert area where air temperature varied between 40 °C around 2 p.m. and 16 °C

around 5 a.m. (local time) with Green Muscle®. Similarly with isolate I91-690, Douro-

Kpindou *et al.* (1995) observed 96 % reduction in counts of treated *Zoncerus variegatus* populations 15 dpa with 2×10^{12} conidia per ha applied to 1 ha plots under conditions of 31.5 to 35 °C at time of treatment in southern Benin. However, Kooyman and Abdalla (1998) calculated an average control efficiency of 68 % of tree locusts in Sudanese plots ranging from 10-25 ha treated with *ca.* 5×10^{12} Green Muscle® conidia per ha with maximum temperatures ranging from well below 30 °C on heavily clouded days to close to 40 °C on sunny days and temperatures in the low 20s recorded in the early morning during the trial.

Milner (1997) also noted considerable variation in field trials in Australia with Green Guard®. Treatment of 50 ha for the wingless grasshopper *P. vittatum* with 4×10^{12} conidia per ha required 7 days for high levels of mortality where daily maximum temperatures were hot (approaching 40 °C) compared to 21 days for sites (5 ha) where temperatures did not exceed 20 °C. Hunter *et al.* (1999) estimated less than 10 % survival of treated migratory locust bands in open grassland and crops in Australia 15-18 dpa of $3\text{-}4 \times 10^{12}$ conidia per ha of the same isolate. Air temperatures during these trials ranged from mild (28-32 °C) to hot ($\geq$ 35 °C).

5.3   **Historical weather data in the Prairie provinces of Canada and potential for**
M. anisopliae **var.** *acridum* **application**

Weather data, where available, were obtained for the years 1975 to 2005 from nine agriculture research weather stations located in Alberta, Saskatchewan, and Manitoba. The meteorological variables included daily values of maximum air temperature, minimum air temperature, sunshine hours, and precipitation. Means for the months of June, July, and August over the thirty years were calculated for each variable

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at each station with the MEANS procedure of SAS (SAS Institute, 2005) (Appendices 3-6).

5.3.1 Air temperature

For each location, July was the warmest month and was significantly warmer than the other two months (Appendix 3). The means for all three months ranged from a low of 18.6 °C for June at Stavely, AB to 26.9 °C for July at the Onefour, AB station. These temperatures fall within the 15-35 °C range for growth of *M. anisopliae* var. *acridum*, indicating that high temperatures will probably not be a factor that precludes pathogen efficacy in a temperate environment. The minimum daily high temperature was recorded at Lethbridge, AB (2.7 °C) and the maximum was 40 °C at Morden, MB. Although outside the range for pathogen development, these temperatures would have a transient impact on the fungus.

June proved to be significantly cooler than July and August, with mean minimum air temperatures ranging from 5.7 °C at Stavely, AB to 14.5 °C at Morden, MB (Appendix 4). These temperatures would certainly inhibit fungal growth but, as with maximum temperatures, probably only temporarily. Of greater concern is the observation of several recordings of temperatures below 0 °C. Eight stations recorded at least one instance of a below freezing temperature in June; all nine stations experienced subzero temperatures in August (range of -0.5 to -7.0 °C). The minimum requirements for duration or magnitude of subzero temperatures necessary for *M. anisopliae* var. *acridum* or grasshopper mortality are unknown.

Under the assumption that native grasshoppers increase their body temperatures three to four degrees above ambient (Lactin & Johnson, 1998) if conditions allow for

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thermoregulation, an adjusted range of ambient temperatures for development of *M.*
anisopliae var. *acridum* would be 15 °C (no thermoregulation) to 31 °C

(thermoregulation would increase host body temperature to 34-35 °C). The FREQ

procedure of SAS (SAS Institute, 2005) was used to calculate frequencies for ambient

temperatures that would preclude growth of *M. anisopliae* var. *acridum* (i.e. maximum

temperatures > 31 °C, maximum temperatures < 15 °C, and mean temperatures < 15 °C).

Results are presented in Table 5-1. For the month of June, all stations have a very small

percentage of days for which the ambient temperature would have been too warm for part

of the day for pathogen growth (> 31 °C). For example, 6.45 % of days in June over 30

years would have had some temperatures too warm in Morden, the location with the

highest mean maximum air temperature (Appendix 3). The number of days in July and

August with too warm temperatures for part of the day increased slightly, but still

remained less than 25 %, suggesting that hot weather will not be a concern for application

of *Metarhizium* for biocontrol. Cool weather will probably be more of a determining

factor in pathogen efficacy as more than half the locations recorded mean temperatures <

15 °C at frequencies greater than 50 % over at least one of the summer months, thus

indicating a delay in fungal development for part of the day. However, there is no

location for which there would be a permanent cessation of *M. anisopliae* var. *acridum*

growth due to cool temperatures during the summer. The data indicate that summer

temperatures in the Prairie provinces of Canada would not be a deterrent to *M. anisopliae*

var. *acridum* efficacy.

Table 5-1. Thirty year summary of frequencies of ambient air temperatures that would preclude the growth of *M. anisopliae* var. *acridum* during June, July, and August in Canada's Prairie provinces

Location	Month(s)	Max > 31 °C	Max < 15 °C	Mean < 15 °C
Lethbridge, AB	June	23 (2.48)[e]	78 (8.40)	446 (48.01)
	July	125 (13.13)	21 (2.21)	145 (15.31)
	August	128 (13.36)	46 (4.80)	242 (25.29)
	summer[d]	276 (9.72)	145 (5.11)	833 (29.28)
Onefour, AB	June	56 (6.02)	67 (7.20)	414 (44.52)
	July	214 (22.27)	16 (1.66)	108 (11.24)
	August	214 (22.27)	28 (2.91)	195 (20.29)
	summer	484 (16.97)	111 (3.89)	717 (25.14)
Vauxhall, AB	June	47 (5.52)	47 (5.52)	358 (42.07)
	July	148 (16.48)	7 (0.78)	122 (13.59)
	August	171 (19.04)	30 (3.34)	207 (23.05)
	summer	366 (13.82)	84 (3.17)	687 (25.95)
Stavely, AB	June	1 (0.13)	192 (24.68)	615 (79.05)
	July	36 (4.44)	62 (7.65)	375 (46.35)
	August	22 (2.72)	92 (11.36)	407 (50.31)
	summer	59 (2.46)	346 (14.43)	1397 (58.31)
Lacombe, AB	June	5 (0.54)	125 (13.44)	641 (68.92)
	July	19 (1.98)	38 (3.95)	395 (41.10)
	August	20 (2.11)	108 (11.38)	497 (52.48)
	summer	44 (1.55)	271 (9.54)	1533 (54.02)
Beaverlodge, AB	June	2 (0.22)	135 (14.64)	642 (70.32)
	July	9 (0.94)	62 (6.45)	453 (47.29)
	August	19 (1.98)	125 (13.02)	552 (57.56)
	summer	30 (1.66)	322 (11.33)	1647 (58.20)
Ft. Vermilion, AB	June	4 (0.83)	41 (8.54)	223 (46.46)
	July	17 (3.43)	15 (3.02)	105 (21.17)
	August	10 (2.02)	71 (14.31)	254 (51.21)
	summer	31 (2.11)	127 (8.63)	582 (39.54)
Scott, SK	June	23 (2.47)	100 (10.75)	498 (53.55)
	July	58 (6.04)	22 (2.29)	239 (24.87)
	August	82 (8.53)	71 (7.39)	371 (38.61)
	summer	163 (5.72)	193 (6.77)	1108 (38.85)
Morden, MB	June	60 (6.45)	57 (6.13)	267 (28.71)
	July	112 (11.65)	8 (0.83)	57 (5.93)
	August	112 (11.67)	13 (1.35)	157 (16.37)
	summer	284 (9.96)	78 (2.74)	481 (16.88)

[d] months of June, July, and August combined
[e] numbers in parentheses represent percentages for the respective month(s) over 30 years

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5.3.2 Sunshine hours

At the time of the summer solstice (June 21) in Canada, the stations at Lethbridge,

Onefour, Stavely, and Vauxhall could expect a 16-hour daylight period with a maximum

approaching 18 hours for the most northerly station at Fort Vermilion (Atlas of Canada,

Natural Resources Canada). Solar radiation has been identified as a key factor in

thermoregulation of grasshoppers (Lactin & Johnson, 1998, 1996b). Stations such as

Lethbridge, Onefour, Stavely, and Vauxhall observed the highest mean values of

sunshine hours, representing more than 50 % of available daylight hours in most months

(Appendix 5). Thermoregulation, therefore, will play a significant, albeit infrequent and

short-termed, role in the determination of pathogen development at higher temperatures

due to low frequencies of maximum air temperatures in excess of 31 °C (Table 5-1).

However, at ambient temperatures suboptimal for fungal growth, solar radiation may

contribute to an increase in host body temperatures to a more optimal range for the

pathogen.

5.3.3 Precipitation

It has previously been shown that initiation (germination) of *M. anisopliae* var.

acridum infection is not dependent upon ambient humidity (Bateman *et al.*, 1993);

however, sporulation and horizontal transmission of the pathogen only occur under high

levels of relative humidity (Arthurs & Thomas, 2001a, 1999; Arthurs *et al.*, 2001;

Thomas *et al.*, 1997). The observed means for precipitation for the months of June, July,

and August over a thirty-year period (Appendix 6) indicate that sporulation is highly

unlikely in the semi-arid Prairies and is supported by observations of a lack of sporulating

cadavers in arid regions of Australia following aerial application of *M. anisopliae* var.

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acridum (D. Hunter, pers. comm.). On the other hand, the impact of observed levels of precipitation should they approach climate normals on sporulation and horizontal transmission is as yet unknown (Table 5-2).

5.4   Lethbridge summer 2003 – a possible scenario for efficacy of *M. anisopliae* var. *acridum* for control of grasshoppers

The most recent significant outbreak of grasshoppers in southern Alberta occurred during the summer of 2003. Agriculture and AgriFood Canada, in conjunction with Alberta Agriculture, Food and Rural Development, established a joint federal/provincial program to compensate producers for costs of grasshopper control. Funding levels were set at $10.5 million and were estimated to cover only one-third of total incurred costs. Economic damage if no control was implemented was estimated at $80 to $100 million in crop losses. At the time, biological methods of control were not available.

June and July are usually considered the target months for control of pest grasshopper species, as early instar nymphs are the preferred target for chemical or biological control. Analysis of daily observed weather in Lethbridge, AB for June and July suggest that environmental conditions may have been highly suitable for control by *M. anisopliae* var. *acridum* (Appendices 7 and 8, also Table 5-3). One-half of the days in June and almost 85 % of those in July had at least 12 hours of the day during which air temperatures were in a range suitable for fungal growth (15-31 °C). June had two days where maximum air temperatures were <15 °C (likely no fungal growth at all on those days) and one day where the maximum was >31 °C (no fungal growth for at least part of the day). There were no days in July where temperatures were too cool for pathogen development for the entire day and nine days where part of the day was too warm for

Table 5-2. Comparison of observed quantities of precipitation to climate normals over a thirty-year period (1975-2005) for June, July, and August in Canada's Prairie provinces

Location	Month	Observed mean (mm)	Normal[f] (1971-2000) (mm)
Lethbridge, AB	June	2.6	63.0
	July	1.3	47.5
	Aug	1.6	45.8
Onefour, AB	June	2.2	48.3[g]
	July	1.2	34.2[g]
	Aug	1.2	38.6[g]
Lacombe, AB	June	2.5	75.7
	July	2.9	89.4
	Aug	2.3	70.8
Beaverlodge, AB	June	2.4	74.5
	July	2.4	70.6
	Aug	2.0	62.9
Scott, SK	June	2.1	62.5
	July	2.1	70.9
	Aug	1.4	43.1
Morden, MB	June	2.9	84.4
	July	2.3	71.2
	Aug	2.3	69.9

[f]data obtained from Environment Canada
[g]data obtained from nearby community of Manyberries, AB

Table 5-3. Summary statistics of days in June and July 2003 at Lethbridge, AB suitable for *in vivo* development of *M. anisopliae* var. *acridum*

	June	July
No. of days where max. temp. < 15 °C	2	0
No. of days where max. temp. > 31 °C	1	9
No. of days suitable for pathogen growth for at least part of day	28	31
No. of days where 15 °C ≤ ambient temp. ≤ 31 °C for ≥ 12 hrs	15	26
Mean daily hours where 15 °C ≤ ambient temp. ≤ 31 °C	11.4	15.3
No. of days with at least part of day suitable for optimum growth	10	27
Mean daily hours where 24 °C ≤ ambient temp. ≤ 27 °C (optimal)	2.7	3.8

development. Of the days in June where temperatures existed at least partially in the 15-31 °C range, the mean daily time frame for this range was 11.4 hours, compared to 15.3 hours in July. Ten days in June had at least part of the day suitable for optimum growth at ambient temperatures of 24-27 °C (range of 8.3-41.1 % of total hours available for fungal development, Fig. 5-3a), with four of these ten days in possession of an additional 1-9 hours at temperatures above 27 °C but not exceeding 31 °C which may or may not have been suitable for pathogen growth depending on influences of cloud cover and solar radiation on host body temperature at the time. Conversely, July had twenty-seven days with temperatures in the optimum growth range (6.7-45.5 % of suitable total hours, Fig. 5-3b) and an additional 1-8 hours at higher temperatures that had the potential to contribute to optimal growth on nineteen days. Sporulation and horizontal transmission were not likely due to low and sporadic episodes of precipitation, except for the occurrence of 21.8 mm of rain over a seven-day period in June (Appendix 7).

Table 5-3 shows that temperature conditions in Lethbridge, AB during June and July 2003 were permissive for *M. anisopliae* var. *acridum* development *in vivo* (range of 15-31 °C) more than 50 % of every 24-hour period. Rapid mortality during an Australian summer is in the order of 8-11 days in the field (D. Hunter, pers. comm.). A model based on the interaction between locust body temperature and air temperature has suggested that daily *Metarhizium* development over a 14-18 hour period is required to achieve mortality that quickly (D. Hunter, pers. comm.). The model has also predicted that mortality due to *M. anisopliae* var. *acridum* application in southern Canada would take two weeks or so, increasing to three weeks or more in the northern Prairies. Analysis of
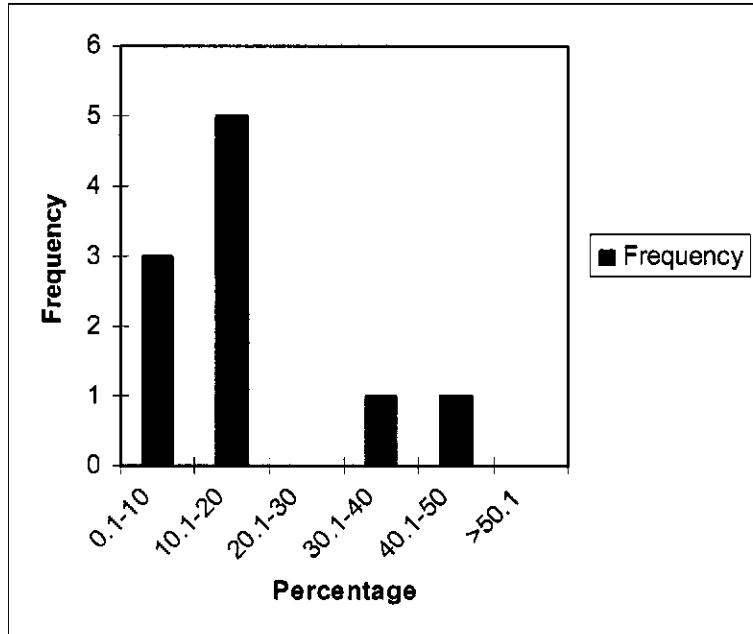
(a) June
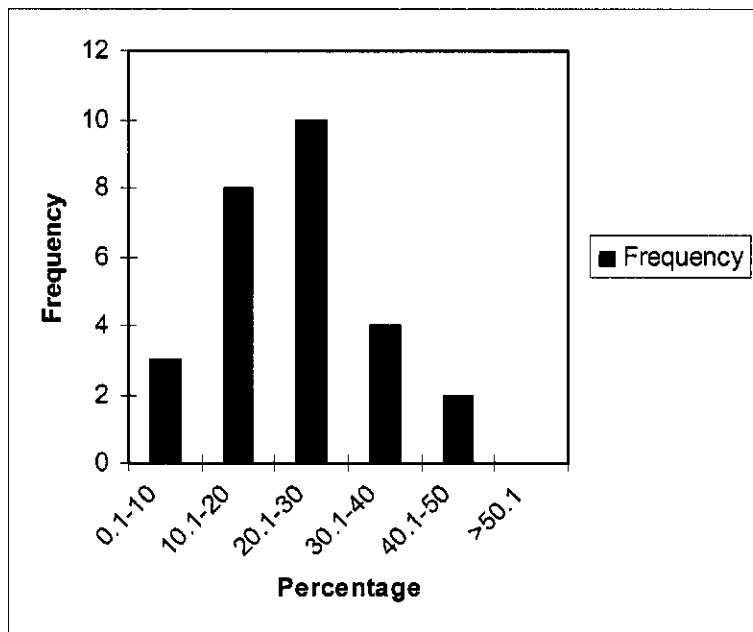
(b) July

Fig. 5-3. Frequency distribution of proportion of total daily hours suitable for *M. anisopliae* var. *acridum* growth *in vivo* at ambient temperatures of 24-27 °C (optimal development)

2003 temperature data for Lethbridge, AB during part of the summer suggests that local

mortality rates may be more rapid than predicted due to increased daily hours available

for pathogen development as a result of the difference in magnitude of elevation of native

grasshopper body temperatures (3-4 °C above ambient) versus the 12-15 °C increase in

locust body temperature above ambient predicted by the model. However, mortality rate

is a result of the interaction of numerous biotic and abiotic variables and it has been

demonstrated that theoretical predictions usually do not extrapolate well to field results.

It may be inferred from these key abiotic factors that summer weather in the Canadian

Prairies supports the hypothesis of efficacy of *M. anisopliae* as part of a biological

control program for grasshoppers and therefore can be considered as a viable alternative

to the chemical control of this significant agricultural pest. As scale-up experiments will

be conducted and documented as part of the registration, the extent of the

entomopathogen's role in terms of efficacy, costs, and health improvement will be

determined.

Chapter 6.   General Discussion and Conclusions

The main objectives of the research in this thesis were to develop and validate a molecular assay for the specific detection of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum*, conduct a soil survey to determine the natural occurrence of *Metarhizium* spp. in southern Alberta soils, screen native isolates for virulence towards grasshoppers, and analyze historical weather conditions in the Canadian Prairies for suitability for implementation of *M. anisopliae* var. *acridum* as a biocontrol agent for indigenous grasshopper pest species.

6.1   Summary of results

A diagnostic PCR assay was developed using DNA primers that targeted the ITS rDNA regions for the specific detection of *M. anisopliae* var. *acridum*. A 420 bp DNA sequence was successfully amplified from *M. anisopliae* var. *acridum* but not from other *Metarhizum* spp. or other soil fungal biota. The highly sensitive assay was also successful in the amplification of specific fungal DNA from infected grasshoppers and soil matrices.

Metarhizium anisopliae isolates were recovered at low incidence (5 out of 20 sites) from southern Alberta soils by direct isolation on semi-selective media and waxworm larvae bait assay. Evidence of *M. anisopliae* presence in soils was further supported by amplification of a 380 bp sequence in nested PCR assays with DNA primers for the specific detection of *M. anisopliae* var. *anisopliae*. Most isolates were identified through DNA sequencing as Group-B strains (Pantou *et al.*, 2003) and were morphologically similar in appearance. One isolate (S54) was identified as a Group-B variant that produced a 670 bp sequence in nested PCR assays with *M. anisopliae* var.

anisopliae-specific primers. Another isolate (20W-5) had a distinct morphological

appearance (Fig. 3-2b) as well as a smaller than expected amplification product of

approximately 250 bp in a PCR assay with general fungal primers TW81 and AB28 (Fig.

3-3). Isolate 20W-5 also failed to produce an amplification product in nested PCR assays

with primers specific for the detection of *M. anisopliae* var. *anisopliae*. No isolates of *M.*

anisopliae var. *acridum* or *M. flavoviride* were recovered from southern Alberta soils and

all soils tested negative for *M. anisopliae* var. *acridum* and *M. flavoviride* DNA by

specific PCR.

The genetic Group-B variant *M. anisopliae* var. *anisopliae* isolate S54 and the

morphologically distinct *M. anisopliae* isolate 20W-5 were screened for virulence

towards grasshoppers in bioassays of laboratory and field-collected insects. Both isolates

induced mycosis in grasshoppers. Isolate 20W-5 produced mortality in 98.7 % of

laboratory-reared treated insects 12 days post-inoculation (LT_{50} = 6.3 days) compared to

100 % mortality 8 dpi (LT_{50} = 4.1 days) for a commercial isolate of *M. anisopliae* var.

acridum. *M. anisopliae* var. *anisopliae* isolate S54 caused 98.6 % mortality 12 dpi (LT_{50}

= 6.7 days) in treated laboratory-reared insects. In bioassays of field-collected

grasshoppers, exposure to isolate S54 resulted in 100 % mortality 7 dpi (LT_{50} = 4.4 days)

compared to 100 % mortality 9 dpi (LT_{50} = 4.7 days) from treatment with the commercial

isolate. Isolate 20W-5 was not evaluated in bioassays of field-collected insects due to

poor sporulation on culture media.

Maximum air temperatures, minimum air temperatures, sunshine hours, and

precipitation data from nine weather stations were analyzed over a thirty-year period

(1975-2005) for occurrences that would preclude use of *M. anisopliae* var. *acridum* as a

biocontrol agent in the Prairie provinces. The analysis indicated that Canadian temperate

weather conditions were suitable for *M. anisopliae* var. *acridum* development and

initiation of infection. Low levels of ambient humidity were projected to be key factors

in sporulation and horizontal transmission of the fungus. It was hypothesized that

mortality rates induced by fungal infection in temperate zones might equal or exceed

those observed in field trials in tropical regimes.

6.2   Conclusions

In Canada, the Pest Management Regulatory Agency (PMRA) has issued a

comprehensive set of guidelines for the registration of microbial pest control agents and

products (PMRA, 2001). These guidelines outline the data required in terms of

characterization of the microbial control product (origin, derivation, and taxonomic

identification as well as biological properties such as level of natural occurrence,

distribution, habitat, and host range) in addition to human health and safety testing

(various routes of exposure and levels of infectivity), food and feed residue studies,

environmental fate (effects on indicator non-target species and persistence in the

environment), and value assessment (identification of potential benefits to and efficacy in

sustainable crop and pest management systems). The guidelines were established after

consultation with interested parties in the biotechnology, agri-food, and forestry sectors,

and have been harmonized with the US directive mandated by the Environmental

Protection Agency (EPA).

Research described in this thesis has contributed important data necessary to

fulfill some of the requirements for registration of a microbial pesticide in Canada. A

molecular assay for the specific detection of *M. anisopliae* var. *acridum* will permit

differentiation of the introduced exotic organism from native inhabitants, provide knowledge about the course of infection in target and nontarget organisms, and provide information on the fate of the biocontrol agent in the environment.

Ecological concerns over the release of exotic organisms are a driving force in a search for indigenous biocontrol agents. Further, uncertainty regarding issues behind regulatory decisions will frequently preclude the use of nonindigenous agents. The identification of native strains of *Metarhizium*, especially one with a unique built-in genetic fingerprint, that demonstrate high virulence towards native grasshopper pest species will ease some of the impediments to registration by providing precision tools for monitoring and tracking the fate of the pest control product in the target population and in the environment. Moreover, there is a higher probability of better ecological fitness with native isolates and, thereby, a higher probability of successful application.

These studies are an important advancement towards investigation of a suitable pathogen for biocontrol. More work is required to identify impacts on non-targets, since it has been demonstrated that individual isolates can differ significantly in host specificity. Compatibility of the pathogen with agroecosystems management practices also need to be determined as part of the multidisciplinary approach in integrated pest management strategies. Scientists, governments, industry, and interested stakeholders need to interact in a positive cooperative program to ensure delivery of a biological alternative for efficacious and environmentally sustainable control of an agroecosystem pest of economic significance.

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Appendix 1. Partial nucleotide sequence for large subunit ribosomal RNA gene and ribosomal intergenic spacer region for *Metarhizium anisopliae* var. *anisopliae* isolate 6W-2

```
   1 ccttgttgtt acgatctgct gagggttagc cgttcttcgc ctcgatttcc
  51 ccaatatcag cgcatcccgt ttcgcggggc gggtgttggg gttagagcca
 101 ccgagtagtg gccgcaggct tgaggagagc cgccgagtgg tggttcggga
 151 gggaaaatct gccaagctca aaggtcaggc aagcgagagg tttaccgggt
 201 cggaaagtcg ggcgagtaaa aagatatacc aagtccaaaa tactgccggc
 251 aacatacccg ggaccccgag taagtcgggg aagaagttgg cggatgtttt
 301 tccagccaac tcaactttga ccaatttacc tgcccgagct cacgggtagg
 351 ctgcggttaa tttttggact ttgcggattc actcgaatca attcacagga
 401 agttacctgc caaattgcct gggctcccgg gtaggctata gttgactttt
 451 ggacttggcg gatttttta aacttacttc ccccactata acctgggatt
 501 ttcccaacca tagctcacgg gtaggttagt tgtgatttt ggacttggtg
 551 aaatttctac tttcctgcca agtctagcct **acctgggagc ccaggcaagt**
 601 **cgggcaggta aattccgcca acccggcagg gaactgattg caaattaatt**
 651 **caaccaaatc tgccaagtcc acaaagaaat gacagcctac ccggtaggcc**
 701 **tgtagatttc ccgcgcgatt tcctgcgaag accaaaaagc taggtgtttt**
 751 **agtacttaat ttatataaag atgagttgat tttttgttt ttttatataa**
 801 **ataaattttg cggaaaataa aaaataaacc acgagagcct agtagcggag**
 851 **gcgggccctc gaggtggtcg gtaggtatat aagagagagg gctggacacg**
 901 **cgtcgcgtcg cgtatttcca ccgcacctaa tacttttggc tttagggtag**
 951 **gctgcttgtt** tagaggcgcg ctgaattaga tggtctctta agtgagggga
1001 tttctctgct ggcagttgcc tgtaatccgg gagtccgtgg tggtaaagtc
1051 agctataagg cttgtgtgtg tgtgggtgcg cgggtgtgtg tgccggggcc
1101 ctgtaagtcc cgaccaggaa ccctacaggt cacgtgccga cacagtaaga
1151 cagataagat atacggcacg tgttagtaca cgtgccaata caagtgacg
```

Positions 1-49	large subunit ribosomal RNA, partial sequence
Positions 50-1199	ribosomal intergenic spacer region, partial sequence
Positions 1069-1088	location of 20 bp GT-rich insertion sequence definitive of group-B strains (Pantou *et al.*, 2003)

Letters in bold indicate nucleotide sequence amplified by *M. anisopliae* var. *anisopliae*-specific Ma-IGSspF/Ma-IGS-spR primers.

Appendix 2. Partial nucleotide sequence for large subunit ribosomal RNA gene and ribosomal intergenic spacer region for group-B variant *Metarhizium anisopliae* var. *anisopliae* isolate S54

```
   1 ccttgttgtt acgatctgct gagggttagc cgttcttcgc ctcgatttcc
  51 ccaatatcag cgcatcccgt ttcgcggggc gggtgttggg gttagagcca
 101 ccgagtagtg gccgcaggct tgaggagagc cgccgagtgg tggttcggga
 151 gggaaaatct gccaagctca aaggtcaggc aagcgagagg tttaccgggt
 201 cggaaagtcg ggcgagtaaa aagatatacc aagtccaaaa tactgccggc
 251 aacatacccg ggaccccgag taagtcgggg aagaagttgg cggatgtttt
 301 tccagccaac tcaactttga ccaatttacc tgcccgagct cacgggtagg
 351 ctgcggttaa tttttggact ttgcggattc actcgaatca attcacagga
 401 agttacctgc caaattgcct gggctcccgg gtaggctata gttgactttt
 451 ggacttggcg gattttttta aacttacttc ccccactata acctgggatt
 501 ttcccgacca tagctcacgg gtaggttagt tgtgataagt ccacaaataa
 551 attacagcct acccggtagg cctgtatatt tcccgcgcga tttcctgtga
 601 agaccaaaaa gttaggtgtt ttagtactta atttatatag gtataagttg
 651 attttttgt tttttttata tatataaatt ttgcgggaaa taaaaaataa
 701 accacgagag cccggtagcg gaggcgagcc cttaaggtag tcggtaagta
 751 tataagagag agggctggac acgcgtcgcg tcgcgtattt ccaccgcacc
 801 taatactttg gctttagggt aggctgcttg tttagatgcg cgctgaatta
 851 gatggtctct taagtgaggg gatttctctg ctggcagttg cctgtaatcc
 901 gggagtccgt ggtggtaaag tcagctataa ggcttgtgtg tgtgtgggtg
 951 cgcgggtgtg tgtgccgggg ccctgtaagt cccgaccagg aaccctacag
1001 gtcacgtgcc gacacagtaa gacagataag atatacggca cgtgttagta
1051 cacgtgccaa tacaagtgac g
```

Positions 1-49   large subunit ribosomal RNA, partial sequence
Positions 50-1071   ribosomal intergenic spacer region, partial sequence
Positions 941-960   location of 20 bp GT-rich insertion sequence definitive of group-B strains (Pantou *et al.*, 2003)

Letters in bold indicate nucleotide sequence amplified by *M. anisopliae* var. *anisopliae*-specific Ma-IGSspF/Ma-IGS-spR primers.

Appendix 3. Mean summary statistics for maximum air temperature for weather stations in Alberta, Saskatchewan, and Manitoba during June, July, and August over a thirty-year period (1975-2005)

Analysis Variable : temp_max_air

location	month	N Obs	Mean	N	Std Error	Lower 95% CL[h] for Mean	Upper 95% CL for Mean	Minimum	Maximum
Beaverlodge	6	930	19.525	922	0.138	19.254	19.796	7.000	31.000
	7	961	21.648	961	0.134	21.385	21.911	6.300	32.000
	8	961	20.729	960	0.162	20.411	21.047	6.500	34.500
FortVermilion	6	930	21.845	480	0.202	21.447	22.243	6.300	31.500
	7	961	23.636	496	0.185	23.273	24.000	12.500	34.200
	8	961	21.041	496	0.227	20.594	21.487	5.000	35.000
Lacombe	6	930	20.128	930	0.147	19.840	20.417	7.800	34.300
	7	961	22.443	961	0.135	22.178	22.709	8.100	34.400
	8	961	21.765	949	0.166	21.440	22.090	5.000	34.500
Lethbridge	6	930	21.776	929	0.161	21.460	22.091	7.400	34.000
	7	961	25.378	952	0.153	25.079	25.677	8.700	37.200
	8	961	24.971	958	0.174	24.629	25.313	2.700	38.100
Morden	6	930	23.143	930	0.167	22.816	23.470	8.000	37.000
	7	961	25.941	961	0.134	25.678	26.204	12.500	38.300
	8	961	25.088	960	0.157	24.780	25.396	11.000	40.000
Onefour	6	930	22.550	930	0.170	22.218	22.883	5.900	38.000
	7	961	26.883	961	0.158	26.573	27.192	8.200	37.900
	8	961	26.303	961	0.177	25.956	26.651	4.700	39.000
Scott	6	930	21.324	930	0.160	21.009	21.638	7.500	37.600
	7	961	23.858	961	0.140	23.583	24.133	8.900	37.600
	8	961	23.351	961	0.174	23.009	23.692	9.000	39.400
Stavely	6	930	18.638	778	0.177	18.290	18.985	3.800	33.300
	7	961	22.590	810	0.169	22.258	22.923	5.400	36.100
	8	961	21.861	810	0.183	21.501	22.220	5.600	34.400
Vauxhall	6	870	23.013	852	0.166	22.687	23.339	8.800	36.000
	7	899	26.371	898	0.148	26.080	26.662	9.900	37.400
	8	899	26.060	898	0.179	25.709	26.411	8.800	38.500

[h]confidence level

Data generated by the MEANS procedure of SAS (SAS Institute, 2005).

Appendix 4. Mean summary statistics for minimum air temperature for weather stations in Alberta, Saskatchewan, and Manitoba during June, July, and August over a thirty-year period (1975-2005)

Analysis Variable : temp_min_air

location	month	N Obs	Mean	N	Std Error	Lower 95% CL[i] for Mean	Upper 95% CL for Mean	Minimum	Maximum
Beaverlodge	6	930	7.432	913	0.095	7.245	7.620	-1.000	15.600
	7	961	9.230	958	0.088	9.057	9.403	0.000	17.800
	8	961	8.106	960	0.104	7.902	8.310	-3.000	17.500
FortVermilion	6	930	8.705	480	0.152	8.407	9.004	-0.500	18.000
	7	961	11.197	496	0.128	10.946	11.448	1.500	19.000
	8	961	8.805	496	0.170	8.471	9.140	-3.000	19.500
Lacombe	6	930	7.206	930	0.105	7.000	7.411	-2.500	17.800
	7	961	9.077	961	0.094	8.891	9.262	0.800	18.000
	8	961	7.738	948	0.109	7.525	7.951	-7.000	16.100
Lethbridge	6	930	8.934	930	0.108	8.722	9.146	-2.000	19.800
	7	961	11.060	948	0.097	10.871	11.250	0.000	22.000
	8	961	10.155	958	0.103	9.953	10.357	-2.500	21.500
Morden	6	930	11.766	930	0.130	11.511	12.021	0.500	25.000
	7	961	14.493	961	0.102	14.293	14.693	3.000	25.000
	8	961	13.006	959	0.122	12.766	13.246	-0.500	25.000
Onefour	6	930	9.103	930	0.110	8.887	9.319	-0.600	20.500
	7	961	11.857	961	0.099	11.662	12.051	4.000	21.700
	8	961	11.095	961	0.114	10.872	11.319	-1.000	22.600
Scott	6	930	8.465	930	0.113	8.243	8.688	-1.600	20.500
	7	961	10.577	961	0.100	10.382	10.773	1.500	20.000
	8	961	9.217	961	0.117	8.988	9.446	-2.000	18.800
Stavely	6	930	5.691	778	0.127	5.442	5.939	-5.600	18.300
	7	961	8.257	811	0.135	7.992	8.523	-0.600	20.800
	8	961	7.863	815	0.143	7.582	8.144	-4.000	19.700
Vauxhall	6	870	8.946	856	0.118	8.715	9.177	-0.500	20.400
	7	899	10.731	898	0.103	10.528	10.934	-0.300	20.800
	8	899	9.742	899	0.114	9.518	9.965	-0.500	19.800

[i]confidence level

Data generated by the MEANS procedure of SAS (SAS Institute, 2005).

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Appendix 5. Mean summary statistics for sunshine hours for weather stations in Alberta, Saskatchewan, and Manitoba during June, July, and August over a thirty-year period (1975-2005)

Analysis Variable : sunshine

location	month	N Obs	Mean	N	Std Error	Lower 95% CL[j] for Mean	Upper 95% CL for Mean	Minimum	Maximum
Beaverlodge	6	930	7.187	122	0.403	6.390	7.984	0.000	14.800
	7	961	7.101	134	0.343	6.422	7.779	0.000	14.100
	8	961	6.509	155	0.304	5.908	7.110	0.000	12.700
FortVermilion	6	930	7.455	120	0.341	6.779	8.131	0.000	13.300
	7	961	6.395	124	0.318	5.765	7.025	0.000	13.100
	8	961	5.040	124	0.268	4.510	5.571	0.000	12.200
Lacombe	6	930	4.771	120	0.482	3.817	5.725	0.000	14.800
	7	961	6.375	124	0.460	5.464	7.286	0.000	14.200
	8	961	5.081	124	0.407	4.274	5.887	0.000	13.400
Lethbridge	6	930	9.181	513	0.208	8.772	9.591	0.000	15.500
	7	961	10.512	558	0.168	10.181	10.842	0.000	15.700
	8	961	9.142	558	0.165	8.818	9.467	0.000	14.400
Morden	6	930	.	0	.	.	.	.	.
	7	961	.	0	.	.	.	.	.
	8	961	.	0	.	.	.	.	.
Onefour	6	930	8.381	210	0.313	7.764	8.998	0.000	15.700
	7	961	10.082	217	0.236	9.617	10.547	0.000	15.500
	8	961	8.655	217	0.262	8.140	9.171	0.000	14.500
Scott	6	930	3.370	120	0.370	2.637	4.103	0.000	11.500
	7	961	5.067	124	0.368	4.338	5.796	0.000	11.300
	8	961	4.218	124	0.336	3.552	4.884	0.000	10.000
Stavely	6	930	7.367	120	0.438	6.499	8.235	0.000	14.700
	7	961	9.848	124	0.323	9.210	10.487	0.000	14.500
	8	961	7.456	124	0.390	6.684	8.229	0.000	13.200
Vauxhall	6	870	8.943	389	0.235	8.482	9.404	0.000	15.500
	7	899	10.404	403	0.181	10.049	10.760	0.000	15.300
	8	899	8.599	403	0.188	8.230	8.968	0.000	14.300

[j]confidence level

Data generated by the MEANS procedure of SAS (SAS Institute, 2005).

Appendix 6. Mean summary statistics for precipitation for weather stations in Alberta, Saskatchewan, and Manitoba during June, July, and August over a thirty-year period (1975-2005)

Analysis Variable : precip

location	month	N Obs	Mean	N	Std Error	Lower 95% CL[k] for Mean	Upper 95% CL for Mean	Minimum	Maximum
Beaverlodge	6	930	2.368	924	0.201	1.973	2.762	0.000	110.000
	7	961	2.400	961	0.189	2.029	2.772	0.000	93.200
	8	961	2.046	961	0.156	1.740	2.353	0.000	45.300
FortVermilion	6	930	1.698	480	0.205	1.296	2.100	0.000	41.000
	7	961	1.666	496	0.206	1.262	2.071	0.000	54.200
	8	961	1.988	496	0.239	1.518	2.458	0.000	43.200
Lacombe	6	930	2.515	930	0.182	2.158	2.872	0.000	50.600
	7	961	2.874	961	0.205	2.473	3.275	0.000	63.600
	8	961	2.324	952	0.216	1.901	2.748	0.000	78.200
Lethbridge	6	930	2.579	930	0.241	2.106	3.052	0.000	77.800
	7	961	1.305	961	0.133	1.045	1.565	0.000	43.400
	8	961	1.575	961	0.191	1.201	1.949	0.000	91.600
Morden	6	930	2.926	930	0.221	2.493	3.359	0.000	56.000
	7	961	2.285	961	0.198	1.897	2.674	0.000	82.100
	8	961	2.284	961	0.278	1.739	2.829	0.000	150.000
Onefour	6	930	2.171	930	0.181	1.815	2.527	0.000	45.600
	7	961	1.221	961	0.119	0.988	1.454	0.000	45.000
	8	961	1.201	961	0.123	0.960	1.443	0.000	39.400
Scott	6	930	2.075	930	0.162	1.757	2.392	0.000	54.600
	7	961	2.121	961	0.173	1.781	2.462	0.000	59.000
	8	961	1.414	961	0.128	1.163	1.665	0.000	38.200
Stavely	6	930	3.439	788	0.331	2.789	4.088	0.000	94.600
	7	961	1.991	819	0.201	1.597	2.385	0.000	52.100
	8	961	2.280	820	0.235	1.819	2.741	0.000	62.700
Vauxhall	6	870	2.410	870	0.220	1.977	2.842	0.000	63.400
	7	899	1.129	899	0.107	0.918	1.340	0.000	27.200
	8	899	1.242	899	0.129	0.988	1.496	0.000	62.000

[k]confidence level

Data generated by the MEANS procedure of SAS (SAS Institute, 2005).

Appendix 7. June 2003 observed daily weather data for Lethbridge, AB

Date	Max temp (°C)	Min temp (°C)	Ppt (mm)	Sun (hrs)	Temp range 15-31 °C (hrs)	Temp range 24-27 °C (hrs)	Temp range 27-31 °C (hrs)
June 1	22.4	13.9	5.0	7.2	14	0	0
2	16.7	10.7	11.0	1.8	2	0	0
3	18.6	8.3	0.2	6.5	4	0	0
4	20.1	9.7	0.0	5.4	10	0	0
5	19.1	7.2	0.6	0.6	6	0	0
6	14.2	6.3	0.2	3.9	0	0	0
7	23.4	2.4	0.0	12.2	14	0	0
8	20.7	8.3	2.8	3.5	8	0	0
9	16.3	10.9	0.0	3.5	3	0	0
10	20.4	10.8	3.8	4.0	6	0	0
11	21.0	10.4	0.0	7.1	9	0	0
12	24.0	5.2	0.0	11.3	12	1 (8.3)[1]	0
13	26.1	9.6	0.0	9.7	15	2 (13.3)	0
14	23.1	10.7	0.0	8.5	12	0	0
15	24.2	9.9	0.0	13.6	12	1 (8.3)	0
16	26.5	6.7	0.0	13.9	14	5 (35.7)	0
17	27.9	11.8	0.0	13.2	17	7 (41.1)	1 (5.9)
18	32.7	14.6	0.4	11.8	20	2 (10.0)	4 (20.0)
19	20.9	16.1	0.6	3.3	22	0	0
20	21.1	12.8	4.2	7.2	11	0	0
21	19.3	9.5	6.8	7.3	7	0	0
22	16.9	7.4	4.8	4.8	2	0	0
23	14.1	6.1	4.8	1.7	0	0	0
24	16.9	6.1	0.2	5.8	4	0	0
25	23.3	4.8	0.0	11.9	11	0	0
26	25.3	8.6	0.0	11.1	17	3 (17.6)	0
27	23.4	11.4	0.0	10.6	16	0	0
28	24.8	10.2	0.0	13.9	12	2 (16.7)	0
29	30.3	11.0	0.0	13.1	17	2 (11.8)	8 (47.1)
30	31.0	15.8	0.0	12.4	21	2 (9.5)	9 (42.8)

[1]numbers in parentheses represent percentage of total hours available suitable for growth of *M. anisopliae* var. *acridum*

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Appendix 8. July 2003 observed daily weather data for Lethbridge, AB

Date	Max temp (°C)	Min temp (°C)	Ppt (mm)	Sun (hrs)	Temp range 15-31 °C (hrs)	Temp range 24-27 °C (hrs)	Temp range 27-31 °C (hrs)
July 1	27.6	9.5	0.0	13.7	19	7 (36.8)[m]	1 (5.3)
2	23.2	12.0	0.0	12.7	18	0	0
3	24.9	11.7	0.0	12.1	15	4 (26.7)	0
4	27.5	7.8	0.0	12.6	17	5 (29.4)	4 (23.5)
5	21.4	11.0	3.2	3.3	6	0	0
6	19.6	4.6	0.0	12.5	9	0	0
7	25.9	7.9	0.0	12.2	11	5 (45.5)	0
8	25.4	12.1	0.0	5.1	13	2 (15.4)	0
9	25.6	11.4	0.0	11.8	14	5 (35.7)	0
10	24.6	12.3	0.0	13.4	15	3 (20.0)	0
11	31.6	9.8	0.0	12.9	16	2 (12.5)	8 (50.0)
12	34.5	14.2	0.0	13.2	13	2 (15.4)	2 (15.4)
13	28.5	14.4	0.0	8.5	22	5 (22.7)	4 (18.2)
14	26.6	12.1	0.0	12.9	20	9 (45.0)	0
15	29.8	12.4	0.0	13.0	15	4 (26.7)	6 (40.0)
16	32.9	14.0	0.0	9.8	16	2 (12.5)	5 (31.2)
17	32.9	12.4	0.0	14.1	13	3 (23.1)	4 (30.8)
18	31.0	10.5	0.0	14.0	15	1 (6.7)	4 (26.7)
19	28.5	11.7	0.0	12.8	15	3 (20.0)	5 (33.3)
20	34.8	12.8	0.0	10.6	10	2 (20.0)	2 (20.0)
21	29.0	13.2	0.0	12.5	16	4 (25.0)	6 (37.5)
22	35.3	12.3	0.0	13.0	10	1 (10.0)	2 (20.0)
23	36.9	18.2	0.0	10.9	15	5 (33.3)	0
24	27.0	16.5	0.0	11.1	24	6 (25.0)	0
25	22.1	12.1	0.0	6.0	16	0	0
26	29.4	13.7	0.0	7.6	19	5 (26.3)	4 (21.1)
27	31.8	11.2	0.0	10.4	12	1 (8.3)	5 (41.7)
28	29.6	14.0	1.9	11.7	23	3 (13.0)	6 (26.1)
29	27.2	13.3	0.0	12.0	17	5 (29.4)	3 (17.6)
30	31.3	11.9	0.0	7.8	12	4 (33.3)	3 (25.0)
31	28.5	12.3	0.0	4.3	19	4 (21.1)	4 (21.1)

[m]numbers in parentheses represent percentage of total hours available suitable for growth of *M. anisopliae* var. *acridum*

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