To the Graduate Council:

I am submitting herewith a thesis written by Nongpanga Doungkeaw entitled "Pathogenicity of the Entomopathogenic Fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin against the Potato Aphid, *Macrosiphum euphorbiae* (Thomas), on Tomato Plants." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

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We have read this thesis and
recommend its acceptance:

Accepted for the council:

Associate Vice Chancellor and
Dean of the Graduate School

PATHOGENICITY OF THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* (METCHNIKOFF) SOROKIN AGAINST THE POTATO APHID, *MACROSIPHUM EUPHORBIAE* (THOMAS), ON TOMATO PLANTS

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ABSTRACT

Forty-seven isolates of the entomopathogenic fungus *Metarhizium anisopliae* collected from soil in Tennessee were bioassayed against the potato aphid, *Macrosiphum euphorbiae* Thomas, in the laboratory and the greenhouse. In the laboratory, potato aphids were susceptible to all isolates of *M. anisopliae* and mortality ranged from 43-98%. The six isolates that produced 90% or more aphid mortality were 79.97, 18.97, 44.97, 38.97, 100.97, and 112.97.

The dose-responses of the potato aphids to *M. anisopliae* were evaluated using selected isolates that caused high mortality in the screening tests. Also isolate 67.97 was included since it was used in other experiments and serves as a standard. This isolate, however, caused one of the lowest aphid mortalities in the screening test. Conidial suspensions ranged from 3.0×10^4 to 1.0×10^7 conidia/ml. The estimated median lethal concentration (LC_{50}) of isolates 18.97, 38.97, 44.97, 79.97, 100.97, and 112.97 at 6 days post-inoculation were 1.53×10^5, 2.94×10^5, 3.25×10^5, 1.62×10^5, 5.40×10^5, and 3.71×10^5 conidia/ml, respectively, in experiment I ; and 4.25×10^6, 3.43×10^5, 1.32×10^5, 8.29×10^5, 8.64×10^5, and 1.84×10^5 conidia/ml, respectively, in experiment II. Isolate 67.97 had an LC_{50} of 3.30×10^6 and 4.56×10^6 conidia/ml for experiment I and II, respectively. These results confirmed the low virulence of isolate 67.97 toward the potato aphid. The isolates 38.97, 44.97 and 112.97 affected the potato aphid consistently in both experiments.

A greenhouse trial to test the virulence of *M. anisopliae* against the potato aphids was conducted with the three isolates with the best performances in the dose-

response experiment. The highest mortality of potato aphids, caused by isolate 44.97 at 1.0×10^8 conidia/ml, was 43.4%. Isolate 112.97 caused 39.6 and 34% mortality of aphid at 1.0×10^8 and 5.0×10^7 conidia/ml, respectively. Poor performance in the greenhouse test may be due to environmental conditions which did not favor the fungus.

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CHAPTER I

LITERATURE REVIEW

Introduction

Tennessee has been ranked among the top five states for the production of tomatoes within the Southern Region since 1992 (Peet 1998). The increase in hectareage under tomato production from 390 hectares in 1995 to 520 hectares in 1996 has enabled Tennessee to increase its tomato production (Rutledge 1997). However, due to the numerous pest problems, tomato crop losses also have increased in the same period.

A great number of insects are harmful to agriculture and human beings, and control measures are frequently required. The use of chemical pesticides that are nonselective and hazardous to the environment is common. An alternative way to control insects is with natural enemies, including predators, parasitoids and pathogens, which are killed inadvertently when chemical pesticides are used. Natural enemies are not toxic to humans and higher animals and do not pollute the environment (Andres et al. 1979). Their use in the biological control of pests represents an environmentally sound solution to insect problems in agriculture.

One of the factors that may contribute to increasingly difficult pest problems is the frequent and regular usage of chemical insecticides. This has caused several long-term problems for farmers although it was successful in solving some short-term pest problems. Examples of these problems include the higher resistance or tolerance of

insects to chemical insecticides that make these products less efficient or ineffective. Also, these pesticides affect beneficial insects (Zimmermann 1982). These undesirable problems may increase the chances for grower acceptance of microbial insect control. Entomopathogens are promising agents for control of pests in various vegetable crops. Entomopathogens, unlike chemical pesticides, do not endanger the environment or beneficial organisms, and may provide effective control of insects. The importance of using entomopathogenic fungi for controlling pests has been emphasized in recent years. With the environmental and human health concerns, and the resistance of insect pests to chemical pesticides, alternative pest control methods must be investigated.

Tomato

Biology and Growth Requirement

The tomato plant, *Lycopersicum esculentum* Miller, is classified in the family Solanaceae. Tomatoes are short-lived perennials which are planted as annuals. In greenhouses, tomato plants can live for 24 months or longer. Fruit size varies from small to large and shape varies from round to plum. Tomato fruits can be yellow, orange, pink, red, or even white. Carotenoids, which are produced by yellow and orange tomatoes, are sources of vitamin A. Tomatoes are quite sensitive to low light and adverse temperatures. Although tomatoes grow well over a wide range of temperatures (18 to 30°C), fruit set is very sensitive to high and low temperatures (Peet 1998). Besides temperatures, fertilizer, sunlight, weeds, diseases and insects are the most important factors affecting the tomato production (Berlinger 1986).

Pests on Tomatoes

Several problems may occur in the tomato crop. Weeds reduce yields by competing with the crop for light, water and nutrients. Some weeds are alternative host of insects, pathogens and nematodes that may affect the tomato plant. Common weeds in a tomato field are several species in the family Solanaceae, such as black nightshade and hairly nightshade. The other common weed species includes dodder, johnsongrass, groundcherries and yellow nutsedge (Zalom 1990).

Diseases caused by plant pathogens (biotic disease) or by environmental factors (abiotic disease) are also important in tomato production. The most important diseases, such as damping off, fusarium wilt and southern blight, are caused by fungi. Diseases caused by viruses are the tomato mosaic virus, cucumber mosaic virus, potato mosaic virus and alfalfa mosaic virus (Blancard 1994).

Insects on Tomatoes

Tomatoes are susceptible to insect damage at every stage of the crop. Insect monitoring and identification are essential in order to prevent and control insect attack precisely. Most tomato pests are from the following orders: Coleoptera - flea beetle, *Epitrix hirtipennis* Melsheimer and Colorado potato beetle, *Leptinotarsa decemlineata* (Say); Lepidoptera - tomato hornworm, *Manduca quinquemaculata* (Haworth), fruitworm, *Helicoverpa zea* (Boddie), and armyworm, *Pseudaletia unipuncta* (Haworth); Heteroptera – Southern green stink bug, *Nezara viridula* (Linnaeus) and Homoptera – greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood), and green

peach aphid, *Myzus persicae* (Sulzer), and potato aphid, *Macrosiphum euphorbiae* (Thomas).

Aphids on Tomatoes

Aphids are one of the most important pests in agricultural systems and cause yield losses in many crops worldwide (Wellings et al. 1989). About 4,000 species of aphids have been identified (Dixon 1987). The most severe aphid pests affect agricultural yields because the insects reproduce in large numbers, take nutrients and water from host, and/or transmit viral diseases (Miles 1989).

Biology

Aphids are small, soft-bodied insects that vary greatly in color and host plant. They may have a pair of transparent wings held roof-like over the body, but wingless forms are more common. Aphids suck plant juices; visual symptoms of leaf damage are leaf curl and yellowing. Aphids are recognized by their sucking mouthparts, long, thin legs, long antennae, pear-shaped body and a pair of tube-like structures (called cornicles) arising from the posterior of the abdomen. These cornicles apparently are the ducts of glands that produce alarm odors. Aphids may be winged or wingless and colonies often have both forms.

Aphids excrete a sugary liquid called honeydew, which drips onto plant foliage or other structures and provides a suitable substrate for sooty molds to grow. This fungal growth can further reduce plant and fruit quality. Aphids also may inject poisonous saliva or disease-causing organisms during feeding, and in tomatoes this may

cause a failure of bloom set. Some aphids transmit viruses that cause diseases such as potato virus Y, alfalfa mosaic virus, and cucumber mosaic virus.

Aphid Life Cycle

Aphids reproduce very quickly. The life cycle of most aphids can be as short as 1 to 2 weeks. Females usually reproduce without mating and give birth to living young, usually more females. Males are produced by some species only during the fall months. A typical species will produce several wingless generations in the Spring, followed by a generation of winged forms. These winged aphids fly to other plants where many more wingless summer generations are produced. As the days become shorter and cooler, a generation of winged aphids is produced and flies back to the winter host plant. Some species overwinter as eggs; others survive as immature or adult females (Sanders 1996).

Aphids that attack tomatoes are the cotton aphid, *Aphis gossypii* (Glover), the green peach aphid and the potato aphid. Adult green peach aphids are about 2 mm long. They may be green, pink or black with darker markings. Infestations occur mainly on tomato seedlings in early Spring. The green peach aphid is the main vector of the tomato mosaic virus and alfalfa mosaic virus. Adult cotton aphids are about 2 mm long and may be winged or wingless with pale to dark green in cool season and yellow in hot, dry summer (Hunt and Baker 1980).

The potato aphid may occur in two distinct forms, the green and the pink forms. They occur at any time but later in the growing season than other aphids. They can cause necrotic spot on leaves, distortion of leaves and stems and stunting of the plants

(Zalom 1990). Blackman and Eastop (1984) identified the potato aphid as having the distance between the spiracles on the first and second abdominal segments shorter than half the distance between the spiracles in the second and the third abdominal segments.

The potato aphid, originally from North America, was introduced into Southern Europe in 1917 on imported new potatoes varieties. The aphid populations grow very rapidly and survive during wintertime. This aphid alternates from a primary host in the family Rosaceae to a secondary host in various families including the family Solanaceae (Eastop 1977). Tomato plants are particularly susceptible to yield losses if high infestations occur during 6 to 8 weeks after planting. Populations of more than 40 aphids per gram of tomato plant are needed to reduce fruit weight (Miles 1989).

Biological Control of Aphids

Insect population can be reduced by parasitoids, predators and/or pathogens. Parasitoids lay eggs on or in a host. Larvae hatch and develop at the expense of the hosts which are eventually killed as the developing parasitoid consumes the host's organs or body fluids. Parasitic wasps commonly provide adequate aphid control if they are not killed with chemical insecticide applications and if ants are not allowed to tend the aphid colonies. Predators attack aphids, killing and eating multiple preys. Lady beetles (adults and larvae), green lacewing larvae, and syrphid fly larvae are commercially available for release to control aphids in greenhouses and fields. Pathogens are microorganisms that invade the host's body and cause diseases. The most promising pathogens in aphid control are the fungi *Verticillium lecanii* (Zimmermann)

Viegas, *Beauveria bassiana* (Balsamo) Vuillemin, *Paecilomyces fumosoroseus* (Wize) Brown and Smith and *Metarhizium anisopliae* (Metchnikoff) Sorokin.

Entomopathogenic Fungi

Entomopathogenic fungi may cause insect mortality by nutritional deficiency, invasion and destruction of tissues and/or discharge of toxin. The most distinguishing characteristic of a fungal infection is the appearance of mycelium in or on the affected insect. Common disease symptoms are lack of feeding, weakness and confusion. The host may change color, and the cuticle may exhibit dark spots, at the point where the fungus penetrates.

Verticillium lecanii

Verticillium lecanii conidia are relatively unstable and have to be kept frozen or below 4°C (Boucias and Pendland 1998). This fungus is the most common fungal pathogen causing mortality of aphids under natural environment. The other susceptible insects include whitefly, thrips, grasshopper, and scale insects. In addition, isolates of this fungus may infect plant pathogens, such as powdery mildews and rusts. *V. lecanii* was first commercially produced in 1982 (Milner 1997).

This fungus has been investigated against various aphids including aphids that infest cereal crops and vegetables. At least three isolates of *V. lecanii* affected potato aphids (Askary et al. 1998) and the author believed that *V. lecanii* is a potential microbial control agent for this aphid species. *V. lecanii* is also a promising biological agent against *A. gossypii* and *Aphis fabae* (Scopoli).

Several studies also have been conducted on cereal and grain aphids. Hsiao and Khachatourians (1997) studied the use of *V. lecanii* on host plants susceptible of bird cherry-oat aphid, *Rhopalosiphum padi* (Linnaeus). The results indicated that the fecundity of apterous insects infected with *V. lecanii* was significantly associated with the food plant. A laboratory study on infection of cereal aphids, *Metopolophium dirhodum* (Walker), with *V. lecanii* showed a significant mortality at 17.5°C. Median lethal concentrations (LC_{50}) were calculated at day 9 (Dromph et al. 1996).

In an integrated pest management program, the combination application of the chemical insecticide methomyl and *V. lecanii* was tested against *M. persicae* and *A. gossypi* on peppers, *Capsicum annuum* (Linnaeus), in greenhouses. The combination was more effective than methomyl or *V. lecanii* alone (Fiume 1993). Zukauskiene and Sirvinskas (1993) observed that *V. lecanii* applied in the greenhouse reduced aphid populations on cucumber, tomato and chrysanthemum by 75-92%.

Beauveria bassiana

Beauveria bassiana is known as the white muscardine fungus. It was found on a worker ant embeded in a 25 millon-year-old amber. *B. bassiana* can be easily grown in artificial media such as oatmeal agar or potato dextose agar and maintained viable at ultra-low temperature (-70°C) for a long time (Boucias and Pendland 1998). *B. bassiana* is commonly used to control whiteflies, cotton weevils, aphids, and European corn borers (Milner 1997). *B. bassiana* is currently mass-produced and formulated as commercial mycoinsecticides. Miranpuri and Khachatourians (1993) studied the effect of *B. bassiana* on the green peach aphid, *M. persicae*, infesting canola [rape] plants. Six

day post inoculation, the results showed 72-86% mortality of the aphids following two sprays with conidial concentrations of 10^8 spores/ ml. Under optimal conditions with 80-90% RH and 26-28°C, *B. bassiana* caused 98% mortality of the aphid on cucumbers (Sukhova 1987). Liu et al. (1999) also tested isolates of *B. bassiana* for their virulence and effect on the fecundity of *M. persicae*. The gradual deaths of the infected adults resulted in the decline in aphid reproduction, but infected adults produced offsprings that could survive and develop as normal.

The brown citrus aphid, *Toxoptera citricida* (Kirkaldy), is susceptible to several isolates of *B. bassiana*, with maximum mortality at 78%. The lethal concentration for 50% of the aphid population (median lethal concentration - LC_{50}) for these isolates ranged from 119 to 995 conidia/ml (Poprawski et al. 1999). Dorschner et al. (1991) used an aphid-derived isolate of *B. bassiana* to control hop aphids, *Phorodon humuli* (Schrank), in the laboratory and the field with conidial suspension concentrations ranging from 10^4 to 10^8 conidia/ml. The isolate exhibited high virulence (LC_{50} of 1.37 x 10^5 conidia/ml), and the median lethal time (LT_{50}) decreased with increasing conidial concentration. Aphid-derived isolates of *B. bassiana* were also bioassayed against 6 species of cereal-infesting aphids (Feng et al. 1990). Aphids were immersed in conidial suspensions with 10^4 to 10^8 conidia/ml. The estimated LC_{50} was different for different aphid species: 8.2 x 10^4 conidia/ml for *Diuraphis noxia* (Mordvilko); 2.1 x 10^5 for *Schizaphis graminum* (Rondani); and 2.1 x 10^6 conidia/ml for *Rhopalosiphum maidis* (Fitch).

The LT_{50} of the English grain aphid, *Sitobion avenae* Fabricius, ranged from 3 to 5.4 days for the various strains of *B. bassiana*. Mortality was correlated with spore concentration, volume of spore suspension and number of applications (Miranpuri and Khachatourians 1995a). Miranpuri and Khachatourians (1995b) also applied *B. bassiana* against the berry leaf aphid, *Acyrthosiphon macrosiphum* (Wilson). In laboratory bioassays, various strains of *B. bassiana* caused 90-100% aphid mortality five days after inoculation. Zaki (1998) confirmed that *B. bassiana* was effective against the cowpea aphid, *Aphis craccivora* (Koch), infesting cucumber. Increased fungal dose decreased adult longevity, period of reproduction, and fecundity of this aphid.

Paecilomyces fumosoroseus

Paecilomyces is easily cultured on Sauboraud dextose agar or potato dextose agar (Boucias and Pendland 1998). This fungus is also commercially available in the U.S. for control of aphids and whiteflies. *P. fumosoroseus* has been reported as an effective agent for the microbial control of *D. noxia* (Vandenberg 1996). The LC_{50} and LC_{90} of a strain of *P. fumosoroseus* tested against 3rd-instar *D. noxia* were 1.78×10^3 and 1.43×10^4 conidia/ml, respectively (Mesquita et al. 1996). Sosnowska (1992) conducted field experiments to control *P. humuli* by applying a *P. fumosoroseus* suspension with 10^8 conidia/ml. Mortality was 55.8% and 57.1% after day 3 and 5, respectively.

Metarhizium anisopliae

Metarhizium anisopliae, the green muscardine fungus, has a wide host range. In 1879, Metchnikov isolated this fungus from the beetle *Anisoplia austriaca* (Herbst) and suggested its use as a microbial agent. The colony of *M. anisopliae* appears white when young but as the conidia mature it turns to dark green. *M. anisopliae* has two subspecies, the short-spored form, *M. anisopliae* var. *anisopliae* (conidia 3.5-9.0 μm), and the long-spored form, *M. anisopliae* var. *majus* (conidia 9.0-18.0 μm).

Metarhizium is essentially a soil fungus, and the sunlight and extreme temperatures will affect spore viability. Conidia can be stored at 4°C for several months. *M. anisopliae* conidia penetrate host insects through the outer integument; the fungus gradually infects the other insect parts (Boucias and Pendland 1998).

Cultures of *M. anisopliae* produce the cyclodepsipeptides, destruxins A, B, C, D and E. Destruxins have been considered as potential new commercial insecticides since they are toxic to insects only by ingestion but not through integument penetration (Tanada and Kaya 1993). Destruxin E was shown to affect aphid species differently and *M. persicae* was sensitive to this compound (Robert and Riba 1989).

Insect-Pathogen Relationships

Before entomopathogens can infect an insects' body, they have to penetrate into the host by chemical or physical means, depending on type of entomopathogen. Entomopathogenic fungi may produce enzymes or toxins to reduce barrier properties of the insect cuticle. When conidia of entomopathogenic fungi contact the insect cuticle,

time is necessary for germination and penetration through the cuticle into the insect's body to occur. Once inside, the fungus obtains nutrients from the insect's body (St. Leger 1993). Zacharuk (1973) studied the *M. anisopliae* infection and determined that 2 to 4 days after inoculation, the fungus germinated and penetrated directly through the insect cuticle. *M. anisopliae* reproduced in the host, and spores appeared to cover the host. Butt et al. (1995) described the different sites that *M. anisopliae* infected insects. This fungus directly infected the surface of aphids but entry was at the dorsal elytra of the beetles. Schabel (1975) tested oral infection of *M. anisopliae*. This fungus could germinate in and infect through the digestive system of pale weevil, *Hylobius pale* Herbst.

Most entomopathogenic fungi require appropriated environment such as temperature and humidity to function effectively. The temperature of 28°C was the most effective temperature for rapid growth rate and sporulation of *M. anisopliae* (Shashi et al. 1998). Rombach and Gillespie (1988) mentioned that entomopathogenic fungi required high relative humidity and moderate temperatures for infection of hosts.

Metarhizium anisopliae against Insects and other Arthropods

The virulence of *M. anisopliae* was investigated against the southern cattle ticks, *Boophilus microplus* (Canestrini). Three days following fungal application, infected females were monitored and conidia of *M. anisopliae* were found on cadavers (Bittencourt et al. 1999). Pathogenicity of *M. anisopliae* against the household pest German cockroach, *Blattella germanica* (Linnaeus), was determined using five

concentrations ranging from 8.0×10^7 to 2.0×10^9 conidia/ml. The calculated LD_{50} value was 4.18×10^8 conidia/ml (Pachamuthu et al. 1999).

Several soil insects also have been the targets of studies using *M. anisopliae*. For instance, control of the termite, *Cornitermes cumulans* (Kollar), was conducted in a field study by Neves and Alves (1999), using *M. anisopliae* and the chemical insecticide imidacloprid. When the chemical was combined with the pathogen lower concentrations of the chemical insecticide were required for control of termites. The pathogenicity of *M. anisopliae* to the formorsan subterranean termite, *Coptotermes formosanus* (Shiraki), was reported by Jones et al. (1996), Milner et al. (1996) and Alves et al. (1995). Fungi attacking adults of Colorado potato beetle during hibernation were studied. *M. anisopliae* was the dominant species isolated from meadow soil at 18° and 28°C and from arable soil at 28°C (Mietkiewski et al. 1996).

More recently, the experimentation with *M. anisopliae* has focused on foliar feeding insects and greenhouse pests. Temperatures between 25-30°C were optimum for pathogenic activity and growth rate of *M. anisopliae* on the legume flower thrips, *Megalurothrips sjostedti* Trybom (Ekesi et al. 1999). Field experiments were conducted to evaluate the potential of *M. anisopliae* for biological control of this insect on cowpeas. Concentrations of 1.0×10^{11} and 1.0×10^{13} conidia/ha significantly reduced thrips populations and plant damage (Ekesi et al. 1998). The mortality of turnip maggot, *Delia floralis* (Linnaeus), using *M. anisopliae* in laboratory and greenhouse was 40-50% when the applied conidial concentration ranged from 10^7 to 10^9 conidia/ml (Vanninen et al. 1999). The susceptibility of adult house flies, *Musca domestica*

Linnaeus, and adult fruit flies, *Bactrocera tryoni* Froggatt, to an isolate of *M. anisopliae* was tested and 100% mortality was achieved at 20°–30°C after 7-9 days (Carswell et al. 1998).

Metarhizium anisopliae against Aphids

Ellis et al. (1996) used an integrated program to control aphids in lettuce including four species; *Nasonovia ribisnigri* (Mosley), *M. euphorbiae*, *M. persicae* and *Pemphigus bursarius* (Linnaeus). New insecticides, resistant plants and the entomopathogenic fungus, *M. anisopliae*, were investigated in field experiments, which showed that the fungus was able to kill *P. bursarius*. Hall (1980) confirmed the pathogenicity of the 'Pemphigus' strain of *M. anisopliae* to aphids in the laboratory. In these studies, spore germination, growth and sporulation rates of *M. anisopliae* were slower than those of *V. lecanii*. In laboratory bioassays against the lettuce root aphids only one isolate of *M. anisopliae* killed the aphids consistently. This fungus, isolated originally from a closely related *Pemphigus* species, sporulated profusely on the host cadavers, producing approximately 4.0×10^6 conidia/cadaver at 14 days after treatment. Diseased aphids died attached to plant roots (Chandler 1997).

Objectives

The objectives of this research were to: 1) evaluate the virulence of *M. anisopliae* against the potato aphid on tomato plants; 2) investigate the median lethal concentration of *M. anisopliae* against the potato aphid; and 3) evaluate *M. anisopliae* against the potato aphid in the greenhouse.

CHAPTER II

SELECTION OF ISOLATES OF THE ENTOMOPATHOGENIC FUNGUS *Metarhizium anisopliae* AGAINST THE POTATO APHID, *Macrosiphum euphorbiae*

Introduction

Potato aphid is an important pest in tomatoes. Aphids in large numbers damage mature plants of susceptible varieties. Aphids feed on plant stems and leaves by sucking fluids. The pest status of some aphids is considerably reduced by natural epizootics of fungal disease (Milner 1997). Recently, many studies have been conducted on insect control using *M. anisopliae* which exhibited promising results in both laboratory and field trials.

Pathogenicity and virulence of *M. anisopliae* against nymphs of the pear psylla, *Cacopsylla pyricola* (Foerster), were studied using a detached-leaf bioassay. Fungal treatments resulted in 42.3-54.7% mortality after 7 days at 10^7 conidia/ml (Puterka et al. 1994). The possible use of *M. anisopliae* for control of the black vine weevil, *Otiorhynchus sulcatus* (Foerster), was examined, and certain isolates showed potential for control of this pest on ornamental crops (Moorhouse et al. 1993).

In the laboratory, experiments evaluated the pathogenicity of the 'Pemphigus' strain of *M. anisopliae*. In these studies, spore germination, growth, and sporulation rates of *M. anisopliae* were slower than those of *V. lecanii* (Hall 1980). In laboratory bioassays against the lettuce root aphid, *P. bursarius*, only one isolate of *M. anisopliae*

killed the aphids consistently. This fungus was isolated originally from a closely related *Pemphigus* species. It sporulated profusely on host cadavers, producing approximately 4.0×10^6 conidia/cadaver 14 days after treatment, and diseased aphids died attached to plant roots (Chandler 1997).

Differences in susceptibility of aphids to *M. anisopliae* isolates reflected the response of the pathogen to insect cuticular cues. More conidia adhered to and germinated readily on the cuticular surfaces of live aphids. *M. anisopliae* also penetrated the cuticle directly or with appressorium production (Butt et al. 1995).

In this study the virulence of 47 isolates of *M. anisopliae* was tested against potato aphids in laboratory bioassays. The isolates with best performance were selected for use in future studies.

Materials and Methods

Metarhizium anisopliae Isolates

A total of 47 *M. anisopliae* isolates, collected from soil in Tennessee, was used in this study. Each isolate was subcultured by transferring mycelium and conidia from the original culture onto SDAY medium (Sauboraud dextrose agar 65 g plus 5 g yeast extract/1 liter deionize water) (Difco) then incubated at 25°C for 14 days. Three plates of each isolate were prepared and conidia were collected, weighed, and kept in the refrigerator until used. To prepare fungal suspensions, conidia were added to 10 ml of sterile water containing 0.02% Tween 80 in a 50 ml flask. Conidial concentration was

determined using a hemocytometer and the suspension was diluted to a concentration of 1.0×10^7 conidia/ml.

To determine viability of conidia, fungal conidia which attached at the tip of a glass droppler were spread on a petri dish (100 x 15 mm) with SDAY medium. After 20–24 hours incubation at 25°C, the germinated and ungerminated conidia in five randomly chosen visual fields under 250X microscope were counted. Approximately 200 conidia were observed per plate (2 plates/isolate) and percent germination (viability) was calculated.

Plants

Tomato plants (variety Malgrobe) were grown in a greenhouse with a light regime of 16:8 h (L:D) at varying temperatures with mean of approximately 25°C. These plants were used to rear aphids and to provide a food source for aphids during bioassays.

Insects

The laboratory stock of the potato aphid used in this study was derived from aphids infesting tomato plants in the field. The colony was started in May 1998 with aphids collected from a tomato field at The University of Tennessee, Knoxville Experiment Station. The colony was maintained in cages in the same greenhouse where tomato plants were grown. Trays with tomato plants were added to the cages as food source for the aphids. A fresh tray of tomato plants was added to a cage approximately

once a month, and the old tomato plant tray was removed after the aphids moved to or were transferred with a camel's hair brush to the new plants.

Bioassay Chamber

Transparent plastic boxes (31.5 x 25.5 x 10 cm) with moist paper towel on the bottom were used to hold bioassay chambers and to maintain saturated humidity during experiments. A cylindrical plastic cup (473 ml) with a fine screen lid was used for each replicate. Inside each plastic cup there was a vial (10 ml) filled with water, covered with parafilm, and holding a tomato leaf with 3-5 leaflets. The vial was held in the center of the plastic cup by a plastic disk that prevented movement of the vial with the tomato leaf.

Aphid Bioassay

Aphids were collected from excised infested tomato leaves. Aphid were transferred to a small plastic cup (SOLO P400, 118 ml) with fine mesh screen covering both the lid and the bottom. This cup was used as an inoculation chamber. At least 15 apterous adult aphids were inoculated by immersing the inoculation chamber containing the aphids for 10 s in a conidial suspension (1.0×10^7 conidia/ml). The inoculation chamber was then removed from conidial suspension, and excess suspension was absorbed by paper towels. Each treated aphid was individually transferred onto a tomato leaf in bioassay chambers (15-18 aphids/chamber). Eight bioassay chambers were placed into the transparent plastic box then incubated at 25°C with constant light. Four

replicates were prepared per treatment. Control treatments consisted of aphids immersed in sterile water containing 0.02% Tween 80.

Tomato leaves were changed if they wilted during experiment by individually transferring aphids with brush sterilized in 70% alcohol to the new tomato leaf. If there was any condensation on the plastic cup walls, it was removed during observation each day to avoid entrapment of aphids in water drops. Newborn and dead aphids were removed from the leaf daily. Aphid mortality was recorded daily over 6 days. The number of dead aphids was recorded for each experimental unit, and cadavers were individually transferred to wells in microtiter plates. These plates were incubated for 3 days under saturated humidity to allow sporulation of the infecting fungus. The production of *M. anisopliae* conidia on aphid cadavers confirmed fungus-induced mortality.

Statistical Analysis

Cumulative percent mortality was calculated for each replicate. Corrected mortalities were calculated using Abbott's formula (Abbott 1925). Abbott-corrected mortalities after 6 days were analyzed using ANOVA, and means were separated using Fisher's protected least significant difference.

Results

The mean viability of conidia used in the experiments was 96% (range 92-98%). The average conidial production weight was approximately 0.4 g/plate (Table 2.1). The growth rate and sporulation varied among the isolates used.

Table 2.1. Mean conidial production and Abbott-corrected percent mortality 6 days after inoculation of the potato aphid, *Macrosiphum euphorbiae*, treated with conidial suspensions containing 10^7 *Metarhizium anisopliae* conidia/ml.

Strains	No. isolates	Conidia production g/plate ***	% Mortality±SE *
1	74.97	0.40	43 ± 16.3 a
2	80.97	0.20	47 ± 4.09 ab
3	14.97	0.45	54 ± 8.98 a-c
4	57.97	0.20	57 ± 16.5 a-d
5	2.97	0.20	59 ± 15.4 a-e
6	173.97	0.30	61 ± 9.44 a-f
7	**67.97****	**0.20**	**63 ± 7.15 a-g**
8	169.97	0.25	64 ± 9.00 a-h
9	103.97	0.40	65 ± 11.3 a-h
10	163.97	0.40	68 ± 4.49 b-i
11	81.97	0.45	69 ± 7.58 b-i
12	162.97	0.45	70 ± 4.08 b-i
13	121.97	0.50	70 ± 10.2 b-j
14	84.97	0.40	70 ± 6.38 b-j
15	182.97	0.30	71 ± 10.2 c-j
16	66.97	0.25	72 ± 11.0 c-k
17	87.97	0.40	72 ± 2.12 c-k
18	11.97	0.30	72 ± 12.8 c-k
19	196.97	0.45	73 ± 9.72 c-l
20	180.97	0.50	76 ± 13.1 c-l
21	88.97	0.40	76 ± 5.05 c-l
22	61.97	0.50	77 ± 8.01 c-l
23	64.97	0.10	77 ± 8.01 c-l

(Continued next page)

Table 2.1. (Continued)

Strains	No. isolates	Conidia production g/plate***	% Mortality ±SE *	
24	102.97	0.50	78 ± 10.9	d-l
25	190.97	0.45	78 ± 4.80	d-l
26	164.97	0.50	79 ± 11.6	d-l
27	27.97	0.40	79 ± 6.00	d-l
28	12.97	0.35	79 ± 14.0	d-l
29	9.97	0.65	81 ± 8.50	e-l
30	83.97	0.20	81 ± 6.76	e-l
31	22.97	0.45	82 ± 6.96	e-l
32	78.97	0.35	83 ± 7.36	e-l
33	177.97	0.30	83 ± 4.84	f-l
34	99.97	0.20	84 ± 5.50	f-l
35	212.97	0.45	84 ± 5.25	f-l
36	63.97	0.60	86 ± 5.53	g-l
37	160.97	0.45	87 ± 7.79	g-l
38	49.97	0.40	88 ± 8.72	h-l
39	62.97	0.50	88 ± 5.10	h-l
40	174.97	0.40	90 ± 4.29	i-l
41	101.97	0.40	90 ± 6.08	i-l
42	**79.97**	**0.50**	**90 ± 5.63**	**i-l**
43	**18.97**	**0.35**	**90 ± 3.33**	**i-l**
44	**44.97**	**0.45**	**94 ± 3.46**	**j-l**
45	**38.97**	**0.30**	**95 ± 4.75**	**k-l**
46	**100.97**	**0.40**	**98 ± 2.25**	**l**
47	**112.97**	**0.45**	**98 ± 2.25**	**l**

* means followed by the same letter are not significantly different (Fisher's protected least significant test).

** isolates in bold were used in experiments described in following Chapter.

*** based on total conidial production from 3 100 x 15 mm petri plates with SDAY.

In screening experiments, cadavers were first observed on the third day post inoculation. Infected aphids died attached to the tomato leaf or fell down on the container plastic disk. *M. anisopliae* isolates used in this experiment tended to kill potato aphids fairly uniformly. Fungal mycelium and conidial production on cadavers were observed after cadavers had been incubated for 3 days, and most of cadavers had profuse sporulation.

Cumulative control mortality after 6 days was 13% (range 5-15%). Most of isolates of *M. anisopliae* caused mortality greater than 50%, except isolates 74.97 and 80.97 that caused 43 and 46.5% mortality, respectively. The virulence of *M. anisopliae* ranged from 43 to 98% mortality after 6 days (Table 2.1).

According to their virulence to the potato aphid, *M. anisopliae* isolates could be divided into three groups. The first group caused low mortality of the potato aphids from 43 to 65%. The second group caused intermediate mortality from 68-73%. The third group, representing 60% of the isolates used, caused mortality of potato aphid between 76 to 98%. There were six isolates, 79.97, 18.97, 44.97, 38.97, 100.97, and 112.97, that killed greater than 90% of the aphids.

Discussion

Conidial yields of different isolates were estimated for future reference only. No attempt was made to compare isolates based on conidial yields on culture medium. However, apparently no relation between conidial yield and virulence could be inferred from the data. Some isolates produced a large amount of conidia but low pathogenicity

against potato aphids. Low conidial yields may affect the ability of the disease to spread in the host population (Hall 1980), and should be an important parameter in selecting isolates for use in biological control programs.

The selection of the most suitable fungal isolates is an important factor in the microbial control of pests, and effective bioassays and techniques are essential for valid tests. The bioassays in this study were simple and convenient. Most equipments were easily produced including the inoculation chamber and the bioassay chambers, and no special tools for spraying the conidial suspensions were needed. The difficulty in the bioassay was the gentle transfer of potato aphids onto plants. Aphids are soft-bodied insects that need careful handling which is time-consuming. Using a tomato leaf that was dipped into a vial with water provided fresh plant for the aphids to feed, unlike the leaf disc bioassay (Feng et al. 1990). The advantage of this technique is that aphids can feed on a fresh leaf that provides enough fluids and nutrients to the insect.

For the treatment with the conidial suspension, immersion of the aphids using the inoculation chamber was simple. The conidial suspensions were drawn off quickly when the inoculation chamber was placed on paper towel. This was as suitable as draining the suspension off by suction (Hall 1976).

The *M. anisopliae* infection that caused aphid mortality was described as deriving from direct penetration into insect cuticle (Butt et al. 1995). Zacharuk (1973) explained the infection process of this fungus as follows: *M. anisopliae* released enzymes to digest the insect epicuticle which enhanced the possibility of fungal infection.

With cumulative aphid mortalities ranging from 43 to 98%, all *M. anisopliae* isolates tested killed more potato aphids after 6 days than isolates applied to the chrysanthemum aphid, *Macrosiphoniella sanborni* (Gillette), on chrysanthemum leaves (Hall 1980). The results from experiments conducted by Poprawski et al. (1999) also confirmed the pathogenicity of *M. anisopliae* isolates against the brown citrus aphid with mortalities ranging from 45 to 89%, which are similar to the results reported here.

The selection of *V. lecanii* isolates against the aphid *M. sanborni* conducted by Jackson et al. (1985) revealed isolates causing mortality from 0 to 90%. Only seven isolates caused greater than 50% aphid mortality with concentration of 10^6 conidia/ml at 6 day post inoculation. The range of aphid mortality in these experiments was not as wide as that reported by Jackson et al. (1985). This fact can be due to a greater degree of similarity among our *M. anisopliae* isolates, perhaps due to their isolation from Tennessee soil only.

Isolates with the best performances, killing more than 90% of the potato aphids, were selected for use in future experiments. These isolates were 79.97, 18.97, 44.97, 38.97, 100.97, and 112.97. Isolate 67.97, which showed good performance in tests against potato wireworms in the field but had low mortality in this study, was also selected for other experimentation against *M. euphorbiae*.

The screening tests of *M. anisopliae* in laboratory demonstrated that the potato aphid was susceptible to various *M. anisopliae* isolates. Some isolates of this fungus can cause high mortality of the potato aphid if suitable condition for fungal infection exist.

CHAPTER III

DOSE-RESPONSE OF THE POTATO APHID, *Macrosiphum euphorbiae*, TO THE ENTOMOPATHOGENIC FUNGUS *Metarhizium anisopliae*

Introduction

Aphids are one of the most important pests in the agricultural systems. The potato aphid has a broad host range, attacking many crops including potato, eggplant, pepper, pea, bean, and tomato. Heavily-infested plants may turn brown and die from the top as the aphids suck plant sap for their nutrients. The new growth and young plants become curled or stunted.

Concern about the negative effects of chemical insecticides, such as insect resistance and pollution of the environment, have increased the interest on alternative strategies for pest control. The use of entomopathogenic fungi as biological agents has received much attention recently. Many commercial entomopathogenic fungi, including *V. lecanii*, *B. bassiana* and *M. anisopliae*, are available (Milner 1997).

The entomopathogenic fungus *M. anisopliae* has a wide host range. *M. anisopliae* has been recovered from soil of natural habitats in many areas. Agricultural use of this fungus include experiments with vegetable pests, such as lettuce root aphid (Chandler 1992), and brown citrus aphid (Tsai et al. 1999). These studies have shown that *M. anisopliae* has potential as a microbial agent in controlling these insects.

The pathogenicity of *M. anisopliae* (isolates V208 and V245) was evaluated against the stem flea beetle, *Psylliodes chrysocephala* (Butt et al. 1994). The estimated

LC_{50} of both isolates was a concentration of 1.0×10^6 conidia/ml. The LT_{50} values for V208 and V245 at 1.0×10^7 conidia/ml were 10.1 and 9.3 days, respectively. Chandler (1997) reported the virulence of *M. anisopliae* against the lettuce root aphid. Only one strain of *M. anisopliae* killed lettuce aphids, and the LC_{50} value at 10 days post-inoculation was 2.45×10^6 conidia/ml.

In this study, isolates of *M. anisopliae* with the best performance from the previous experiment (Chapter II) were tested against the potato aphid at several doses. The objective was to determine LC_{50} of the isolates. Comparison of LC_{50} values allows selection of best candidates for use in the field and greenhouse.

Materials and Methods

Metarhizium anisopliae Isolates

Seven isolates of *M. anisopliae* selected in the previous experiment were used in this study. Three SDAY plates of each isolate were prepared, and conidia were collected and kept in the refrigerator. To prepare fungal suspensions, fungal conidia were added to 10 ml of sterile water containing 0.02% Tween 80 in a 50 ml flask. Conidial concentrations were determined using a hemocytometer, and the conidial suspensions were diluted to concentrations of 3.0×10^4, 1.0×10^5, 3.0×10^5, 1.0×10^6, 3.0×10^6, and 1.0×10^7 conidia/ml. Viability of the conidia was determined as described previously in Chapter II.

Plants and Insects

Plants were grown and insects were reared as described in Chapter II.

Bioassay Chamber and Aphids Bioassay

The bioassay procedures for this experiment were the same as described in Chapter II. Aphid mortality was recorded daily over 6 days. The number of dead aphids were recorded, and cadavers were individually transferred to wells in microtiter plates and observed as described previously. Two experiments were conducted. In the first experiment, all concentrations of the same isolate were tested simultaneously. In the second experiment, similar concentrations of all isolates were tested simultaneously.

Statistical Analysis

Cumulative mortalities at day 6 were corrected using Abbott's formula (Abbott 1925) and used to calculate LC_{50} by using POLO PC®, which is a probit and logit analysis software for IBM compatible computers (LeOra software 1987, and Russell et al. 1977). The software estimated concentrations needed to kill 50% of the potato aphid population, i.e., the median lethal concentration (LC_{50}). Treatment means were separated by comparison of the 95% confidence intervals for the LC_{50}.

Results

The viability of all isolates used in bioassays was greater than 92%. Some condensation occurred on plastic disk inside the bioassay container but did not impact in to aphid population.

The virulence of isolate 18.97 (Figure 3.1) was the highest among all isolates in experiment I, causing 92% cumulative mortality of potato aphids. In contrast, in experiment II, cumulative aphid mortality dropped to 70% with the concentration of 1.0

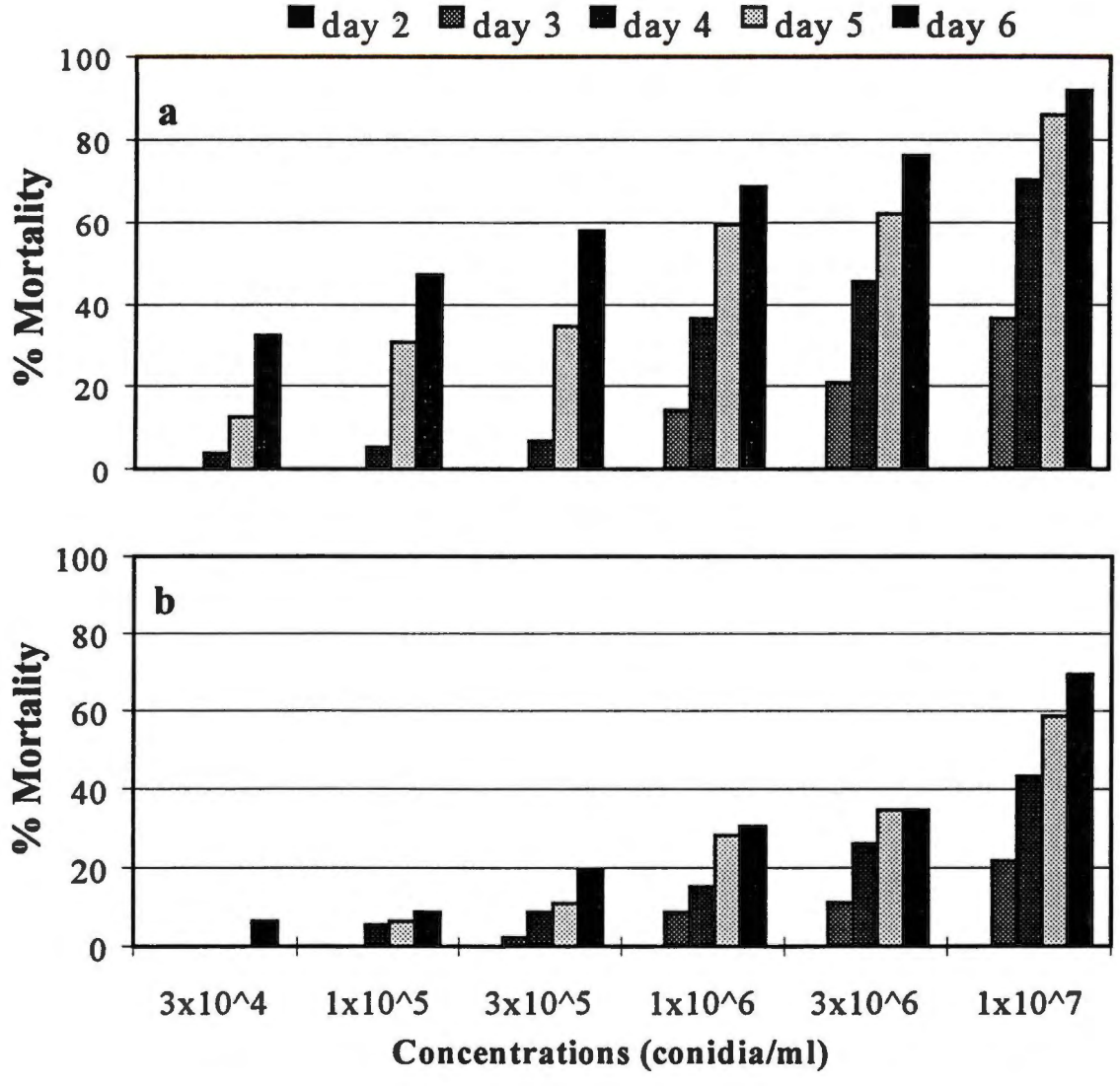


Figure 3.1. Percent Abbott-corrected mortality of the potato aphid, *M. euphorbiae*, treated with *M. anisopliae* isolate 18.97 suspensions with 3.0×10^4 to 1.0×10^7 conidia/ml: (a) experiment I, (b) experiment II.

x 10^7 conidia/ml at 6 days post inoculation. With an increase in fungal concentrations there was an increase in aphid mortality (Figure 3.1).

Cumulative mortality caused by isolate 38.97 (Figure 3.2) was 96 and 98% with the highest dose in experiments I and II, respectively. A clear response to increasing concentration was obtained in both experiments and mortality rose slowly with increased concentrations of conidia. The mortality of aphids caused by isolates 44.97 (Figure 3.3) was similar in experiment I and II with increasing concentrations killing increasing percentage of aphids. Mortalities at day 6 were 94 and 91% of the high conidial concentration for experiment I and II, respectively.

Isolate 67.97 caused low mortality of aphids in both experiments. The highest mortalities were only 66 and 59% in experiment I and II, respectively (Figure 3.4). A response to increasing concentration was not clear in experiment I, but it seemed more defined in experiment II.

Mortality of aphids caused by isolate 79.97 (Figure 3.5) was similar in experiments I and II. The cumulative mortality with the highest conidial concentration was 87% in experiment I and 90% in experiment II. The virulence of isolate 100.97 (Figure 3.6) against aphids was high in experiment I, with 95% cumulative mortality at the highest conidial concentration, but it decreased in experiment II, with maximum mortality of 69%. Isolate 112.97 (Figure 3.7) showed high maximum mortality in both experiments, with 97 and 96% mortality in experiment I and II, respectively, with application of a concentration of 1.0 x 10^7 conidia/ml.

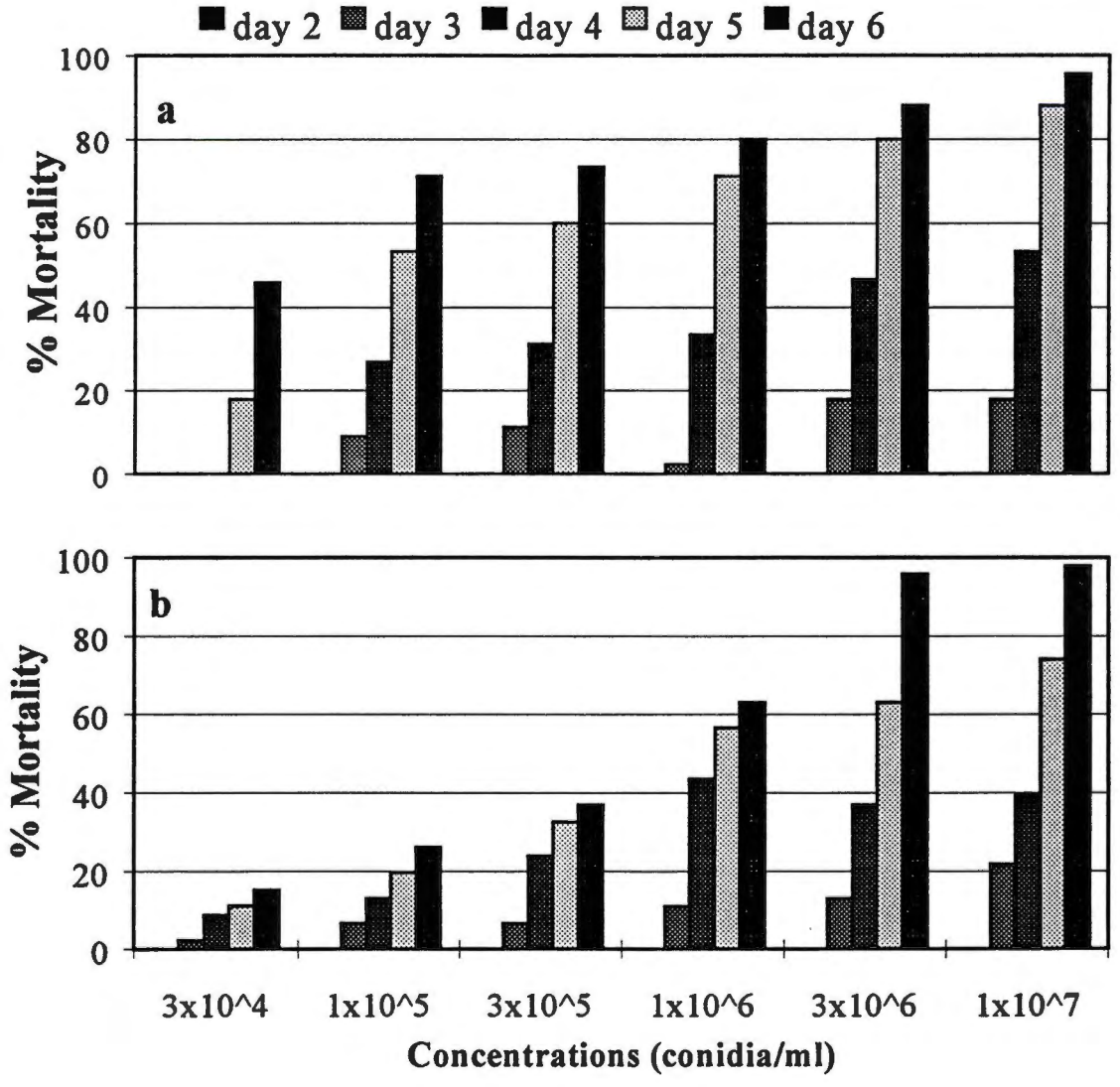


Figure 3.2. Percent Abbott-corrected mortality of the potato aphid, *M. euphorbiae*, treated with *M. anisopliae* isolate 38.97 suspensions with 3.0×10^4 to 1.0×10^7 conidia/ml: (a) experiment I, (b) experiment II.

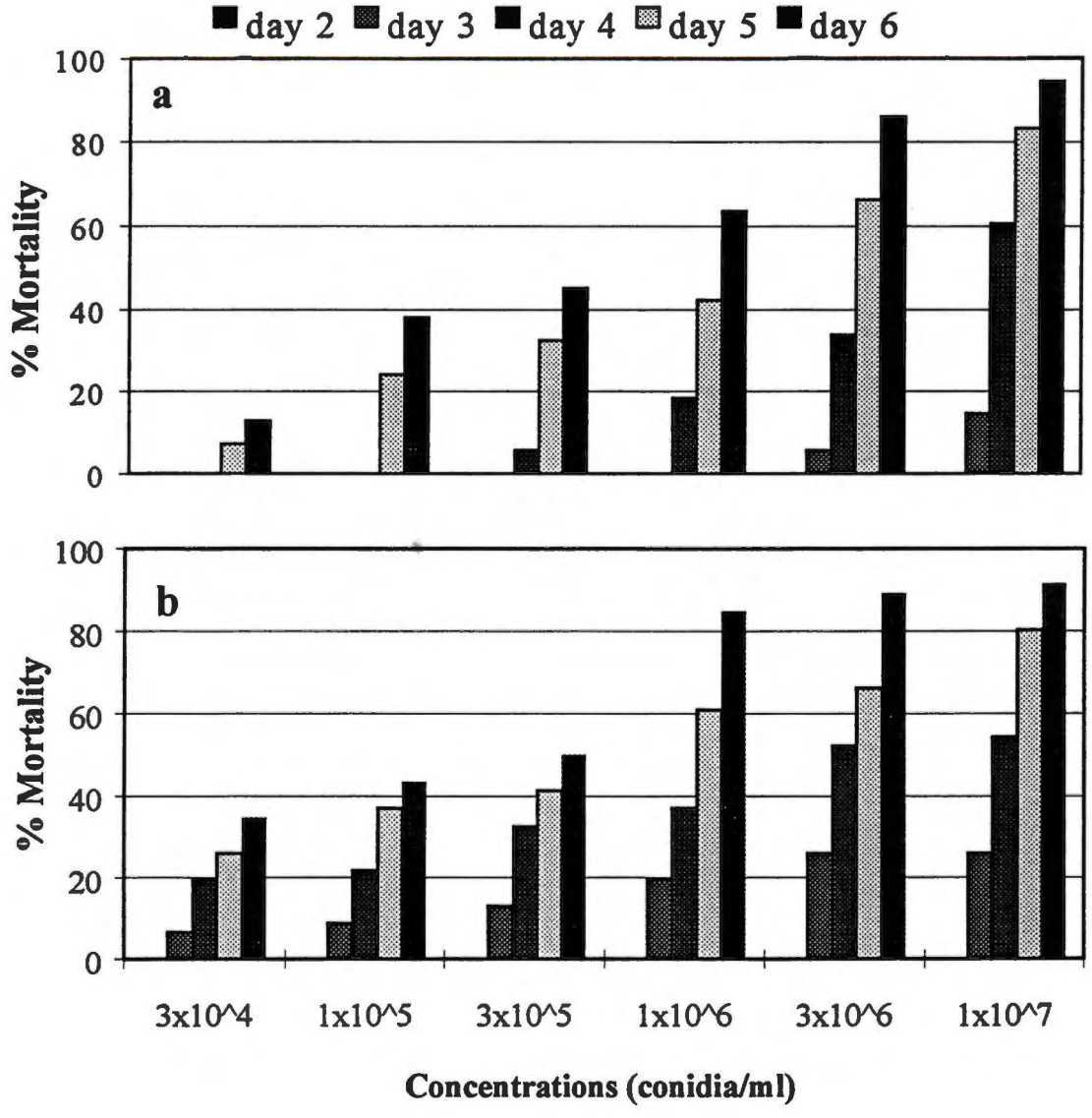


Figure 3.3. Percent Abbott-corrected mortality of the potato aphid, *M. euphorbiae*, treated with *M. anisopliae* isolate 44.97 suspensions with 3.0×10^4 to 1.0×10^7 conidia/ml: (a) experiment I, (b) experiment II.

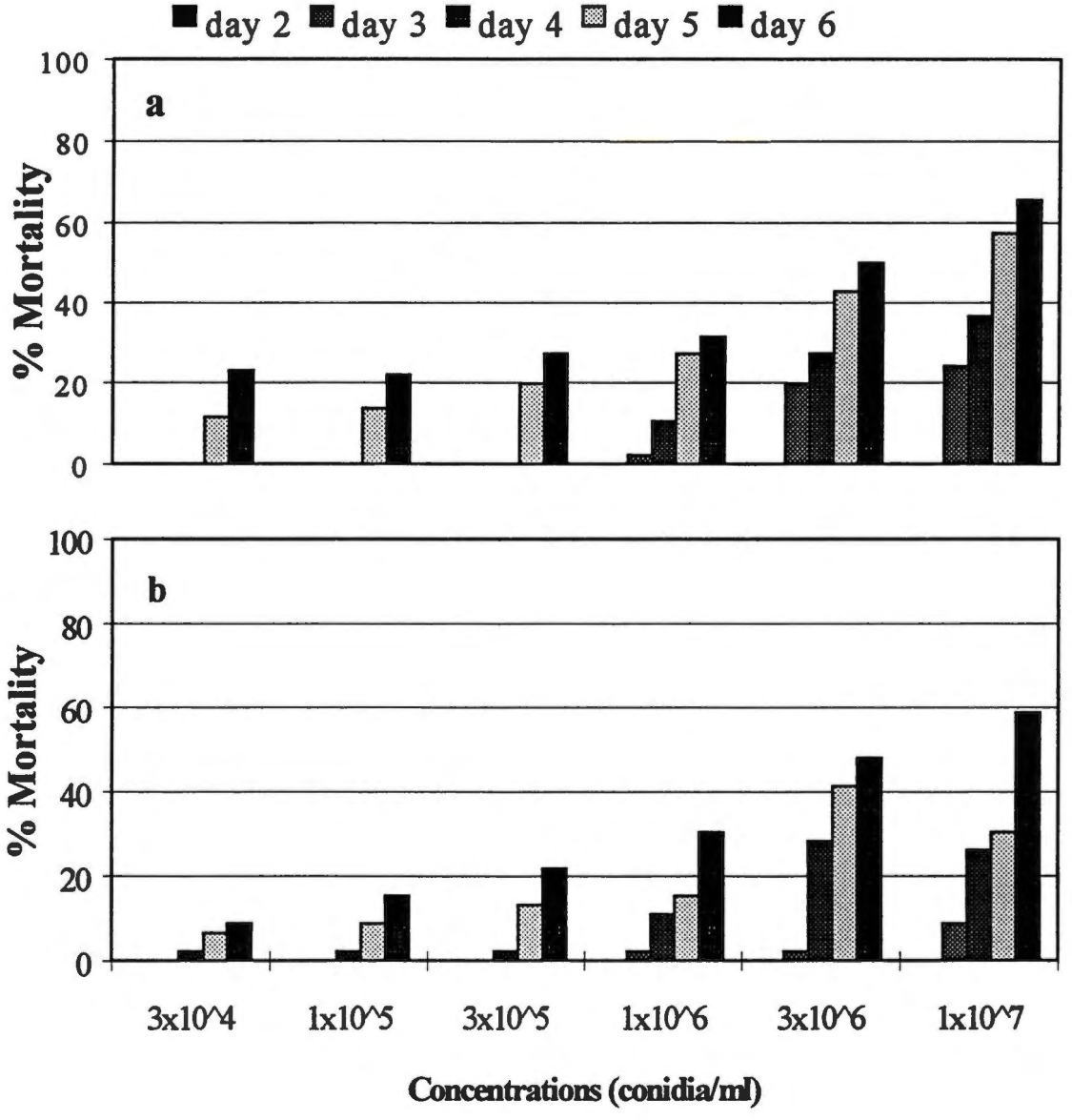


Figure 3.4. Percent Abbott-corrected mortality of the potato aphid, *M. euphorbiae*, treated with *M. anisopliae* isolate 67.97 suspensions with 3.0×10^4 to 1.0×10^7 conidia/ml: (a) experiment I, (b) experiment II.

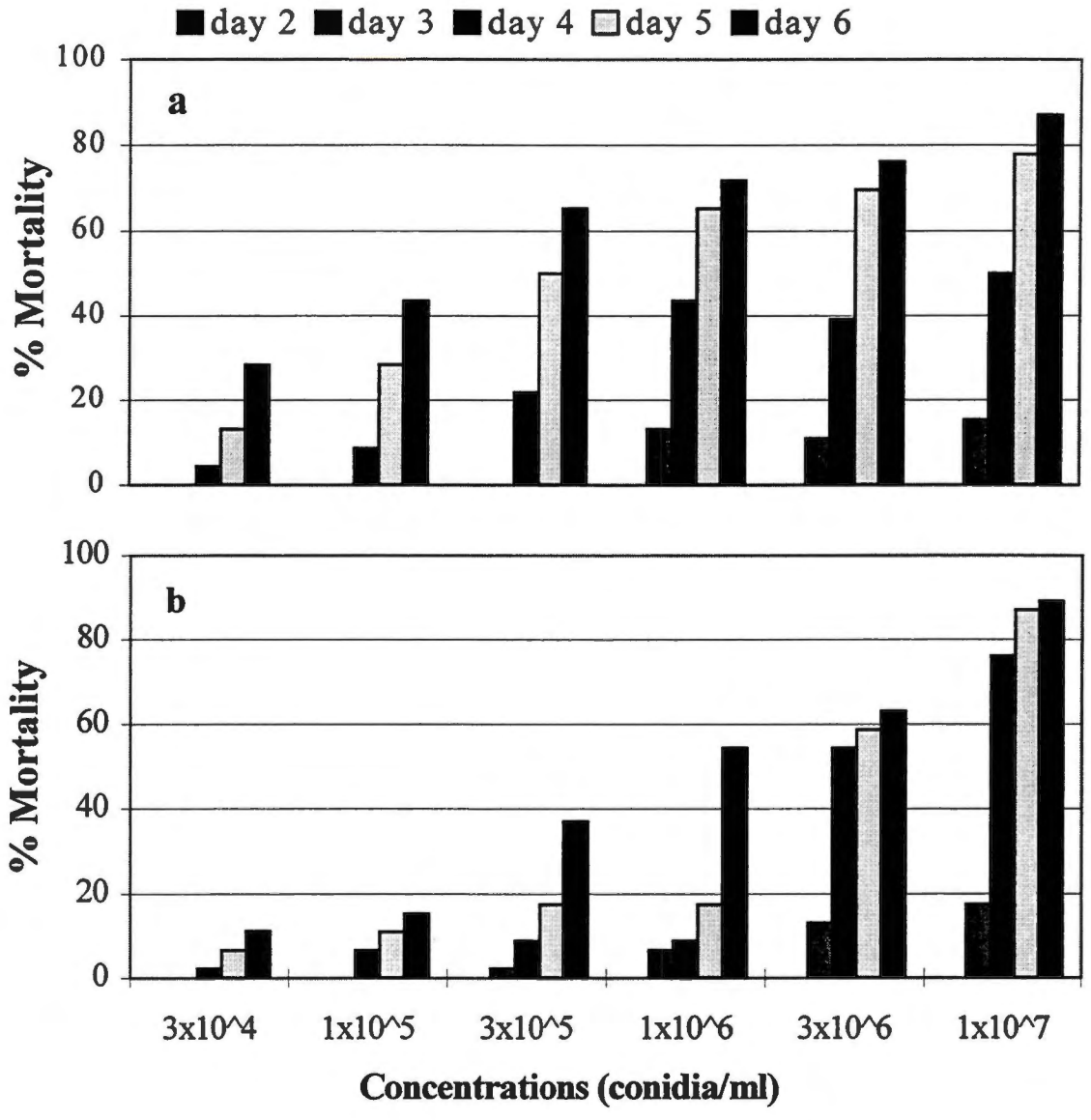


Figure 3.5. Percent Abbott-corrected mortality of the potato aphid, *M. euphorbiae*, treated with *M. anisopliae* isolate 79.97 suspensions with 3.0×10^4 to 1.0×10^7 conidia/ml: (a) experiment I, (b) experiment II.

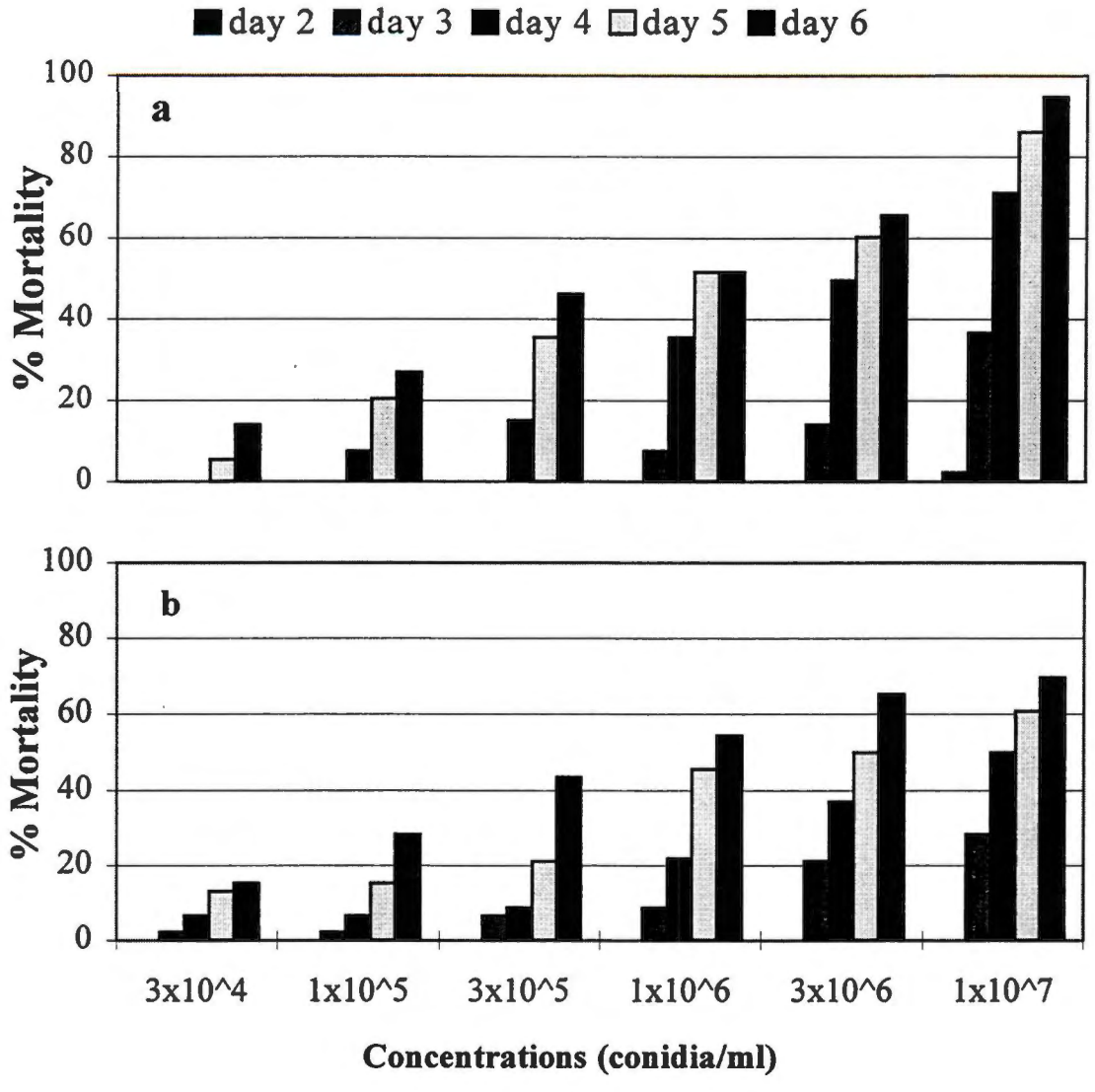


Figure 3.6. Percent Abbott-corrected mortality of the potato aphid, *M. euphorbiae*, treated with *M. anisopliae* isolate 100.97 suspensions with 3.0×10^4 to 1.0×10^7 conidia/ml: (a) experiment I, (b) experiment II.

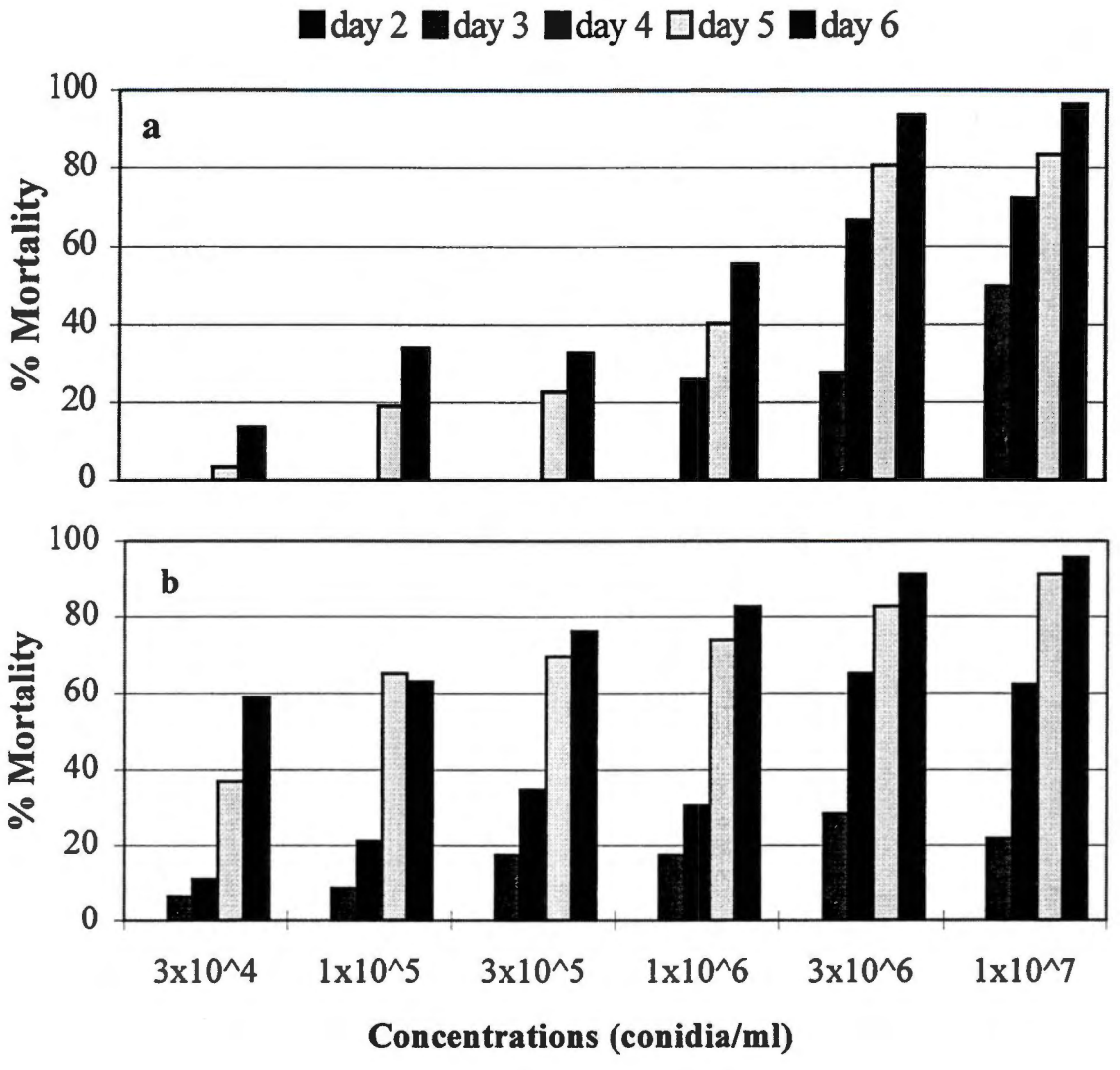


Figure 3.7. Percent Abbott-corrected mortality of the potato aphid, *M. euphorbiae*, treated with *M. anisopliae* isolate 112.97 suspensions with 3.0×10^4 to 1.0×10^7 conidia/ml: (a) experiment I, (b) experiment II.

The LC_{50} of all isolates at 6 days post inoculation is shown in Table 3.1. Isolate 18.97 was highly effective in experiment I with LC_{50} of 1.53 x 10^5 conidia/ml. However, isolate 18.97 was one of the two least effective isolates in experiment II with LC_{50} of 4.25 x 10^6 conidia/ml. LC_{50} for isolate 67.97 reflected the low effectiveness of this isolate in both experiments, being 3.30 x 10^6 and 4.56 x 10^6 conidia/ml for experiment I and II, respectively. The LC_{50} for 38.97, 44.97, 79.97, 100.97, and 112.97 were not significantly different at 95% CI in experiment I. Differences existed among these isolates in experiment II, but isolates 38.97, 44.97 and 112.97 were still highly effective, with LC_{50} below 4.0 x 10^5 conidia/ml, as in experiment I.

Discussion

The bioassay procedure used was not difficult but it required careful attention in transferring aphids from the colony to the inoculation chambers and the bioassay chambers. This procedure was time-consuming which limited the number of treatments that could be handled each time. However, the bioassay procedures were completely adequate for the estimation of LC_{50}, and the two experiments had similar results, except for a few exceptions.

The maximum mortalities of the aphids treated with isolate 18.97 were different in the two experiments (92% and 70% in experiment I and II, respectively). Conidial viabilities were 96% in experiment I and 92% in experiment II, so quality of the conidia does not seem to be the cause for the observed differences. Besides isolate 18.97, isolate 100.97 also had decreased virulence to aphids in experiment II. In addition, 100.97

Table 3.1. Median lethal concentrations LC_{50} (conidia/ml) from *Metarhizium anisopliae* isolates applied against the potato aphid, *Macrosiphum euphorbiae*, based on cumulative mortality 6 days after fungal application.

Isolates	Experiment I		Experiment II	
	LC_{50}	95% CI	LC_{50}	95% CI
18.97	1.53×10^5 a*	$0.94\text{-}2.27 \times 10^5$	4.25×10^6 c*	$1.94\text{-}16.5 \times 10^6$
38.97	2.94×10^5 ab	$0.59\text{-}6.98 \times 10^5$	3.43×10^5 ab	$1.59\text{-}7.11 \times 10^5$
44.97	3.25×10^5 ab	$2.00\text{-}5.07 \times 10^5$	1.32×10^5 a	$0.35\text{-}3.00 \times 10^5$
67.97	3.30×10^6 c	$1.05\text{-}60.8 \times 10^6$	4.56×10^6 c	$2.82\text{-}8.69 \times 10^6$
79.97	1.62×10^5 ab	$0.74\text{-}2.90 \times 10^5$	8.29×10^5 b	$5.09\text{-}14.1 \times 10^5$
100.97	5.40×10^5 bc	$2.43\text{-}12.1 \times 10^5$	8.64×10^5 b	$5.83\text{-}13.2 \times 10^5$
112.97	3.71×10^5 ab	$1.31\text{-}9.78 \times 10^5$	1.84×10^5 a	$0.63\text{-}3.69 \times 10^5$

* Means within the same column followed by the same letter are not significantly different based on comparisons of 95% CI.

killed the aphids rapidly in experiment I with dead aphids occurring on the second day post inoculation, while all other treatments only had dead aphids starting on the third day after inoculation.

Isolates 18.97 and 100.97 may have lost their virulence due to the genetic shifts during culturing of the isolates, or to the inappropriate conidial harvesting and storage method. Changes did not affect viability of the conidia but affected their ability to cause disease on insects. It is also possible that the application of isolates 18.97 and 100.97 during experiment II was faulty, affecting performance of the fungal isolates. Both 18.97 and 100.97 showed high virulence in previous experiments described in Chapter II. These isolates should be tested again using newly-produced conidia so result can be confirmed.

Isolate 67.97 was selected among those that caused low aphid mortality in experiments described in Chapter II. Dose-response experiments confirmed its low virulence to the potato aphid. This isolate should not be used for control of the potato aphid unless other characteristics could justify the use of an isolate with low virulence.

Overall, the mortality of the aphids by all isolates in experiment I was greater than in experiment II. The median lethal concentration (LC_{50}) of *M. anisopliae* was different among isolates. The most effective isolates, which showed low LC_{50} and killed aphids equally well in both experiments, were selected to be tested in the greenhouse. These were isolates 38.97, 44.97, and 112.97.

The LC_{50} estimates obtained were usually around 10^5 conidia/ml. This value compares favorably with other values reported in the literature in relation to the use of entomopathogenic fungi against aphids. The estimated LC_{50} of *B. bassiana* and *V. lecanii* were 8.2×10^4 and 4.1×10^5 conidia/ml respectively against the Russian wheat aphid (Feng et al. 1990). Dorschner et al. (1991) estimated that the LC_{50} of *B. bassiana* toward the hop aphid was 1.37×10^5 conidia/ml. Estimates of LC_{50} obtained with *M. anisopliae* isolates against the potato aphid were apparently lower than those established before for other related insects (Butt et al. 1994, Chandler 1997). These results can be an indication that these isolates are well adapted to the potato aphid. These comparisons suggest that the *M. anisopliae* isolates used in dose-response experiments are at least as effective as other fungal isolates used in aphid control. The use of these isolates in the field and greenhouse studies is warranted as part of the development of a biological insecticide to use in commercial tomato production.

CHAPTER IV

THE PATHOGENICITY OF THE ENTOMOPATHOGENIC FUNGUS *Metarhizium anisopliae* AGAINST THE POTATO APHID, *Macrosiphum euphorbiae*, IN THE GREENHOUSE

Introduction

Entomopathogenic fungi are potential microbial agents for control of insects pests in greenhouse and field crops. The conditions that favor fungal infection include the virulence of entomopathogens, the density of the susceptible hosts, and optimum environmental conditions. Generally, entomopathogenic fungi are favored by appropriate high temperatures and humidities when infecting insects (Shashi et al. 1998). *M. anisopliae* has optimum temperature at approximately 28°C, at which temperature rapid growth rate and extensive sporulation occur. Rombach and Gillespie (1988) and Milner and Bourne (1983) also reported that entomopathogenic fungi require high relative humidity and moderate temperatures for host infection and conidial production.

Greenhouse experiments have been conducted by several researchers to determine virulence of entomopathogenic fungi toward greenhouse pests. Commercial mycopesticides containing *V. lecanii*, *B. bassiana*, and *P. fumosoroseus* are available to control pests in greenhouse.

In the greenhouse, conditions with 100% relative humidity for at least 36 hours are required for *V. lecanii* infection on the aphid *M. persicae* to occur in significant

levels (Milner and Lutton 1986). Zimmermann (1982) also reported that *V. lecanii* infected *M. persicae* on bean, sweet pepper and eggplants in the greenhouse but the fungus had no effect on insects at high temperatures (40-45°C). *V. lecanii* was recommended to use in the greenhouse to control aphids on chrysanthemum and control the greenhouse whitefly on cucumber (Rombach and Gillespie 1988). Several *M. anisopliae* isolates are virulent against the greenhouse whitefly (Malsam et al. 1998).

In greenhouse experiments, *M. anisopliae* applied to control the pecan weevil, *Curculio caryae* (Horn), caused only 49% mortality of adult weevils (Gottwald and Tedders 1983). Vanninen et al. (1999) studied the pathogenicity and efficacy of *M. anisopliae* against the third-instar larvae of *D. floralis*. This author reported that a concentration of 1 $x10^8$ conidia/ml reduced the root damage of head cabbage by 20-70%. Borisov and Ushchekov (1997) tested *M. anisopliae* against nightshade leafminer *Liriomyza bryoniae*, another pest of greenhouse crops. They reported that *M. anisopliae* was effective in reducing adult emergence from soil by 60-88%. Helyer et al. (1995) treated the western flower thrips, *Frankliniella occidentalis* (Pergande), with *M. anisopliae* as a pre-pupation treatment and observed 74.5% mortality of this insect.

Metarhizium anisopliae has shown the potential to control various insects in greenhouse including the green peach aphid. In this study, the pathogenicity of the entomopathogenic fungus *M. anisopliae* was evaluated against the potato aphid infesting tomato plants in the greenhouse.

Materials and Methods

Metarhizium anisopliae Isolates

Metarhizium anisopliae isolates 38.97, 44.97 and 112.97 were selected for evaluation based on their low LC_{50} and high virulence toward the potato aphid (see Chapter III). Conidia from agar plates were kept in the refrigerator and checked for viability before use in experiments as described previously. Preparation of fungal suspensions followed procedures described in the previous chapters. Conidial concentrations were counted under microscopy using a hemocytometer, and the conidial suspensions were diluted to concentrations of 1.0×10^7, 5.0×10^7 and 1.0×10^8 conidia/ ml.

Tomato Plants

Tomato plants were grown in a greenhouse at varying temperatures and with mean approximately 25°C. Experimental units were maintained on a separate bench in the same greenhouse. The tomato variety used was Monopal which is susceptible to potato aphids. These plants, which were also used to rear aphids, were planted in a tray with Pro-Mix® potting mixture. Pots with plants were maintained in a tray with approximately 1 cm layer of water to maintain soil humidity. Three-week old tomato plants were used in this experiment.

Insect Rearing

Potato aphids, *M. euphorbiae*, were reared as described in Chapters II and III.

Experimental Unit

This experiment consists of nine treatments (three fungal isolates x three conidial suspensions) and a control (sprayed with 0.02% Tween 80 in water). Four replications of each treatment were prepared. Each experimental unit contained 2-3 tomato plants in a small round pot (7.5 cm diameter). Ten aphids were transferred onto each experimental unit and allowed to feed and reproduce for 10 days on the tomato plants. After this period, excess aphids were removed, and 15 adult aphids were left on tomato plants in an experimental unit as the initial population to be used in experiment. Four experimental units, representing four replicates, were contained within a tray.

Spray Application

The laboratory spray unit "Chromist" (Gelman Science Inc.) was used to spray conidial suspensions onto aphids on tomato plants. Preliminary tests were done to estimate the amount of conidial suspension to be used in experiments. Five ml of conidial suspension were sprayed onto each experimental unit. During application, each unit was moved to the clean area, and a thick paper was used as a barrier to prevent conidial contamination of other plants.

Statistical Analysis

The numbers of dead aphids were recorded at 1, 4, 7 and 10 days after application. Each dead aphid was removed from the tomato plants and placed on wells of a microtiter plate to confirm fungal infection as described in previous chapters. Mortality values were corrected using Abbott's formula (Abbott 1925) and corrected

cumulative mortality after 10 days was analyzed. Aphid mortality was analyzed using ANOVA with factorial design. Isolates and doses were used as main effects. Means were compared using Fisher's protected LSD range test.

Results

Greenhouse conditions varied during the day around a mean temperature of 25°C, and a relative humidity around 80%. On the control plants, mean cumulative aphid mortality after 10 days was 10%. Aphids that survived in all treatments reproduced as usual, but population growth rate was low during the experimental period.

Aphids that died were attached to tomato leaves and stems. *M. anisopliae* isolates caused aphid mortality ranging from 6 to 43% in the greenhouse (Table 4.1). Isolate 38.97 produced significantly lower mortalities than isolates 44.97 and 112.97. Isolate 38.97 killed 6, 21 and 30% of aphids at suspensions of 1.0×10^7, 5×10^7 and 1.0×10^8 conidia/ml, respectively. Isolates 44.97 and 112.97 were equally effective in controlling potato aphids, killing an average over all doses of 33 and 31% of aphids, respectively.

Significant differences among concentrations were documented. The dose response showed increasing mortalities when concentrations increased from 1.0×10^7, to 5.0×10^7, and 1.0×10^8 conidia/ml. Isolate 44.97 at the highest concentration (1.0×10^8 conidia/ml) caused the highest mortality, killing 43.4% of aphids, but this value was not significantly different from the maximum mortality with isolate 112.97 (39.6%).

Table 4.1. Mortality of the potato aphid *Macrosiphum euphorbiae* 10 days after treatment with different conidial dose of different isolates of *Metarhizium anisopliae*.

Isolates	Concentration	Abbott-corrected % ± SE mortality
38.97	1.0×10^7	5.7 ± 2.2 a*
	5.0×10^7	20.8 ± 2.2 bc
	1.0×10^8	30.2 ± 3.6 cde
44.97	1.0×10^7	28.3 ± 4.9 bcd
	5.0×10^7	28.3 ± 2.2 bcd
	1.0×10^8	43.4 ± 3.8 f
112.97	1.0×10^7	18.9 ± 1.9 b
	5.0×10^7	34.0 ± 4.7 def
	1.0×10^8	39.6 ± 3.1 ef

* means with the same letters are not significantly different at P=0.05 (Fisher's protected least significant test).

With the lowest dose (10^7 conidia/ml), isolates 44.49 and 112.97 killed significantly more aphids than isolate 38.97. At a concentration of 5 x 10^7 conidia/ml, 112.97 was the most effective isolate. Increasing aphid mortality with increase in concentration was apparent when isolates 38.97 and 112.97 were used. With isolate 44.97, that was not the case since the two lowest doses caused the same aphid mortality (28.3%). The aphid mortality curves (Figure 4.1 a-c) showed that mortality increased until the seventh day post inoculation, but, in general, mortalities did not increase afterward.

Discussion

Isolates of *M. anisopliae* killed far less potato aphids in the greenhouse experiment than was expected, based on laboratory studies. Factors that favor high virulence of the entomopathogenic fungi are the high density of pathogen, high pest populations, and certain environmental conditions such as high humidity and moderate temperature. The conditions in the greenhouse were such that temperatures may have reached levels much above the optimum for *M. anisopliae*, therefore, preventing successful control of aphids. Also, populations of aphids on plants was maintained low by removal of nymphs, and the removal of dead aphids prevented secondary cycling of the fungus. The fungus sporulated on the dead aphids that were placed in wells of microtiter plate, and the conidia could have served as inoculum for infection of the other aphids on plants if sporulation had occurred.

In this experiment, the data were inadequate to estimate the median lethal time (LT_{50}) and the median lethal concentration (LC_{50}). Also, because of time constraints, the experiment could not be repeated so that results could be confirmed. Results from

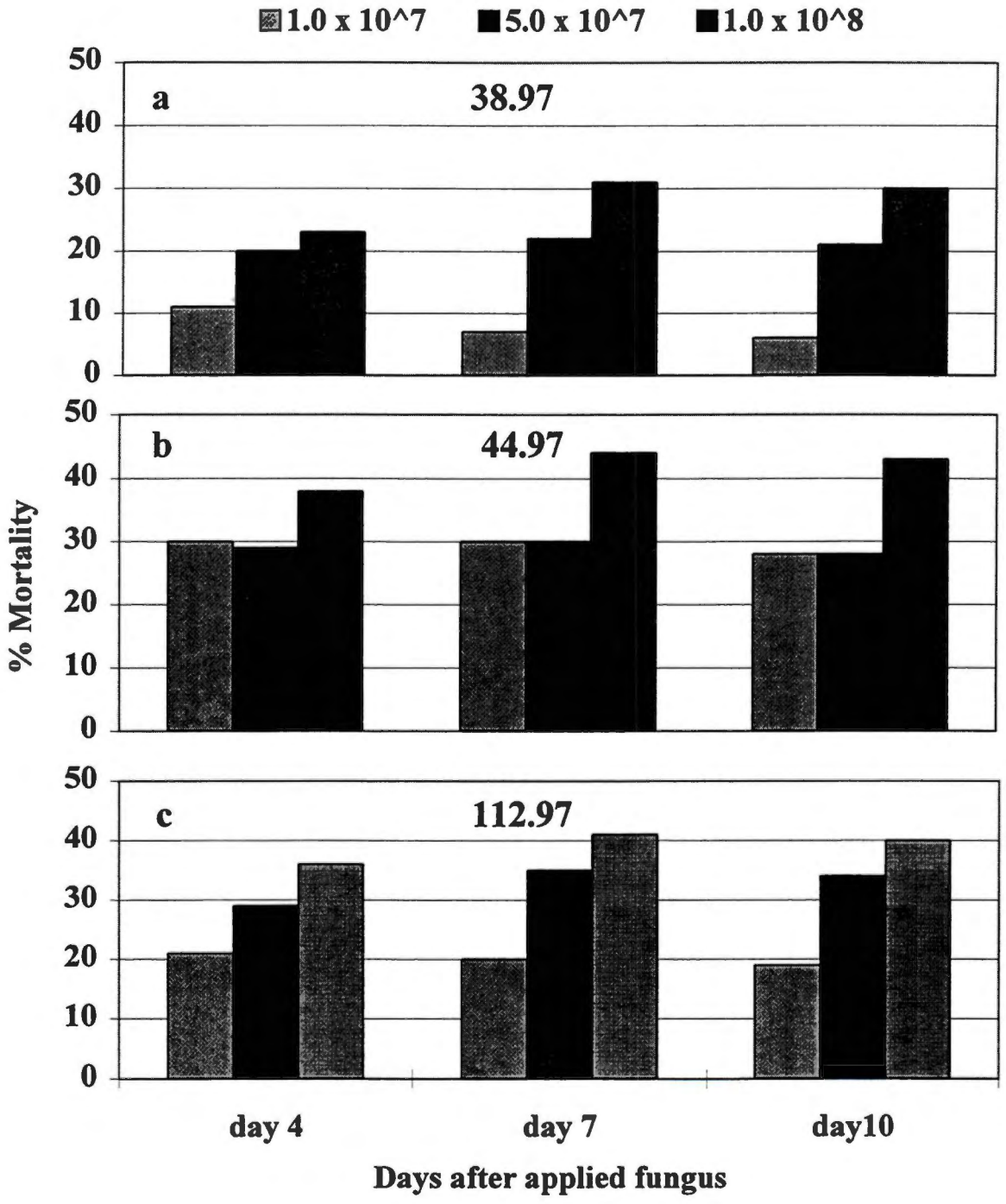


Figure 4.1 Mortality of the potato aphid, *M. euphorbiae*, by *M. anisopliae* isolates 38.97 (a), 44.97 (b), and 112.97 (c) at 4, 7 and 10 days after application of concentrations of 1.0×10^7 , 5.0×10^7 and 1.0×10^8 conidia/ml to tomato plants in greenhouse.

this greenhouse experiment compared unfavorably with other greenhouse experiments. For instance, the use of *M. anisopliae* on pecan to control pecan weevils caused only 49% mortality of this pest (Gottwald and Tedders 1983). The optimum conditions for *V. lecanii* against green peach aphid were 20°C and 100% RH in the greenhouse, which allowed 94.5% of aphid mortality 4 days after application (Milner and Lutton 1986). In this experiment, mortality increased slowly in the aphid population. It may be necessary to repeat the application of fungus to adequately control the aphid population.

CHAPTER V

CONCLUSIONS

The bioassay used in this study, despite its simplicity, was time-consuming due to the required handling and transferring of the small soft-bodied aphids. Also, modifications are needed to reduce condensation in the bioassay chambers and prevent accidental death of aphids that get trapped in condensation drops.

In this study, the entomopathogenic fungus *M. anisopliae* infected, and caused high mortality of the potato aphid in laboratory test. All forty-seven isolates of *M. anisopliae* infected *M. euphorbiae* but their virulence varied greatly. More than forty isolates caused mortality greater than 60% as shown in chapter II. The differences in virulence of the fungal isolates toward aphids suggest that isolates of this fungus have specific characteristics. Some results also suggest that virulence may change during the storage of conidia in refrigerator. The virulence of isolate 18.97 and 100.97 decreased when kept for 6 months in refrigerator despite no change in viability. Research is needed on techniques to maintain pathogenicity of conidia in cold storage.

Studies on the response to increasing conidial concentrations are very important if *M. anisopliae* will be used as a microbial agent for control of potato aphids. An appropriate concentration is needed for development and application of commercial product. Results from laboratory experiments demonstrated that *M. anisopliae* has the potential to control the potato aphid. Results from the limited greenhouse experiment did not produce very satisfactory results. Further experimentation is needed before control of the potato aphid using *M. anisopliae* in the greenhouse and the field is

attempted. More information on the appropriate environment for fungal infection and the effects of the greenhouse environment on *M. anisopliae* are also needed. These conditions may have great impact on the host's susceptibility to *M. anisopliae.* The development of application methodology and conidial formulation are perhaps the most important factors to consider. These factors affect how conidia adhere to the insect cuticle and how readily the fungus penetrates the insect integument. Adhesion and penetration are critical steps in the entomopathogenic fungus life cycle, and may determine the level of isolate virulence. Although the virulence of *M. anisopliae* in the greenhouse was lower than that in the laboratory bioassays, the possibility of using this fungus in the control of the potato aphid should not be discarded.

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