Virulence of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) to Sugarbeet Root Maggot (Diptera: Ulidiidae) Larvae

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ABSTRACT

Initial comparisons of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* indicated that *M. anisopliae* had greater potential for control of sugarbeet root maggot, *Tetanops myopaeformis,* larvae than *B. bassiana.* In subsequent screening of several *M. anisopliae* strains (ATCC 22099, ATCC 56096, ATCC 60335, ATCC 16085, ATCC 62176), ATCC 62176 affected the greatest larval mortality at 14 days after exposure (DAE). Follow-up bioassays and survival probability analyses on ATCC 62176 indicated rate responses with the strain, and demonstrated that higher ($\geq 2.58 \times 10^5$ conidia/ml) dosages increased larval mortality in comparison with controls. In one bioassay, lethal time (LT_{50}) values for 2.58×10^5, 2.58×10^6, and 2.58×10^7 conidia/ml were 16, 10, and 7 DAE, respectively. Respective LT_{50} values for 2.58×10^5, 2.58×10^6, and 2.58×10^7 conidia/ml were 15, 9, and 7.5 DAE in a second bioassay. Survival likelihood at 12 DAE was 0% for root maggot larvae exposed to 2.58×10^7 conidia/ml, and $\leq 15\%$ after

exposure to a concentration of 2.58 x 10^6 conidia/ml. Lower (≤ 2.58 x 10^4 conidia/ml) concentrations did not achieve 50% larval mortality, indicating those concentrations would not likely cause sufficient mortality under field conditions. These results demonstrate that *M. anisopliae* is a virulent fungal pathogen of sugarbeet root maggot larvae, and strains differ in virulence. This work also establishes a virulence baseline and a target concentration for using *M. anisopliae* strain ATCC 62176 as a potential bioinsecticidal organism for root maggot management. Field trials are needed to identify efficacious rates, grower-adoptable formulations, and optimal application timing for maximizing the efficacy of this entomopathogen against this important insect pest of sugarbeet.

Additional key words: *Tetanops myopaeformis*, *Beta vulgaris*, *Beauveria bassiana*, bioassay, entomopathogenic fungus, insect-pathogenic fungus, bioinsecticide

The sugarbeet root maggot (SBRM), *Tetanops myopaeformis* Röder, is the most serious insect pest of sugarbeet, *Beta vulgaris* L., in the Red River Valley of North Dakota and Minnesota (Yun, 1986). The insect is also a major sugarbeet pest in Montana, Wyoming, Colorado, Nebraska, Idaho, and southwestern Canada (Yun, 1986; Cooke, 1993). Larvae develop through three instars as they feed on the surface of sugarbeet roots. Most larval feeding typically occurs from mid-June through August. Extensive early season feeding injury by larvae can sever small taproots, frequently leading to the death of affected plants under dry soil conditions. Yield losses of up to 40% could be common in portions of the Red River Valley in the absence of control measures (Campbell *et al.*, 1998).

Sugarbeet producers have depended primarily on organophosphate and carbamate insecticides to manage the SBRM for three decades. Two organophosphate insecticides, terbufos (Counter 15G [granular]; BASF Corporation, Research Triangle Park, NC) and chlorpyrifos (Lorsban 4E and 15G; Dow AgroSciences, Indianapolis, IN), are typically applied to a combined total of over 95% of the treated acres in the Red River Valley of North Dakota and Minnesota (Luecke and Dexter, 2003). The intensive use of a single insecticide mode of action has exerted significant selection pressure on these populations for the development of insecticide-resistant root maggot strains. This threat, coupled with concerns regarding the potential negative impacts of conventional chemical insecticides on the environment, has prompted a

renewed interest in biological control agents as components of integrated programs for managing SBRM populations (Campbell *et al*., 2000).

The green muscardine fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin (Deuteromycotina: Hyphomycetes) is a widely distributed insect pathogen with a diverse arthropod host range (Tanada and Kaya, 1993). In addition, successful *M. anisopliae* infection has been achieved with adult and immature stages of insect development (Ferron, 1978). Virulence of the entomopathogen has been demonstrated in larvae of the house fly, *Musca domestica* L., (Barson *et al*., 1994), plum curculio, *Conotrachelus nenuphar* (Herbst), (Tedders *et al*., 1982), and in larval and adult Mexican fruit flies, *Anastrepha Ludens* (Loew) (Lezama-Gutiérrez *et al*., 2000). Campbell *et al*. (2000) used barley, *Hordeum vulgare* L., inoculum to apply *M. anisopliae* to sugarbeet for protection from sugarbeet root maggot feeding injury. Control provided by some *Metarhizium* treatments under moderate to light root maggot pressure was equal to that obtained with the insecticide chlorpyrifos; however, the volume of barley inoculum required to achieve that level of control made it an impractical formulation for use in commercial sugarbeet production. Although the findings of Campbell *et al*. (2000) suggested that *M. anisopliae* had the potential for reducing yield losses caused by the SBRM, the virulence of the fungus in the insect had not been quantified. We conducted laboratory bioassays and used survival probability analyses to characterize the effect of *M. anisopliae* concentration and time on SBRM survival, and to explore the potential of the fungus as a tool for managing this important insect pest.

MATERIALS AND METHODS

The initial exploratory experiment involved screening two entomopathogenic fungi, *M. anisopliae* and *Beauveria bassiana* (Balsamo) Vuillemin, against third-instar SBRM larvae to identify the pathogen with greater potential as an SBRM biological control agent. The *M. anisopliae* strain examined, ATCC 22099, was characterized by Jong and Edwards (1991) as having virulence to a variety of insect species. The *B. bassiana* evaluated was an increase from Naturalis-L (Troy Biosciences, Inc. Phoenix, AZ), a commercial strain of the organism. Third-instar sugarbeet root maggots used in bioassays were obtained from untreated sugarbeet fields in the northern Red River Valley during August, and stored in moist sand at 4± 1^{0}C until needed. The experimental unit was a 35 x 10 mm sterile culture dish containing 7 ml of sterile fine (ca. 0.1 mm particle size) sand and five SBRM larvae. A phosphate-buffered saline solution comprised of 10 mM sodium

phosphate, pH 7.0, 0.85% sodium chloride, and 1.14% Tween 80™ (Sigma Chemical Co., St. Louis, MO) was used to apply each fungus treatment in 3-ml aliquots of 2.3 x 10^7 viable conidia/ml to sand in the culture dishes. A 3-ml buffer-only treatment was included as a control. Each dish was infested with five live larvae, incubated in total darkness at 24± 1^0C, and observed daily for larval mortality. This initial screening indicated that *M. anisopliae* had greater potential as a candidate organism for control of SBRM than *B. bassiana* (Table 1). Therefore, *M. anisopliae* became the focus of the remainder of this investigation.

***M. anisopliae* Strain Testing.** Subsequent trials involved the screening of five *M. anisopliae* strains for virulence in SBRM larvae. This testing also was carried out to provide an indication of variation within the species. The following strains were obtained from the American Type Culture Collection (Rockville, MD at time of culture acquisition; currently located in Manassas, VA) for use in these bioassays: 1) ATCC 22099, isolated from a moth, *Thaumetopoea wilkinsoni* Trams, in Israel; 2) ATCC 56096, from the spittlebug, *Aeneolamia albofasciata* Lall., in Mexico; 3) ATCC 60335, isolated from another spittlebug species, *Deois flavopicta* Stål, in Brazil; 4) ATCC 16085, isolated from Canadian soil; and 5) ATCC 62176, collected from soybean cyst nematode, *Heterodera glycines* Ichinohe, in Illinois (Jong and Edwards, 1991). The spore concentration used for all strains was 2.3 x 10^7 conidia/ml. A treatment with no fungi was included as a control. The study was run three times with five replicates per run, and evaluations were carried out on a total of 450 third-instars. Dead larvae were counted at 4, 14, 21, and 28 days after exposure to the *M. anisopliae* strains. Data were analyzed by using PROC GENMOD (SAS Institute, 1999) with the log-odds ratio or logit as the dependent variable. Single-degree contrasts were used to compare

Table 1. Mortality of third-instar SBRM larvae at 5-day intervals after exposure to 2.3 x 10^7 viable conidia/ml of *M. anisopliae* and a commercial *B. bassiana* strain.

Days after exposure	Mortality† (%) ± SE	
	***M. anisopliae* (ATCC 22099)**	***B. bassiana* (Naturalis-L)**
5	12.3 ± 1.8	5.7 ± 2.0
10	61.1 ± 3.6	15.2 ± 3.2
15	94.6 ± 1.4	28.7 ± 5.5
20	99.5 ± 0.2	40.6 ± 6.6
25	100.0 ± 0.0	46.9 ± 3.6

†Four percent mortality in control larvae through 15 days after treat-

all possible pairs of fungus strains, and the Wald Chi-square test (Allison, 1999) was used to indicate differences among strains.

ATCC 62176 Virulence. ATCC 62176 was selected for additional virulence studies because of its superior performance (Table 2) relative to the other strains evaluated. Fungi were incubated for 10-14 days in the dark at 25±1^{0}C on petri dishes (15-mm deep by 100-mm diameter) containing potato dextrose agar (PDA). A mixture of conidia and hyphae was obtained by scraping the surface of each colony with a sterile spatula. Colonies were combined into a 1% (vol: vol) Tween 80™ suspension and homogenized by using a Vortex Maximix II™ shaker (Barnstead/Thermoline, Dubuque, IA). Conidial concentrations were determined visually by using a Bright-Line™ Improved Neubauer hemacytometer (Hausser Scientific, Horsham, PA). Concentrations were initially adjusted to 1 x 10^8 conidia/ml, and serial dilutions were prepared to obtain additional concentrations of 10^7, 10^6, 10^5, 10^4, and 10^3 conidia/ml. The six *M. anisopliae* concentrations and a 1% (vol:vol) Tween 80™ control (i.e., no fungus) were applied to eight replications of ten field-collected larvae in a completely randomized design. Thus, observations were made on 80 maggots for each of the seven treatments, and a total of 560 maggots were used in each of two bioassays (I and II). Blue filter paper (9-cm diameter) was placed into inverted 9-cm petri dish lids and dampened with 4 ml of sterile distilled water. Third-instar SBRM were immersed in conidia suspensions for two seconds and subsequently placed on the filter paper (Goettel and Inglis, 1997). Petri dish bases were then inverted onto lids and sealed with parafilm to retain humidity. Controls were established by

Table 2. Mortality of third-instar SBRM larvae at 4, 14, 21, and 28 days after exposure to isolates of *M. anisopliae*, and percentage of dead larvae with confirmed mycosis.

ATCC isolate no.	Mortality† Days after exposure 4	14	21	28	Mycosis confirmed
-	- - - - - - - -	- - - - - - - -	-%-	- - - - - - - -	- - - - - - - -
62176	0.0 a	84.0 a	100.0 a	100 a	100.0
56096	4.0 a	65.3 b	100.0 a	100 a	100.0
16085	2.7 a	68.0 b	97.3 a	100 a	97.3
60335	0.0 a	53.3 bc	100.0 a	100 a	98.7
22099	4.0 a	46.7 c	98.7 a	100 a	98.7

† Mortality percentages within a column sharing a letter are not significantly different ($\alpha = 0.05$) according to the Wald Chi-square test

immersing larvae in the same manner in a 1% (vol:vol) Tween 80™ solution. All dishes were maintained at 25± 1°C in constant darkness. Larvae were not fed during the experiments. Filter paper was moistened daily with 0.5 ml of sterile distilled water to maintain high humidity in the dishes (Lezama-Gutiérrez *et al.*, 2000). Mortality was recorded at 24-h intervals for 18 d after exposure (DAE). Dead larvae were removed each day to prevent horizontal contamination. A subsample (10%) of dead larvae from each treatment was placed into a 15 x 100-mm diameter petri dish containing PDA to determine if death resulted from mycosis. Symptoms of mycosis in cadavers included distention and rigidity, a mottled rusty brown coloring, and development of *M. anisopliae* hyphae on the exterior surface of the integument.

Conidial viability was determined by plating 0.2 ml of the suspension and counting colony-forming units 7 d later using the methods of Goettel and Inglis (1997). Adjusted concentrations used as treatments were 2.58×10^7, 2.58×10^6, 2.58×10^5, 2.58×10^4, 2.58×10^3, and 2.58×10^2 viable conidia/ml. The LIFETEST procedure (SAS Institute, 1999) was used to compute larval survival estimates via the Kaplan-Meier product-limit method, and the log-rank statistic was used to determine homogeneity of larval survival functions between *M. anisopliae* treatments and the controls (Fisher and Belle, 1993). Survival functions also were used to generate quartile estimates of larval survival for each treatment and, thereby, establish LT_{50} values for the fungus on SBRM larvae.

RESULTS

***M. anisopliae* Strain Testing.** In screening of *M. anisopliae* strains, 100% mortality was observed in third-instar SBRM larvae by 28 days after exposure to conidia, irrespective of strain tested (Table 2). Mortality rates of control larvae (not exposed to *Metarhizium*) were 1.3, 12, 20, and 21.3 % at 4, 14, 21, and 28 DAE, respectively. Although all five *M. anisopliae* strains were found to be capable of killing root maggot larvae, differences in mortality among strains were evident at 14 DAE. Although ATCC strains 62176, 56096, and 60355 caused 100% mortality by 21 DAE, exposure to ATCC 62176 resulted in significantly greater larval mortality than the other *M. anisopliae* strains at 14 DAE. Mortality at 14 DAE in larvae exposed to strain 62176 was about 19% higher than in those exposed to ATCC 56096, the strain that caused the second-highest mortality percentage. This indicated that 62176 had the potential to kill larvae quicker and perhaps that it would be more efficacious than the other strains tested. Differences in the time required to kill larvae could mean the difference between success and failure as a

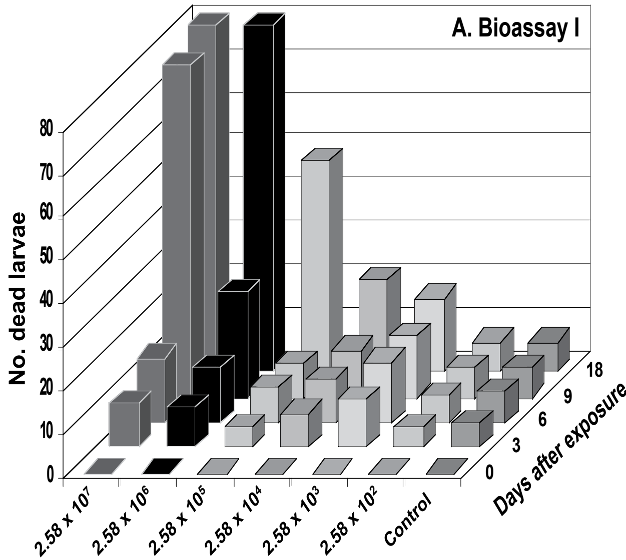
bioinsecticidal control organism. Therefore, ATCC 62176 was selected for additional testing.

ATCC 62176 Virulence. Concentrations of 2.58 x 10^5, 2.58 x 10^6, and 2.58 x 10^7 conidia/ml significantly (χ^2 = 43.99; df = 1; P < 0.0001, χ^2 = 135.72; df = 1; P < 0.0001, and χ^2 = 130.78; df = 1; P < 0.0001, respectively) reduced SBRM survival compared to the controls in Bioassay I (Table 3). The lower dosages, 2.58 x 10^3 and 2.58 x 10^4 conidia/ml, also significantly reduced larval survival (χ^2 = 4.68; df = 1; P = 0.0305 and χ^2 = 7.90; df = 1; P = 0.0049, respectively) in comparison to the control in Bioassay I. The lowest (2.58 x 10^2) dose was the only *M. anisopliae* treatment in Bioassay I that did not have a significant impact on larval mortality (χ^2 = 0.00; df = 1; P = 0.9956). Data from Bioassay II closely corresponded to and confirmed the findings from the first bioassay. Concentrations of 2.58 x 10^5, 2.58 x 10^6, and 2.58 x 10^7 conidia/ml caused highly significant (χ^2 = 33.29; df = 1; P < 0.0001, χ^2 = 122.95; df = 1; P < 0.0001, and χ^2 = 139.16; df = 1; P < 0.0001, respectively) reductions in larval survival when compared to the control. Survival by larvae exposed to 2.58 x 10^4 conidia/ml or less, however, was not significantly different (χ^2 = 1.77; df = 1; P = 0.1829, χ^2 = 0.95; df = 1; P = 0.3305, and χ^2 = 0.00; df = 1; P = 0.9624, respectively) from untreated control larvae. The three lowest (2.58 x 10^2, 2.58 x 10^3, and 2.58 x 10^4 conidia/ml) concentrations of *M. anisopliae* failed to achieve 50% larval mortality during the 18-d evaluation period in either bioassay (Fig. 1.A. and Fig. 1.B.). Therefore, lethal time (LT_{50}) values were not estimable for those treatments. Larval mortality exceeded 50% for the three higher concentrations. This permitted calculation of quartile estimates of mortality via the LIFETEST procedure and thereby provided LT_{50} estimates for those concentrations (Table 4). Results for lethal time estimates from the two bioassays were consistent. For example, LT_{50} values for the 2.58 x 10^5, 2.58 x 10^6, and 2.58 x 10^7 conidia/ml concentrations were 16, 10, and 7 DAE, respectively, in Bioassay I, and 15, 9, and 7.5 DAE, respectively in Bioassay II. Both bioassays also demonstrated a rate response for the fungus. Larvae exposed to the high (2.58 x 10^7) concentration of *M. anisopliae* were killed in about one-half the time of those exposed to 2.58 x 10^5 conidia/ml. Early (0 to 3 DAE) larval mortality occurred in all treatments, irrespective of *M. anisopliae* presence or concentration, and was thus regarded as background mortality. This was confirmed by postmortem examinations of the cadavers that indicated only a small number (7.4 and 0% in Bioassays I and II, respectively) of those larvae dead within the first 3 d of the experiment were killed as a result of *M. anisopliae* infection. Non-infected larvae were flaccid, black

Table 3. Log-rank test for equality of *M. anisopliae* dosages with untreated controls for survival of third-instar SBRM larvae in two 18-day bioassays.

	Bioassay I			Bioassay II		
Dosage (conidia/ml)	**Total live larvae**†	χ^2	***P***‡	**Total live larvae**†	χ^2	***P***‡
2.58×10^7	0	130.78	<0.0001	0	139.16	<0.0001
2.58×10^6	0	135.72	<0.0001	4	122.95	<0.0001
2.58×10^5	31	43.99	<0.0001	38	33.29	<0.0001
2.58×10^4	59	7.90	0.0049	73	0.00	0.9624
2.58×10^3	63	4.68	0.0305	76	0.95	0.3305
2.58×10^2	73	0.00	0.9956	77	1.78	0.1829
Control	73	----	----	73	----	----

†Number of surviving larvae 18 d after exposure to treatment; n = 80 (eight replications of ten larvae per treatment).
‡ Comparison of dosage strata to the untreated control using the log-rank test (Fisher and Belle, 1993). Values are not considered significantly different where probability (*P*) exceeded 0.05.



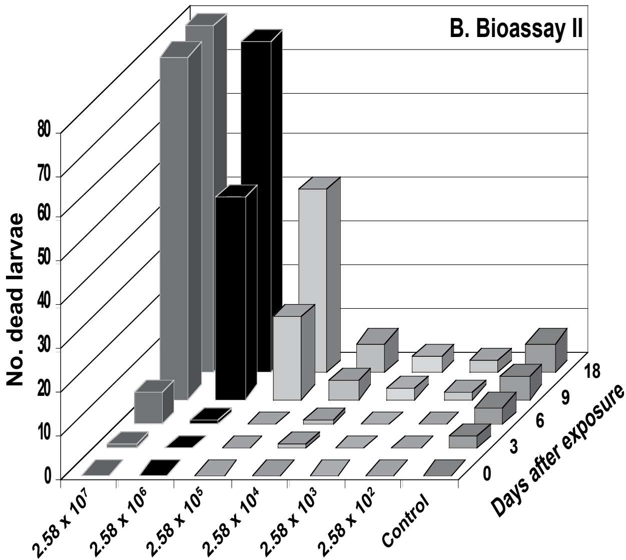


Fig. 1. Cumulative mortality of third-instar SBRM larvae over time after exposure to *M. anisopliae* conidia in Bioassays I (A), and II (B).

Table 4. Estimated LT_{50} values for third-instar SBRM larvae exposed to *M. anisopliae*.

Dosage (conidia/ml)	df	Bioassay I		Bioassay II	
		LT_{50} (days)	Confidence limits (95% CI)	LT_{50} (days)	Confidence limits (95% CI)
2.58×10^7	1	7.0	7.0, 8.0	7.5	7.0, 8.0
2.58×10^6	1	10.0	10.0, 11.0	9.0	9.0, 10.0
2.58×10^5	1	16.0	14.0, 18.0	15.0	11.0, 19.0
2.58×10^4	1	NE†		NE†	
2.58×10^3	1	NE†		NE†	
2.58×10^2	1	NE†		NE†	

† Nonestimable: larval survival exceeded 50% and, therefore, precluded calculation of lethal time (LT_{50}) value estimates (LIFETEST, SAS Institute, 1999).

in color, and did not display symptomology consistent with mycosis. Four larvae exposed to the high (2.58 x 10^7 conidia/ml) concentration in Bioassay I were killed within 4 DAE, and all displayed symptoms of an *M. anisopliae* infection. Larval mortality resulting from mycosis exhibited typical symptomology. Infected cadavers were distended and rigid, had a mottled rusty-brown coloring, and *M. anisopliae* hyphae were protruding from the exterior surface of their integument.

The LIFETEST procedure indicated a survival probability of over 80% for up to 6 DAE in larvae exposed to the highest (2.58 x 10^7 conidia/ml) concentration of the fungus in the two bioassays (Figs. 2.A. and 2.B.). At 7 DAE, likelihood of survival in larvae subjected to 2.58 x 10^7 conidia/ml dropped sharply to 37.5 and 50% in Bioassays I and II, respectively. In Bioassay I, larvae exposed to the highest concentration of *M. anisopliae* conidia had a 0% likelihood of survival at 10 DAE. The highest concentration of the fungus in Bioassay II resulted in a low (1.25%) survival probability for larvae at 9 DAE, and likelihood of survival was reduced to 0% by 12 DAE. Correspondingly, larval survival expectancy increased as *M. anisopliae* conidial concentrations decreased. Survival probability in larvae treated with the 2.58 x 10^7 conidia/ml rate remained above 70% for up to 8 DAE in both bioassays, although likelihood of those larvae remaining alive at 12 DAE dropped to 15.0 and 6.25% in Bioassays I and II, respectively. Bioassay I results suggested a survival probability of 0% by 18 DAE for larvae exposed to 2.58 x 10^6 conidia/ml. Similarly, the data from Bioassay II indicated that larvae exposed to the 2.58 x 10^6 concentration would have only a 5% likelihood of surviving from 14 through 18 DAE. The 2.58 x 10^5 conidia/ml concentration resulted in survival probabilities of 56.2 and 51.3% for larvae through 14 DAE according to Bioassays I and II, respectively, and larvae were 38.7 and 47.5% likely to survive for up to 18 DAE at that concentration. Additionally, larvae exposed to concentrations lower than 2.58 x 10^4 conidia/ml were more than 70% likely to survive for at least 18 days.

DISCUSSION

The results of this investigation demonstrate that *M. anisopliae* is highly virulent to SBRM larvae and that strains differ in virulence. The three highest concentrations (2.58 x 10^5, 2.58 x 10^6, and 2.58 x 10^7 conidia/ml) of the most virulent strain (ATCC 62176) of *M. anisopliae* resulted in significantly increased larval mortality when compared to controls. A rate response was evident in that exposure to

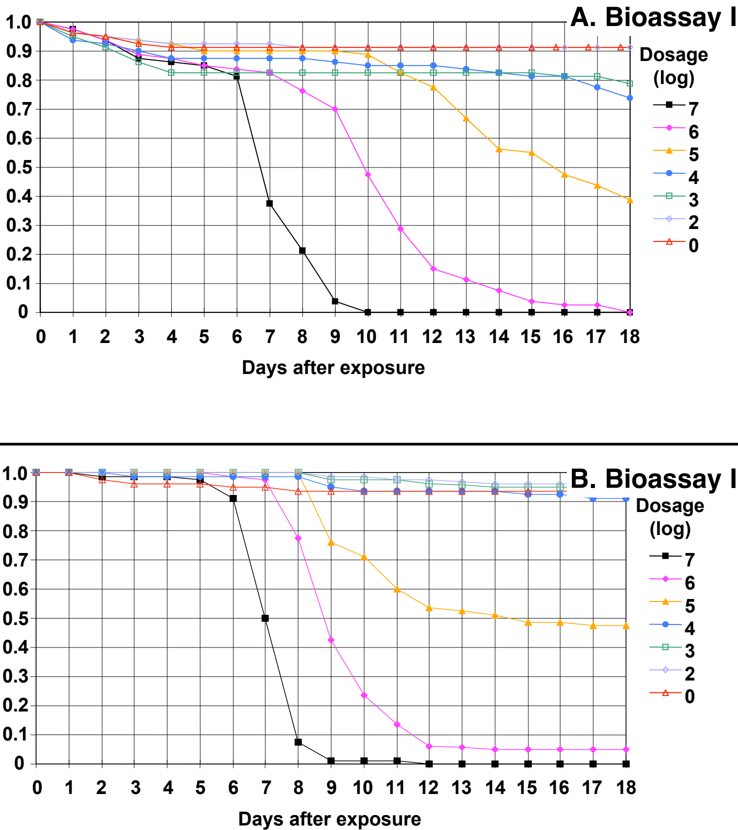


Fig. 2. Survival probability curve of third-instar SBRM larvae over time after exposure to *M. anisopliae* conidia in bioassays I (A), and II (B).

2.58 x 10^6 and 2.58 x 10^7 conidia/ml caused distinctly greater larval mortality than lower concentrations. The 2.58 x 10^2 conidia/ml treatment did not increase mortality of SBRM larvae in comparison to the control treatment. Additionally, concentrations of 2.58 x 10^3 and 2.58 x 10^4 conidia/ml were inconsistent in causing larval mortality.

Previous investigators have observed high levels of virulence in *M. anisopliae* against a diversity of arthropod pests. Zhioua *et al.* (1997) observed 100% mortality in *Ixodes scapularis* Say 14 d after being exposed to a concentration of 10^8 *M. anisopliae* conidia/ml. Chandler (1997) estimated the LC_{50} of *M. anisopliae* conidia on lettuce root aphids, *Pemphigus bursarius* (L.), to be 2.45 x 10^6 conidia/ml at 10 d post-inoculation.

According to Barson *et al.* (1994) the most pathogenic organism in the least time against larvae of the house fly, *Musca domestica* L., in intensive animal units was *M. anisopliae*. They observed 100% mortality in fly larvae in just 6 d after application of the conidia.

The ability to infect and kill first-instar larvae would increase the likelihood of early control and, consequently, effective protection from root injury. Preliminary trials in this investigation, as well as those of Smith and Eide (1995), demonstrated high levels of infection and mortality from *M. anisopliae* in first- and third-instar SBRM larvae. The primary bioassays in our research generated LT_{50} estimates ranging from 7 to 7.5 DAE for the high dosage (2.58 x 10^7 conidia/ml), with nearly 0% likelihood of SBRM larval survival by 10 DAE. Larvae exposed to 2.58 x 10^5 conidia/ml had about a 54% average likelihood of surviving up to 14 days and a 43% chance of surviving up to 18 DAE. Additionally, concentrations of 2.58 x 10^4 *M. anisopliae* conidia/ml or lower were not efficacious against SBRM larvae in this investigation.

These findings suggest that exposure to concentrations higher than 2.58 x 10^5 *M. anisopliae* conidia/ml may be required to provide sufficient protection against SBRM larvae. Only five *M. anisopliae* strains were examined in these bioassays. Thus, the potential exists for identifying additional strains with equal or superior virulence in future screening efforts. The relationship between *M. anisopliae* conidial concentration and virulence demonstrated in these trials will be an important consideration for development of *M. anisopliae*-based bioinsecticide formulations for SBRM management. Because these results are from a series of controlled laboratory bioassays, caution should be exercised in attempting to speculate on the potential efficacy of the fungus at controlling field populations of the insect. Findings from our laboratory studies provide no indication regarding the potential stability or persistence these strains would have in the environments they would likely be subjected to under field conditions. Our results do, however, demonstrate that conidial concentration is an important consideration for the use of entomopathogenic fungi, and should facilitate the design and implementation of field tests to determine appropriate application rates, placement methods, and timing to enhance the effectiveness of *M. anisopliae* as a bioinsecticidal organism for management of this major economic pest of sugarbeet.

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