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Production of microsclerotia of the fungal entomopathogen *Metarhizium anisopliae* and their potential for use as a biocontrol agent for soil-inhabiting insects☆

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ABSTRACT

Microsclerotia (MS), overwintering structures produced by many plant pathogenic fungi, have not been described for *Metarhizium anisopliae*. Three strains of *M. anisopliae* – F52, TM109, and MA1200 – formed MS in shake flask cultures using media with varying carbon concentrations and carbon-to-nitrogen (C:N) ratios. Under the conditions of this study, all strains produced MS, compact hyphal aggregates that become pigmented with culture age, in addition to more typical blastospores and mycelia. While all strains formed desiccation tolerant MS, highest concentrations (2.7–2.9 $\times$ 10^8 L^{-1} liquid medium) were produced in rich media with C:N ratios of 30:1 and 50:1 by strain F52. All three strains of *M. anisopliae* produced similar biomass concentrations when media and growth time were compared. Strain MA1200 produced higher concentrations of blastospores than the other two strains of *M. anisopliae* with highest blastospore concentrations (1.6 and 4.2 $\times$ 10^8 blastospores ml^{-1} on days 4 and 8, respectively) in media with the highest carbon and nitrogen concentrations. Microsclerotial preparations of *M. anisopliae* containing diatomaceous earth survived air-drying (to <5 % moisture) with no significant loss in viability. Rehydration and incubation of air-dried MS granules on water agar plates resulted in hyphal germination and sporogenic germination to produce high concentrations of conidia. Bioassays using soil-incorporated, air-dried MS preparations resulted in significant infection and mortality in larvae of the sugar beet root maggot, *Tetanops myopaeformis*. This is the first report of the production of sclerotial bodies by *M. anisopliae* and provides a novel approach for the control of soil-dwelling insects with this entomopathogenic fungus.

Introduction

For over 60 y, chemical pesticides have been the prevalent tool for insect, weed, and plant disease control. Interest in the use of biologically based pest control measures has been brought about by the development of pest resistance to many chemical pesticides coupled with public concerns about the adverse impact of widespread chemical use on human health, food

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safety, and the environment (Moore & Prior 1993; Auld & Morin 1995). In the late 19th century, Metchnikoff was the first to describe *Metarhizium anisopliae* ''green muscardine'' infections on the cereal cockchafer and to suggest the use of the microorganism as a biological control agent for insects (Zimmermann *et al.* 1995). Subsequent studies showed that an application of *M. anisopliae* conidia could be used to kill the cereal cockchafer and the sugar beet weevil *via* direct infection. Since that time, hundreds of fungi have been identified and are being developed as biological control agents for various insect and weed pests (Copping 2004).

The entomopathogenic fungus *M. anisopliae* has been reported to infect more than 100 insects including a number of soil-dwelling insects: *Reticulitermes* and *Coptotermes* spp., *Diabrotica* spp, *Otiorhynchus sulcatus*, *Diaprepes abbreviatus*, *Popillia japonica*, and *Rhizotrogus majalis* (Krueger *et al.* 1992; Schwarz 1995; Krueger & Roberts 1997; Bruck 2005). The choice of pursuing soil-dwelling insects as targets for biological control *versus* insects on the phylloplane is tempting. The deleterious effects of UV radiation and removal by rainfall are avoided in the soil. Temperatures are moderated in the soil, and soil moistures above the permanent wilting point of plants are well within the optimal range for survival and even growth of many microorganism, especially the fungi. Commercially important targets include root weevils, soil grubs, rootworms, wireworms, fruit flies, and root maggots (Bruck 2005; Chandler & Davidson 2005; Kabaluk *et al.* 2005; Krueger & Roberts 1997; Vanninen *et al.* 1999). Commercial interest in using *M. anisopliae* to control soil-dwelling insects has resulted in pest control formulations based on liquid culture-produced mycelial pellets, encapsulated fungal biomass, or solid substrate-produced conidia, either in aqueous suspension or on a nutritive or non-nutritive granular carrier (Andersche *et al.* 1995; Schwarz 1995; Storey *et al.* 1990).

Infection by this and related entomopathogenic ascomycetes involves the percutaneous penetration into an insect's body from conidia attached to and germinating on the insect's cuticular surface. As a preliminary step, the conidia must contact the insect cuticle in sufficient numbers to infect and kill the host. Thus, conidial concentration and distribution in the soil must be adequate so that insects moving through the soil acquire a fatal dose of conidia (Bruck 2005; Hu & St. Leger 2002; Jaronski 2007). While aqueous or dry suspensions of conidia can be mixed into soil, homogenous distribution through the soil matrix is difficult. A preferred, more practical formulation is a granular preparation, much like the many ''at-planting'' formulations of chemical insecticides. With this approach, numerous foci of large numbers of conidia are created. An insect crawling through the soil needs to encounter one or a few foci (granules) to acquire an infectious and fatal dose of conidia. In addition, granular formulations are also more suitable for standard ''at-planting'' application equipment.

For persistence in soil and decaying plant material, many plant pathogenic fungi produce sclerotia; i.e., compact hyphal aggregates, often melanized, that are highly resistant to desiccation. These propagules serve as the overwintering structure for fungi (Cooke 1983; Coley-Smith & Cooke 1971). Sclerotial bodies have also been shown to be produced by a limited number of entomopathogenic fungi including *Cordyceps*, *Hirsutella*, and *Synnematium* species (Evans & Samson 1982; Speare 1920). Microsclerotia (MS), 200–600 μm diameter sclerotial particles of fungal plant pathogens such as *Colletotrichum truncatum* and *Mycoleptodiscus terrestris* have been produced in high concentration in submerged liquid culture fermentation (Jackson & Schisler 1995; Shearer & Jackson 2003, 2006). For use as bioherbicides, MS of these pathogens of weedy plants have shown value as persistent, infective propagules in soil and in aquatic environments (Boyette *et al.* 2007; Shearer & Jackson 2006).

Our study initially focused on evaluating liquid culture production methods for stable propagules of various strains of the entomopathogenic fungus *M. anisopliae* for use in soil to control the sugar beet root maggot (*Tetanops myopaeformis*). We discovered that the *M. anisopliae* strains under study produced not only blastospores and mycelia, but also melanized MS. We evaluated different liquid culture nutritional environments, measured biomass accumulation, and blastospore and MS yields. Desiccation tolerance was evaluated by air-drying MS and by measuring their ability to germinate hyphally and/or sporogenically upon rehydration. The biocontrol efficacy of soil-incorporated microsclerotia of *M. anisopliae* was evaluated based on their ability to infect and to kill larval sugar beet root maggots in soil.

Materials and methods

Cultures and growth conditions

Three strains of *M. anisopliae* var. *anisopliae* were used in this study: a commercial strain, F52 (ATCC 90448, Novozyme Biologicals, Salem VA), MA1200 (ATCC 62176, originally isolated from *Heterodera glycines* egg cysts), and TM109 (ARSEF5520, originally isolated from *Delia floralis*). All these strains have been under evaluation as mycoinsecticides for the control of *T. myopaeformis* (sugar beet root maggot) and were passaged through larvae of that insect. Each re-isolation was from a single colony and respective strains were cultured twice on agar media before storage at −80 °C.

For liquid culture work, single spore isolates of each strain of *M. anisopliae* were grown on potato dextrose agar (PDA) for three weeks at room temperature. The sporulated agar plate was cut into 1 mm^2 agar plugs and stock cultures of these agar plugs stored in 10 % glycerol at −80 °C. Conidial inocula for liquid culture experiments were produced by inoculating PDA plates with a conidial suspension from the frozen stock cultures and growing these cultures at room temperature (~22 °C) for 2–3 wks. Conidial suspensions were obtained from sporulated PDA plates by rinsing plates with 10 mL of a solution of 0.04 % polyoxyethylene sorbitan mono-oleate (Tween® 80, Sigma). All liquid cultures were inoculated with a conidial suspension of *M. anisopliae* to provide an initial concentration of 5×10^6 conidia ml^{-1} in the culture broth.

The six liquid media tested were composed of a basal salts medium supplemented with trace metals and vitamins (Jackson *et al.* 1997) and various combinations of glucose and acid-hydrolyzed casein (Casamino acids®, Difco Laboratories, Detroit, MI, USA). The defined basal salts solution used in all

liquid cultures contained per litre of de-ionized water: KH_2PO_4, 4.0 g; $CaCl_2 \cdot 2H_2O$, 0.8 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; $CoCl_2 \cdot 6H_2O$, 37 mg; $MnSO_4 \cdot H_2O$, 16 mg; $ZnSO_4 \cdot 7H_2O$, 14 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thiotic acid, 500 μg each; and folic acid, biotin, vitamin B_{12}, 50 μg each. In Table 1, the amounts of glucose and acid-hydrolyzed casein and the corresponding carbon concentration and carbon-to-nitrogen (C:N) are given for each medium tested. Carbon concentration and C:N ratio calculations were based on 40 % carbon in glucose and 53 % carbon, 8 % nitrogen in acid-hydrolyzed casein.

All cultures were grown as 100 ml cultures in 250 ml baffled, Erlenmeyer flasks at 28 °C and 300 rev. min^{-1} in a rotary shaker incubator (INNOVA 4000, New Brunswick Scientific, Edison, NJ, USA). Flasks were hand-shaken frequently to minimize mycelial growth on the flask wall. At two, four, and eight days post-inoculation, samples were taken to measure biomass accumulation, blastospore concentration, and MS concentration. For each experiment, duplicate samples were made from each flask on each sampling date, and three replicate flasks for each media were used. All experiments were repeated at least twice.

Analysis of biomass, MS, and blastospore production

For biomass accumulation measurements, one ml of whole culture broth was collected from culture flasks and the biomass was separated from the spent medium by vacuum filtration onto pre-weighed filter disks (Whatman GF/A, Maidstone, England). Dry weight accumulation was determined by drying the biomass and filter disk at 60 °C to a constant weight prior to measurement. Blastospore concentrations were determined microscopically using a hemocytometer.

For MS concentration measurements, 100 μl of culture broth was placed on a glass slide and overlaid with a large, 24 × 50 mm coverslip (Fisher Scientific, USA). All the MS on the slide were counted microscopically. Only discrete, compact hyphal aggregates larger than 50 μm in diameter were counted as MS. Culture broth was diluted as appropriate for ease in counting MS. During culture broth sampling and dilution, MS suspensions were constantly vortexed to ensure homogeneity. Wide-bore, 10 mL pipettes (Falcon #357504, Becton Dickinson, Franklin Lakes, NJ, USA) or wide-bore, 1000 μL plastic pipette tips (tip removed with razor blade to create wider opening) were used for all sampling. A hemocytometer could not be used for counting MS due to the large size (>300 μm) of some MS. All microscopic analyses were conducted using an Olympus BH-2 microscope with Nomarski optics.

Table 1 – Carbon concentration (g l^{-1}) and carbon-to-nitrogen (C:N) ratio in liquid media used to assess the growth, propagule formation, and yields for different strains of *M. anisopliae*

C g l^{-1}	C:N	Glucose (g l^{-1})	Casamino acids (g l^{-1})
8	10:1	10.0	10.0
8	30:1	16.6	3.4
8	50:1	18.0	2.0
36	10:1	45.0	45.0
36	30:1	75.0	15.0
36	50:1	81.0	9.0

After growing the *M. anisopliae* cultures for eight days, diatomaceous earth [DE (HYFLO®, Celite Corp., Lompoc, CA, USA)] was added to the combined fungal biomass of the three flasks in each treatment at a concentration of 5 g DE 100 ml^{-1} culture broth. The MS–DE mixture was vacuum-filtered in a Buchner funnel using Whatman No. 54 filter paper. The filter cake was broken up by pulsing in a blender (Mini Prep® Plus, Cuisinart, Stamford, CT, USA), layered in shallow aluminum trays, and air-dried overnight in the air flow within a biological containment hood. The moisture content of the MS–DE preparation was determined with a moisture analyzer (Mark II, Denver Instruments, Arvada, CO, USA). When *M. anisopliae* formulations dried to a moisture content ≤5 %, they were vacuum packed in synthetic polyethylene bags with a vacuum packer (Multivac C 100, Sepp Haggenmüller, Wolfertschwenden, Germany) and stored at 4 °C. For bioassays against the sugar beet root maggot in soil, dry MS–DE granules 0.6–1.7 mm were used. These granules were obtained by collecting granules that passed a 12 mesh screen and were caught on a 30 mesh screen. All experiments were repeated at least twice.

Upon rehydration, MS–DE preparations of *M. anisopliae* germinated hyphally (germ tube formation) and sporogenically (production of conidial masses on the surface of the MS). Microsclerotial viability (hyphal germination) and conidial production (sporogenic germination) were determined for air-dried, MS–DE preparations by sprinkling 25 mg of the unsieved MS–DE formulation onto the surface of a water agar plate. Two water agar plates were used for each treatment. Following a 24 h incubation period at 28 °C, 100 MS–DE granules on each plate were examined with a stereo microscope (Olympus, model SZH10, Planapo 1× objective) for hyphal germination as a measure of viability. To enumerate conidial production, the incubation of the water agar plates was continued for a total of eight days at 28 °C. Each water agar plate was then flooded with 5 ml of sterile water and the conidia were dislodged from the MS–DE granules using a sterile loop. After the conidia were dislodged, the available liquid was pipetted from each plate, and the liquid volume measured. The concentration of conidia in the pipetted liquid was measured microscopically using a hemocytometer and the total number of conidia per plate calculated. To determine the number of conidia of *M. anisopliae* produced g^{-1} of air-dried MS–DE preparation, the number of conidia harvested per plate was divided by the weight of the dried MS–DE preparation added to each water agar plate (0.025 g).

Assessment of biological efficacy

A bioassay was performed to evaluate the entomopathogenic efficacy of MS–DE granules of *M. anisopliae* strains F52, MA1200, and TM109 produced in Medium 5 (Table 1) against SBRM larvae. The bioassays were conducted using a clay soil from a sugar beet field in Sidney, MT, USA (sand:silt:clay ratio of 11:33:56). The MS–DE granules (0.6–1.7 mm), were incorporated into dry soil at the rate of 116 mg 100 g^{-1} soil. This rate was based on a theoretical concentration of MS–DE granules in a 3-cm wide zone just above seed level along the row if

MS–DE granules were applied at the rate of 16.8 kg ha^{-1} using a ''modified in-furrow'' application (Boetel *et al.* 2006) with standard farm equipment. After MS–DE granule incorporation, the soils were moistened with reverse-osmosis water to an end point of 15 % Field Capacity (previously determined), and the water potentials were determined with an Aqualab® moisture meter (Decagon Products, Pullman WA). The resulting soil moistures (0.982–0.983 A_w equivalent to a matric potential of −2.32 to −2.47 MPa) were sufficient for fungal outgrowth and sporulation. An untreated control soil was prepared simply by wetting an additional aliquot of soil, without MS–DE granules, with the same amount of water. Each treated and control soil was then dispensed equally into three 60cc Solo® plastic condiment cups with lids. The cups were sealed and placed on a layer of water-moistened paper towel (to maintain humidity) in a large, lidded plastic container, and incubated at 25 °C. After 1 week, the soils were infested with 10, third-instar SBRM larvae cup^{-1}. These larvae were field-collected in diapause, non-feeding yet still motile, and stored in moist sterile sand at 3–4 °C for several months prior to use. Larval mortality was determined weekly for two weeks. Each treatment was replicated three times.

Statistical analysis

Mean values for biomass, blastospore, and MS yields from culture growth and for conidial yields from air-dried MS–DE granules were analyzed by ANOVA with Tukey–Kramer mean separation tests ($P \leq 0.05$) using JMP® (SAS, Cary, NC, USA). ANOVA was restricted to culture growth data for individual strains of *M. anisopliae* and to data for each sampling time. Comparison of data on conidial yield was restricted to each strain of *M. anisopliae*. For statistical analyses of bioassay data, all mortality data were adjusted for control mortality, when necessary, by application of Abbott's correction (Abbott 1925) and then subjected to angular transformation before further analysis (Zar 1998). Data were then subjected to ANOVA and significantly different means identified with Tukey's HSD test when significant treatment effects were identified (Zar 1998).

Results

Biomass, blastospore, and MS yields and morphology

Biomass accumulation by the three strains of *M. anisopliae* followed the predicted pattern in which fungus grown in media that contained 8 g l^{-1} carbon produced lower biomass concentrations when compared to those grown in media with 36 g l^{-1} carbon (Table 2). When comparing cultures grown in media with differing C:N ratios, biomass accumulation was not affected by nitrogen content for those grown in media with a carbon concentration of 8 g l^{-1} suggesting that these media were carbon-limited. For all strains of *M. anisopliae* grown in media containing 36 g l^{-1} carbon, biomass accumulation was significantly higher after 4 and 8 d growth for cultures grown in lower C:N ratio (higher nitrogen content) media, suggesting that growth was limited by nitrogen (Table 2).

Table 2 – Comparison of biomass production by strains of *Metarhizium anisopliae* after 2, 4, and 8 d growth at 28 °C and 300 rev. min^{-1} in various liquid culture media

Metarhizium anisopliae Strain (Medium)		Carbon (g l^{-1})	Carbon-to-Nitrogen Ratio (C:N)	Biomass (mg ml^{-1})		
				Day 2	Day 4	Day 8
MA1200	(1)	8	10:1	2.5a[a]	5.5d	3.8d
	(2)	8	30:1	2.5a	4.5d	5.0d
	(3)	8	50:1	1.5a	4.5d	4.4d
	(4)	36	10:1	2.7a	22.2a	26.6a
	(5)	36	30:1	3.5a	14.8b	18.9b
	(6)	36	50:1	2.1a	10.0c	13.3c
F52	(1)	8	10:1	1.1c	8.2d	3.2c
	(2)	8	30:1	3.4a	5.4e	5.5c
	(3)	8	50:1	1.7c	4.0e	3.5c
	(4)	36	10:1	2.0b,c	22.6a	33.0a
	(5)	36	30:1	3.8a	19.7b	21.6b
	(6)	36	50:1	3.2a,b	11.8c	18.3b
TM109	(1)	8	10:1	0.8c	5.2c	4.5d
	(2)	8	30:1	0.7c	3.1c,d	3.8d
	(3)	8	50:1	0.6c	2.2d	4.0d
	(4)	36	10:1	1.0b,c	12.7a,b	30.5a
	(5)	36	30:1	1.7a	13.7a	24.0b
	(6)	36	50:1	1.5a,b	10.6b	14.0c

a For each strain and each sample day, mean values followed by the different letters are significantly different using Tukey–Kramer HSD.

Blastospore formation was highest in *M. anisopliae* strain MA1200 in all six media and at all growth times tested (Table 3). On days 4 and 8 post-inoculation, the highest blastospore concentrations (1.6 and 4.3 × 10^8 blastospores ml^{-1}, respectively) were measured in cultures of *M. anisopliae* strain MA1200 in carbon-rich media with the highest nitrogen content (Table 3). Strains F52 and TM109 produced relatively low blastospore concentrations in all media tested.

The formation, yield, and melanization of MS by *M. anisopliae* were strain- and medium-dependent (Table 4). While MS formation could be seen in all media and with all three strains of *M. anisopliae*, highest MS concentrations were measured in cultures of strain F52 on day 8 post-inoculation in carbon-rich media with C:N ratios of 30:1 and 50:1, 2.7 and 2.9 × 10^5 MS ml^{-1}, respectively (Table 4). Microsclerotia formed by *M. anisopliae* strain F52 in media with a C:N ratio of 50:1 were generally more melanized compared to MS formed in media with lower C:N ratios. The morphogenesis of microsclerotia in liquid culture by cultures of *M. anisopliae* strain F52 generally followed a pattern whereby conidia germinated within 24 h to form a homogenous mycelial mass which increased in density for 2 d (Fig 1). After 2 d growth, mycelia began to aggregate to form sclerotial bodies which became increasingly compact and melanized as culturing continued. Hyphal diameter increased and cells became more irregular in shape during sclerotia morphogenesis (data not shown). Following 8 d of liquid culture incubation, culture viscosity decreased as sclerotia formed, and an abundance of highly melanized microsclerotia was observed, particularly in rich media low in nitrogen [Fig 1 (C:N ratios 30:1 and 50:1)]. Microsclerotia formed by cultures of *M. anisopliae* varied in size but were generally 50–300 μm in diameter.

Table 3 – Comparison of blastospore production by strains of *Metarhizium anisopliae* after 2, 4, and 8 d growth at 28 °C and 300 rev. min^{-1} in various liquid culture media

Metarhizium anisopliae Strain (Medium)		Carbon (g l^{-1})	Carbon-to-Nitrogen Ratio (C:N)	Blastospores (blastospores ml^{-1}) × 10^5		
				Day 2	Day 4	Day 8
MA1200	(1)	8	10:1	26.8a,b[a]	0.9c	61.3c
	(2)	8	30:1	2.8b	6.5c	5.8c
	(3)	8	50:1	0.5b	4.5c	7.7c
	(4)	36	10:1	149.3a	1,616.7a	4,266.7a
	(5)	36	30:1	119.7a,b	515.0b	1,600.0b
	(6)	36	50:1	94.3a,b	180.8c	323.3c
F52	(1)	8	10:1	0.0b	1.3b	6.1b
	(2)	8	30:1	0.0b	0.9b	0.9b
	(3)	8	50:1	0.0b	0.1b	0.8b
	(4)	36	10:1	0.3a,b	3.2b	7.3b
	(5)	36	30:1	0.2a,b	1.3b	70.8a
	(6)	36	50:1	0.4a	26.8a	17.1b
TM109	(1)	8	10:1	0.8a	0.0a	2.7b
	(2)	8	30:1	0.0a	0.0a	1.2b
	(3)	8	50:1	0.0a	0.0a	0.8b
	(4)	36	10:1	0.0a	14.5a	43.0a,b
	(5)	36	30:1	4.80a	12.1a	68.9a
	(6)	36	50:1	0.3a	7.9a	32.6a,b

a For each strain and each sample day, mean values followed by the different letters are significantly different using Tukey–Kramer HSD.

Table 4 – Comparison of microsclerotia (MS) production by strains of *Metarhizium anisopliae* after 2, 4, and 8 d growth at 28 °C and 300 rev. min^{-1} in various liquid culture media

Metarhizium anisopliae Strain (Medium)		Carbon (g l^{-1})	Carbon-to-Nitrogen Ratio (C:N)	Microsclerotia (MS ml^{-1})[a] × 10^4		
				Day 2	Day 4	Day 8
MA1200	(1)	8	10:1	3.1a	10.6a,b	15.3a
	(2)	8	30:1	0.5b	2.7c	6.4b,c
	(3)	8	50:1	0.5b	2.4c	4.9c
	(4)	36	10:1	1.7a,b	5.7b,c	12.0a,b
	(5)	36	30:1	2.3a,b	7.7b,c	9.3a,b,c
	(6)	36	50:1	2.1a,b	15.3a	14.7a
F52	(1)	8	10:1	0.8b	10.5a	5.3b
	(2)	8	30:1	1.7b	6.4a,b	11.7b
	(3)	8	50:1	2.3b	5.0b	8.5b
	(4)	36	10:1	8.0a	10.3a	9.3b
	(5)	36	30:1	7.9a	11.0a	27.0a
	(6)	36	50:1	9.5a	6.8a,b	29.0a
TM109	(1)	8	10:1	0.0a	2.0a,b	1.2a
	(2)	8	30:1	0.7a	1.2b	1.8a
	(3)	8	50:1	0.6a	0.2b	1.0a
	(4)	36	10:1	0.2a	1.9a,b	5.3a
	(5)	36	30:1	0.5a	2.0a,b	3.7a
	(6)	36	50:1	0.3a	4.9a	3.9a

a For each strain and each sample day, mean values in columns followed by the different letters are significantly different using Tukey–Kramer HSD.

Air-dried, MS–DE preparations from 8-d old cultures of all the strains of *M. anisopliae* tested survived the drying process with no significant loss in viability except those cultures grown in a carbon-poor medium with low nitrogen content [8 g l^{-1} carbon, 50:1 C:N ratio (Table 5)]. Viability was determined microscopically as hyphal germination after 24 h incubation (Fig 2). Conidia production by air-dried, MS–DE preparations for all strains of *M. anisopliae*, regardless of media, was greater than 1×10^8 conidia g^{-1} dried formulate (Table 5). In general, dried MS–DE preparations from carbon-rich media (Media 4, 5, and 6) produced higher numbers of conidia than MS–DE preparations derived from media with lower carbon concentrations. In addition, cultures of *M. anisopliae* grown in carbon-rich media produced more biomass (Table 2), which in turn resulted in higher mass yields for those dried MS–DE preparations (data not shown).

Assessment of biological efficacy

Seven days after SBRM larvae were introduced into the treated soils, mean cumulative mortalities ranged from 23.3 to 53.3 % (Table 6); control mortality was 0 %. Overall, there was a tendency to higher SBRM mortality by strain F52 than the other two strains of *M. anisopliae*, although the mortality was not significantly different. By 14 d after larvae had been introduced into the MS-treated soils, all *M. anisopliae* strains produced cumulative larval mortalities of 90–100 %. After 14 d incubation, there were no significant differences in SBRM larvae mortality among the strains of *M. anisopliae* tested.

Discussion

The liquid culture production of conidia, blastospores, and mycelia of *M. anisopliae* has been evaluated in numerous studies (Andersche *et al.* 1995; Booth *et al.* 2000; Fargues *et al.* 2001; Jenkins & Prior 1993; Krueger *et al.* 1992; Kleespies & Zimmermann 1998; Pereira & Roberts 1990; Vega *et al.* 2003). In this study, we describe the liquid culture production of microsclerotia (MS) by the entomopathogenic fungus *M. anisopliae*. These undifferentiated, melanized, compact hyphal aggregates have been reported as overwintering structures for many plant pathogenic fungi and a limited number of entomopathogenic fungi (Coley-Smith & Cooke 1971; Speare 1920; Evans & Samson 1982). Sclerotia (including small sclerotia, ''microsclerotia'') persist in soils and decaying plant material and often serve as the source of infection for emerging susceptible plant seedlings in subsequent growing seasons. Our previous studies have shown that MS of the fungal plant pathogens *C. truncatum* and *M. terrestris* can be produced in submerged liquid culture (Jackson & Schisler 1995; Shearer & Jackson 2006). The finding that strains of the entomopathogen *M. anisopliae* produce MS in liquid culture was unexpected but may be related to the soil-inhabiting nature of *M. anisopliae* (Zimmermann 2007; Klingen *et al.* 2002). It is interesting to note that the MS produced by *M. anisopliae* strain F52 in liquid culture were considerably smaller (50–300 μm in diameter) and were produced in much higher concentration (~50×) than MS produced by *C. truncatum* or *M. terrestris* (Jackson & Schisler 1995; Shearer & Jackson 2006). These characteristics may be beneficial for *M. anisopliae*'s development as a bioinsecticide in terms

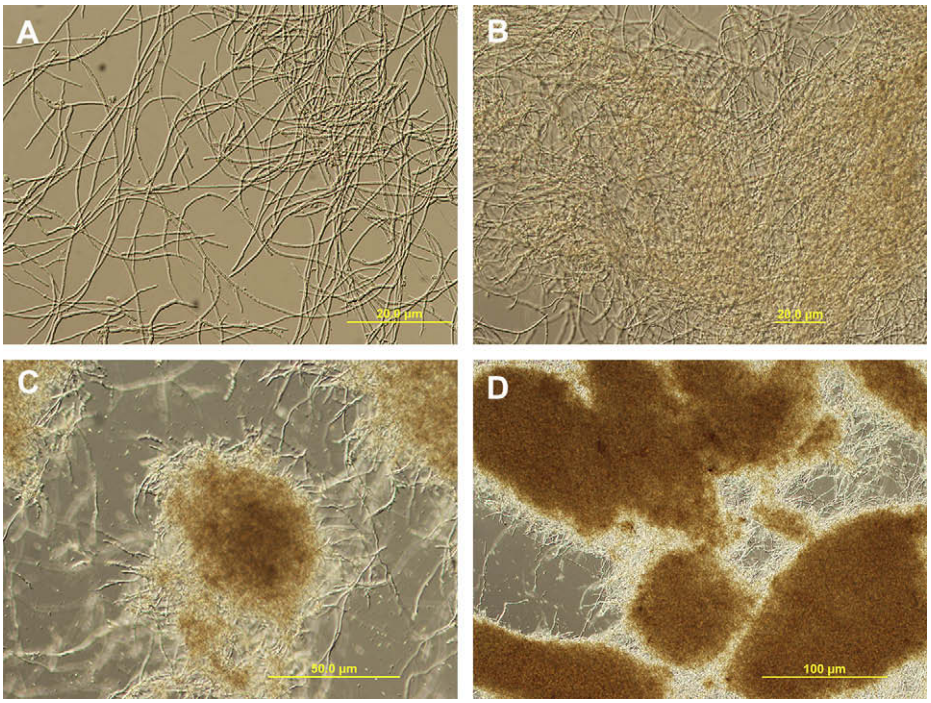

Fig 1 – Development of sclerotia in aerated liquid cultures of *Metarhizium anisopliae* grown in a rotary shaker incubator, 300 rpm and 28 °C. Cultures were grown in rich media (36 g carbon l^{-1}) with a C:N ratio of 30:1. Conidial inocula germinated to produce a homogenous hyphal mass (A) after 24 h growth. As cultures aged, hyphae aggregated to form sclerotial bodies (B) Day 2, (C) Day 4, (D) Day 7.

Table 5 – Desiccation tolerance and conidia production capability of air-dried microsclerotia (MS) preparations of *Metarhizium anisopliae*. Dried MS preparations (25 mg) were sprinkled onto water agar plates and incubated at 28 °C

Metarhizium anisopliae Strain (Medium)		Carbon ($g l^{-1}$)	Carbon-to-Nitrogen	Hyphal Germination[a] (% granules)	Sporogenic Germination[b] (conidia g^{-1} dried MS preparation) × 10^7
MA1200	(1)	8	10:1	100a[c]	24.0b
	(2)	8	30:1	100a	29.5b
	(3)	8	50:1	87b	28.0b
	(4)	36	10:1	100a	42.3b
	(5)	36	30:1	100a	97.5a
	(6)	36	50:1	99a	21.5b
F52	(1)	8	10:1	99a	53.5b
	(2)	8	30:1	96a	64.5b
	(3)	8	50:1	99a	46.5b
	(4)	36	10:1	100a	114.5a
	(5)	36	30:1	100a	82.5a,b
	(6)	36	50:1	97a	82.3a,b
TM109	(1)	8	10:1	85a,b	18.1b
	(2)	8	30:1	93a,b	15.4b
	(3)	8	50:1	46b	16.0b
	(4)	36	10:1	100a	81.3a,b
	(5)	36	30:1	100a	94.3a
	(6)	36	50:1	100a	63.8a,b

a After 24 h incubation, germ tube extension from MS granules was microscopically evaluated.
b Conidia production by dried MS formulations was measured after 8 d incubation.
c For each strain and each sample day, mean values in columns followed by the different letters are significantly different using Tukey–Kramer HSD.

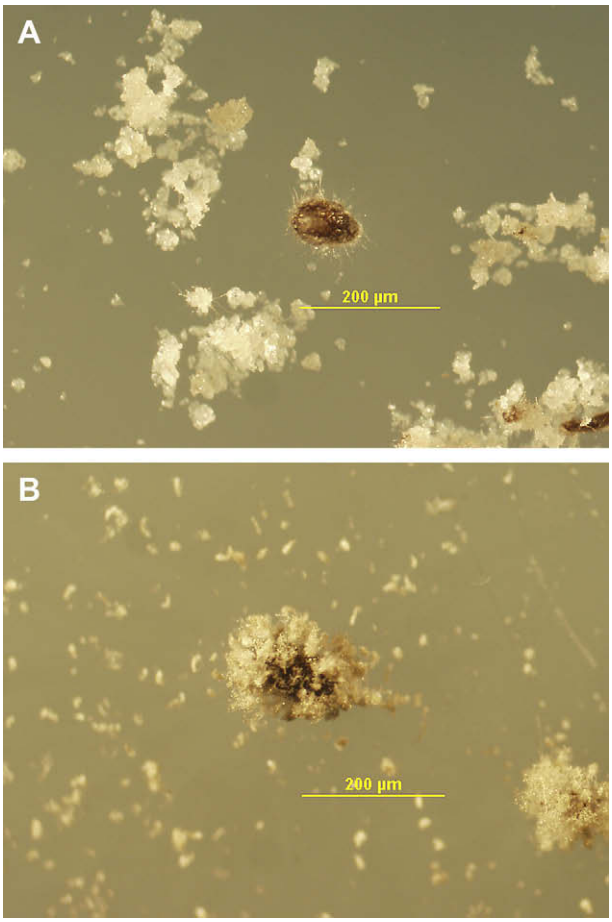

Fig 2 – Hyphal (A) and sporogenic (B) germination of air-dried microsclerotia of *M. anisopliae* F52 following 24 and 192 h incubation, respectively, on water agar at 28 °C.

Table 6 – Efficacy of microsclerotial granules of three strains of *Metarhizium anisopliae* produced in Medium 5 against third-instar sugar beet root maggot (SBRM) larvae (*Tetanops myopaeformis*) in a laboratory bioassay using non-sterile clay soil

	Larval SBRM Mortality (% mean cumulative mortalities)	
Metarhizium strain	7 d after treatment	14 d after treatment
MA1200	23.3 (5.77)a[a]	90 (10.0)a
F52	53.3 (15.28)a	100 (0)a
TM109	36.7 (5.77)a	100 (0)a
Untreated Control	0.0 (0.0)b	0.0 (0.0)b

a Mean values in columns followed by the different letters are significantly different (Tukey's HSD test, $P = 0.05$).

of lower production costs and better distribution in soil for contact with foraging insect pests.

Reports pertaining to the environmental association of strains of *M. anisopliae* with soil type and not necessarily insect host suggest that the persistence of this fungus in these soils may be unrelated to the insect host (Bidochka *et al.* 2001; Quesada-Moraga *et al.* 2007). The higher colony-forming unit counts of *M. anisopliae* found in association with plant roots and root exudates suggest that these fungi may be capable of survival in soils without an insect host (Hu & St. Leger 2002). The ability of *M. anisopliae* strains to produce an overwintering propagule such as a sclerotium would certainly be an ecological advantage. The general thought is that *M. anisopliae* persists in the soil in the form of conidia that are prevented from germinating by microbial-generated fungistasis (Jaronski 2007). The ability of MS of *M. anisopliae* to germinate sporogenically to produce conidia confounds this assumption. This is particularly true given the fact that most studies concerning the presence of *M. anisopliae* in soil have been conducted by baiting with susceptible insects or by serial soil dilution plating onto *Metarhizium*-selective media to identify colony-forming units of *M. anisopliae* (Meyling & Eilenberg 2006; Klingen *et al.* 2002; Quesada-Moraga *et al.* 2007; Keller *et al.* 2003: Hu & St. Leger 2002). Microscopic studies of soil are needed to determine the presence or absence of MS structures of *M. anisopliae* and, if present, their ability to produce conidia by sporogenic germination in soil, root exudates, or decaying insect cadavers.

Previous studies on the liquid culture growth and propagule formation by *M. anisopliae* showed that the relative degree of blastospore or conidium production was strain-dependent (Vega *et al.* 2003). Our studies demonstrated this variability in propagule formation among *M. anisopliae* strains with strain MA1200 producing relatively high concentrations of blastospores under these conditions while strain F52 produced the highest concentrations of MS (Tables 3 and 4). For commercial use, the concentration of blastospores produced by *M. anisopliae* strain MA1200 was low ($1–2 \times 10^8$ range ml^{-1}) and required a 4-d fermentation time. In comparison, the entomopathogenic fungus *Paecilomyces fumosoroseus* (Wize)[renamed *Isaria fumosorosea*] can produce more than 1×10^9 blastospores ml^{-1} in submerged culture in 2 d (Jackson *et al.* 2003). Furthermore, it is unlikely that vegetative blastospores would persist in soil to provide long-term benefit in controlling soil-dwelling insects.

In contrast, yields of MS by *M. anisopliae* strain F52, $\sim 3 \times 10^8$ l^{-1} after 4 d growth, are superior to MS yields for submerged cultures of the plant pathogenic fungi *C. truncatum* and *M. terrestris* (Jackson & Schisler 1995; Shearer & Jackson 2006). Our studies showed that the medium highest in carbon and with low to intermediate levels of nitrogen produced the most rapid melanization of MS of *M. anisopliae*. Research with *C. truncatum* suggested that the formation and melanization of MS produced in liquid culture were associated with nitrogen and carbon depletion, respectively (Jackson & Schisler 1995). Additional studies with *M. anisopliae* cultures are needed to evaluate the impact of nitrogen and carbon depletion on MS formation, melanization, stability in soil, and insect biocontrol efficacy. While the results of our laboratory bioassays showed that significant and complete control of third-instar SBRM larvae was obtained with MS–DE granules in clay soil, their practical application to SBRM control will require additional bioassays to assess the impact of soil type and soil moisture level on biocontrol efficacy. Laboratory and field experiments using MS-containing granules to

control the SBRM have demonstrated the utility of using these granules as a control tool for this insect pest (Jaronski & Jackson 2008).

The liquid culture production, desiccation tolerance, and sporogenic germination of MS of *M. anisopliae* suggest that these propagules may be well suited for control of soil-dwelling insects. The ability of *M. anisopliae* to produce sclerotial bodies may also provide insight into the soil-dwelling habit of this fungus. As with other fungi that produce sclerotia under the controlled conditions inherent to liquid culturing, a model is now available for discerning the processes involved in the differentiation of *M. anisopliae* hyphae to produce sclerotia under gnotobiotic conditions. Understanding and developing this biocontrol approach for soil-dwelling insects should provide MS preparations of *M. anisopliae* with distinct advantages over the use of spore- or mycelium-based biocontrol products.

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