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Comparative growth kinetics and virulence of four different isolates of entomopathogenic fungi in the house fly (*Musca domestica* L.)

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ABSTRACT

Virulence (speed of kill) of a fungal entomopathogen against a particular host insect depends on biological properties of the specific isolate-host combination, together with factors such as fungal dose. How these intrinsic and extrinsic factors affect the actual pattern and extent of fungal growth *in vivo* is poorly understood. In this study we exposed adult house flies (*Musca domestica* L.) to surfaces treated with high and low doses of *Beauveria bassiana* (isolates BbGHA and Bb5344), *Metarhizium anisopliae* (strain MaF52) and *M. anisopliae* var. *acridum* (isolate Ma189) and used quantitative real-time PCR with species-specific primers to examine the relationship between fungal growth kinetics and virulence. At the highest dose, all fungal isolates killed flies significantly faster than controls, with BbGHA, Bb5344 and MaF52 roughly equivalent in virulence (median survival time (±SE) = 5.0 ± 0.10, 5.0 ± 0.08 and 5.0 ± 0.12 days, respectively) and Ma189 killing more slowly (MST = 8.0 ± 0.20 days). At the lower dose, effective virulence was reduced and only flies exposed to isolates BbGHA and Bb5344 died significantly faster than controls (MST = 12 ± 1.36, 15 ± 0.64, 18 ± 0.86 and 21.0 ± 0.0 days for BbGHA, Bb5344, MaF52 and Ma189, respectively). Real-time PCR assays revealed that flies exposed to surfaces treated with the high dose of spores had greater spore pickup than flies exposed to the low dose for each isolate. After pickup, a general pattern emerged for all isolates in which there was a significant reduction of recovered fungal DNA 48 h after exposure followed by a brief recovery phase, a stable period of little net change in fungal sequence counts, and then a dramatic increase in sequence counts of up to three orders of magnitude around the time of host death. However, while the patterns of growth were similar, there were quantitative differences such that higher final sequence counts were recovered in insects infected with the most lethal isolates and with the higher dose. These results suggest that variation in virulence between isolates, species and doses is determined more by quantitative rather than qualitative differences in fungal growth kinetics.

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1. Introduction

Individual isolates of entomopathogenic fungi can vary greatly in their virulence to a given insect host but what factors contribute to this variation often remain unclear. Kershaw et al. (1999) hypothesized that differences in isolate virulence can be attributed to the position the phenotype of a particular isolate occupies on a continuum between two main strategies; an isolate may produce a large amount of toxins or may focus their energy into vegetative growth. Both *Beauveria bassiana* and *Metarhizium anisopliae* have been shown to produce metabolites within insect hosts with effects ranging from paralysis to immunosuppression (Gillespie and Claydon, 1989; Hajek and St. Leger, 1994; Hung and Boucias, 1992; Kershaw et al., 1999). While modern molecular techniques have facilitated progress in understanding the biochemistry of fungal metabolites and how they contribute to virulence in insects (Kershaw et al., 1999; Mazet and Boucias, 1996), relatively few studies have attempted to relate virulence to differences in growth kinetics between fungal isolates. That is, in addition to the toxic effects of metabolites, fungi could kill insects via vegetative growth, with death occurring when fungal hyphae penetrate vital organs, block the flow of hemolymph, or sap the nutritive resources from the host (Clarkson and Charnley, 1996).

To date, many studies evaluating growth kinetics have only considered growth *in vitro* (Fargues et al., 1997; Fargues et al., 1992). However, these methods do not subject the fungus to the same nutritional environment or immune pressure present in an insect host and, therefore, may not be a good representation of natural growth kinetics. Accurate, quantitative *in vivo* measurements of fungal growth are difficult to obtain; most often this

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has been done using microscopic methods to examine the concentration of blastospores and/or hyphal fragments in the hemolymph of infected insects (Ouedraogo et al., 1997; Pendland et al., 1993). Unfortunately, these techniques may yield inaccurate results in both early and later stages of infection, as fungal infection may not be evident in the hemolymph for up to 3 days following exposure to spores and blastospores and hyphal fragments may be too numerous to count during later stages of infection (Ouedraogo et al., 2003).

Recently, several researchers have developed quantitative PCR (qt-PCR) protocols to quantify the growth of pathogenic fungi in plants (Boyle et al., 2005; Gachon and Saindrenan, 2004) and insects (Bell et al., 2009), providing a highly sensitive tool to model the dynamics of fungal infection from the point of exposure to the death of the host. Using this application, it is possible to evaluate differences in growth kinetics between fungal isolates and, when paired with bioassays, determine if growth rates can be used to explain the virulence of a particular isolate.

The aim of the current study was to compare the differences in virulence and *in vivo* growth kinetics of four different isolates of entomopathogenic fungi against the house fly (*Musca domestica* L.). Understanding the factors that contribute to the virulence of fungal entomopathogens is important for understanding the ecological and evolutionary basics of insect–pathogen interactions.

2. Materials and methods

2.1. House flies

The house flies used in this study were derived from the Cornell University CS strain, an insecticide susceptible strain. The flies were reared and maintained in an environmental chamber under standard insectary conditions at 27 °C with a 12:12 light: dark photoperiod. Fly eggs were collected by placing a small cup (50 g) of larval diet (wheat bran, Manno-Pro™ calf protein supplement, baker's yeast and water) into fly stock cages for 2 h. Roughly 300 mg of eggs were placed into 1 l of larval medium contained in a 1.5 l plastic container and larval development was monitored daily. Upon pupation, pupae were collected by gently shaking them from the surface of the diet into plastic (~50 ml) soufflé cups. Cups containing pupae were then placed in clean stock cages until eclosion ceased. Adult flies were provided access to food, consisting of a 1:1 ratio of powdered milk and granulated sugar, and water *ad libitum*.

2.2. Fungal isolates

Four isolates of mitosporic Ascomycete entomopathogenic fungi were used: two strains of *B. bassiana* (strains GHA and ARSEF 5344, hereafter BbGHA and Bb5344) and two strains of *M. anisopliae* (F52 and IMI330189, hereafter MaF52 and Ma189) as detailed in Table 1. These isolates were chosen because they represent two different species of fungi that have been shown to vary greatly in their fundamental virulence to range of insect species, including dipterans such as house flies (Lecuona et al. 2005) and mosquitoes (Bell et al. 2009). The fungal isolates used for this study were maintained in long-term storage at −80 °C on microporous beads (Pro-Lab Diagnostics, Austin, Texas, USA) at Penn State University. Fungi were recovered by placing one or two beads onto Potato dextrose agar (Oxoid, UK) or Sabouraud dextrose agar (SDA) (Oxoid, UK) in 9 cm diameter Petri dishes or slopes in 25 ml universal bottles and incubated at 25 °C for 10 days. Spores were then propagated using a diphasic culture system as listed in Jenkins and Goettel (1997). Briefly, conidia were harvested from slopes or plates to make a spore suspension of approximately 1×10^6 conidia ml^{-1} in sterile 0.05% w/v Tween 80 (Sigma) in distilled water. This suspension was then used to inoculate liquid culture medium (4% d-Glucose, 2% yeast extract [Oxoid, UK] in tap water), which was then incubated on a rotary shaker (160 rpm) at 24 °C for 3 days. The resulting suspension was used to inoculate sterile, moistened barley flakes (Bob's Red Mill, Milwaukie, Oregon, USA). Once the sporulate reached <20% moisture content, the conidia were harvested using a Mycoharvester (Acis Manufacturing, Devon, UK) and were placed in glass dishes and dried over silica gel at 24 °C. Upon reaching 5% moisture content, a small sample of conidia from each isolate was taken for quality analysis and the remaining powder was sealed in foil-laminated envelopes with a small sachet of silica gel and stored at 5 °C until use.

Table 1
Fungal isolates and origins.

Fungal isolate	Geographic location	Source
Beauveria bassiana GHA (BbGHA)	USA	Coleoptera: Chrysomelidae
Beauveria bassiana 5344 (Ba5344)	USA	Diptera: Muscidae
Metarhizium anisopliae anisopliae F52 (MaF52)	Austria	Lepidoptera: Olethreutidae
Metarhizium anisopliae var. acridum IMI330189 (Ma189)	Niger	Orthoptera: Acrididae

Spore formulations were prepared by suspending dry, pure spore powder into a mixture of mineral oils (80% Shel-Sol: 20% Ondina oil). The formulation was homogenized by vortexing for 30 s and sonicating for an additional 30 s to break up aggregates of spores. The concentration of spores for each formulation was counted using an improved Neubauer Hemocytometer and the volume adjusted until the desired concentration was obtained. Concentrations used in this experiment consisted of a high dose (1×10^9 spores/ml) and a low dose (1×10^7 spores/ml). All preparations proved to have germination rates of over 90% as assessed by plating on SDA.

2.3. Application of fungal spores to exposure surfaces

Spore formulations were applied to 9 cm circles of HP™ Color-Laser paper using an artist's airbrush sprayer. Paper circles (hereafter referred to as exposure substrates) were taped inside a 0.25 m^2 spray area on the rear wall of a chemical fume hood. The formulation was then loaded into the reservoir of the air brush and sprayed to give an equivalent volume application rate of 20 ml/m^2. Care was taken to keep the airbrush sprayer moving at a constant rate of speed and distance from the exposure substrates during the application process to ensure even coverage. Additionally, four exposure substrates were sprayed with blank oil to serve as positive controls, while another set of four was left unsprayed to serve as negative controls. After spraying, the exposure substrates were removed from the wall of the hood, set into the lids of 9 cm petri dishes and allowed to dry at room temperature overnight.

2.4. Exposing flies to fungal spores

Houseflies were removed from stock colonies using a battery-powered insect aspirator (BioQuip Inc.). All flies were from the same stock cage, and between 2 and 3 days old at the time of exposure. Flies were anesthetized with CO_2 and forty flies of mixed sex were then placed into the bottom of a standard size petri dish. The lid of the dish containing an exposure substrate was then placed over the anesthetized flies. Flies recovered from the CO_2 within 3–5 min and started to walk and groom themselves, at which time the dish was flipped over to encourage the flies to walk and rest on the exposure substrate. Flies remained enclosed in the dishes for

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