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Fusarium pathogenesis investigated using *Galleria mellonella* as a heterologous host

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Abstract

Members of the fungal genus *Fusarium* are capable of manifesting in a multitude of clinical infections, most commonly in immunocompromised patients. In order to better understand the interaction between the fungus and host, we have developed the larvae of the greater wax moth, *Galleria mellonella*, as a heterologous host for fusaria. When conidia are injected into the hemocoel of this Lepidopteran system, both clinical and environmental isolates of the fungus are able to kill the larvae at 37°C, although killing occurs more rapidly when incubated at 30°C. This killing was dependent on several other factors besides temperature, including the *Fusarium* strain, the number of conidia injected, and the conidia morphology, where macroconidia are more virulent than their microconidia counterpart. There was a correlation in the killing rate of *Fusarium* spp. when evaluated in *G. mellonella* and a murine model. *In vivo* studies indicated *G. mellonella* hemocytes were capable of initially phagocytosing both conidial morphologies. The *G. mellonella* system was also used to evaluate antifungal agents, and amphotericin B was able to confer a significant increase in survival to *Fusarium* infected-larvae. The *G. mellonella-Fusarium* pathogenicity system revealed that virulence of *Fusarium* spp. is similar, regardless of the origin of the isolate, and that mammalian endothermy is a major deterrent for *Fusarium* infection and therefore provides a suitable alternative to mammalian models to investigate the interaction between the host and this increasingly important fungal pathogen.

Keywords

Fusarium; heterologous host; fusariosis; thermotolerance; Galleria; antifungal agent

Introduction

Fusarium species are ubiquitous in nature, commonly found throughout soils and capable of growth on a wide range of substrates (Nelson et al. 1994). They have been isolated from

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diverse climatic conditions but are prevalent in temperate and tropical environments (Nelson et al. 1994). These fungi represent an important genus of plant pathogens capable of causing disease such as blights, scabs, and root rots on several agriculturally important crops (Desjardins 2003). *Fusarium* spp. are well known for the production of a vast number of harmful secondary metabolites termed mycotoxins that are responsible for several maladies in livestock and humans after consuming contaminated food sources (Bennett and Klich 2003; Desjardins 2003; Nelson et al. 1994).

Members of the genus *Fusarium* cause a wide breath of clinical manifestations, most commonly as superficial infections of the skin and/or nail(s), however they are also able to cause locally invasive and disseminated infections and are currently one of the most common etiological agents of filamentous fungal infections (Nucci and Anaissie 2007; Walsh et al. 2004). Fusariosis in immunocompromised patients, particularly those with neutropenia, has a poor prognosis and a corresponding high mortality rate (Nucci and Anaissie 2007).

There are numerous members of the anamorphic genus of *Fusarium*, however members of the *Fusarium solani* species complex (FSSC) and the *Fusarium oxysporum* species complex (FOSC) are the most frequently isolated from clinical laboratory specimens (Nucci and Anaissie 2007). There are ~50 phylogenetically distinct species within the FSSC, including at least 20 which have been isolated from mycoses (O'Donnell et al. 2008). Two of the less common species involved in infections are *F. verticillioides* and *F. proliferatum* (Nucci and Anaissie 2007), however these species may be the most prevalent in certain geographical locations (Tortorano et al. 2008).

One limitation in studying the pathogenesis of *Fusarium* spp. is the availability of suitable hosts to study fungal virulence. Two mouse models have been developed for *Fusarium* spp. (Legrand et al. 1991; Mayayo et al. 1999) however the ethical issues and expense associated to utilize these models have hindered their use in the laboratory. To circumvent these issues, the larvae of the greater wax moth, *Galleria mellonella*, have been developed as a model host to investigate virulence of *Fusarium* spp. This organism has served as a model host for several other fungal pathogens of clinical importance (Cotter et al. 2000; Mylonakis et al. 2005), including the aspergilli (Leger et al. 2000; Reeves et al. 2004). Importantly, many of the virulence factors of these medically important fungi required to infect a mammalian host are also necessary for *G. mellonella* infection (Brennan et al. 2002; Mylonakis et al. 2005). Through the use of this pathosystem, we have uncovered that *Fusarium* spp. are more virulent at lower temperatures, and *Fusarium* isolates from clinical specimens and isolates identified as plant pathogens are both able to kill *G. mellonella*.

Materials and methods

Strains and media

Fusarium isolates were obtained from the clinical mycology laboratory at Massachusetts General Hospital (MGH) or the Fungal Genetics Stock Center (McCluskey et al. 2010) (Kansas City, MO) (Table 1) and were maintained on potato dextrose agar (PDA) plates at room temperature. To obtain conidia for assays, *Fusarium* isolates were inoculated to V8 agar plates (10% V8 juice, 2.2% agar) containing 45μg/mL kanamycin, 100 μg/mL ampicillin, and 100 μg/mL streptomycin to prevent potential bacterial contamination and kept at room temperature for 6 days. To harvest the conidia, phosphate buffered saline (PBS) was added to the V8 plates and agitated, the suspended conidia was filtered through two layers of cheese cloth, and the conidia collected by centrifugation. Conidia were washed twice with PBS, suspended in 2 mL of PBS, the concentration determined by counting on a hemocytometer, and adjusted to the desired final concentration by diluting in PBS. To obtain

microconidia from isolates producing macroconidia on V8 medium (Fs CI-1 and FGSC 9935), the isolates were inoculated into 50 mL potato dextrose broth (PDB) and allowed to grow for 5 days on a shaker at room temperature. Conidia were then filtered through two layers of cheese cloth to remove mycelia, washed twice with PBS, and suspended in 2 mL of PBS. The number of microconidia was determined by counting on a hemocytometer and adjusted to the desired final concentration by diluting in PBS. This method produced suspensions of >95% microconidia.

Species identification of clinical *Fusarium* spp. isolates

The *Fusarium* isolates obtained from the clinical mycology laboratory at MGH were initially identified by sequencing of the translation elongation factor (TEF) 1α coding region. This was accomplished by PCR using genomic DNA of the *Fusarium* isolate as template using the primers EF1 and EF2 (O'Donnell et al. 1998) which was then cloned into pGEM-T Easy (Promega, Madison, WI) and bi-directionally sequenced at the MGH DNA sequencing core facilities.

Isolates belonging to the FSSC or FOSC were further subjected to multilocus sequence typing. DNA fragments of the FSSC isolates encoding the internal transcribed spacer and portions of the nuclear large-subunit rRNA (rRNA) and the second-largest subunit of RNA polymerase (*RPB2*) were amplified as previously described (O'Donnell et al. 2008). A DNA fragment of the FOSC isolate containing the nuclear ribosomal DNA intergenic spacer region (FoITS) were amplified as described in O'Donnell et al (2009). PCR products were cloned in pGEM-T Easy and sequencing was conducted at the MGH sequencing core facilities using vector primers (O'Donnell et al. 2009; O'Donnell et al. 2008), and MLST group determined using the results of a blastn analysis at NCBI or the FUSARIUM-ID database (O'Donnell et al. 2010).

G. mellonella killing assays

G. mellonella caterpillars in the final instar larval stage of development (Vanderhorst, Inc., St. Marys, OH) were stored in the dark and used within seven days from receipt of the shipment. Sixteen randomly chosen *G. mellonella* larvae (330 ± 25 mg body weight) were used per condition in all assays. All experiments included two control groups: the first group of larvae was mock inoculated with 10 μL of PBS to observe for killing due to physical trauma from the injection, and the second group did not receive any injection. A 10 μl Hamilton syringe was used to inject 10 μl of the inoculum into the hemocoel of each larva via the last left proleg. Before injection the area was cleaned using an alcohol swab. After injection, caterpillars were incubated in plastic containers at 30 or 37°C, and the larval survival scored daily where larvae were considered dead by an absence of movement in response to touch. Experiments that had more than two dead caterpillars in either control group were discarded and repeated. All *G. mellonella* killing experiments were performed at least twice, and representative experiments are presented. In order to simplify some of the figures, the PBS control group may have been omitted.

Radial growth assay

In order to determine if the ability of the *Fusarium* strains to grow at physiologically relevant temperatures had a role in *Galleria* killing, radial growth assays were conducted at 30°C and 37°C. *Fusarium* isolates were inoculated to 2% water agar plates and allowed to grow for three days at room temperature. Agar plugs (~5mm) were removed from the growing colony margins and inoculated to 10% V8 juice plates with the mycelial side of the plug directly on the V8 agar surface. Inoculated plates were placed in the dark in 30°C or 37°C incubators and scored after seven days of growth. The growth assay is based on two

independent replicates. The percent inhibition of radial growth was calculated and standard deviation determined.

Murine model of infection

The murine virulence assays were conducted as on immunosuppressed mice using three clinical Fusarium strains (Fs CI-1, Fs CI-2, and Fo CI-1). Briefly, neutropenia was induced in male CF-1 mice (Charles River Laboratories, Wilmington, MA) weighing ~30g one day prior to inoculation with *Fusarium* spores. Immunosuppression was induced by a single intraperitoneal dose of 200 mg/kg of cyclophosphamide (Sigma). Groups of eight mice were inoculated by a lateral tail vein injection with 100 μL of a 10^7 conidial suspension (10^6 conidia inoculated per mouse). Animal survival was monitored twice daily, and moribund animals or those in distress were sacrificed by CO_2 asphyxiation.

Fusarium surface fluorophore labeling

Fusarium conidia were fluorescently labeled by selectively linking Alexa Fluor 647 succinimidyl ester to primary amines located on the conidial surface. Alexa Fluor 647 succinimidyl ester was reconstituted in dimethylformamide (100 mg/mL). Prior to fluorescent labeling, conidia were washed in phosphate buffered saline (PBS) 3 times at 4000 g for 1 minute. After the PBS washes, conidia were suspended in 500 μL of PBS, and 3 μg of dye was mixed with the pathogen at 37°C for 30 minutes. During the labeling/ incubation, the spores were shaken for 10 sec every 10 min to prevent them from settling in the bottom of the tube. The dye-conidia mixture was then washed again 3 times in PBS (4000 rpm for 1 minute) and kept on ice and protected from light until use in the imaging experiments.

Fusarium-infected *G. mellonella* hemolymph

Alexa Flor 647-labeled *Fusarium* conidia were inoculated into *G. mellonella* as described above and incubated at 37°C. Twelve hours post-inoculation, hemolymph was collected from the larvae into cold insect physiologic saline as previously described (Fuchs et al. 2010). After centrifugation, the hemocytes were washed twice in 500 μL of Grace's insect medium (Lonza, Walkersville, MD) and suspended in 300 μL Grace's medium. The hemocytes were then permitted to adhere to the slide chamber for 1 hr.

Confocal microscopy

Spinning-disk confocal microscopy was performed on a Nikon Ti-E inverted microscope equipped with a CSU-X1 confocal head (Yokogawa) that provides scan speeds of up to 2,000 frames per second. A Coherent, 4 W, continuous-wave laser was used as an excitation light source to produce excitation wavelengths of 647 nm. To acquire high quality fluorescence images, a high magnification, high numerical aperture (NA) objective, used for Total Internal Reflection Imaging (TIRF), was used (Nikon, 100X, 1.49 NA, oil immersion). A piezo stage (Prior Instruments, Rockland, MA) capable of X, Y, and Z movement was used for z-stack acquisition. A halogen light source and an air condenser (0.52 NA) were used for bright field illumination and a polarizer (Nikon, MEN51941) and Wollaston prisms (Nikon, MBH76190) were used to acquire differential interference contrast (DIC) images. Images were acquired using an EM-CCD camera (Hamamatsu, C9100-13) capable of acquiring high-resolution images under low light levels with high quantum efficiency. Emission light from the sample was collected after passage through the appropriate emission filters (Semrock, Rochester, NY). Image acquisition was performed using MetaMorph software (Molecular Devices, Downingtown, PA).

Determination of the minimal inhibitory concentration of antifungal agents

The minimial inhibitory concentration (MIC) for fluconazole, amphotericin B, and mancozeb, against the *Fusarium* spp. was determined spectrophotometrically using RPMI 1640 media (Mediatech, Inc., Manassas, VA) following the established broth microdilution method of the Clinical and Laboratory Standards Institute document M38-A (National Committee for Clinical Laboratory Standards 2002). All antifungal compounds were obtained from Sigma-Aldrich (St. Louis, MO).

Evaluation of antifungal agents in the *G. mellonella-Fusarium* system

The same technique of inoculation for the experiments that include antifungal efficacy evaluation were conducted as described above. Administration of the antifungal agent to be evaluated was accomplished by an injection consisting of 10 μL of the compound into the right last proleg of the larvae. Antifungal agents (amphotericin B, fluconazole, and mancozeb) were diluted in PBS, and each larva was administered a single compound by a separate injection. Amphotericin B (1.5 mg/kg), fluconazole (14 mg/kg), or mancozeb (0.25 mg/kg) treatments were administered a single time on the same day of inoculation. *G. mellonella* caterpillars that were injected twice with PBS were used as a control group for these experiments.

Statistical analysis

Kaplan-Meier survival curves were plotted using STATA 6, and a P value of <0.05 was considered to be statistically significant. For the *Fusarium-Galleria* experiments conducted at different temperatures, the differences in survival and the hazard ratio were calculated by running a Cox proportional hazards regression model (Cox regression) considering the treatment or intervention (*Fusarium* isolates), the source of the isolates (clinical and plant isolates), and the incubation temperature (30°C and 37°C) as independent variables. The hazard function was considered as the dependent variable. The Cox regression procedure allows the comparison between clinical versus plant isolates and the incubation temperatures of 30°C versus 37°C on larval survival while simultaneously taking into account the effect of each individual *Fusarium* isolate on *Galleria* survival.

Results

Killing of *G. mellonella* by *Fusarium* conidia

When *Fusarium* conidia are inoculated into the last proleg of the larvae, the time of survival of the infected insect was dependent on the number of spores inoculated, regardless if the *Fusarium* isolate was from a clinical (Fig 1A) or an environmental source (Fig 1B). After *G. mellonella* were injected with 10^6 conidia and incubated at 37°C, the larvae began dying after 2 days and were usually all dead within 4–6 days. Inoculating 10^7 conidia/larvae into the hemocoel resulted in an increased rate of *G. mellonella* killing and usually no larvae survived longer than two days (data not shown). By the time of death, larvae were deeply melanized as observed with other fungal pathogens (Cotter et al. 2000).

Killing of *G. mellonella* by *Fusarium* spp. is temperature dependent

This study is the first instance where virulence of *Fusarium* spp. has been evaluated at two temperatures in a single host system, and reveals that *Fusarium* spp. are more pathogenic at temperatures lower than 37°C. Isolates from clinical sources were capable of killing *G. mellonella* within 3–4 days post inoculation when incubated at 30°C (Fig 2A), where the time necessary to result in 50% killing ($TD^{50\%}$) ranged from ~1–2 days for all clinical isolates included in the assay. However, most of these clinical isolates were unable to cause significant mortality one week or more after inoculation with 1×10^5 conidia and incubated

at 37°C (Fig 2B). The exception to this observation was an isolate of *F. solani* (Fs CI-1) which was collected from a blood culture of a patient with invasive fusariosis. *G. mellonella* inoculated with Fs CI-1 consistently had a $TD^{50\%}$ ~1–3 days, whereas the other clinical isolates were unable to yield a $TD^{50\%}$ of less than one week.

Virulence of a collection of environmental *Fusarium* isolates was also evaluated in *G. melonella* (Fig 2C; Table 1). As seen with the clinical isolates, there was a marked decrease in *G. mellonella* survival when the larvae were incubated at 30°C with a $TD^{50\%}$ ranging from ~1–2 days (Fig 2C). Interestingly, all isolates included in this study were capable of killing *G. mellonella*, including *F. cerealis* and *F. culmorum* which are not normally associated with clinical cases of fusariosis (Fig 2C). Conversely, when larvae were incubated at 37°C post-inoculation, most of the isolates were unable to cause any significant degree of killing, although *F. oxysporum* f.sp. *lycopersici*, a pathogen from tomato, was highly virulent in *G. mellonella* (a $TD^{50\%}$ of 1.5–3 days) where all the larvae succumb to the fungal infection within four days post-inoculation (Fig 2D).

There was no significant difference between the virulence of *Fusarium* isolates from clinical sources and environmental isolates when the *G. mellonella* were incubated at either 30°C (P=0.267, hazard ratio = 0.68, CI (0.34–1.34)) or 37°C (P=0.57, hazard ratio =0.77, CI (0.31–1.87)) post inoculation. However, there is a significant difference in the survival of the larvae when placed at 30°C versus 37°C after inoculation with the *Fusarium* spp., which was evident regardless if the fungus was from a clinical (P= 0.002, hazard ratio = 4.03, CI(1.65–9.80)) or environmental origin (P=0.00, hazard ratio =5.86, CI (2.58–13.27)).

The difference in virulence of the *Fusarium* spp. on *G. mellonella* when incubated at 30 and 37°C could be due to various reasons, including host factors and the ability of the fungus to germinate and grow within the larvae. Radial growth assays were conducted to assess the thermotolerance of the *Fusarium* isolates at higher temperatures. There was a distinct correlation between the inhibition of radial growth at 37°C and the ability of the fungus to kill *G. mellonella* larvae at 37°C. All of the *Fusarium* isolates displayed reduced growth at 37°C, including those isolated from invasive clinical specimens (Table 2); however, Fs CI-1 was the most thermotolerant of the invasive clinical isolates (58.5±4.6% inhibition) and FGSC 9935 was the most thermotolerant of the environmental isolates (64.2±6.7% inhibition), both of which were able to kill *G. mellonella* at 37°C.

A positive correlation exists between *G. mellonella* and a murine model to assess the virulence of *Fusarium* spp

Three clinical *Fusarium* isolates (Fs CI-1, Fs CI-2, and Fo CI-1) were selected to evaluate their pathogenicity in a murine model to determine if their virulence is correlated to the results observed in *G. mellonella*. Both of the *F. solani* isolates were highly virulent in a mouse model while Fo CI-1 did not cause any morbidity within the experimental timeframe (Fig 3), a result consistent with previous findings (Mayayo et al. 1999). Importantly, a positive correlation was present between the rate of killing of these *Fusarium* isolates. Fs CI-1 was the most virulent in a murine model, and Fo CI-1 the least (Fig 3), similar to the results observed using *G. mellonella* followed by incubation at 37°C (Fig. 2B). The median time of death of mice inoculated with Fs CI-1 was 72 h whereas those inoculated with Fs CI-2 was 120 h (Fig 3).

Morphology of the conidia is involved in *G. mellonella* killing

Many *Fusarium* spp. are capable of producing two types of conidia, microconidia and macroconidia. In our inoculation assays, we observed isolates producing either predominantly microconidia or a mixed population of these conidial morphologies.

Interestingly, the clinical isolate which displayed the highest *G. mellonella* killing at 37°C, Fs CI-1, produced a greater number of macroconidia than microconidia suggesting conidial morphology may play a role in establishing disease.

In order to investigate the role the spore type has in killing, Fs CI-1 and the environmental isolate *F. oxysporum* f.sp. *lycopersici* were selected for additional assays, as each of the conidia morphologies could be separated to >90% of the desired conidia morphology by culturing on different media. There was a distinct difference in survival between larvae inoculated with the different conidia for both *Fusarium* isolates, where larvae inoculated with macroconidia died within three days while the majority of larvae challenged with microconidia survived (*P* value <0.0001; Fig 4).

As *Fusarium* spp. macroconidia are multicellular (having ~4–5 cells per conidia), the bias of macroconidia virulence could be due to increased cell germination within this inoculum. *Fusarium* macroconidia typically germinate from either one or, more commonly, both terminal spore cells (Harris 2005), resulting in approximately twice the amount of germination tubes produced. However, when twice the amount of microconidia compared to macroconidia were injected into the *G. mellonella* larvae (2×10^5 microconidia vs. 1×10^5 macroconidia), the macroconidia were significantly more virulent (*P*= <0.0001; Fig 4). Additionally, the amount of fungal biomass between microconida and macroconidia could have a role in the observed difference in *G. mellonella* killing. When microconidia were inoculated at a concentration five times that of macroconidia (5×10^5 microconidia vs. 1×10^5 macroconidia), the larvae inoculated with macroconidia still died more rapidly than the higher concentration of microconidia (*P*= <0.0001; Fig 4).

Superficial and invasive clinical *Fusarium* isolates are virulent in *G. mellonella*

As previously mentioned, *Fusarium* spp. are capable of causing superficial localized infections at the extremities of immunocompetent patients. The *G. mellonella-Fusarium* spp. pathosystem was utilized to investigate if fungal isolates associated with these superficial infections were as virulent as isolates from an invasive infection. When *G. mellonella* larvae were challenged with 1×10^5 conidia from fusaria isolated from superficial infections, killing occurred regardless of the severity of the clinical infection from where the strain was isolated (Fig 5). An isolate of *F. solani* (Fs CI-3) isolated from a nail culture was capable of killing 100% of the infected larvae within 72 hrs, a rate that is comparable to the most virulent systemic *Fusarium* isolate obtained from a blood culture (the $TD^{50\%}$ for Fs CI-3 was ~1.5 days versus a $TD^{50\%}$ for Fs CI-1 of ~1–2 days). Despite being isolated from a superficial infection site, Fs CI-3 also exhibited a relatively high degree of thermotolerance (47.4±0.2% inhibition; Table 2).

Interaction of *Fusarium* conidia and hemocytes

The hemolymph of *G. mellonella* larvae challenged with *Fusarium* spp. was collected and examined by confocal microscopy 16 hours after injection with conidia (Fig 6). Macroconidia of the fungus are able to be phagocytosed despite their size (Fig 6A and B). Large obstructions in the hemolymph are sequestered through a process known as nodulation where multiple hemocytes adhere and surround the object (Lavine and Strand 2002), in this case a germinating *Fusarium* conidium (Fig 6C). Hemocyte phagocytosis also occurs in response to encountering microconidia in the hemolymph (Fig 6D and E).

Assessment of antifungal agents in *G. mellonella*

Few clinically used antifungal agents have efficacy against *Fusarium* spp., although the polyene amphotericin B has been shown to be effective against several *Fusarium* spp. (Al-Abdely 2004; Carneiro et al. 2011; Muhammed et al. 2011; Nucci and Anaissie 2007). The

G. mellonella-Fusarium spp. pathosystem was used to evaluate single dose treatments of two clinically relevant antifungal agents; amphotericin B and the azole fluconazole which has limited efficacy against *Fusarium* spp. Following administration of 14 mg/kg fluconazole there was no significant increase in survival of the larvae, conversely a dose of 1.5 mg/kg of amphotericin B was able to increase survival of *Fusarium*-infected larvae (Fig 7). No significant difference in larval survival was observed for other assayed concentrations of the antifungal agents (amphotericin B 0.5–2.0 mg/kg; fluconazole 3.5–28 mg/kg; data not shown). There was a correlation between the survival of the infected *G. mellonella* and the *in vitro* MIC of the antifungal agents (MIC for isolate Fo CI-1: Flu 256 μg/mL; AmpB 2 μg/mL).

In order to assess the possibility that *G. mellonella* can be used as an alternative host to detect compounds with antifungal efficacy against *Fusarium*, the *Fusarium-Galleria* system was used to evaluate the antifungal efficacy of a compound not used clinically against *Fusarium* spp. Mancozeb, a fungicide used as a protectant in agriculture against *Fusarium* spp., was evaluated in *G. mellonella* (Fig 7). Previously this compound had been reported to inhibit *Fusarium* spp. growth (Allen et al. 2004; Fravel et al. 2005), which was confirmed having an MIC of 4 μg/mL against isolate Fo CI-1. Despite antifungal activity of the compound, treatment of infected *G. mellonella* larvae with a single dose of ranging from 0.07–0.53 mg/kg of mancozeb resulted in no significant difference in survival when compared to the PBS control (Fig 7).

Discussion

The diverse host range of *Fusarium* spp. has been well documented, however these fungi are best known for the ability to cause devastating crop losses as plant pathogens and contaminating feed with mycotoxins (Desjardins 2003; Desjardins and Proctor 2007). *Fusarium* spp. have been described as entomopathogenic fungi on a wide range of insects, including members of the order Lepidoptera (Hajek et al. 1993; Teetor-Barsch and Roberts 1983). Taking advantage of the diverse host range of these pathogens we show that the greater wax moth provides a suitable alternative host to evaluate virulence of *Fusarium* spp. isolated from various sources.

G. mellonella has served as a heterologous host for several medically important fungi, including *Candida* spp., *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Aspergillus flavus* (Cotter et al. 2000; Jackson et al. 2009; Leger et al. 2000; Mylonakis et al. 2005; Reeves et al. 2004). *Fusarium* spp. were able to form a lethal infection when injected into the hemocoel and incubated at lower temperatures. Several secondary metabolites from *Fusarium* spp. have been shown to have insecticidal activity and therefore may play a role in *G. mellonella* larvae killing (Claydon et al. 1977, 1979; Mule et al. 1992; Strongman et al. 1988). In addition, the morphology of the conidia was important for virulence in this pathosystem (Fig 3), however the precise mechanism conferring the higher virulence of macroconidia is unknown. The observed difference in virulence from the two conidia morphologies compounded with the fact that the environmental condition of the *Fusarium* isolate may influence the type of conidia the fungus produces, suggests that the surrounding environment may play an indirect role in the virulence of *Fusarium* on the larvae. It should be noted though that the virulence of the two different conidia morphologies was conducted for only two isolates (Fs CI-1 and FGSC 9935), as it was not possible to obtain inoculums of a single conidial morphology for the other isolates.

The amenability of *G. mellonella* to various temperatures, including physiological relevant temperatures, has made the arthropod ideally suited as a heterologous host for *Fusarium* spp. Unlike other clinically relevant fungal pathogens, *Fusarium* spp. displayed an inversely

related temperature-virulence relationship, where there was increased virulence when incubated at 30°C than at 37°C. This increase in virulence at lower temperatures may explain the prevalence of localized skin infections and onychomycosis caused by *Fusarium* spp. in immunocompetent and immunocompromised patients (Gupta et al. 2000; Nucci and Anaissie 2002). These skin infections usually are located on the patient's extremities such as the toes (Nucci and Anaissie 2007), a location that would be lower than physiological temperature, therefore potentially favoring *Fusarium* spp. colonization.

The observed decrease in *G. mellonella* killing at mammalian physiological temperatures compared to 30°C suggests that many *Fusarium* spp. have the capability to cause disease, however the frequency of invasive infections by these fungi may be curtailed by the higher internal temperatures of mammals. A study of 4802 fungal isolates found that an additional ~6% of the fungi were unable to grow for each increase of 1°C within the 30–40°C temperature range (Robert and Casadevall 2009). Therefore, thermotolerance could potentially be one of the limiting factors for an increased incidence of invasive fusariosis, and conversely mammalian endothermy a factor in limiting members of this fungal group from causing disease. Although, host endothermy alone can not account for the reduced incidence of *Fusarium* spp. in the clinic, as many of these isolates can grow at 37°C (Mehl and Epstein 2007; Sugiura et al. 1999), and therefore other factors, in particular the immune system of the host, play a major role in host's defense against the fungus (Carneiro et al. 2011; Dignani and Anaissie 2004; Nucci and Anaissie 2007).

A decreased number of neutrophils in patients is a major risk factor for fusariosis. The equivalent immune cells in *G. mellonella*, contained in the hemolymph, are a collection of several different types of hemocytes, of which plasmatocytes and granulocytes are involved in phagocytosis, nodule formation, and encapsulation (Lavine and Strand 2002). Furthermore, studies have shown that the hematocyte density within the hemolymph was inversely correlated with the degree of virulence of the fungal pathogen that was inoculated (Bergin et al. 2003). The hemocytes are capable of phagocytizing *Fusarium* conidia, and may provide a suitable pathosystem to evaluate the host immune response to *Fusarium* infection *in vivo*. Furthermore, *G. mellonella* is capable of producing a number of antimicrobial peptides (Brown et al. 2009), some of which have been shown to have antifungal activity against *F. graminearum* and *F. oxysporum* (Brown et al. 2008). In the study by Brown *et al.* (2008), the spores of *F. oxysporum* were found to be more tolerant of the *G. mellonella* moricin-like antimicrobial peptides than those of *F. graminearum* (Brown et al. 2008), an observation which may account for the consistent more rapid killing of *G. mellonella* larvae by both clinical and environmental isolates of *F. oxysporum* (Fig 2).

Fusarium isolates able to cause disease in humans are prevalent within the surrounding environment (Anaissie et al. 2001; Mehl and Epstein 2008; O'Donnell et al. 2004; Zhang et al. 2006). Multilocus analysis of members of the FSSC from clinical and environmental sources revealed that many isolates from both sources had shared haplotypes which suggests that clinical infections caused by these fusaria originally were obtained from the environment (Zhang et al. 2006). This notion is further supported from studies with *F. solani* f. sp. *cucurbitae* race 2 strains which were obtained from clinical specimens and from infected plant tissue. Regardless of the source of the fungus, all isolates were pathogenic on cucurbits and were able to grow at human body temperature. Furthermore, isolates from clinical sources were fertile with those from plants, demonstrating they comprise a single species (Mehl and Epstein 2007). Our results using *G. mellonella* as a host for several species of *Fusarium* show a similar trend. Isolates from both clinical and plant/environmental sources were able to kill *G. mellonella* larvae (Fig 2), further supporting there is little difference in the virulence of *Fusarium* regardless of the source.

As *Fusarium* spp. are capable of infecting a diverse array of hosts, their virulence in human hosts could be a result of traits that have been selected by the fungus persisting in the environment, a concept termed “accidental virulence” (Casadevall and Pirofski 2007). All of the *Fusarium* isolates included in this study were able to kill the larvae of *G. melonella*. Therefore it possible that passage of *Fusarium* through insects in the environment has selected for traits which allow the fungus to overcome the immune response of the invertebrate, thereby conferring virulence to other potential animal hosts. The correlation between the killing rate of the *Fusarium* isolates in the *G. mellonella* and mice models suggest that virulence factors maybe conserved between these hosts supporting the notion that these factors may have evolved by “accidental virulence” (Fig 3).

Currently, there are a limited number of antifungal agents available to treat fusariosis (Muhammed et al. 2011). Of these compounds, amphotericin B provides the broadest efficacy to multiple *Fusarium* spp. (Nucci and Anaissie 2007; O’Donnell et al. 2008); however newer azoles, such as posaconazole and voriconazole, have some antifungal efficacy for *Fusarium* spp. (Nucci and Anaissie 2007; O’Donnell et al. 2008). *G. mellonella* provides a suitable alternative to identify and evaluate the efficacy of antifungal agents *in vivo* instead of animal models. Furthermore, since the larvae have significantly less body mass than their murine counterpart, *G. mellonella* is highly suited for instances where a limited amount of compound is available to evaluate the antifungal efficacy against *Fusarium* species.

The use of *G. mellonella* has an alternative host for *Fusarium* spp. provides a suitable system to further investigate host-pathogen interactions and to evaluate the antifungal efficacy of compounds against *Fusarium* species. This system demonstrated that *Fusarium* spp. from both clinical and environmental sources are more virulent at temperatures lower than 37°C and that conidia morphology is involved in establishing disease in *G. mellonella*.

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Research Highlights

- The greater wax moth, *Galleria mellonella*, is a suitable alternative host for *Fusarium* spp.
- Thermotolerance is important for pathogenicity of *Fusarium* spp.
- Macroconidia of Fusarium are more infectious than microconidia

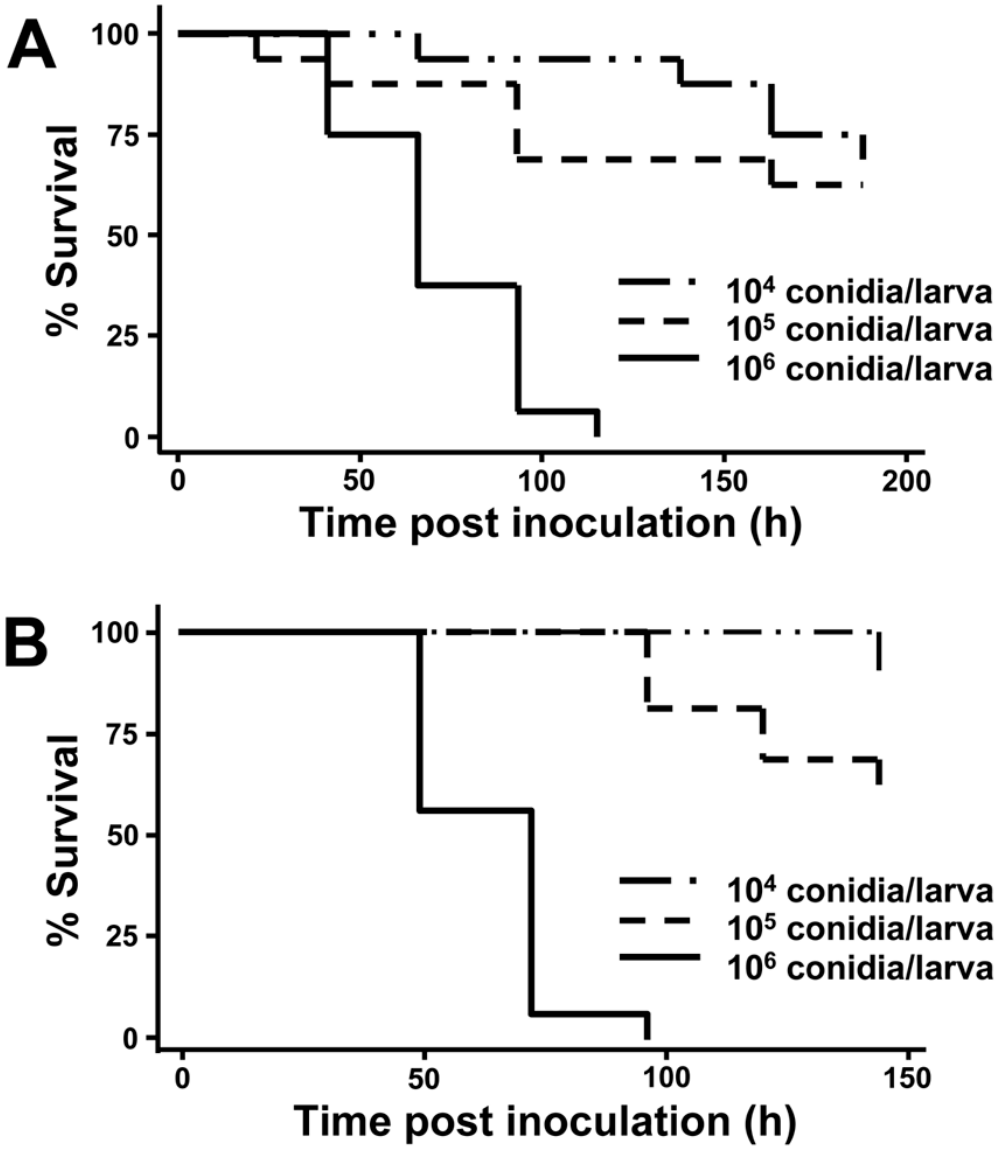

Fig 1.

Killing of *G. mellonella* larvae is dependent on the number of conida inoculated. Kaplan-Meier survival of larvae inoculated with number of a mixture of predominately microconidia indicated for Fo CI-1 (A) and *F. solani* FGSC 9596 (B).

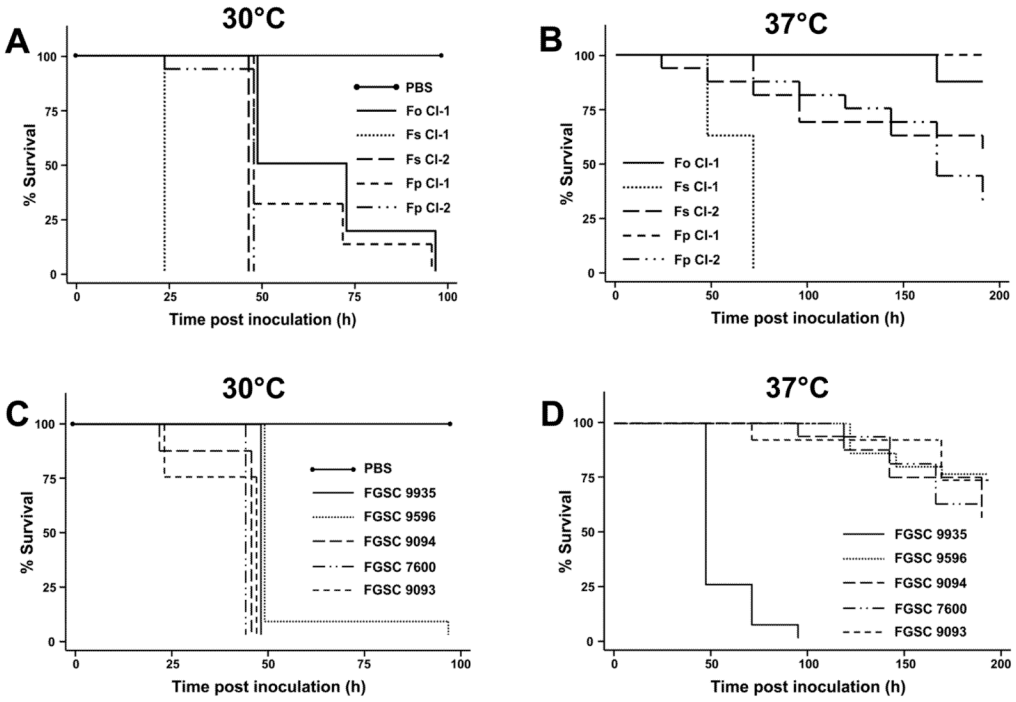

Fig 2.

Killing of *G. mellonella* by clinical and established environmental *Fusarium* isolates. Kaplan-Meier survival of larvae following injection with 1×10^5 of conidia (Fs CI-1, FGSC 9093, FGSC 9094, FGSC 9935 were predominately macroconidia while the other isolates were predominately microconidia) and incubation at 30°C (A and C) and 37°C (B and D). In some instances, lines were offset slightly to allow them to be visible.

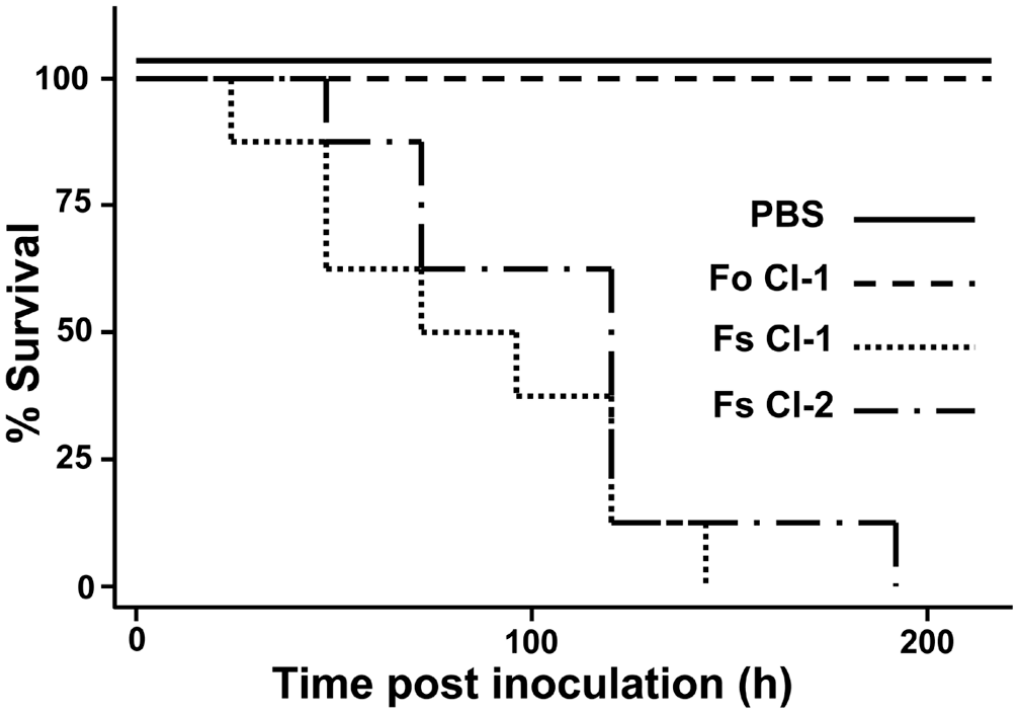

Fig 3.

Clinical isolates of *Fusarium* spp. evaluated in a murine model. Kaplan-Meier survival of neutropenic mice inoculated with 10^6 conidia of two *F. solani* isolates (Fs CI-1 and Fs CI-2) and one *F. oxysporum* isolate (Fo CI-1).

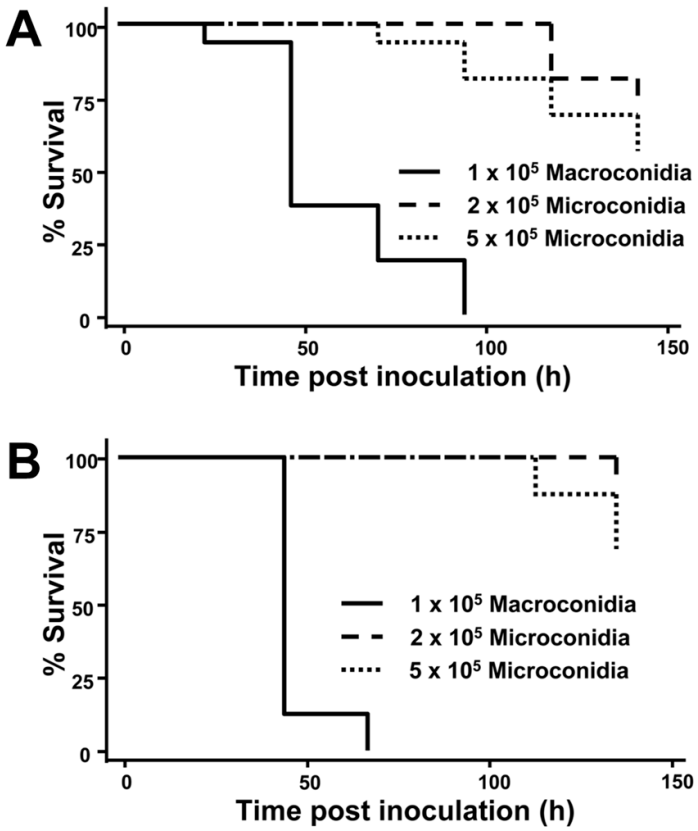

Fig 4.

Fusarium conidia morphology is involved in virulence on *G. mellonella*. Survival of larvae was dependent on the morphology of the inoculated *F. solani* conidia of the clinical isolate Fs CI-1 (A) and the environmental reference isolate FGSC 9935 (B) and placed at 37°C. Statistical analysis of larval survival after injection with 1×10^5 macroconidia compared to 2×10^5 or 5×10^5 microconidia resulted in a *P* value < 0.0001 for either isolate. The differences in survival of larvae injected with the two microconidia concentrations resulted in *P* values = 0.22 and 0.069 for Fs CI-1 (A) and FGSC 9935 (B), respectively.

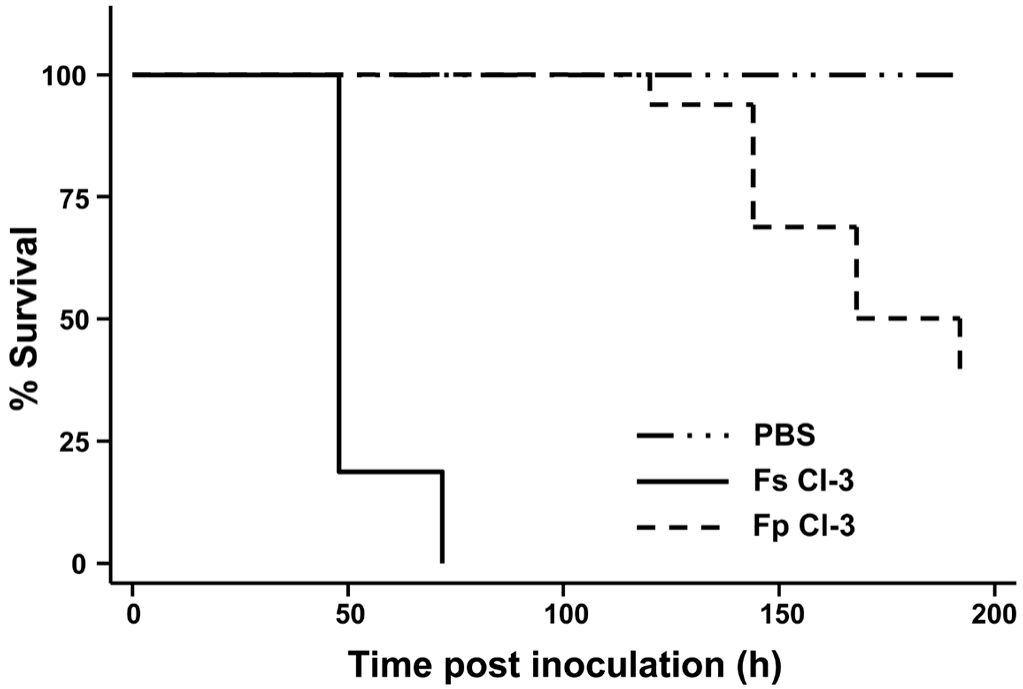

Fig 5.

Clinical *Fusarium* isolates from superficial infections are capable of killing *G. mellonella*. Kaplan-Meier survival of larvae following injection with 1×10^5 conidia and incubation at 37°C. Fs CI-3 produced mostly macroconidia.

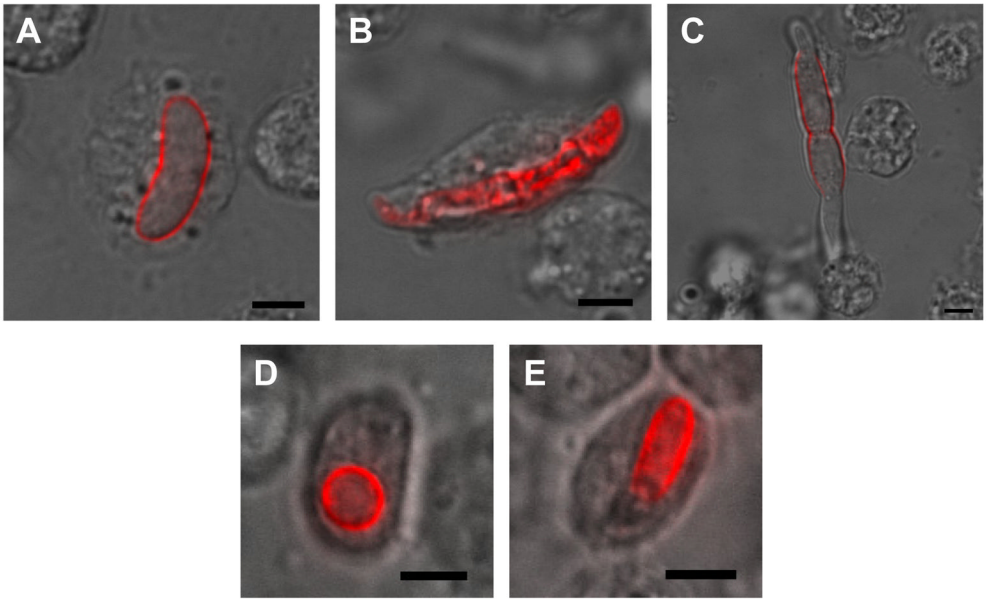

Fig 6.

G. mellonella hemocytes associate with *Fusarium* conidia. Macroconidia of Fs CI-1 after phagocytosis by hemocytes (A and B). A germinating Fs CI-1 conidium with multiple hemocytes associated initiating nodulation (C). Microconidia of Fo CI-1 phagocytosed by hemocytes (D and E). Scale bars represent 5 μm.

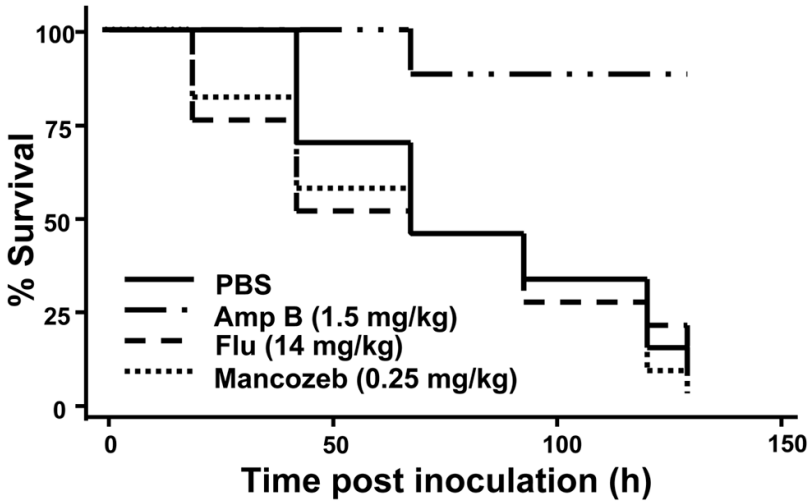

Fig 7.

Amphotericin B prolongs survival of *Fusarium*-infected *G. mellonella*. Percent survival of larvae challenged with 1.5×10^6 conidia of Fo CI-1 followed by treatment with the indicated antifungal agents. Abbreviations: Amp B, amphotericin B; Flu, fluconazole.

Table 1

List of *Fusarium* isolates used in this study.

Isolate abbreviation	Identification	Details	Source	Reference(s)
Fo CI-1	*F. oxysporum* MLST 33	Clinical isolate	Groin tissue culture	this study
Fs CI-1	*F. solani* MLST 2	Clinical isolate	Blood culture	this study
Fs CI-2	*F. solani* MLST (3+4)	Clinical isolate	Bronchial lavage	this study
Fp CI-1	*F. proliferatum*	Clinical isolate	Bronchial wash	this study
Fp CI-2	*F. proliferatum*	Clinical isolate	Sputum	this study
Fs CI-3	*F. solani* MLST 2	Clinical isolate	Nail	this study
Fp CI-3	*F. proliferatum*	Clinical isolate	Nail	this study
	F. solani f.sp. *pisi*	Plant pathogen	FGSC 9596	(Coleman et al. 2009; Kistler and VanEtten 1984)
	F. oxysporum f.sp. *lycopersici*	Plant pathogen	FGSC 9935	(Ma et al. 2010)
	F. culmorum	Plant pathogen	FGSC 9094	(Bottalico and Perrone 2002; O'Donnell et al. 1998; Parry et al. 1995)
	F. cerealis	Plant pathogen	FGSC 9093	(Bottalico and Perrone 2002; O'Donnell et al. 1998)
	F. verticillioides	Plant pathogen	FGSC 7600	(Ma et al. 2010; Xu et al. 1995)

Abbreviations are as follows: Fo = *F. oxysporum*, Fs = *F. solani*, Fp = *F. proliferatum*, FGSC = Fungal Genetics Stock Center

Table 2

Comparison of radial growth of *Fusarium* isolates at 30 and 37°C.

	Percent inhibition of radial growth at 37°C relative to radial growth at 30°C[a]
Fo CI-1	90.7±4.9
Fs CI-1	58.5±4.6
Fs CI-2	67.7±5.8
Fs CI-3	47.4±0.2
Fp CI-1	84.5±3.8
Fp CI-2	89.6±0.6
Fp CI-3	89.0±3.1
FGSC 9596	68.6±5.5
FGSC 9935	64.2±6.7
FGSC 9094	100[b]
FGSC 9093	100[b]
FGSC 7600	82.5±2.0

[a] Average and standard deviation of two independent assays.

[b] FGSC 9093 and FGSC 9094 were unable to grow when incubated at 37°C.