A *Falciformispora senegalensis* grain model in *Galleria mellonella* larvae

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Abstract

Eumycetoma is a subcutaneous implantation mycosis often found in the foot. One of the hallmarks of eumycetoma is the formation of grains. These grains are either black or white and the consistency and morphology differs per causative agent. The two most common causative agents of black-grain eumycetoma are *Madurella mycetomatis* and *Falciformispora senegalensis*. Since grains cannot be formed *in vitro, in vivo* models are needed to study grain formation. Here, we used the invertebrate *Galleria mellonalla* to establish an *in vivo* grain model for *F. senegalensis.* Three different *F. senegalensis* strains were selected and four different inocula were used to infect *G. mellonella* larvae, ranging from 0.04 mg/larvae to 10 mg/larvae. Larval survival was monitored for 10 days. Grain formation was studied macroscopically and histologically. Efficacy of antifungal therapy was determined for itraconazole, amphotericin B and terbinafine. A concentration of 10 mg *F. senegalensis* per larvae, was lethal for the majority of the larvae within 10 days. At this inoculum, grains were formed within 24 hours after infection. The grains produced in the larvae resembled those formed in human patients. Amphotericin B given at 1 mg/kg 4h, 28h and 52h after infection prolonged larval survival. No enhanced survival was noted for itraconazole or terbinafine. In conclusion, we developed a *F. senegalensis* grain model in *Galleria mellonella* larvae in which grains were formed which were similar to those formed in patients. This model can be used to monitor grain formation over time and to study antifungal efficacy.

Lay Summary

Within eumycetoma lesions, the causative agents are embedded in grains. However the grains differ per causative agent. In this study we developed a grain model of *Falciformispora senegalensis* in the larvae of *Galleria mellonella.* This model can in the future be used to study the efficacy of novel antifungal agents.

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Introduction

Mycetoma is a neglected tropical disease, characterized by large subcutaneous lesions, most often in the feet. The disease has been reported to be endemic in 102 countries with a high prevalence in countries located between 30 degrees North and 15 degrees South of the equator[1, 2]. Mycetoma can be caused by either of bacteria (actinomycetoma) or fungi (eumycetoma)[2]. Actinomycetoma is most commonly caused by the actinomycetes *Nocardia brasiliensis, Actinomadura madurae* and *Streptomyces somaliensis*[2]. Eumycetoma is most commonly caused by *Madurella mycetomatis, Falciformispora senegalensis, Trematosphaeria grisea* and *Scedosporium boydii[3]*.

One of the characteristic features of mycetoma are the granules called grains[4]. Actinomycetoma grains are often white, yellow or red, depending on the causative agent, while eumycetoma grains are often black or white. The color of the grain is dependent on the production of melanin and/or pigments by the causative agents. Of the four most common causative agents of eumycetoma, *M. mycetomatis, F. senegalensis* and *T. grisea* all form black grains, while *S. boydii* forms white grains[3]. Despite the fact that the majority of the eumycetoma causative agents produce black grains, the grain morphology per species differs in histology sections. First, the grains of *M. mycetomatis* generally are larger in size than those of *F. senegalensis* or *T. grisea*[3, 5]. *M. mycetomatis* forms grains which are 1.5-5 mm in size, while the grains of *F. senegalensis* are 0.5-2 mm in size [3, 5]. Second, within many of the black grains an anamorphic matrix called cement material is surrounding the fungal hyphae. In the *M. mycetomatis* grains, this cement material is found throughout the grains, while in *F. senegalensis* and *T. grisea* the cement material is only found in the peripheral region of the grain. The center is often non-pigmented and consists of a loose network of hyphae [3, 5]. Surrounding the grain, usually a large zone of inflammatory cells is seen. These often include neutrophils. The inflammation reaction itself is encapsulated by collagen [3, 5].

Additionally, *in vitro* susceptibility towards the commonly used antifungal agents varies for the different causative agents. *M. mycetomatis* hyphae are susceptible towards the azoles with median

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minimal inhibitory concentrations (MICs) of 0.03 µg/ml for itraconazole and 0.004 µg/ml for ravuconazole, while MICs of 0.125 µg/ml and 0.25 µg/ml respectively are found for *F. senegalensis* [6, 7].

It is difficult to determine if the differences in *in vitro* susceptibility and the difference in grain morphology will have a direct impact on therapeutic efficacy since grains cannot be formed *in vitro.* Previously we demonstrated that *M. mycetomatis* grains can be formed in the invertebrate *Galleria mellonella* [8]. In sixth instar *G. mellonella* larvae, grains were formed as early as 4h after inoculation and after 72h mature grains were seen which resembled the grains in human patients [8]. Furthermore, when *M. mycetomatis* infected *G. mellonella* larvae were treated with amphotericin B or itraconazole, a similar outcome was obtained as in the *M. mycetomatis* mouse model [9, 10]. Therefore the invertebrate *G. mellonella* can be considered a good *in vivo* screening model to study *M. mycetomatis* grain and determine therapeutic efficacy.

Since grains are different for the causative agents of eumycetoma, new models for the other fungi are needed. In this study, we developed a *F. senegalensis* grain model in *G. mellonellla.* For this we tested the grain forming potential of three different *F. senegalensis* strains in *G. mellonella* larvae and compared the grain formation with the grains formed in human. This model can in the future be used to study the grain formation as well as therapeutic response towards antifungal agents for eumycetoma caused by *F. senegalensis.*

Material and methods

Galleria mellonella larvae

Sixth instar *G. mellonella* larvae from SA.G.IP (Bagnacavallo, Italy) were purchased from www.forelshop.be (Baal, Belgium). The larvae were raised under shadow protection at a

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temperature of approximately 25 °C. Active larvae without any discoloration and weighing 500 mg were selected for the experiments.

Fungal strains

F. senegalensis strains CBS132257, CBS197.79 and CBS198.79 were used in all experiments (Figure 1). Strains were identified to the species level by ITS sequencing. All isolates were obtained from the Westerdijk Fungal biodiversity center (Utrecht, The Netherlands) and maintained in the ErasmusMC on Sabouraud agar (BD, USA) at 37°C.

Infection of *G.mellonella* larvae

To generate hyphal fragments which could be injected into *G. mellonella* larvae, mycelia from F. *senegalensis* were obtained from 2 week old cultures grown on Sabouraud agar plates and inoculated in RPMI1640 culture medium supplemented with L-glutamine (0.3 g/liter; capricorn-scientific, Germany), 20mM mopholinepropanesulfonic acid (MOPS; Sigma, USA) and chloramphenicol (100 mg/liter; Oxoid, Basingstroke, United Kingdom). After 2 weeks incubation at 37°C, hyphae were collected by filtering them through a 0.22 µm filter (Nalgene, Abcoude, The Netherlands) and washed with phosphate-buffered saline (PBS; Gibco, USA). To obtain hyphal fragments, the washed hyphae were sonicated for 10 seconds at 20 µm (Soniprep, Beun de Ronde, The Netherlands) as previously also done for *M. mycetomatis* [8]. To establish the lethal dose, different inocula were prepared in PBS. These were: 10 mg/larvae, 4 mg/larvae, 0.4 mg/larvae and 0.04 mg/larvae. 40 µl was injected into the last left pro-leg of the larvae with an insulin 29G U-100 needle (BD diagnostics, Sparsk, USA). Uninfected controls were injected with 40 µl PBS. 15 larvae per group were used, and every test was replicated three times. Larvae were placed in a petridish

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containing Whattman paper and incubated at 37 °C in a normal incubator. Survival was monitored for 10 days.

Grain formation and histology

In order to observe the grain formation over time, in a separate experiment, larvae were infected and 5 larvae per group were dissected to observe grain formation visually. To be able to monitor grain formation over time microscopically, for an additional 5 larvae per time point also histology slides were prepared as previously described [8]. In short, larvae were injected via the last right pro-leg with 100 µl 10% buffered formalin and moved into 50 ml tubes filled with buffered formalin in order to fixate the tissue. After 24h incubation, the larvae were dissected into two parts and incubated for at least another 48h before they were further processed for histology via standard methods. The histology sections were stained with hematoxylin and eosin (HE) and Grocott to visualize the grains. To compare the histology sections with those from human, the grains were compared to pictures obtained from the Centers for Disease Control and Prevention [11] and published cases [12, 13]

CFU counting

To assess the number of colony-forming units of each inoculum, 50 µl undiluted, 50 µl 1:10 diluted and/or 50 µl 1:100 diluted inoculum was spread on an Sabouraud agar plate and incubated for 7 days at 37°C. After incubation the number of colonies was counted and the number of CFU per ml was determined.

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Viability of the grain

In order to determine the viability of the grains, grains from CBS132257 infection larvae were isolated and inoculated on Sabouraud agar plates containing Penicillin-Gentamycin and incubated at 37°C for 14 days. Growth was checked every day. When growth was observed, the identity of the fungal isolate was confirmed by ITS sequencing. The sequence was deposited in Genbank under **OR095724.**

Antifungal susceptibility testing

The antifungal susceptibility against itraconazole, amphotericin B and terbinafine were determined according to the previously published protocols[7]. In short the mycelia were sonicated for 10s at maximum power and incubated at 37°C including RPMI 1640 working medium containing 0.35 g/L L-glutamine (Capricorn-scientific, Germany) and 1.98 mM 4-morpholinepropane sulfonic acid (MOPS; Sigma, USA). After 7 days, The mycelia were harvested by 5 minutes centrifugation at 3400 rpm and again sonicated for 10 s at 10 µm. The resulting hyphae were further diluted to obtain a working inoculum consisting of a hyphal suspension of 68%-72% transmission at 660 nm (Novaspec II; Pharmacia Biotech) in RPMI 1640 working medium. One hundred microliter of this suspension was pipetted into round-bottom 96-well plates (Fisher Scientific, USA). In each well, 1 µl of the diluted compounds were added to reach final drug concentrations of 0.03 to 16 µg/ml for itraconazole (Janssen pharmaceutica, Beerse, Belgium) and amphotericin B (Bristol-Myers Squib, USA), and 0.125 to 64 µg/ml for terbinafine (Sigma, Missouri, USA). Then, 20 µl of a resazurin (R7017, Sigma Aldrich, The Netherlands) working solution(0.15mg/ml) was added to the wells before incubation. A deep blue color was noted in all wells at the start of incubation. Then the plate was incubated for 7 days at 37°C. After incubation, 100 µl of supernatant was moved into a flat-bottom 96-well plate (Greiner

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Bio-One, The Netherlands). In the wells where the fungus was not inhibited in growth, the dark blue color of resazurin was changed to the pink color of resorufin. The intensity of the color was measured with spectrophometer (Epoch 2, BioTek, USA) at 600 nm.

Antifungal treatment

In order to determine the efficacy of currently used antifungal agents, *G. mellonella* were infected with 10 mg/larvae *F. senegalensis* as described above. On 4h, 24h, 52h after infection 15 larvae per experiment were treated with either 1 mg/kg amphotericin B (Fungizone, BristolMyers Squibb, Utrecht, The Netherlands), 5.7 mg/kg itraconazole (Janssen Pharmaceuticals, Beerse, Belgium), 7.14 mg/kg terbinafine (Sigma, Missouri, USA) or PBS. Antifungal agents were administered in a 20 µl volume, each time via a different proleg with an insulin 29G U-100 needle (BD diagnostics, Sparsk, USA). Larval survival was monitored for 10 days. Every treatment was replicated 3 times.

Statistical analysis

To compare the survival rate for the different experimental groups a Log-Rank test was performed. A p-value <0.05 was considered significant. All calculations were performed in GraphPad Prism 8.

Results

Survival of *G. mellonella* larvae infected with *F. senegalensis*

A dose dependent survival was noted for three different *F. senegalensis* isolates in *G. mellonella* larvae (Fig.2). After infection with concentrations of 0.04 mg/larvae and 0.4 mg/larvae, almost all larvae remained alive during the 10 day observation period (Log-Rank, $p>0.05$, for all strains tested).

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When larvae were infected with 4 mg/larvae or 10 mg/larvae, enhanced mortality was noted. The lowest survival rates were noted when larvae were infected with strain CBS197.79 (Figure 2A), followed by strain CBS132257 (Figure 2B) and strain CBS198.79 (Figure 2C). Although working with wet-weight did result in reproducible inocula (Figure 2D), the final CFU/ml did differ between strains. An inoculum of 10 mg/larvae corresponded to $7.95 \times 10^5 - 9.3 \times 10^5$ for strain CBS197.79, $3.63 \times 10^5 - 5.52 \times 10^5$ CFU/ml for strain CBS132257 and $1 \times 10^5 - 1.915 \times 10^5$ for CBS198.79, indicating that there was a correlation between CFU and pathogenicity.

Grain formation during infection

To determine if *F. senegalensis* was able to form grains in *G. mellonella* larvae, larvae were dissected at several time points. Macroscopically, grains were observed after 24h in all larvae investigated (Table 1). After 4h, black grains were noted in only 2 out of 5 larvae in the group infected with 0.04 mg *F. senegalensis* CBS132257 and in only 3 out of 5 larvae for strain CBS198.79 and in 4 out of 5 for strain CBS197.79. At the higher doses grains were also observed at 4h after infection. To check if the grains were viable, the grains were inoculated on agar and after 14 days growth was observed. The morphology of the isolate was similar to that of the original *F. senegalensis* isolate and the identity was confirmed by ITS sequencing (Genbank OR095724). To study the different stages of grain formation over time in more detail, histology slides were also prepared. When larvae were infected with 10 mg/larvae, after 4 hours of infection hyphal masses were noted which were surrounded by inflammatory cells (Figure 3A and 3F). There was no clear grain structure noted at this point of time. After 24 hours the shape of the grain is formed; in the periphery a more dense hyphal mycelium is present (Figure 3B and 3G). Surrounding the grain, inflammatory cells and a capsule were noted. After 96 hours, a melanised red-brownish cement material is observed at the periphery of the grain (Figure 3C and 3H). Loose fungal hyphae and some individual host cells are found in the center of the grain and a light cement material is noted as well. This cement material is not as dense as in the

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periphery. Furthermore the grain is surrounded by a collagen-like capsule and inflammation cells. At 7 days after infection the grain resembles the grain noted after 96 hours. Again a more dense cement material is noted at the periphery of the grain and a capsule and host cells are surrounding the grain(Figure 3D and 3I). However, individual host cells are no longer observed in the center of the grain. The more dense cement material at the periphery of the grain was also noted in grains obtained from patients, as presented by Dr. Ajello (Figure 3E)[11]. After 10 days no larvae were surviving and the grain morphology could not be studied.

In vivo efficacy of itraconazole, terbinafine and amphotericin B in *F. senegalensis* infected *G. mellonella* larvae

To determine the efficacy of commonly used antifungal agents in our model, *G. mellonella* larvae were infected with *F. senegalensis* and treated with amphotericin B, itraconazole and terbinafine. Larvae were treated on three consecutive days. The MICs for the two *F. senegalensis* strains were quite comparable. Both strains had an MIC of 4 µg/ml for amphotericin B. Lower MICs were observed for itraconazole (1 µg/ml for strain CBS197.79 and 0.5 µg/ml for strain CBS133257) and terbinafine (0.125 µg/ml for strain CBS197.79 and 0.25 µg/ml for strain CBS133257)(Table 2). Enhanced survival was noted on day 10 in larvae infected with strain CBS197.79 and treated with amphotericin B (Figure 4A, Log-Rank, $p=0.043$). For strain CBS132257, enhanced survival was only noted during treatment (Figure 4B, day 4, Log-Rank, $p=0.046$), not at day 10, the end of the observation period (Log-Rank, $p=0.238$). Treatment with itraconazole and terbinafine did not result in enhanced survival in *F. senegalensis* infected *G. mellonella* larvae (Log-Rank, $p>0.05$ in all conditions).

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Discussion

In this study, we developed a *F. senegalensis* grain model in *G. mellonella* larvae. As in the *M. mycetomatis* grain model in *G. mellonella,* black grains were formed early in the infection and resembled those seen in human patients [8]. However there were differences noted between the two models. First of all, the time to form a mycetoma grain differed between the two causative agents. For *M. mycetomatis,* only 4 hours were needed to form an early grain [8]. For *F. senegalensis* this took longer. At 4 hours after inoculation *F. senegalensis* hyphae were indeed clustered but a grain structure was not observed yet.

Also the morphology of the mycetoma grain differed in the two *G. mellonella* grain models. In the *F. senegalensis* grain model, the distribution of cement material was different from that observed in the *M. mycetomatis* grain[8]. In *F. senegalensis* grains the cement material was only found in the periphery, while in *M. mycetomatis* grains this was more evenly distributed [14]. In the *F. senegalensis* grain the central portion of the grain consists of a the loose network of hyphae, while *M. mycetomatis* grains are compact [5]. These differences are similar to the differences seen in human grains and suggest that despite the evolutionary distance between *G. mellonella* larvae and humans, grain formation occurs in a similar way. The immune system of *G. mellonella* does not have an adaptive immune system but their innate immune system is similar to that of humans[15-20]. Thus, the innate immune system seems to be necessary for grain formation although the pathogen determines the shape of the grain since the form of *F. senegalensis* and *M. mycetomatis* grains clearly differs in the same host. Recently, proteomics [21] and transcriptomics [22] studies have been performed in the *G. mellonella* larval model to unravel the processes leading to grain formation in *M. mycetomatis* infected larvae. In these studies metal acquisition of the pathogen from the host seemed to be an important process in grain formation [21-23]. To determine which of these

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processes are similar and which are different between the two pathogens will offer us more insight in the process of mycetoma grain formation in the future and might lead to novel therapeutic targets.

Despite the clear differences in grain morphology, similarities were found in the in vivo efficacy of antifungal agents. In our study we demonstrated that only amphotericin B had some therapeutic efficacy in *F. senegalensis* infected *G. mellonella* larvae. No enhanced survival was noted for itraconazole or terbinafine. In *M. mycetomatis* infected *G. mellonella* larvae amphotericin B also resulted in significantly prolonged larval survival and itraconazole did not [9]. An outcome similar to what was observed in mice [10]. This indicates that despite the morphological differences, amphotericin B can penetrate in a similar fashion in these grains. For *M. mycetomatis* infected *G. mellonella* larvae, prolonged larval survival was noted when larvae were treated with 7.14 mg/kg terbinafine, however, in our experiments this terbinafine dosage did not prolong the survival of *F. senegalensis* infected larvae. This can have several reasons. The first reason could be differences in antifungal susceptibility. The two *F. senegalensis* strains had very low MICs for terbinafine. Only 0.25 to 0.125 µg/ml were needed to inhibit strain CBS197.79 and strain CBS132257, respecitively. In contrast, *M .mycetomatis* strain mm55, the strain used in the *M. mycetomatis G. mellonella* grain model, was not inhibited at 100 µM (32.7 µg/ml)[24]. Other *M. mycetomatis* isolates were more susceptible than mm55 as 8 µg/ml terbinafine is needed to inhibit the growth of 50% of all isolates tested [25]. This was much higher than the MICs observed for *F. senegalensis* and therefore not very likely the reason behind this difference. A second explanation could be that the thick peripheral cement material noted in *F. senegalensis* could be a strong physical barrier for terbinafine. For other fungal species, such as *Microsporum canis* and *Trichophyton mentagrophytes*, a 1000 to 2000-fold increase in MIC was observed between planktonic cells and biofilms[26], suggesting that the presence of this thick cement material is the most likely explanation for the difference in efficacy. For *M. mycetomatis,* currently an Open Source drug discovery program is available to identify novel drugs for mycetoma. However, in order to identify drugs which will also have efficacy against other

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causative agents it might be wise to include *F. senegalensis* in the screening cascade as well. With the development of this *F. senegalensis* grain model in *G. mellonella* larvae a first step is made for this.

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Table 1: *F. senegalensis* grain formation in surviving larvae over time

	Grain formation as macroscopically observed in surviving larvae				
	4h	**24h**	**3 days**	**7 days**	**10 days**
CBS132257					
0.04 mg/larvae	2/5	5/5	5/5	5/5	5/5
0.4 mg/larvae	4/5	5/5	5/5	5/5	5/5
4 mg/larvae	5/5	5/5	5/5	5/5	5/5
10 mg/larvae	5/5	5/5	4/4	2/2	no survival
CBS197.79					
0.04 mg/larvae	4/5	5/5	5/5	5/5	5/5
0.4 mg/larvae	5/5	5/5	5/5	5/5	5/5
4 mg/larvae	5/5	5/5	5/5	5/5	5/5
10 mg/larvae	5/5	5/5	4/4	3/3	no survival
CBS198.79					
0.04 mg/larvae	3/5	5/5	5/5	5/5	5/5
0.4 mg/larvae	5/5	5/5	5/5	5/5	5/5
4 mg/larvae	5/5	5/5	5/5	5/5	5/5
10 mg/larvae	5/5	5/5	5/5	3/3	no survival

5 larvae per group were dissected after 4h, 24h, 3days, 7days, 10days infection, and grain formation was observed visually. No survival means that there were no viable larvae left. This could be due to death, or the formation of cocoons[15].

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Table 2: MICs for 4 isolates *Falciformispora senegalensis* against 3 antifungal agents and strain from CBS132257 infected larvae body

Isolates	Itraconazole MIC mean (ug/ml)	Amphotericin B MIC mean (ug/ml)	Terbinafine MIC mean (ug/ml)
CBS197.79	1	4	0.125
CBS198.79	0.125	2	0.125
CBS132257	0.5	4	0.25
CBS132257 from infected larvae	0.5	8	0.25

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Figure 1:

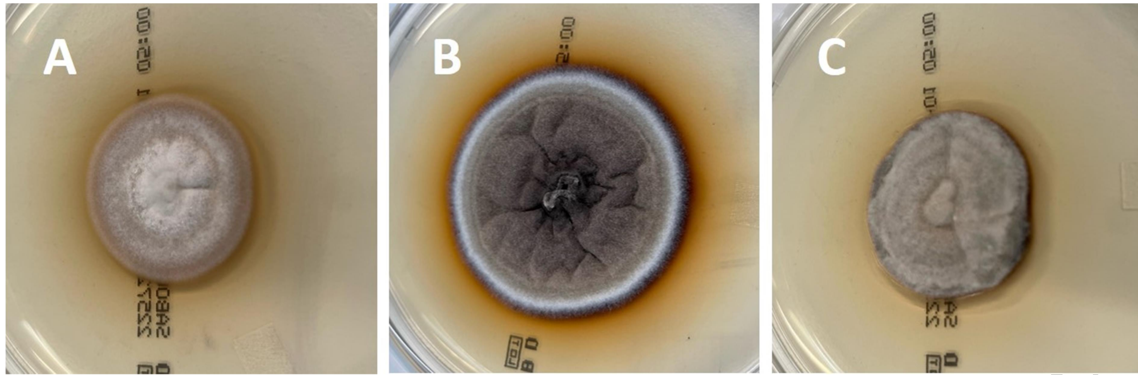

Figure 1: The colonial morphology of the three *F.senegalensis* strains selected to infect *G.mellonella* larvae. *F. senegalensis* strain CBS197.79 (A), CBS132257 (B), CBS198.79(C) grown on Sabouraud agar for 2 weeks at 37°C.

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Figure 2:

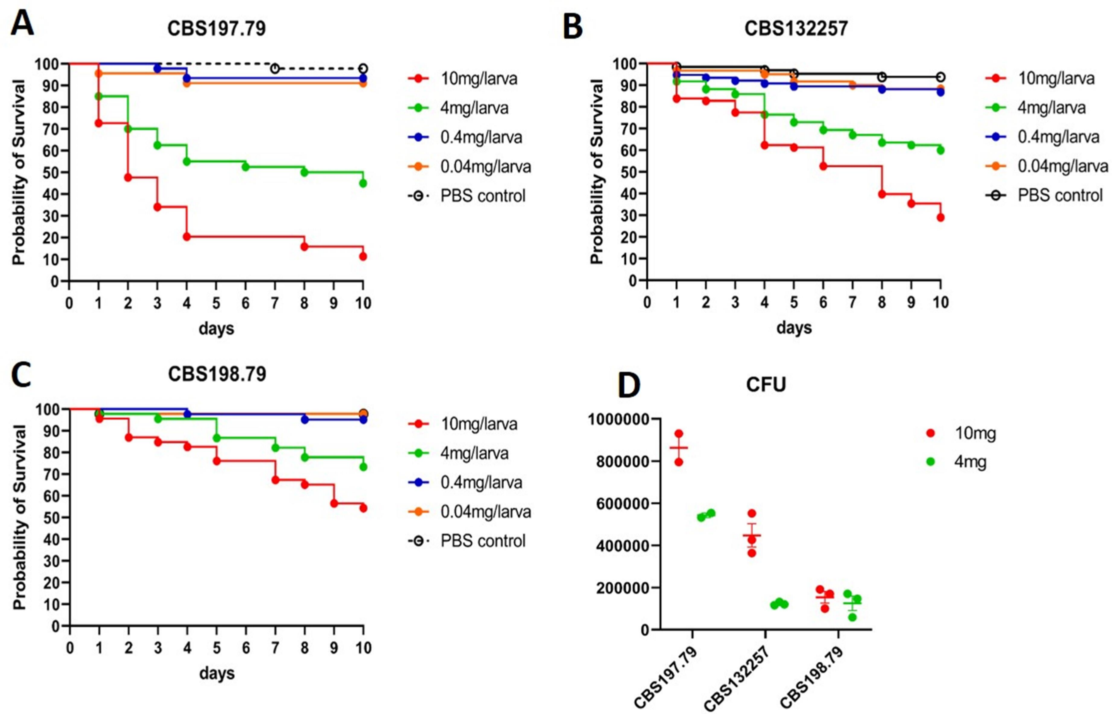

Figure 2: survival curve of three *F.senegalensis* grains and CFU counting. The survival curves for CBS197.79 (panel A), CBS132257 (panel B) and CBS198.79 (panel C) are shown for four different inocula per strain. The inocula are displayed as follows: 10 mg/larvae in red, 4 mg/larvae in green, 0.4 mg/larvae in blue and 0.04 mg/larvae in orange. The survival of the non-infected larvae are shown as a black dashed line. In panel D, the number of colony forming units are shown. The CFU/ml for three independent experiments are shown for the inocula of 10mg/larvae (red) and 4 mg/larvae (green). The CFU counting was performed after 7 days incubation at 37°C.

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Figure 3:

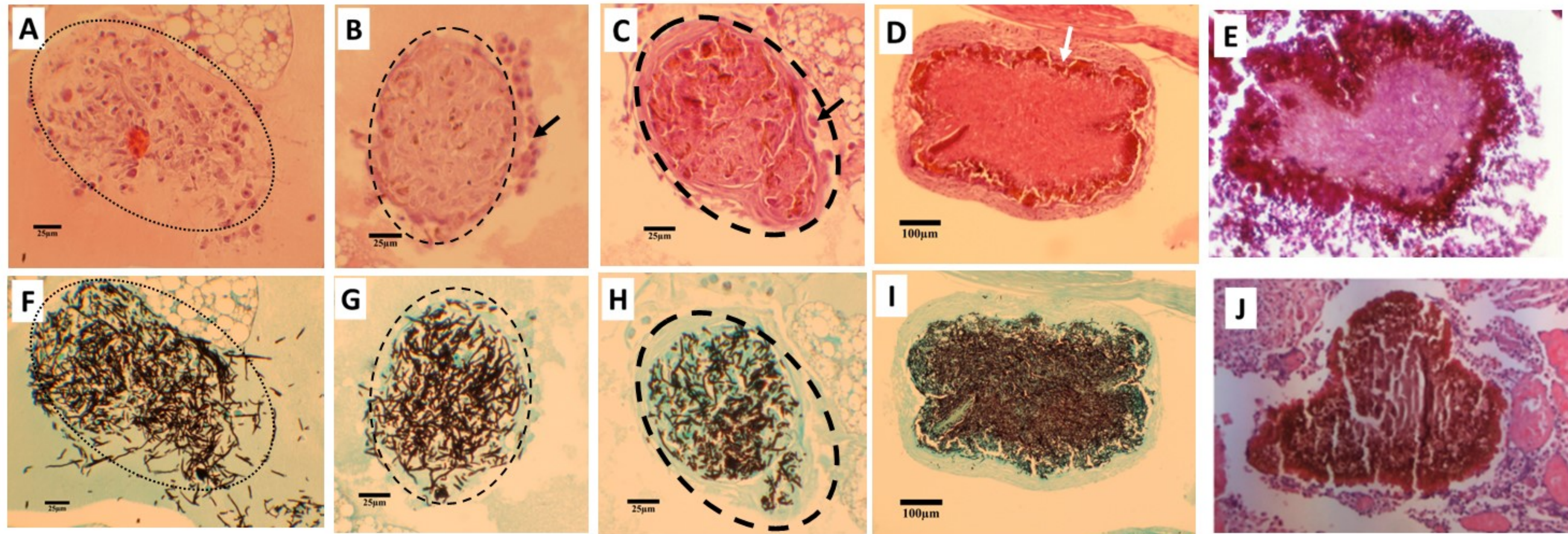

Figure 3: grain formation over time as observed by histology. Grain formation was observed using HE staining (panels A-D) and Grocott staining (panels F-I). In this panel, the grain formation for strain CBS132257 infected with 10 mg per larva is noted. After 4 hours of infection hyphal masses (black strained with Grocott as seen in panel F) were noted which were surrounded by inflammation cells (Panel A). After 24h, a more clear grain structure is noted within the black dotted line (panels B and G) and immune cells are attracted to the grain shown with black arrow in panel B. After 96 hours (panels C and H), a collagen-like capsule surrounds the grain and immune cells shown with black arrow are still clearly seen surrounding the grain. A mature *F. senegalensis* grain is noted after 7 days of infection (panels D and I). In panel D it is seen that a very dense cement material is present at the periphery of the grain shown

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with white arrow. The center of the grain is less densely melanised. Also a thick capsule is noted around the grain. The scale bar in panels A-C and F-H is 25µm, while the scale bar in panels D and I is 100µm. Panels E and J were cited from reference 11 and 8. Panel E shows the grain formed by *F.senegalensis* in an infected patient by HE staining, while Panel J shows a grain formed by *M.mycetomatis* in an infected patient. In Panel E the *F.senegalensis* grain from patient shows a very dense material in the periphery and a less densely melanised material in the center of the grain. The *M. mycetomatis* grain from a patient in panel J shows cement material through the whole grain.

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Figure 4:

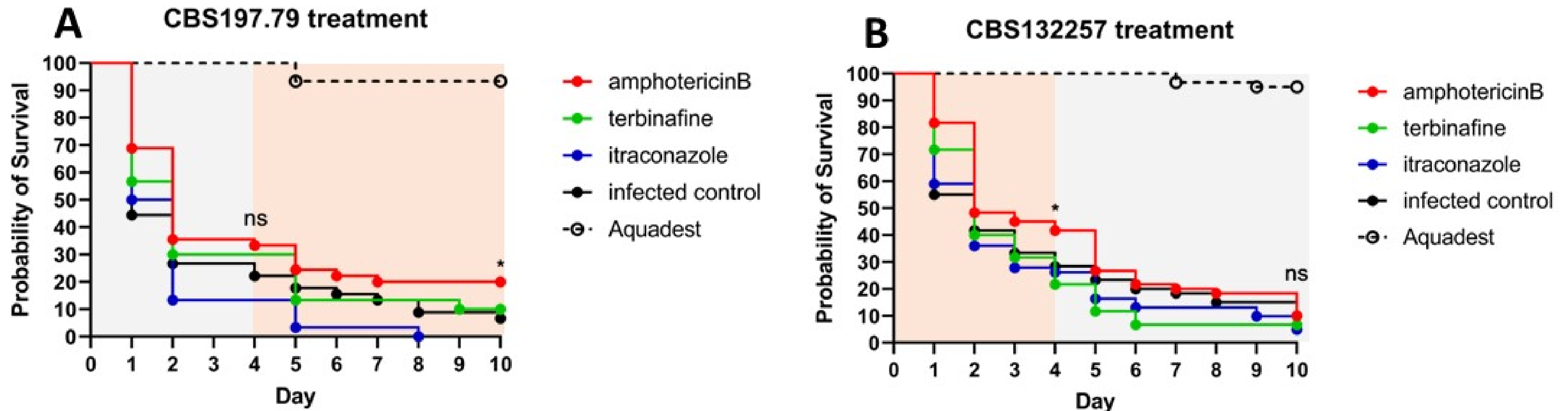

Figure 4: Survival curves after treating *F. senegalensis* infected *G. mellonella* larvae with different antifungal agents. In panel A, the survival curves of strain *F. senegalensis* CBS197.79 are shown. In panel B, the survival curves of strain *F. senegalensis* CBS132257. Larvae were infected with 10 mg/kg *F. senegalensis* and treated 4h, 28h and 52h after infection with either PBS (black line), 1 mg/kg amphotericin B (red line), 7.14 mg/kg terbinafine (green line) or 5.7 mg/kg itraconazole (blue line). In panel A, amphotericin B prolonged the survival of CBS197.79 infected larvae survival within 10 days (p value =0.043(*)), but not after 4 days (p value =0.089 (ns)). In panel B, amphotericin B prolonged CBS132257 infected larvae survival within 4 days (p value =0.046 (*)), but not for 10 days (p value =0.238 (ns)). Treatment with terbinafine or itraconazole didn't prolong the survival or CBS132257 or CBS197.79 infected larvae.

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