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Galleria melonella as an experimental *in vivo* host model for the fish-pathogenic oomycete *Saprolegnia parasitica*

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ABSTRACT

Oomycetes are eukaryotic pathogens infecting animals and plants. Amongst them *Saprolegnia parasitica* is a fish pathogenic oomycete causing devastating losses in the aquaculture industry. To secure fish supply, new drugs are in high demand and since fish experiments are time consuming, expensive and involve animal welfare issues the search for adequate model systems is essential. *Galleria mellonella* serves as a heterologous host model for bacterial and fungal infections. This study extends the use of *G. mellonella* for studying infections with oomycetes. Saprolegniales are highly pathogenic to the insects while in contrast, the plant pathogen *Phytophthora infestans* showed no pathogenicity. Melanisation of hyphae below the cuticle allowed direct macroscopic monitoring of disease progression. However, the melanin response is not systemic as for other pathogens but instead is very local. The mortality of the larvae is dose-dependent and can be induced by cysts or regenerating protoplasts as an alternative source of inoculation.
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1. Introduction

Oomycetes are eukaryotic organisms that are morphologically similar to fungi but genetically related to brown algae (Baldauf et al., 2000). The vast majority of oomycete species are pathogenic to animals and plants, significantly affecting the agri- and aquaculture industries, but also responsible for wiping out wild populations (van West, 2006). The most devastating oomycete affecting salmon farming is *Saprolegnia parasitica* because it infects fish eggs as well as salmonid fish (Stueland et al., 2005; van West, 2006; van den Berg et al., 2013). However, also other fish in fresh water and arthropods such as crayfish and river insects are susceptible to *S. parasitica* and other Saprolegnia species (Krugner-Higby et al., 2010; Sarowar et al., 2014). The disease caused by Saprolegniaceae is called saprolgeniosis (or saprolegniasis) and once the infection is established, cotton-like white to grey patches on the skin, especially around the fins, as well as in the gills can be observed with destruction of the epidermis (Khoo, 2000; Bruno et al., 2011). While the infection progresses, fish become lethargic and lose their equilibrium (Bruno et al., 2011). Final death of the fish occurs due to an osmotic shock caused by haemodilution (Roberts, 1993; Torto-Alalibo et al., 2005).

While research on plant pathogenic oomycetes allows *in planta* infection assays without ethical objections (Kanneganti et al., 2007; Bhaskar et al., 2009), studies on fish pathogens such as *S. parasitica* on its native salmonid hosts are expensive, time consuming and raise animal welfare issues. Moreover, it is difficult to replicate the complex conditions under which infections naturally occur (Kales et al., 2007; Minor et al., 2014). Due to the economic impact of *S. parasitica*, *Saprolegnia diclina* and other *Saprolegnia* species in hatcheries, eggs are also frequently used for challenge experiments (Songe et al., 2016; Eissa et al., 2013). However, these studies aim more at replicating conditions of outbreaks on eggs specifically than investigating the host–pathogen interaction and insights of the defence system gained from egg experiments cannot simply be transferred to fish. Another way to study the immune response to saprolegniosis is the work with cell lines (Kales et al., 2007; de Bruijn et al., 2012; Belmonte et al., 2014); although the humoral immunity component is absent in cell line studies (Magnadóttir, 2006). Hence, a reliable and simple model system to screen potential compounds against Saprolegnia species and to study virulence factors under controlled conditions is needed.

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Due to their low cost, easy handling, short reproductive cycles and simple housing, insects and nematodes have become increasingly popular as alternative model hosts in infection assays (Cook and McArthur, 2013). For many fungal pathogens, such as the human opportunistic pathogen *Candida albicans*, the greater wax moth (*G. mellonella*) is used as an alternative approach to classical mouse model system (Chamilos et al., 2007). The insects are economical, simple to maintain and viable at a wide range of temperatures allowing experimentation at temperatures more similar to the natural conditions in freshwater ecosystems (10–20 °C). *G. mellonella* can be used without ethical concerns and results can be obtained within days. Similar to the skin of vertebrates, the cuticle of *G. mellonella* provides the first defence barrier against pathogens (Magnadóttir, 2006). In the insect itself, the pathogen has to overcome the interconnected cellular and humoral immune response contained in the haemolymph, the blood analogue of insects (Matha & Áček, 1984; Brennan et al., 2002). Hence, the insect immune system comprises important similarities to the vertebrate immune system at a structural and functional level (Zhao and Kanost, 1996; Rock et al., 1998; Wittwer et al., 1999; Kavanagh and Reeves, 2004; Lemaitre and Hoffmann, 2007) and therefore provides a potential model system to study the pathogenicity of *S. parasitica*.

Here we report on the use of *G. mellonella* as a novel *in vivo* model to study animal pathogenic oomycetes. The virulence of two *Saprolegnia* species was determined through the mortality rates of infected insects. Progression of infection was monitored enzymatically and by visual inspection as well as histological examinations. Since the production of zoospores or cysts can be challenging, especially for newly discovered species, we show that protoplasts can also be used as an inoculum.

2. Material and methods

2.1. Galleria mellonella

Sixth instar larvae of *G. mellonella* (Lepidoptera: pyralidae) (Livefood UK Ltd. Somerset, UK) were kept in wood shavings in the dark at 12 °C. Prior to a challenge experiment, healthy insects without black or grey marks visible on the skin and a swift response to stimulation (being flipped on their backs) were separated and used. Twenty larvae for each group were inoculated with 10 μL of cysts or protoplasts or as a negative control with 10 μL PBS via the last left pro-leg into the haemolymph with a 10 μL microsyringe (VWR, 549-0199) as described elsewhere (Mylonakis et al., 2007; Bergin et al., 2006). Following inoculation, larvae were incubated at 24 °C (optimal *in vitro* growth temperature for *S. parasitica*) in the dark. Insects were investigated as indicated. An insect was scored as dead when it did not show a response to physical stimulation with forceps.

2.2. Oomycete strain maintenance, zoospore/cyst and protoplast production

S. parasitica C65 (CBS223.65) was originally isolated from young pike (*Esox lucius*), *S. parasitica* N12 (VI-02736) originated from parr of Atlantic salmon in Scotland (Lochailort) (Jiang et al., 2013). *Saprolegnia delica* (DON160516) was isolated from caddisfly larvae (*Rhyacophila dorsalis*) sampled from the river of Don near Monymusk, Scotland. *Phytophthora infestans* (P88069) was originally isolated from tomato in the Netherlands (Kamoun et al., 1998). Stock cultures of each strain were kept at 12 °C on potato-dextrose agar. For growth tests, agar plugs (⌀ 0.5 cm) were cut from the stock cultures and mycelial growth was tracked over time.

For the production of zoospores, an agar plug of mycelia was grown in pea broth (125 g pea/L) at 24 °C. Sporulation of 3 d old mycelia was induced by washing 3 times with deionised water followed by 24 h incubation in Tap:tank water (sterile water from a fish tank diluted 1:2 in tap water) at 24 °C. Zoospores were separated from sporulating mycelia through 40 μm cell strainers and encysted during the washing procedure.

For the production of protoplasts, 24 h old mycelium was digested in 0.5 M sorbitol supplemented with cellulose (5 mg/mL, Sigma–Aldrich, C8546) and lysing enzymes (5 mg/mL, from *Trichoderma harzianum*, Sigma–Aldrich, L1412) at 200 rpm at 25 °C for 1 h. Protoplasts were separated from non-degraded mycelia through 40 μm cell strainers and washed 3 times in 0.5 M sorbitol to remove digestive enzymes and small debris from the disrupted mycelia. Before injections, protoplasts were allowed to regenerate in LB medium at 24 °C for 2 h.

For injections into *G. mellonella*, cysts and protoplast were concentrated by centrifugation, counted and diluted in PBS to the final concentration as indicated.

2.3. Histological examination

Insect preparation for paraffin embedding was performed essentially as described elsewhere (Perdoni et al., 2014). Briefly, 50 μL of 4 % PFA/PBS was injected into the last right pro-leg before immersing insects in 4 % PFA overnight at 4 °C. Fixed insects were embedded in either paraffin or OCT embedding matrix (Cellpath, KMA-0100-00A). Samples were cut in 10 μm sections on a cryostat (Leica 1850) at −20 °C.

Staining with GMS (Grocott methamine-silver) was performed by the NHS Grampian Biorepository (Aberdeen, Scotland). Briefly, slides were deparaffinised, hydrated and oxidised in 2 % chromic acid for 5 min. Treatments in 1 % sodium metabisulfit (1 min), quickly in methenamine silver solution and 0.5 % gold chloride followed before a final incubation in 5 % sodium thiosulfate (3 min). Counterstaining was performed with working light green solution. Slides were thoroughly rinsed with water between individual steps.

For PAS stain slides were deparaffinised, hydrated and oxidised in 0.5 % periodic acid schiff (PAS) for 5 min. Followed by an incubation in Schiff reagent for 15 min and a counter stain with Mayer's Haematoxylin Solution for 1 min. Slides were thoroughly rinsed with water between individual steps. Slides were scanned on a Zeiss Axio Scan Z1.

For whole mount microscopy insects were opened and the cuticle spread out on a glass slide after removing internal organs. Samples were inspected on an inverted microscope and images were taken using a Lumenera Infinity2 camera.

2.4. DNA extraction

For PCR amplification, DNA was isolated from mycelia and infected larvae of *G. mellonella*. A piece of pea-broth-cultured mycelium or a cut larvae were transferred into 2 mL tubes with glass beads (425–600 μm) and 800 μL DNA extraction buffer (Sarowar et al., 2014) containing RNAseA and homogenized in a FastPrep-24™ 5G tissue homogenizer (MP Biomedicals, 4 × 40 s, 6 m/s, 2 min breaks). After disruption samples were incubated at 55 °C for 30 min followed by a centrifugation to remove debris at 10,000 × g for 10 min. The supernatant was used for DNA extraction by a phenol-chloroform method as described elsewhere (Zelaya-Molina et al., 2011). Briefly, for DNA extraction from insects an additional phenol extraction step was performed. DNA was precipitated in 1 mL isopropanol overnight at −20 °C and pelleted by centrifugation at 4 °C. Pellets were washed twice with 70 %

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