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When aspergillosis hits the fan: Disease transmission and fungal biomass in diseased *versus* healthy sea fans (*Gorgonia ventalina*)

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ABSTRACT

Sea fan aspergillosis, first reported in the Caribbean in the 1990s, is one of the best-characterized coral diseases. The disease is named after *Aspergillus sydowii*, which was proposed as the sole pathogen. Here, inoculation of healthy fans in aquaria with *A. sydowii* failed to induce purpling and tissue necrosis, the characteristic signs of aspergillosis. Grafting experiments *in situ*, using diseased and healthy tissues, failed to induce permanent purpling in most cases, suggesting that fans have developed resistance to aspergillosis. The temporary purpling is likely a non-self-recognition response and not an exclusive criterion to diagnose aspergillosis. For the first time, we quantified fungal biomass in fans demonstrating that ergosterol concentration in healthy tissues was generally higher than that in the diseased tissues, contrary to our expectations for a fungal-induced disease. These results suggest that there are still considerable gaps in our understanding of aspergillosis, coral-associated fungal communities and coral non-self-recognition responses.

Introduction

Coral diseases are on the rise and pose a substantial threat to the health of reefs. Since the first reported case in the 1970s (Antonious 1973), at least 29 coral diseases have been described worldwide, three-quarters of them from the Caribbean (Mydlarz *et al.* 2008). Three decades of studies have accumulated a wealth of information on the potential consequences for marine ecosystems (Green & Bruckner 2000; Rosenberg & Ben-Haim 2002). However, remarkably little is known about their etiology and pathology (Work & Aeby 2006; Work *et al.* 2008), and even less is known about the immune responses of corals to pathogens.

Aspergillosis of sea fans (*Gorgonia* spp.) is among the best-characterized diseases of corals. It is named after the saprotrophic fungus *Aspergillus sydowii*, which was reported as the sole causative agent (Smith *et al.* 1996; Geiser *et al.* 1998). At present, 'aspergillosis' is used to refer to diseases not only in *Gorgonia* spp. but also in other gorgonians from the Caribbean and the tropical Pacific (Smith & Weil 2004). Necrotized tissue and purpling are considered to be characteristic signs of aspergillosis (Petes *et al.* 2003; Alker *et al.* 2004; Mullen *et al.*

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2004; Kim et al. 2006). Disease transmission may occur *via* contact with infected colonies (Jolles et al. 2002) as demonstrated by *in situ* grafting experiments (Smith & Weil 2004) or by free fungal propagules, as suggested by inoculation experiments with pure strains of *A. sydowii* (Smith et al. 1996; Geiser et al. 1998; Dube et al. 2002). In recent years, however, some studies have suggested that *A. sydowii* may not be the sole pathogen implicated in 'aspergillosis' (Toledo-Hernández et al. 2007, 2008; Zuluaga-Montero et al. 2010). In addition, tissue purpling may be a non-specific immune response rather than a specific sign of 'aspergillosis'. For example, tissue purpling may result from physical contact with fouling organisms such as *Millepora* sp. and macroalgae or from predation (Alker et al. 2004).

Unfortunately, the extent of fungal colonization of sea fans (healthy or diseased) has never been quantified using either molecular approaches (e.g. qPCR) or biochemical markers (e.g. ergosterol). Ergosterol is the principal fungal-specific sterol in the cell membranes of higher fungi, similar to cholesterol in animals (e.g. Gessner & Chauvet 1993; Gessner et al. 2007). Ergosterol is routinely used as a specific biomarker for fungi in both terrestrial and aquatic environments that allows estimation of fungal biomass when the mycelium and its substratum or host cannot be easily separated (e.g. Newell 1992; Gessner & Chauvet 1993). However, estimates of fungal biomass associated with corals are currently lacking.

In this study, we assessed the susceptibility of the Caribbean sea fan *Gorgonia ventalina* to *A. sydowii* by performing inoculation experiments similar to those that helped to establish *A. sydowii* as the pathogen of sea fans (Smith et al. 1996; Geiser et al. 1998). We also repeated the grafting experiments used to postulate the transmissibility from diseased to healthy sea fan tissue and signs of aspergillosis (i.e. necrotic tissue surrounded by a purpled ring) (Smith & Weil 2004). We expected that the inoculation and disease tissue grafting trials in this study would invariably induce disease signs in healthy fans (e.g. Smith et al. 1996; Dube et al. 2002). Finally, we compared levels of fungal colonization of healthy and diseased tissues by estimating fungal biomass from ergosterol concentrations. We hypothesized that diseased sea fan tissues will have higher fungal biomass (ergosterol) than healthy tissues due to proliferation of *A. sydowii*.

Materials and methods

Pure culture inoculation experiment in aquaria

Eight healthy *G. ventalina* sea fans (i.e. no lesions, purpling or any overgrowth by fouling organisms) between 600 cm^2 and 900 cm^2 were collected at Escambrón Beach, San Juan, Puerto Rico and brought to the University of Puerto Rico, Río Piedras. In the laboratory, sea fans were placed in individual 72.5 l tanks filled with fresh sea water and checked for the presence of *A. sydowii* by removing and culturing one 1 cm^2 tissue sample from each sea fan following the method of Toledo-Hernández et al. (2007). Morphological characteristics were used to identify the fungi that grew from the tissue samples (Klich 2002). Only one of eight sea fans was found positive for *A. sydowii*, and this sea fan was excluded from the experiment. Six d after establishment in tanks and moments before the inoculation of the sea fans with *A. sydowii*, one tissue wound was inflicted on each colony by scraping tissue with a scalpel down to the axial skeleton. Wounding was intended to enhance contact between fungal hyphae and internal tissue. Wounds were approximately 2 cm^2 and adjacent to the area previously sampled. Experimental fans were inoculated with an *A. sydowii* strain isolated from a sea fan and identified by sequencing the nuclear ribosomal ITS region (GenBank #EU554604, Toledo-Hernández et al. 2008). Inoculation was conducted by attaching a 2 × 2 cm block of glucose peptone yeast extract agar (GPYA) overgrown with mycelium to the wounded area. Approximately 100 000 spores of *A. sydowii* were placed on the agar blocks before the start of the experiment and incubated at 28° C for 5 d only to allow hyphal growth but not yet sporulation. As a control, an uninoculated 2 × 2 cm GPYA block was also attached to each sea fan 2–4 cm from the experimental block. Visual examinations were conducted daily for the next 7 d after which agar blocks were removed. To check if inoculation with *A. sydowii* was successful, tissue samples (1 cm^2 each) were collected from the experimental and control areas of each fan and cultured as described by Toledo-Hernández et al. (2007). Sea fans were monitored for signs of tissue purpling and necrosis for an additional month.

Grafting experiments

Healthy recipients

To test the transmission of the disease (i.e. development of necrotized tissue surrounded by a purple ring of tissue) by allografts of diseased tissue, 20 healthy colonies (having no signs of purpling or necrotized tissue) were tagged at Escambrón Beach. (In this study, 'allograft' is a tissue transplant between colonies which are presumed to be genetically distinct individuals; 'isograft' is a tissue transplant within a single colony.) Tagged colonies ranged in size from 800 cm^2 to 1200 cm^2 and were located within an area of approx. 50 × 100 m and a depth of 1 m. Each sea fan colony received three grafts of 4 cm^2: one purpled and one healthy allograft, and one healthy isograft (Fig 1A, C). The healthy allografts and isografts were used as controls for grafting effects. All grafts were fastened with cable ties, equidistant from one another and near the center of the recipient colonies.

Diseased recipients

To test induction of tissue purpling and necrosis by diseased isografts in already diseased sea fan colonies, six diseased colonies at the same location and with size similar to those from the previous experiment were tagged. As before, each colony received three grafts of 4 cm^2 each: one diseased and one healthy isograft and one healthy allograft (Fig 1B, D). The healthy isografts and allografts were used as controls for grafting effects.

For both experiments, visual examination and photos of each experimental colony were taken every 3–7 d for 6 weeks.

Fungal biomass in healthy and diseased tissues

Ergosterol was extracted from 2 × 2 cm tissue samples taken from 28 *G. ventalina* colonies ranging from 600 cm^2 to 900 cm^2,