Aspergillosis of *Pseudopterogorgia americana*: Increased Host Range of *Aspergillus sydowii* from the Wider Caribbean

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Abstract: Reports of gorgonian diseases have been increasing in the past few years,but, with the exception of *Gorgonia* spp., the etiological agents responsible are generally unknown. In the summer of 1999, populations of *Pseudopterogorgia americana* were observed with lesions and galls, somewhat similar to *Aspergillus sydowii* infections on *Gorgonia ventalina*, in Bermuda. Surveys of three sites were made, repeated the following two years, and compared with sites in the Bahamas. Microscopic observations of affected tissue and subsequent pure culture studies indicated the pathogen was also *A. sydowii*. Over half of the colonies at the Bahamas site were affected, but about 60% of those showing lesions, had 10% or less of the colony affected. Pure culture and inoculation studies confirmed that the pathogen was *A. sydowii*. Isolates from *P. americana* also were pathogenic on *Gorgonia ventalina*. Results show that *A. sydowii* can also infect *P. americana* and that the disease may be widespread among gorgonians.

Introduction

Diseases of coral reef organisms appear to be increasing in frequency and diversity[1, 2]. The result of these epizootics is often devastating[3] and frequently leads to shifts in overall community structures[4]. The approaches to the study of diseases of coral reef organisms and the pathogens responsible are presently an area of intensive study[5-7]. In general, diseases of scleractinian corals are better understood than diseases of gorgonians[7, 8], with the exception of aspergillosis of sea fan corals (*Gorgonia* spp.). A sea fan epizootic was first reported in the 1980's[9-11], but the pathogen was not identified. A less virulent, but more widespread epizootic was reported by Nagelkerken et al.[12, 13] throughout the Caribbean. The pathogen was identified as *Aspergillus sydowii*[14, 15]. Subsequently, it was shown that *Gorgonia* spp. have both cellular[16, 17] and chemical[18] defense mechanisms against *A. sydowii* infections, which may explain why the incidence was high but overall colony mortality was lower than the 1980s epizootic. Less is known about the diseases of other gorgonians. Harvell et al.[19] reported a cyanobacterial infection of *Briareum asbestinum*, which was bleaching-related. Feingold[20] described an apparent cyanobacterial infection affecting 8% of the *Pseudopterogorgia acerosa* colonies sampled in the northern Florida Keys. Similar infections occurred with *P. americana*, although at a lower incidence. The progression of the disease was similar to that described in this paper on *P. americana* from Bermuda and the Bahamas. The purpose of this study was to survey the incidence of *P. americana* disease in Bermuda and to investigate the causal organism. Initial observations were made during a course survey [Pathology of Coastal Organisms, Bermuda Biological Station for Research (BBSR)] and the diseased gorgonian surveys became integrated in ongoing surveys of scleractinian corals throughout the Caribbean[7, 21]. A comparison of incidence was made with reef sites in the Bahamas.

Materials and Methods

Site Description. Hog Breaker is located on the rim reef approximately 10 km off the north shore of Bermuda with an average depth of approximately 8.0 m (Fig. 1). Coral cover is approximately 25%[22, 23], and is dominated by the species *Diploria strigosa*, *D. labyrinthiformis*, *Montastraea franksi*, *M. cavernosa*, *Porites astreoides*, and the hydrozoan *Millepora alcicornis* (Smith 1998). The corals *Favia fragum, Stephanocoenia intersepta, Siderastrea* spp., and *Agaricia* spp. are present, but less common. Additionally, the gorgonians *Gorgonia ventalina, Pseudopterogorgia americana, Pseudoplexaura porosa, Plexaura homomalla, P. flexuosa, Plexaurella nutans* and *Eunicea* spp. are conspicuous members of the reef assemblage. The Cathedral-Gurnet Rock sites are located directly beyond the southeastern opening of Castle Harbor, inside the boilers, at a depth of 5.0 - 8.0 m. The reefs consist of a similar coral reef community to that found at Hog Breaker. Noticeable differences include a reduced abundance of the hydrocoral *M. alcicornis*, and the gorgonian *G. ventalina*, and an increased abundance of *F. fragum* colonies at the Cathedral-Gurnet Rock sites. Castle Harbor is a semi-enclosed inshore basin that is connected, through a small passage on the northwest side, with the

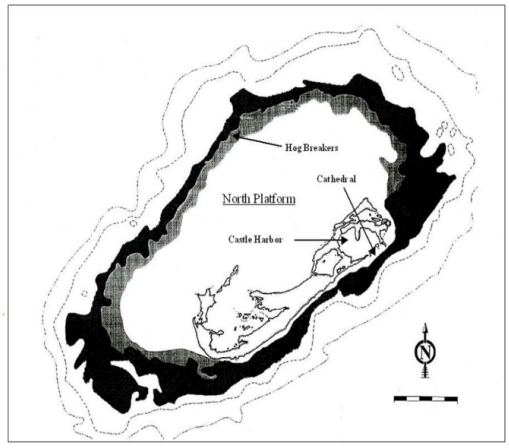

Figure 1. Map of Bermuda showing sampling sites.

north lagoon of the Bermuda platform, and to the open ocean on the southeastern side through a larger passage between several islands[25] (Fig. 1). It is approximately 10.2 km^2 with an average depth of 8.2 m, and a mean tidal range of one meter[26]. Castle Harbor consists of three reef types, fringing inshore reefs, knoll coral-algal reefs, and pinnacle reefs[25-27]; the present study investigated only the latter reef type. The pinnacles are approximately 8.0-10.0 m high with a diameter of approximately 5 - 7 m[25]. The top of the pinnacles is a shallow horizontal surface of only 1.0 - 2.0 m depth, while the vertical sides reach 8.0 - 10.0 m. Frazier[27] noted that they are constructed primarily from Diploria heads. Coral cover on the pinnacles averages approximately 13%[25] and is lower than either Hog Breaker or the Cathedral-Gurnet Rock sites. The dominant coral species on the vertical surfaces of the pinnacles are the branching species *Madracis decactis, M. mirabilis*, and *Oculina diffusa*, and the hydrozoan *M. alcicornis*, while the horizontal coral communities consist of the species *D. labyrinthiformis, D. strigosa, M. cavernosa, M. franksi, P. astreoides*, and *S. intersepta*. Additionally, the species *Isophyllia sinuosa*, *and S. radians*, are found throughout the pinnacles. The dominant gorgonian species are *G. ventalina, G. mariae, P. americana*, with *Pseudoplexaura spp., Plexaura* spp. and *Eunicea* spp. present but not common.

Two sites were selected in the Bahamas. Belt transects (2m x 50m) were conducted on Lindsay's Reef and Rocky Point in San Salvador, Bahamas in 2000, to compare with the Bermuda sites. These two patch reefs are part of an ongoing (now 10 year) survey and are described in McGrath and Smith[28, 29]. Figure 2 shows the location of these reefs.

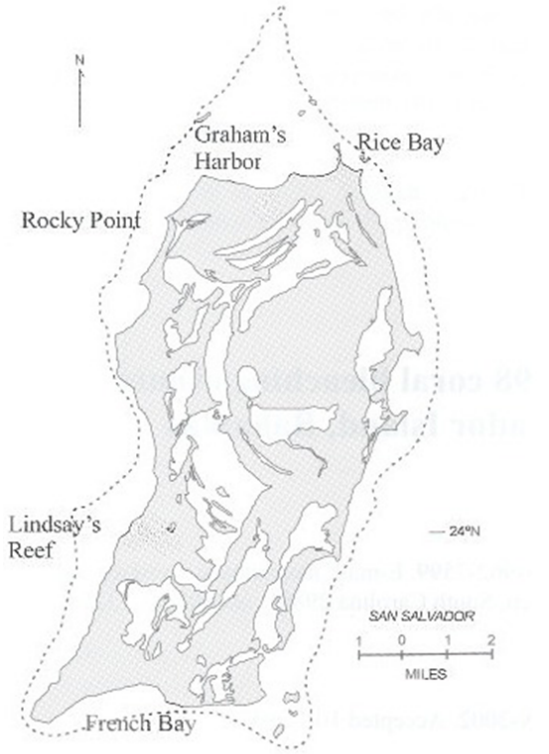

Figure 2. Map of San Salvador Island, Bahamas. The two sampling sites were Lindsay's Reef and Rocky Point.

Surveys. The Bermuda reef sites were surveyed in the summers of 1999, 2000 and 2001. Twelve belt transects (20m x 2m) were made each at the Hog Breaker and Cathedral- Gurnet Rock sites. Divers counted all apparent healthy and affected *P. americana, G. ventalina* and other octocoral colonies within the transects. In 1999, four patch reefs were surveyed in Castle Harbor by dividing each reef into quadrants and counting all apparent healthy and affected *P. americana* colonies. In 2000 and 2001, 2m-wide transects crossed each reef (N to S and E to W) where healthy and affected colonies were counted. Additional counts were made in areas not transversed by the transects. In this way, counts representing the entire reefs were made for three years. For comparison, colonies of *Gorgonia* spp., and other octocorals, were also surveyed. The Bahamian surveys consisted of counting all of the *P. americana, G. ventalina* and *G. flabellum* colonies on a 2 x 50m transect from Rocky Point and all the *G. ventalina* and *G. flabellum* colonies from Lindsay's Reef. In addition, the percentage of colonies affected by the diseases was also estimated.

Microscopy and Isolations. Samples retrieved from the surveys were returned to the lab at BBSR, and subsamples of both healthy and affected colonies observed microscopically. Particular attention was paid to areas

adjacent to degenerating tissue to determine the presence of active possible pathogens. These areas were also plated out on both GASW medium[30] for bacterial comparisons, and YEG (yeast extract glucose seawater medium)[14] to compare fungal isolates. Initial mixed cultures were replated to pure culture and observed microscopically. Samples of diseased and healthy *P. americana* were obtained from Castle Harbor where the incidence of infection was highest. Scrapings from the coenchyme of both healthy and diseased samples were viewed and photographed under a laser confocal microscope. Subsamples of each were plated on solid yeast extract glucose (YEG) and malt extract glucose (MEG) plates supplemented with kanamycin to reduce bacterial growth. Fungal isolates were compared morphologically with known *A. sydowii* isolates, previously characterized using trpC gene sequences[15]. Those appearing to be *A. sydowii* were used in transfection experiments.

Sclerite Counts. In 1999, duplicate tissue samples from affected and unaffected colonies were viewed microscopically (400x) to determine the relative number of pigmented (purple) to unpigmented (clear) sclerites within the coenenchyme. Five samples from each seafan colony were counted. The affected tissue samples were collected from intact tissue less than 2 cm below the degenerating lesion margin. In 2001, sclerites were extracted from 1cm sections of coenenchyme removed from healthy and diseased samples. Organic matter was oxidized in a hypochlorite (1.25 %) solution. Sclerites were then air dried and counted microscopically. The percentage of pigmented sclerites was then calculated for both healthy and diseased samples.

Transfections. Tests to determine transmissibility of the putative pathogens were performed by attaching diseased tissue samples to healthy colonies of *P. americana* and *G. ventalina* using cable ties. After one week, the inoculated tissues were removed, observed using light microscopy and plated on YEG plates. In 2000, segments of affected tissue (about 5cm lengths) were cable tied onto healthy segments within the same colony (to avoid tissue rejection) to determine if the disease was transmissible. Colonies were located in Castle Harbor and 16 replicates of the transfections were made. These were left in situ for five to seven days, after which paired (previously healthy tissue and attached diseased tissue) segments were removed for analysis in the laboratory. In addition, fungal isolates obtained from the 1999 *P. american* isolations, were grown on YEG plates overlain with sterile gauze strips (1 x 3cm). The strips were used to inoculate *Gorgonia ventalina* (six inoculations) also in Castle Harbor, to determine if the fungus was also pathogenic against both hosts. Controls were strips kept on uninoculated YEG plates. In 2001, isolated strains of *A. sydowii* from *P. americana* were used to inoculate *G. ventalina* and *P. americana* in the field and in aquaria. Field inoculations were only performed in Castle Harbor, with fungi isolated from diseased *P. americana* from the same site. The inoculum was obtained by spread plating a spore suspension of the fungus onto YEG plates and overlaying the plate with sterile strips of gauze (1cm X 5cm). After three days of growth the fungal hyphae extended into the fibers of the gauze. Controls were identical except that plates were not inoculated with the fungus. Application of the inoculum consisted of inserting gauze strips into spaces within the colonial mesh in *G. ventalina* and attaching the gauze strips onto branches of *P. americana* using small cable ties.

In the Bahamas, 5 cm x 5 cm square colony segments cut from healthy colonies in the field were suspended in closed aquaria in the lab. Fungal isolates from *P.americana*, *G. ventalina* and reference isolates were prepared (as described above) on gauze strips. The *G. ventalina* (Saba strain) was used as a positive control and the reference strain as a negative control (previously shown to be a nonpathogenic *A. sydowii*). The diameter of the resulting lesion was measured after one week.

Microbial Dehydrogenase. Overall microbial activity of healthy colonies was compared with affected colonies using a tetrazolium reduction assay[31]. Triplicate 5 cm lengths of tissue were cut from affected and apparently healthy colonies. These were placed into vials containing 10 ml of autoclaved seawater. To these, and to sterile seawater controls, 0.25 ml of INT (iodo-nitro-tetrazolium, 1.5 mg/L) was added. Vials were allowed to incubate overnight at room temperature, after which subsamples were measured at 485 nm in a spectrophotometer for the production of formazan.

Results

The prevalence of infected *P. americana*, *G. ventalina* and total octocoral colonies at each site from Bermuda are given in Figure 3. In all cases, the percentage of affected colonies for both species increased from 1999 to 2000, but *P. americana* showed a non-significant decrease in 2001. Among all sites and species, Castle Harbor had a higher prevalence than Hog Breakers or Cathedral. Comparing Bahamian *G. ventalina* surveys with Bermudian surveys (Fig. 4), the overall percentage of diseased colonies was somewhat lower but within the range of what was observed in Bermuda. Most Bahamian diseased colonies had 25% or less of the colony affected by the disease (Fig. 5). The percentage of diseased colonies were higher for *G. ventalina* than for *G. flabellum* at the Rocky Point site, but the opposite was observed at the Lindsay Reef site. The

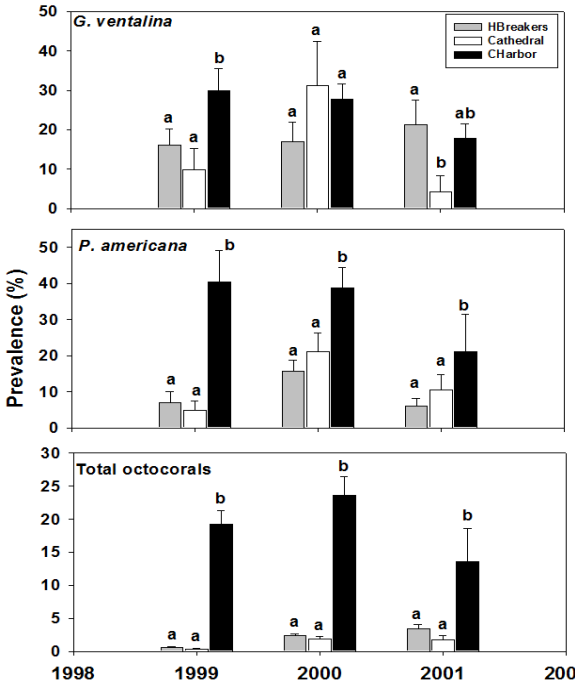

Figure 3. Disease prevalence (percentage of colonies affected) for *G. ventalina*, *P. americana* and total octocorals over a three year period at three sites in Bermuda; Hog Breakers (HBreakers); Cathedral; and Castle Harbor (CHarbor)

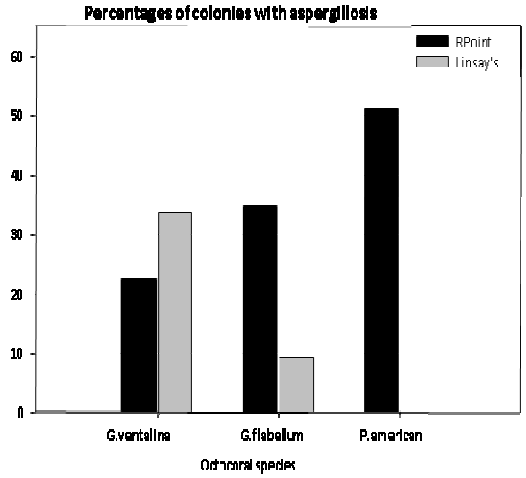

Figure 4. Disease prevalence of three octocorals at Rocky Point (RPoint) and Lindsay's Reef in San Salvador, Bahamas.

prevalence of infection for *P. americana* was much higher in the Bahamas. Microscopic observations of *P. americana* tissue scrapes from affected colonies showed the presence of fungal hyphae, while healthy tissue did not contain hyphae. The appearance of affected tissue was similar to

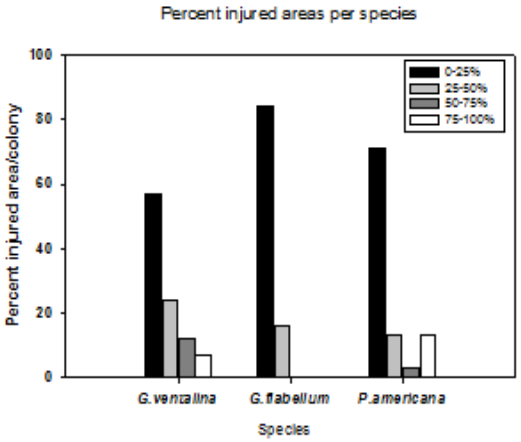

Figure 5. Degree of infection (percentage of colony affected) among octocorals in the Bahamas.

that described by Smith et al.[14] for *G.ventalina*. In addition, diseased samples were covered by thick mucus and the brown coenenchymal tissue was receding, revealing the central axis. Polyps, in diseased tissue, appeared expanded, swollen and disintegrating. Bacteria and fungi obtained from sample platings of healthy and affected *P. americana* colonies, differed significantly in only one respect. A fungus was consistently isolated from affected colony samples that were not observed in plates from healthy samples. The fungus was isolated to pure culture and viewed microscopically. The fungus was morphologically identical to *A. sydowii* as described in Geiser et al.[15]. Subcultures of the *P. americana* fungal isolates were used for transfection experiments the following year, after physiological and metabolic verification of the species[32]. Sclerite counts from *P. americana* taken in 1999, greatly favored pigmented sclerites associated with affected tissue. In fact, no pigmented sclerites were found in surface scans of healthy tissue at all, and the number of pigmented sclerites in affected tissue was significantly higher than clear sclerites (Table 1). The overall ratio of pigmented to healthy sclerites were about 2 to 1, but with considerable variation. Increased ratios of pigmented to nonpigmented sclerites seemed to correspond with the degree of infection, but this was not quantified. Still, compared with healthy tissue, the difference was significant. Results of sclerites counts were similar in 2001 when the sclerites were extracted by oxidizing the tissue rather than performing surface counts (Fig. 6).

Evidence showing transmissibility of affected *G. ventalina* and *P. americana* segments to healthy areas of each corresponding species (Fig. 7) was apparent within two days. Harvested transfected segments showed abundant hyphae in gorgonians transfected with diseased tissue, and degenerating areas near the sources of inocula (Fig. 8B).

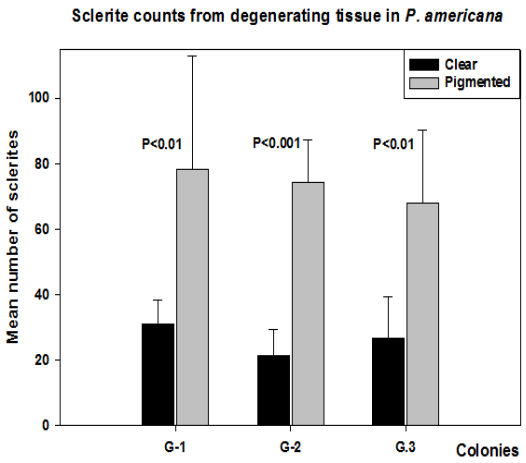

Figure 6. Pigmented and clear sclerite counts from three diseased *P. americana* colonies from Bermuda.

Tissue from colonies exposed to healthy segments contained no fungal hyphae and showed no lesions although some purpling of tissue occurred in some cases (Fig. 7A). Healthy *G. ventalina* colonies inoculated with pure cultures of the *P. americana* isolates also became diseased (Fig.8), while controls did not respond to the gauze strips. All 16 *A. sydowii* inoculations showed evidence of infection (degradation of coenenchyme). Re-isolation of fungi from affected *G. ventalina* was performed and cultures were identical to the inoculum. Aquarium inoculations, in the Bahamas, showed that *P. americana* isolates were as aggressive as *G. ventalina* isolates (Table 2). Overall microbial activity (as measured by tetrazolium reduction) was highest with affected *P. americana* segments. Mean relative absorptions for affected tissue, unaffected tissue and seawater, are given in Table 3. All differences were statistically significant (p=0.0001)

Table 1. Pigmented and non pigmented sclerites counts from *P. americana* from Bermuda.

Sample	**Clear Sclerites**	**Pigmented Sclerites**	**Ratio**
Healthy 1	100	0	-
Healthy 2	100	0	-
Healthy 3	100	0	-
Healthy 4	100	0	-
Healthy 5	100	0	-
Diseased 1	34	72	2.12
Diseased 2	40	74	1.85
Diseased 3	20	35	1.75
Diseased 4	29	132	4.55
Diseased 5	32	78	2.44
Average			**2.45**
Standard Error			**2.45**

Table 2. Diameter (cm) of lesions on *G. ventalina* squares produced during bioassays of *A. sydowii* isolated from *Pseudopterogorgia*.

Fungal strain	Average Diameter (cm) N=4	Standard Error
PACD F1	1.00	0.89
PACD F2	0.78	0.33
PACD C2	0.80	0.22
PAD-UB	0.50	0.24
Control	0	-

Table 3. INT-linked dehydrogenase assays (absorption at 490 nm) of colony segments from seawater, healthy and diseased *P. amaericana*.

	Colony			Mean	Standard Deviation
	1	2	3		
Sea Water	0.139	0.148	0.142	0.14	0.005
Unaffected	0.453	0.373	0.398	0.41	0.04
Affected	0.462	0.521	0.511	0.50	0.03

Conclusions

A comparison of the prevalence of diseased *P. americana* and *G. ventalina* between Bermuda and the Bahamas shows that the Bahamas have a higher prevalence of disease, but it appears to have increased for *P. americana* in Bermuda from 1999 to 2000. Disease prevalence has been monitored for some time in the Bahamas[33] but these are the first temporal measurements of sea fan disease in Bermuda, and the first systematic study of the *P. americana* epizootic to be characterized in this much detail.

Other octocorals with apparent lesions were counted, and studies are presently under way to confirm the identities of the possible pathogens. The presence of *A. sydowii* adjacent to lesions in *P. americana* indicates that the host range of *A. sydowii* exceeds the genus *Gorgonia*. That fungal isolates were found in basically all of the affected tissue sampled, but not in healthy tissue, supports this observation. Among the isolates obtained, all had similar morphology[15] and metabolic profiles[32]. Based on these results, the fungi can be confirmed as being *A. sydowii*. Although not all *A. sydowii* cause lesions in gorgonians[34], inoculation experiments in Bermuda and the Bahamas showed that isolates from diseased *P. americana* could cause lesions in both healthy *P. americana* and *G. ventalina*.

The increased occurrence of pigmented verses non-pigmented sclerites in the coenenchyme of *G. ventalina* was shown to be a characteristic of

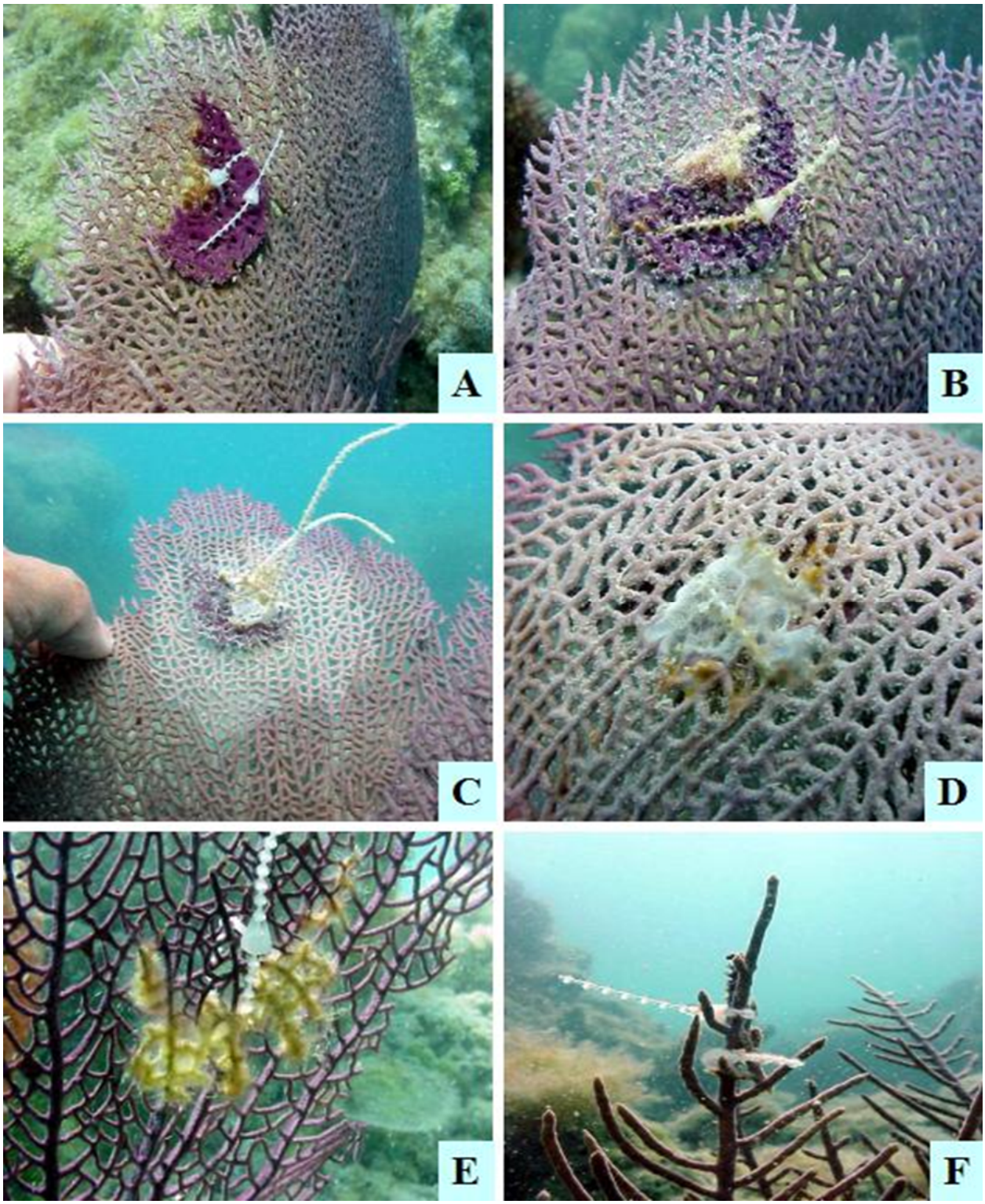

Figure 7. Colony to colony inoculations (transfections). A; Control, healthy *G. ventalina* inoculated on healthy *G. ventalina* tissue. B; diseased *G. ventalina* inoculated on healthy *G. ventalina* tissue (after one day). C; diseased *G. ventalina* inoculated on healthy *G. ventalina* tissue (after two days). D and E; Fungus from diseased *P. americana* inoculated on healthy *G. ventalina* tissue. F; Fungus from diseased *G. ventalina* inoculated on healthy *P. americana* tissue.

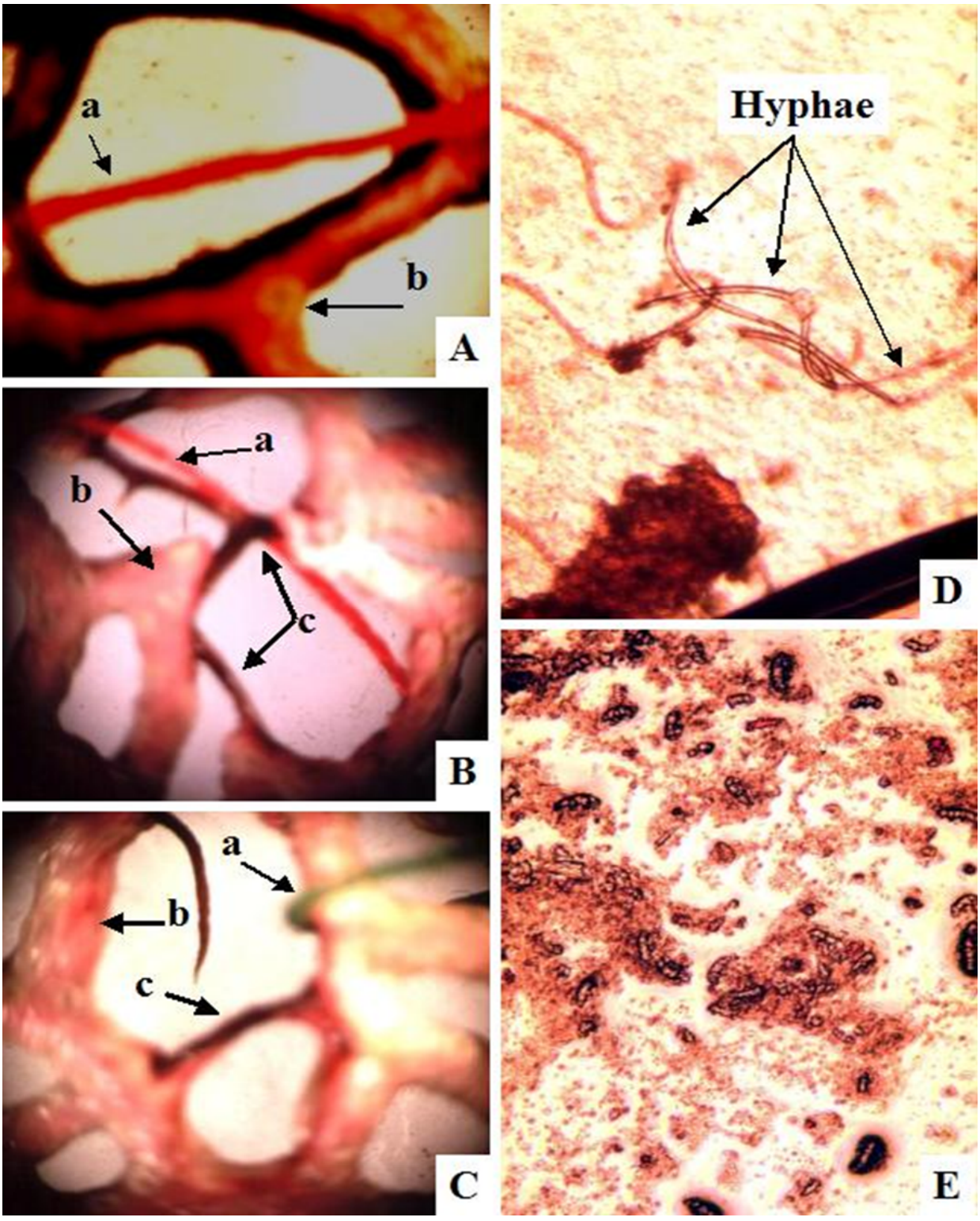

Figure 8. Photomicrographs of healthy *G. ventalina* inoculated with fungal strains from diseased *P. americana*. A; (a) shows fungal hypha and (b) shows the axial tissue not yet degraded. B; (a) fungal hypha, (b) axial tissue not yet degraded, (c) shows the degrading axis. C; (a) fungal hypha, (b) axial tissue not yet degraded, (c) shows the degrading axis. (d)D; coenenchymal tissue with fungal haphae. E; healthy coenenchymal tissue.

aspergillosis[16]. Similarly, pigmented sclerites were much higher in affected *P. americana* tissue. In fact, only clear sclerites were observed in healthy tissue. It has been suggested that the production of increased pigmented sclerites occur as a result to injury or infection in *G. ventalina*[17]. This also seems to be the case for *P. americana*. The purple pigment in *G. ventalina* is a carotenoid[35].

Overall microbial activity, as measured by tetrazolim reduction, was significantly higher in affected tissue. This was also reported by Ritchie et al.[36] for bleached coral tissue compared with healthy tissue. It is likely that increased activity results from the breakdown of coenenchyme and polyps, which may then be used as a nutritional source for a variety of microorganisms.

In summary, this study has shown that *A. sydowii* is the pathogenic agent responsible for the primary disease of *P. americana* in Bermuda and the Bahamas. The host range of the pathogen may not be limited to the genera *Gorgonia* and *Pseudopterogorgia*. Instead, aspergillosis may be a common disease among gorgonians. Studies are underway to determine if the host range is even larger.

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