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STUDY OF SECONDARY METABOLITES ASSOCIATED WITH VIRULENT AND NON-VIRULENT STRAINS OF *ASPERGILLUS SYDOWII*: SEA FAN PATHOGEN

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ABSTRACT

During the past decade, two sea fan species, *Gorgonia ventalina* and *Gorgonia flabellum*, have been enduring an epizootic caused mainly by the disease known as Aspergillosis. Earlier research has identified the fungus, *Aspergillus sydowii*, as the causal organism. Pathogenic strains of *A. sydowii* have been isolated from several different locations throughout the Caribbean. Secondary metabolites from these pathogenic strains were extracted, and separated by High Performance Liquid Chromatography (HPLC). Chemical profiles of the pathogenic strains were then compared with those of marine strains collected in Venezuela, as well as terrestrial strains. Results showed some differences between metabolites detected in our pathogenic strains and those of reference strains.

INTRODUCTION

Prior to the early 1990's there were only two well-documented cases of diseases affecting coral. In the past ten years there have been a number of apparently novel diseases observed affecting hard corals. The causal agents of most of these diseases are still unknown (Richardson 1998; Harvell *et al*, 1999). Aspergillosis has been well characterized and is known to affect two species of gorgonian corals. Symptoms of this disease entail purpling of the infected tissue and lesions of exposed skeleton as a result of lost tissue (Nagelkerken *et al*, 1997a&b). When diseased tissue was examined under a microscope, fungal hyphae were found in the area around the lesions and were not observed in healthy tissue. Phenotypic and genetic analysis revealed that the fungus was most similar to *Aspergillus sydowii* (Geiser *et al*, 1998; Smith *et al*, 1996; Smith *et al*, 1998). This same species of fungus was isolated from sea fans from the Bahamas, Trinidad, Saba, the British Virgin Islands, and the Florida Keys. When these strains were introduced to healthy sea fans, they produced disease symptoms similar to those earlier described. Re-infection with controls and reference strains of *A. sydowii* did not result in

disease. After re-isolating the fungus from the diseased sea fans, it was determined that this marine strain of *A. sydowii* was the causal agent (Geiser *et al*, 1998; Smith *et al*, 1996; Smith *et al*, 1998). *A. sydowii* is a common terrestrial fungus, although it is salt tolerant and has been isolated from the marine environment. It previously has not exhibited widespread pathogenicity or mass mortality to marine or terrestrial organisms (Geiser *et al*, 1998). However, *Aspergillus* species have been known to become opportunistic pathogens. *A. fumigatis* is a human pathogen that causes disease in immune compromised individuals. Current research has shown that the pathogenic strains of *A. sydowii* have a different metabolic profile from that of reference strains (Alker *et al*, 2001).

This paper describes how secondary metabolites produced by four pathogenic strains of *A. sydowii* (that were isolated from diseased sea fans) compared to two non-pathogenic reference strains from the Northern Regional Research Laboratory (NRRL) collection, as well as ten strains of *A. sydowii* isolated from the marine environment (Venezuela) and ten terrestrial strains from the IBT collection (Table 1).

Table 1. *A. sydowii* strains used in this study. NRRL 244 & 249 are reference strains known to be non-pathogenic. FK2, FK11, SS-7, and SS-2 are pathogenic strains. KIR strains are of marine origin and IBT strains are of terrestrial origin.

Isolate	Isolated from
NRRL 244	Fish from Japan
NRRL 249	Silk thread in Philadelphia
FK 2	Florida Keys #1
FK 11	Florida Keys #2
SS-7	San Salvador #1
SS2	San Salvador #2
KIR ###	Venezuela marine collection
IBT ####	IBT terrestrial collection

METHODS

Pathogenic strains of *A. sydowii* were isolated from samples of diseased gorgonian tissue by the standard methods. All isolates were cultured on Yeast Extract Sucrose (YES) and marine agar media, (0.5 % yeast extract, 0.5 % peptone, 1.0 % glucose made in artificial sea water (pH 7.5), and were incubated for fourteen days at 25°C.

Cultures were analyzed by the simple agar plug method for intra- and extracellular metabolites (Filtenborg *et al*, 1993). Ten plugs were taken from each isolate (one 9 cm agar plate), and extracted with a mixture of ethyl acetate : chloroform : methanol (3 : 2 : 1) containing 1 % formic acid (2 ml). The solutions were evaporated to dryness under N_2. The extracts in methanol (2 ml) were allowed to stand for 0.5 h, filtered (13 mm GHP 0.45 µm, Waters) and subjected to HPLC analysis. This was performed by injection of the sample (10 µl) on a Waters analytical HPLC equipped with a 3 µm Hypersil BDS C18 column and diode array detection (PDA). The PDA detector measures full UV-VIS spectra (200-600 nm) for each peak detected at 254 nm. A gradient from 15 % to 100 % acetonitrile was run at a flow rate of 1 ml/min for 43 minutes. The performance of the analyses procedure is checked using alkylphenones (Frisvad, 1989).

RESULTS

The HPLC profiles characterizing the secondary metabolites (chemical fingerprints) of the 27 isolates of *A. sydowii* grown on YES medium were compared. Wide ranges of metabolites are produced by the isolates originating from the diseased gorgonian (figure 1). The profiles of the reference isolates are more similar to the profile of the control sample (non-inoculated agar plate) (figure 1), than the profiles of the pathogen isolates. Furthermore, the profiles

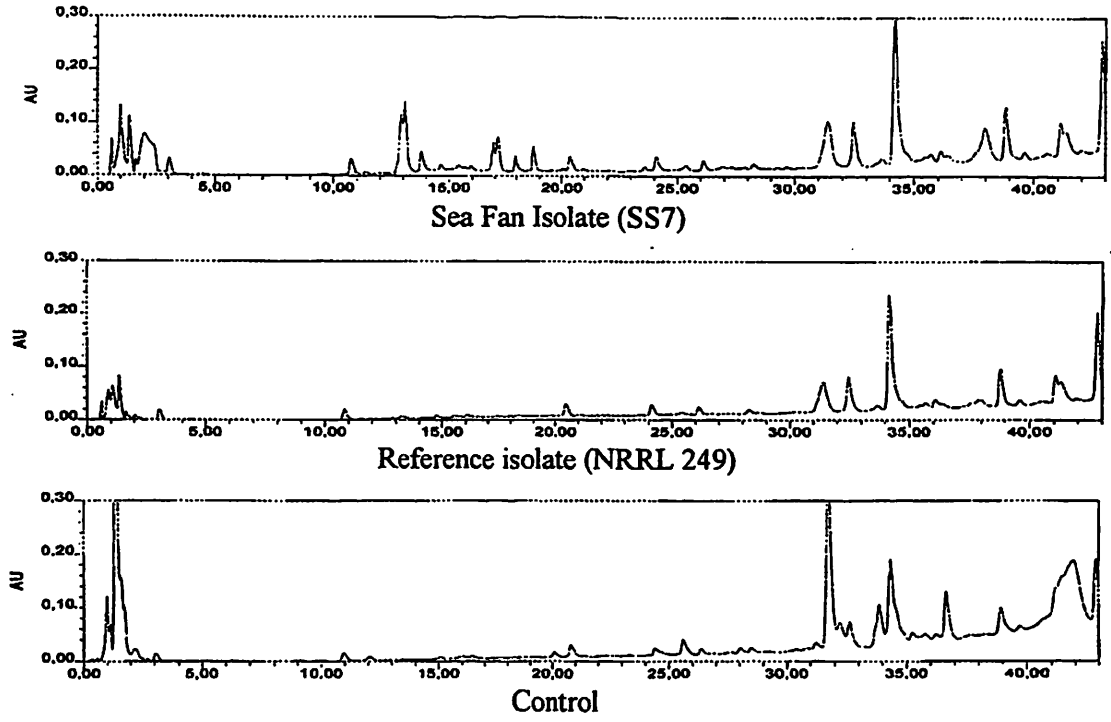

Figure 1: Chromatograms of a virulent strain (SS&), a known avirulent reference strain (NRRL 249), and a YES media control.

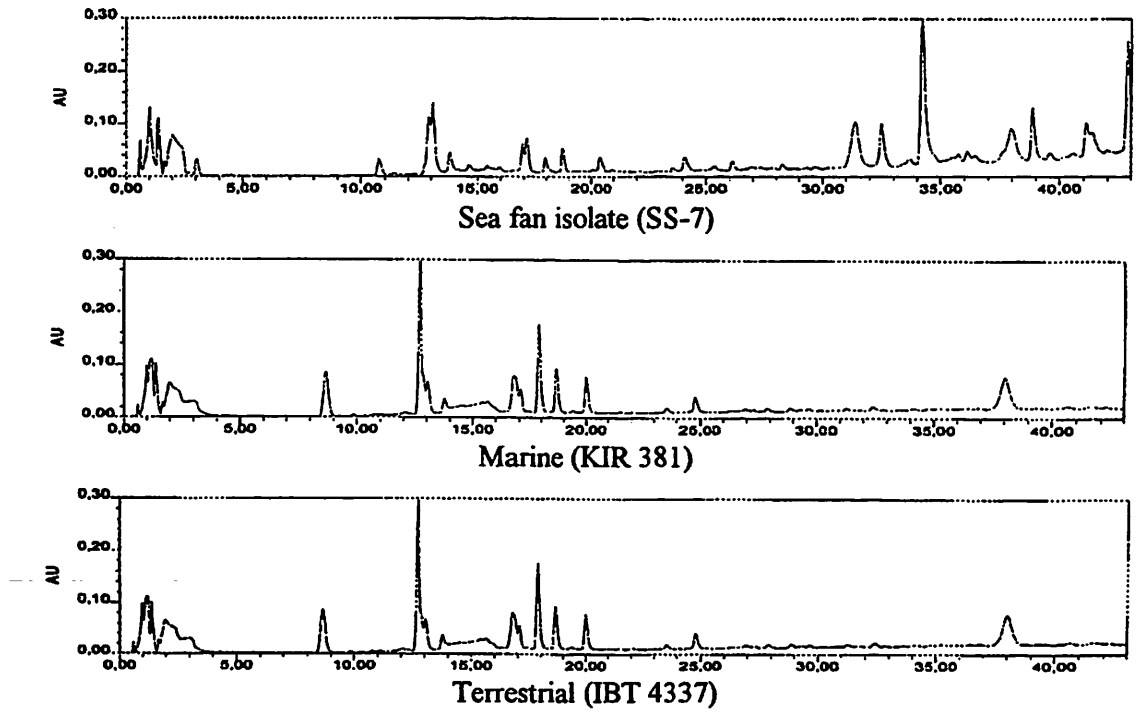

Figure 2. Chromatograms of sea fan, marine, and terrestrial isolates.

of the pathogen isolates are similar to the profiles of the marine-derived and terrestrial isolates, viewed below 30 minutes (figure 2). Peaks appearing after 30 minutes are also detected in the control sample, and are treated as "blind peaks". The overall profile observed for the isolates investigated (except reference isolates) is a typical profile expected for *A. sydowii*, stated by Frisvad (pers. comm.). The chemical profiles of *A. sydowii* cultivated on marine agar media differ from the profiles detected from cultivation on YES media (figure 3).

DISCUSSION

The production of secondary metabolites is strongly dependent on the medium used. YES is an optimal medium for the expression of all types of mycotoxins (except alkaloids and naphthoquinones) (Frisvad *et al*, 1989). This medium was chosen in order to obtain a broad profile of metabolites, allowing a good basis for comparison of the individual profiles of secondary metabolites. The isolates were cultivated on the marine agar media to mimic the environment inhabiting the pathogenic isolates. Very few investigations address the problem whether species, well known from the terrestrial environment, are true inhabitants in the sea. If they are truly adapted to life in the sea, the crucial question is whether they differ in the expression and content of metabolites as compared to their terrestrial counterparts. Cultivated on identical medium, the *A. sydowii* isolates investigated (except reference isolates) express identical metabolite profiles. The reference isolates could in time have lost their ability to produce secondary metabolites. When sea salt is present in the medium, the total number of metabolites is reduced. Furthermore, no new metabolites are expressed in detectable amounts.

Further research must be done to determine if secondary metabolites are involved in the disease process of Aspergillosis of gorgonians. However, this research appears to support the theory that the sea fan pathogen has a terrestrial origin. Currently the marine and terrestrial strains of *A. sydowii* used in these experiments are being tested for pathogenicity. Also the metabolites indicated in the HPLC profiles are in the process of being identified.

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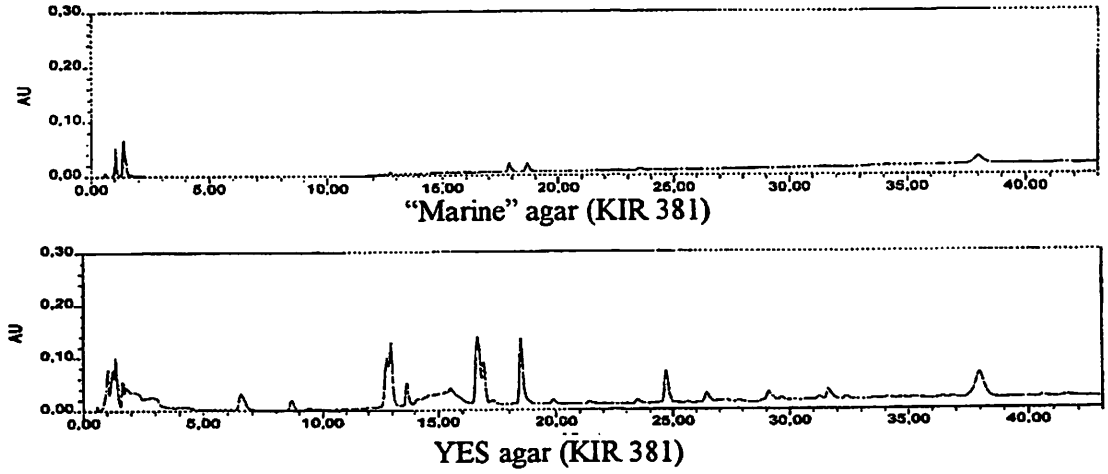

Figure 3: Chromatograms of the same isolate on the two different media.

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