POTENTIAL OF FUNGAL ENDOPHYTES *FROM Thalassia testudinum* Bank ex K.D. Koenig AS PRODUCERS OF BIOACTIVE COMPOUNDS

by

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Thesis submitted in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCES
in
Biology

UNIVERSITY OF PUERTO RICO
MAYAGÜEZ CAMPUS
2008

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ABSTRACT

Sea grass beds of *Thalassia testudinum* provide an essential habitat for marine species of economic importance. Previous studies have demonstrated the presence of mycelial endophytic fungi from undamaged tissue of *T. testudinum*, however the role of the endophytic mycoflora and its potential as producers of bioactive compounds is unknown. The main goal of this research was to identify the endophytic fungi producing bioactive compounds and their possible role in relation to the health of *T. testudinum*. Samples from damage and undamaged leaves of *T. testudinum* from Playa Buyé in Cabo Rojo, Puerto Rico were collected and surface sterilized with sodium hypochlorite (0.5%) and rinsed with distilled water. Each leaf was fragmented and the pieces were plated in Petri dishes containing Marine Agar and incubated at 25ºC for 7 days. A total of 8 genera and 13 species were isolated, including *Acremonium strictum*, *Aspergillus niger*, *A. fumigatus*, *A. flavus*, *A. versicolor*, *A. ustus*, *Botrytis cinerea*, *Cladosporium cladosporioides*, *Colletotrichum* spp., *Curvularia lunata*, *Curvularia brachyspora*, *Penicillium chrysogenum*, *Penicillium janthinellum*, and *Scopulariopsis asperula*. Bioassays were performed with filter discs impregnated with fungal extracts. A bacteriostatic effect was observed in cultures of some opportunistic and endophytic bacteria, suggesting the prophylactic role and the production of bioactive compounds of these endophytic fungi associated to *T. testudinum*. Host specificity test were conducted suggesting a non-pathogenic role of the endophytic fungi tested in relation with the health of the host plant.

RESUMEN

Las praderas de *Thalassia testudinum* proveen un hábitat esencial para especies de importancia económica. Estudios preliminares han demostrado la presencia de hongos endófitos miceliales en tejido de *T. testudinum*, pero el rol de la micoflora endofítica y su potencial como productores de compuestos bioactivos es desconocido. El objetivo principal de esta investigación fue determinar la diversidad de micoflora endófita productora de compuestos bioactivos asociada a *T. testudinum* y determinar su posible rol en relación a la salud de estas plantas. Muestras de hojas sin ningún tipo de daño y dañadas de *T. testudinum* de la playa Buyé en Cabo Rojo, Puerto Rico fueron colectadas y esterilizadas con hipoclorito de sodio (0.5%) y enjuagadas con agua destilada. Cada hoja se fragmentó y los pedazos obtenidos se colocaron en platos Petri con Agar Marino y se incubaron a 25ºC por 7 días. Aislamos un total de 8 géneros y 13 especies, incluyendo *Acremonium strictum*, *Aspergillus niger*, *A. fumigatus*, *A. flavus*, *A. versicolor*, *A. ustus*, *Botrytis cinerea*, *Cladosporium cladosporioides*, *Colletotrichum* spp., *Curvularia lunata*, *Curvularia brachyspora*, *Penicillium chrysogenum*, *Penicillium janthinellum* y *Scopulariopsis asperula*. Se realizaron bioensayos con discos impregnados con filtrados extraídos de los hongos. Se observó un efecto bacteriostático en cultivos de bacterias oportunistas y endófitas, sugiriendo el rol profiláctico y la producción de compuestos bioactivos de estos hongos endófitos asociados a *T. testudinum*. Se llevaron a cabo pruebas de patogenicidad, que sugieren un rol no patogénico de los hongos endofitos utilizados en relación a la salud de la planta hospedera.

DEDICATORY

I dedicated the product of my hard work and dedication to two people that helped and accompanied me during my master degree years, Mr.Angel A. Cruz Afanador and Dra. Sandra L. Maldonado Ramírez.

Angel, thanks for your love, patient, support, but above all, for the courage to continue ahead. We are the proof that no matter what happened, the desires and courage to move ahead makes you stronger. Remember that I will love you always no matter what happens.

Sandra, thanks for giving me the opportunity to work in the laboratory and to expand my knowledge not only in the scientific world, but in all life aspects. You have been more than my professor counselor; you are a part of my family, an angel in my way. One day you told me I could do it. Now I want to accomplish greatest things in life.

I will never end thanking God for the opportunity for meet and share beautiful moment, with these two special people. They make the world a better place to live in.

ACKNOWLEDGMENTS

I want to thank God, for the strength and opportunity to growth spiritually and professionally during all these years.

To my parents for giving me their support during all the stages of my life, for their love, and inspiration.

I will forever be grateful to Sandra Maldonado for all her support and advice since my undergraduate years. To my graduate committee, Dr. Rafael Montalvo and Dr. Aníbal Quintana for their direction and positive advice during my research.

To my friend Selimar Malavé for her help at the laboratory, for her motivation, and emotional support during these years.

Thanks to Hernán Torres for all his help during the collection of the samples, also for his support and motivation. To Carlos Virella and Angel Cruz for helping with the collection of the samples, and to Jessica Castellanos for her help during the second sampling.

Thanks to Mrs. Magaly Zapata, laboratory technician at the Microbiology Laboratory and to Dra. Mildred Zapata for providing the phytopathogeneic bacterial cultures, and to Carolyn Rivera at the Tropical Mycology Laboratory for all the technical support.

To Mr. José Almodóvar, technician at the Microscopy Center of the Biology Department for his collaboration with the photographic material and for his professional and personal support. Thanks to Dr. Wood and Aaron Miller from the University of Utha for their collaboration with the analysis of the bioactive compounds analysis.

Thanks to laboratory and study partners Orlisa Crespo, Gualberto Rosado, Juan A. Rivera, Julio O. Acevedo, Gloria Natal, Somsismith Deechaleune, Roberto Román, Coraly Morales, and Miguel Ortiz for all their help and for being there during the good and tough times, most of all for the friendship. Also, thanks to Dra. Gladys O. Ducodray for her support.

I am also very grateful to the people and institutions that helped, supported, and guided me along the way and without whom this opportunity would not have been possible. The Department of Biology of the UPR Mayagüez Campus for the facilities, the Department of Natural and Environmental Resources authorized permits (DRNA: 04-IC-045) for this research, and UPR Sea Grant College Program (Award Number 535406) for providing materials for the research on bioprospecting.

Last but not least, I want to thank Anniesther Betances, Jomayra Marrero, Sadja Gaud, Ixia Ortiz, Betsy Colón, Hernán Torres Maldonado, and Carmen Torres Maldonado for being part of my life and giving me the light I need.

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1. INTRODUCTION

For centuries plants have been an invaluable source of bioactive compounds used as medicine. Recently, the nature of many of these compounds is known. Since the discovery of penicillin as a secondary metabolite with antibiotic properties produced by a *Penicillium griseofulvum*, multiple studies has focus in search of microorganisms that serve as new sources of bioactive compounds. The need to identify new resources has carried us to study the marine environment searching for novel sources of bioactive compounds.

Seagrass beds are a complete marine ecosystem that provides habitat for numerous epiphytes such as diatoms, algae, fungi, and many kinds of invertebrates. Many components of the vegetal tissue such as calcium, phosphorus, potassium and magnesium serve as a potential food source for many organisms (Vicente et al., 1978) and microorganism. Wilson (1998) documented the presence of endophytic fungi in the tissue of *Thalassia testudinum* Banks ex. K.D. Koenig, *Halodule bermudensis* Kützing, and *Syringodium filiforme* Kützing.

Thalassia testudinum is the most abundant and common seagrass in the Caribbean. It is distributed through the north of the Gulf of Mexico to the north coast of South America. It distribution depends on several ecological factors such as temperature, depth, turbidity of water, salinity, and the effect of the surge (Moore, 1963). *Thalassia* beds developed better in shallow calm plains of the sublitoral, mainly over muddy or

sandy substrate. They are found from the lower interstitial to depths of 25 meters. Nevertheless, the densest meadows are found in about 10 meters of depth and in areas of salinities between 25 and 40% (García Ríos, 1990).

Endophytes are a group of organisms, fungi or bacteria, which live asymptomatically in the internal tissues of a plant. The first endophytic fungi was discovered in 1940, but only until recently their importance was recognize. They live as mitosporic fungi inside the host plant, producing spores or cloning themselves. Their behavior is unknown but they have been described as benign parasites and/or true symbionts. Studies have proved that they have the capacity to produce toxins in response to plagues that benefit the host plant (Schardl et al., 1997, Meijer and Leuchtmann, 1999), contributing to their growth and protection. Nevertheless, information on the nature of this relationship in seagrasses is lacking.

One of the least studied biochemical systems in nature is the relationship between symbiotic microorganisms and their host plant. For instance, it appears that all higher plants are host to one or more endophytic microbes. These microbes include fungi, bacteria, and actimomycetes that primarily reside in the tissues beneath the epidermal cell layers and the host tissues are transitorily symptomless (Stone et al., 2000). Currently, there are no studies aimed to determine the role of the endophytic mycoflora associated to *T. testudinum*, and their potential as producers of bioactive compounds that affect the health of these plants.

The principal objective of this research was to determine the role of the endophytic mycoflora with the potential to produce bioactive compounds associated to *T. testudinum* and the relationship among them. Bioassays were performed to determine the capacity of the exudate producing endophytic fungi to inhibit or promote opportunistic bacterial growth. We also determine the infection rate for the depth of the sample and the leaf section.

2. LITERATURE REVIEW

2.1 *Thalassia testudinum*

Thalassia testudinum is the most strong and abundant seagrass of the Caribbean. It is found forming extensive monospecific meadows or coexisting with other seagrasses such as *Halodule wrightii* and *Syringodium filiforme*. Seagrasses beds are significant habitat for numerous marine producers, consumers, and decomposers. These meadows support a high species diversity many of which are the young stages of important commercial species. Seagrasses are also important in the deposition and stabilization of substrate, and indirectly reduce water turbidity of the overlying water (García-Ríos, 1990).

Thalassia possesses strong rhizomes that grow generally to depths among 3 to 15 cm under the sediment. The rhizomes are born short but at regular intervals, among each 9 to 13 knots. From the bud they develop groups of three to seven leaves. The superior part of the buds and the basal one of the leaves are surrounded by a case. Roots also developed from the rhizomes. Marine seagrasses roots are more reduced than those of terrestrial plants, and the absorption of water and salts is share by the leaves and the other structures (García-Ríos, 1990).

Thalassia testudinum shows sexual reproduction by seeds and vegetative propagation by rhizomes. Dense *Thalassia* beds are found in muddy to sandy shallow clam areas. However, it can also growth exposed to wave surge and heavy sands. They are found from the intertidal zones to 25 meters of depth. Nevertheless, the dense

meadows are found at depths of 10 meters and salinities between 25 to 40% (Dawes, 1986). Grazing by the marine urchins and the shortage of incident light seem to be the factors that determine the maximum limits of depth for this species (Vicente et al., 1978).

Thalassia testudinum is distributed from the north of the Gulf of Mexico to the north coast of the South America, including the Caribbean. In Puerto Rico, meadows of *T. testudinum* are complex communities with a diverse assembly of organisms. They provide a diversity of habitats composed by different portions of the dense vegetation such as the portion of the leaves exposed to the light and the ones exposed to the shadows, and different water movements. This composition has an effect in the diversity of organisms associated permanently to the substrate (García-Ríos, 1990).

2.2 Endophytes

Endophytes are an interesting group of organisms associated with several plant species. In general, the endophytic infection is inconspicuous and asymptomatic in terrestrial and aquatic plants. Histological approaches have shown that the microbial colonization can be internal. Several studies have reported them in sterile tissue or amplified the genetic material of the fungi or bacteria in the tissue of the colonized plant (Bacon and White, 2000).

Species composition and distribution of the endophytic associations inside and among host, and the effect of the colonization in the health of the host are issues of common interest among investigators. There is evidence that suggest that endophytic fungi represent a great deposit of genetic diversity and a rich source of undescribed

species (Reddlin and Carries, 1996). Two potential applications attributed to the endophytes include potential agents for biological control that provided protection to the host plant and a source of secondary metabolites for medicine (Bacon and White, 2000). But these applications are still uncertain for terrestrial plants and are unknown for marine seagrasses.

2.2.1 Endophytic fungi

Fungi are important components in every ecosystem, intimately associated with crucial processes like the decomposition, recycling, and transportation of nutrients in many environments. Endophytic fungi were described for first time in 1940 (Carris and Redlin, 2000), but the importance of their role was recognizing much later. They live as imperfect fungi most of the time and have been described as benign parasites or true symbionts. It has been suggested that they can influence the distribution, ecology, physiology, and biochemistry of the host plants (Sridhar and Raviraja, 1995).

2.2.2 Diversity of endophytic fungi

The idea that diversity can have strong effects on ecosystem processes was first suggested, among others, by Darwin. Theoretical models and experimental test exploring relationships between diversity and ecosystem properties have proliferated during the

past decade (Naeem, 2002). Experiments have revealed important functions for diversity in some cases, including the enhancement of primary productivity (Tilman, et al., 1997b), nutrient retention (Tilman et al., 1997a), nutrient flow (Cardinale et al., 2002), water availability (Calderia et al., 2001), and resistance to pathogen invasion (Levine and D'Antonio, 1999). A mechanism proposed to underlie diversity ecosystem functioning relationships include the selection effect, whereby communities with higher diversity have a greater probability of including a species with a strong effect on the community and complementarily effects (Loreau et al., 2001). The diversity of endophytes is manifested not only in the specificity of the hosts and their morphology, but also in the types of benefits that they offer to the host (Bacon and White, 2000).

Plants that belong to Coniferaceae, Ericaceae and Graminaceae familie have been the most intensively sampled. Oomycetes and some fungi classified as Ascomycota, and Basidiomycota have been isolated as endophytes form different regions and (Redlin and Carris, 1996). Endophytic fungi are poorly known in the tropical regions, making the estimates of endophytic fungal species conservative. Many researches such as Gamboa et. al., (2002) tested strategies for sampling endophytic fungi in tropical plants. They compared the number of fungi isolated from leaf pieces that constantly are divided into increasingly small fragments. His study determine that there is a relationship between the size of the fragments and the number of fungi isolated, concluding that reducing the size and increasing the number of leaf fragments will increase the number of fungal species isolated.

2.2.3 Relationship between endophytic fungi and the host plant

The relationship between endophytic fungi and host plants still uncertain. The association may be variable form host to host, depending on the environmental conditions. Endophytes may be important for the protection of the host plant during biotic (e.g. herbivores or parasites) or abiotic (e.g. drought) stress situations (Redlin and Caris, 1996).

Studies addressed that endophytic fungi can spread in a plant tissue for many generations through the seed (Boursnell, 1950). He determined that the host plant needed the fungi for the development of the seeding and for general nutritional benefit, been both in a mutualistic relationship.

2.3 Bioactive compounds

Bioactive compounds are secondary metabolites that cause some effect in living organism. They include substances with therapeutic value (antibiotics, antitumors, antivirals, antimicrobials, and inmunosupresors), citotoxic agents, pesticides, insecticides, growth inhibitors, and sexual repellents, among others. The bioactive compounds may be synthetic or natural products extracted from plants, animals or microorganisms (Verpoorter, 1998).

These compounds vary widely in their chemical structure and function. One example are the phenolic compounds, including flavonoids, which are present in almost

all plants and have been studied extensively in cereals, legumes, nuts, olive oil, vegetables, fruits, tea, and red wine (Etherton et al., 2002). Over the period of 1981 through 2002, 16.4 % of new chemical synthetic entities were derived from natural products (Newman et al., 2003).

2.3.1 Production of bioactive compounds in marine environments

The first notable discovery of biologically active compounds molecules from a marine source was in the early 1950's. Compounds with antiviral activity named as C-nucleosides, spongouridine, and spongothymidine were isolated and identified from the Caribbean sponge, *Cryptotheca crypta*. Synthetic analog studies eventually led to the development of other bioactive compounds such as cytosine arabinoside (Ara-C), clinically useful as an anticancer agent, and just about 15 years later, together with Ara-A as an antiviral agent (Schwartsmann et al., 2002).

These studies have clearly demonstrated that the marine environment could be a source of novel bioactive compounds. The systematic investigation of marine environments as sources of novel biologically active agents only began in earnest in the mid 1970's, and many discoveries yielded to novel chemical classes (Petrichtcheva et al., 2003).

More than 600 secondary metabolites have been isolated from marine algae, catalogued as terpenes (60%), fatty acids (20%), and metabolites with nitrogenous

compounds and compounds of mixed biosynthesis (10%). Many of these compounds are bioactive and have been extensively studied using laboratory and pharmacological assays. However, their natural functions under ecologically realistic conditions have been investigated only recently (Thompson et al., 1998).

Marine microorganisms can be considered a new source of prominent bioactive compounds. Although no major therapeutic drugs have yet been developed from the sea, several compounds have so far entered clinical trials as anticancer drugs. Research on marine natural products in the last three decades has also brought to discoveries of many chemically and biologically interesting molecules. Some of them (e.g., kainic acid, okadaic acid, tetrodotoxin, manoalide, palytoxin, etc.) have become indispensable tools in biochemical research and played significant roles in the recent advance of life science (Higa et al., 2001).

Recent studies suggest that some bioactive compounds isolated from marine animals (e.g. sponges and molluscs) and marine plants are related with natural products structurally similar to metabolites from microorganism that live internally or externally in some of them (Kobashi and Ishibashi, 1993). This suggested the microbial origin of some of these compounds. The complexities of these associations in marine organisms, especially in seagrasses, like *Thalassia testudinum*, make it more difficult to establish if there is a definitive biosynthetic source of natural marine products.

The isolation of a small lactone, leptosphaerin from *Leptosphaeria oraemaris* by Schiehser et al., (1986) suggested that marine fungi might represent important resource for unique metabolites. Also, the useful chemical, gliovictin, was isolated from the

marine fungus, *Asteromyces cruciatus* (Shin and Fenical, 1987). Lin et al., (2002), Chen et al. (2003), and Krohn and Riaz (2004) revealed the presence of secondary metabolites from marine fungi, such as *Verruculina enalia*, *Kandelia candel*, and *Xylaria* sp. isolated from mangroves. The compounds found showed potent and diverse antifungal activities, antitumor activities, and acetylcholine esterase inhibitors, respectively.

These discoveries support the idea that organisms, plants, and sediments associated to marine ecosystems represent important environments for the isolation of microorganisms capable of producing bioactive compounds (Castillo–Machalskis et al., 2006). Even so, the role of the endophytic fungi in seagrasses and the importance of these fungi in the local ecology are unknown**.**

2.3.2 Production of bioactive compounds by endophytic fungi

Limited studies on endophytes have revealed an interesting novel array of bioactive compounds. Biological diversity implies chemical diversity, due to the constant chemical innovation that exists in the ecosystems where the evolutionary race for surviving is active. Tropical forests are a notable example of this type of environment. The great competence, the limited resources and the selective pressures of the selection can change or induce the production of bioactive compounds (Aracari, et al., 2005). By comparison these bring a high probability that marine environments have invaluable resources of bioactive compounds.

Bills et al., (2002) describe a metabolic distinction between tropical and templates endophytes through statistical analysis in which compares the number of remote natural bioactive products from endophytes in tropical regions with those from temperate origin. They found that endophytes from tropical regions presented a mayor activity of natural products than the endophytes from template regions.

Besides, they observed a number of significant tropical endophytes producing a great number of active secondary metabolites, as compared with other studies (Bills et al., 2002). These observations suggested the importance of the host plant in the general metabolism of endophytic fungi. Ergot alkaloids 2–18 were discovered in cultures of *Neotyphodium* endophytes. These metabolites were later demonstrated to be neurotoxic to insects and mammals, suggesting an herbicide role for the fungi. Although terrestrial fungi have been a major resource, but studies that develop the potential of marine fungi are scarce.

Bérdy (2005) establish that a total number of 50,000 microbial metabolites may be known and approximately 8,600 come form bioactive compounds from fungi, representing 38% of all microbial products (Table 1). From the early nineties the number of bioactive compounds isolated from various filamentous and other microscopic and higher fungal species had continuously increased up to more than 50% by the turn of the millennium.

Table 1. Approximate number of bioactive microbial natural products (2002) according to their producers (reproduced from Bérdy, 2005).

Source	Antibiotics	Other bioactive metabolites	Total bioactive metabolites	Used in human therapy	Inactive metabolites
Bacteria	2900	900	3800	10-12	3000 to 5000
Actinomycetales	8700	1400	10100	100-120	5000 to 10000
Fungi	4900	3700	8600	30-35	2000 to 15 000
Total	16500	6000	22500	140-160	20000 to 25000

2.3.3 Methodologies for the isolation of bioactive compounds from endophytic fungi

The isolation and the purification of bioactive compounds from endophytes happen to be a difficult process. The most common methodology involves the fungal growth in a liquid media and chromatographic techniques such as thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) (Hundley, 2005).

3. METODOLOGY

3.1 Sampling area and sample collection

Samples of *Thalassia testudinum* were obtained from Buyé beach in Cabo Rojo, Puerto Rico. A total of 18 samples were pulled and hand collected from the muddy substrate, consisting on a piece of rhizomes with at least two leaves. Two samplings were conducted, one on February 2006 (M1) and the other on June 2006 (M2). The sampling area is moderately impacted by human activities such as aquatic motorboats and bathers. The shore is rocky, with rocky bottoms end reefs, inhabited a diverse population of fishes and sea urchins. The sand is white and the water is crystaline.

The study area was divided in twelve quadrants of 10 x 10m (Figure 1). The exact location lies between 18º 02' 45.3" N and 67º 12' 11.5" W, as determined by a global position system (Asunto ®). Salinity measures were taken with a refractometer (model RHS-28), as well as temperature and depth measurements before collecting each sample. The samples were selected haphazardly inside the perimeters of each quadrant. Thirteenth samples of *T. testudinum* were collected; twelve from the quadrants previously divided and the thirteen sample was obtained haphazardly from inside the study area. The samples were kept in sterile plastics bags and transported for processing to the laboratory of Aerobiology and Endophytic Fungi at the Biology Department of the University of Puerto Rico, Mayagüez Campus (UPRM).

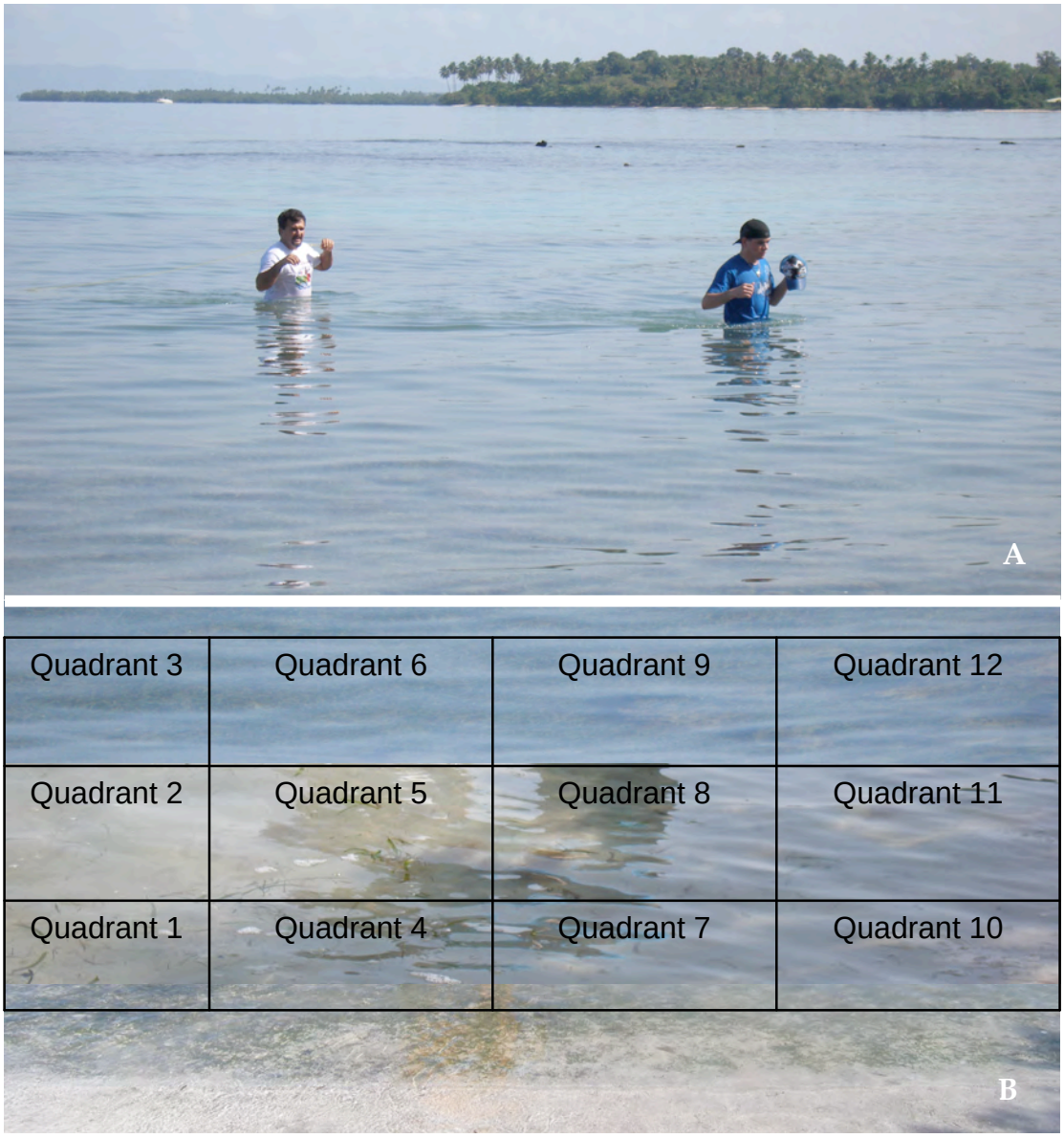

Figure 1. Study area (A) and sampling quadrants (B).

3.2 Processing of sample

At the laboratory two rhizomes with at least four leaves were selected from each quadrant. Four leaves were removed from each rhizome; 2 undamaged (healthy looking) and 2 damaged (showing foliar stains, chlorotic symptoms or necrosis). The leaves were surface sterilized with a solution of sodium hypochlorite (0.5%) for one minute, to eliminate epiphytic organisms, spores or mycelium fragments (Petrini, 1986) and rinsed with distilled water for one minute twice.

Every leaf was cut in three sections: apex, center, and base (Figure 2A) with a sterile dissection knife. Each section was cut in 3 pieces and each piece was subsequently cut in 3 sections (1 x 1 cm). The 9 pieces obtained for each section were placed in groups of 3 in 3 Petri dishes (100 x 15 mm) containing Marine Agar (MA, 55.1 g l-1, Difco) (Figure 2B) supplemented with chloramphenicol (4 ml/l-1) to avoid bacterial growth. Plates were labeled according to sample number (M1 or M2), quadrant number (C1, C2, C3, etc.), plant number (Pl1 or Pl2), and leaf type (undamaged or damaged). Each plate was sealed with parafilm and incubated at 25˚C during almost 30 days or until growth was observed.

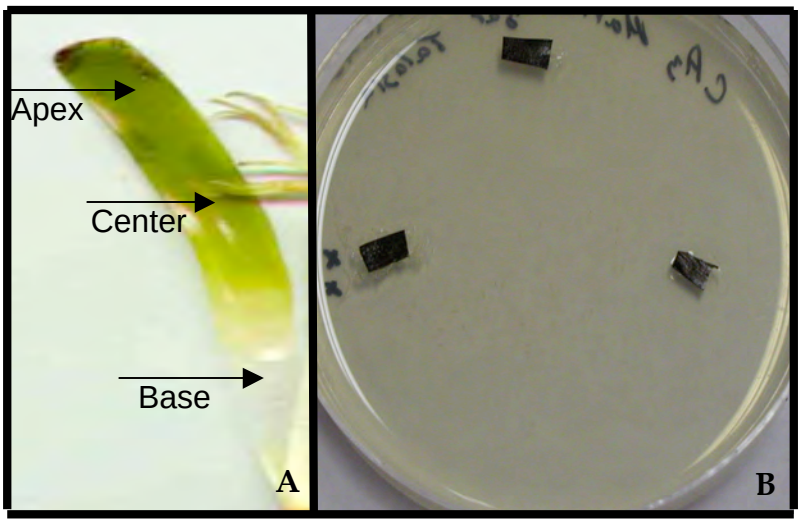

Figure 2. Undamaged leaf of *T. testudinum* showing leaf sections (A) and a Petri dish containing MA with inoculated fragments (B).

3. 3 Isolation of fungal endophytes

Colonies from mycelial fungi growing around the edge of each fragment were inoculated in Petri dishes (60 x 15 mm) with Oatmeal Agar (OA, 72.5 g l-1, Difco), Malt Extract Agar (MEA, 33.6 g l-1, Difco), MA, and Potato Dextrose Agar (PDA, 39 g l-1, Difco) and incubated at 25˚C for 7 days or until growth was observed. We documented mycelial endophytic fungi isolated by digital photography (Camedia C-7070) and characterized each colony macroscopically (form, color, elevation, and texture).

Semipermanent slides were prepared with lactophenol for microscopic characterization. The morphological characteristics were observed with light and interdiferential contrast microscopy (Nomarsky) utilizing an Olympus BH2 microscope. Photographs and measurements of the structures were obtained using a digital camera and a computer program (SPOT Insight®). Fungi isolated in pure cultures were identified

using the taxonomic keys of Ellis (1971), and Barnett and Hunter (1987).

3.4 Bioassays

Pure cultures with exudates of *Aspergillus fumigatus*, *Botrytis cinerea*, A*cremonium strictum*, *Cladosporium cladosporioides*, *Curvularia lunata*, and *Penicillium janthinellum* were selected. Using 50ml beakers, we inoculated individually 6 different species of the endophytic fungi in Malt Extract Broth (MEB). Cultures were incubated in a shaker at 25ºC for 2 weeks at 120 rpm. After incubation the fungal cultures were filtered and the filtrates used to impregnate blank sterile disks. Impregnated disks were used for further bioassay. A beaker containing only MEB was incubated as a control.

Bioassays were conducted with human and plant opportunistic bacteria, including *Escherichia coli*, *Burkhodenia cepacia*, *Erwinia chrysanthemi*, *E. caratovora pv. caratovora, Pseudomonas aeruginosa, P. cichorii, Xanthomonas axonopodis, X. axonopodis pv. translucens, X. campestris pv. campestre, X. campestris pv. phascali* and *X. campestris pv. fuscans*. Bacterial cultures were provided by Dra. Mildred Zapata from the Department of Crop Protection (UPR-M) and Mrs. Magaly Zapata from the Department of Biology (UPR-M).

Each bacterial culture was transferred to Petri dishes (60 x 15 mm) with Tryptic Soy Agar (TSA, 40.0 g l-1, Difco) and incubated at 25°C for 24 to 48 hours. From fresh cultures we inoculated using a sterile culture swab (BBL^{TM} Becton Dickinson) in seven Petri dishes (100 x 15 mm) containing TSA and challenged against four different fungal

filtrates and a control disk. We also tested these 6 fungal extracts against 8 endophytic bacteria isolated from undamaged tissue of *T. testudinum* from the Solar Saltern and 6 from *Puerto de la Libertad* in Vieques Island.

The cultures were incubated at 25°C for 24 hours. Then we documented interactions between the filtrates and the test microorganisms. To maintain the viability of the bacterial strain and avoid mutations, these were criopreserved at -80˚C (Simione, 1998).

3.4.1 Alternate antibacterial screening

Pure cultures of *Salmonella* and *Pseudomonas* were reactivated in 20ml of a liquid media an incubated for 24 hours. After the incubation period, 100µl of each bacteria were plated on separate culture plates of Milton Hueller's Agar (MHA). The inoculum was evenly distributed using glass beads to create a bacterial field. For the bioassays, six paper disks cut in half were placed on each of the plates containing the bacterial field of *Salmonella* and *Pseudomonas*. Then, 10µl of concentrated extracts 7-12 were placed on each of the discs on the bacterial field.

A similar process was followed for the fungal exudates, except there was not enough sample in any of the tubes and the concentrations were slightly adjusted. About 4 drops of 100% ethanol to dilute the samples were added to provide enough samples to test. Due to the ethanol in the samples, exudates could not be directly added onto the bacterial field. Instead, twelve half-disks on a sterilized glass surface received 10µl of exudate/ethanol sample and allowed the ethanol to evaporate.The procedure was repeated

a second time placing 10µl of each exudate/ethanol sample onto each of the twelve discks. After the second addition of the exudate/ethanol sample the discks were placed onto the MHA culture plates that contained the bacterial field. For sample 3, that contained a larger volume of sample the exudate was added to the discks using a capillary open at both ends. No ethanol was added to this sample. The plates containing the twelve discs saturated with each exudate sample were incubated for about 16 hours, to promote bacterial growth. After the incubation period, plates were evaluated to determine if inhibition of bacterial growth has occurred (Hundley, 2005).

3.5 Separation of bioactive compounds

3.5.1 High Performance Liquid Chromatography

Curuvlaria lunata and *Penicillium janthinellum* exudates were diluted in 100% EtOH to make about a 50:50 exudate/ETOH mix. Then 10 µl of the 50:50 exudate/EtOH mix were drew into a syringe and deposited into the HPLC. The HPLC started with 100% H_2O as the solvent, and the HPLC system was set to increase acetonitrile by 10% every 10 minutes. At the 60 minute point, the HPLC system increase the acetonitrile concentration to 100% and was run for 15 more minutes, for a total of 75 minutes. Every 2 minutes fractions were collected to test against Gram-negative bacterial species. The same exact procedure was repeated three times and each time fractions were collected every two minutes (Hundley, 2005).

After collecting the individual fractions three times each, an anti-bacterial test (Sec. 3.4.1) on each fraction was performed using the protocol previously described. Paper discks were used and let the fractions, which contained acetonitrile, sufficient time to evaporate before placing the saturated discs in contact with the bacterial field.

3.5.2 Mass spectral analysis (MSA)

MSA were run on an Agilent LC/MS equipped with a time of flight (TOF) electrospray mass spectrometer and diode array detector. The mass spectral data was analyzed using a computer program that allowed to look at the intensity and the absorbance of the compound (Hundley, 2005).

3.6 Host specificity tests

Due to permit limitations and possible damage to the marine ecosystem, we only carried out *in vitro* and *in vivo* laboratory host specificity tests. Twenty-four samples with undamaged leaves and part of the rhizomes were collected form Buyé beach; 18 for the *in vivo* test and 6 for the *in vitro* test. To allow the maintenance and development of the *in vivo* samples we created a controlled system in the laboratory (Figure 4) to recreate natural growing conditions for *T. testudinum*. Six containers were constructed (22 x 9.2 cm.) with the appropriate dimensions for the plants and its substrate.

Water taken from Buyé beach in plastic containers was let to rest for a period of 30 days, and 2 L were filtered (Millipore, 0.115ml, 0.1μm filter) for each container. Aeration was provided to avoid the deterioration of the plants. Also alternated 12 hours cycles of light and darkness were provided by an artificial light (n:vision, 150W), simulating the natural cycles to which the plants are exposed. Salinity (25-30%) and temperature (25-27˚C) parameters were taken daily and regulated with distilled sterile water.

Figure 3. Samples used in the host specificity tests.

Three plants samples (shoots) with part of the rhizome and developed undamaged leaves (Figure 3), were placed in each container (Figure 4B). Plants were fixed to a piece of dead coral to secure them at the bottom of the container. Experimental plants (2) were marked with a yellow tape as infected and the third was kept as control. Also separated leaves were placed in containers with 200 ml of filtered sea water (Figure 4A) at room temperature and lighting.

The infection procedure was conducted after the plants were stabilize in the system previously described. Pure cultures of the six endophytic fungi were transferred to Potato and Carrot Agar (PCA) and incubated for 7 days. After the incubation period all the mycelium was removed and suspended in 100 ml of sterile water with 3 drops of Tween 20 (Sigma Chemical Co.) and mixed gently (Vélez-Rodríguez, 2001). Then 1/2cc were injected into the experimental leaf tissue with a syringe (Becton Dickinson Insulin Syringe 28G1/2 (0.36mm x 13mm)). The concentration of conidia/ml was determined utilizing a hematocitometer (Hausser Scientific, 3120).

Figure 4. Container with individuals leaves (A) and controlled system for *in vitro* pathogenicity tests (B and C).

3.6 Analysis of results

3.6.1 Fungal endophytes isolated

A total of 1404 pieces were processed for each sampling, 702 from undamaged leaves and 702 from damaged leaves. We compared the species of mycelial endophytic fungi identified from the undamaged leaves with the species identified from the damaged leaves. We calculated the total infection rate (pieces infected / 2808) and for each sampling (total of peaces infected / 1404). Also, we calculated the infection rate per leaf region (pieces infected in each region / 468) and leaf type (pieces infected in the leaf type undamaged/damaged leaf/ 702). To show if the rates of infection are independent of the sampling or of the region of the leaf we carried out an independence Chi² (χ^2) test with a level of confidence of 95 % using Infostat ® (Student version 2006). We calculated the relative frequency for each genus of mycelial endophytic fungi identified in each sampling (number of times that genus or specie was isolated / total of fungi isolated initially in the sampling) (Espola, 2005). The data is presented with graphics generated with Microsoft Excel ® (Version 9.0).

3.6.2 Bioassays

Bioassays were performed by documenting the presence or absence of an inhibition zone around the impregnated filter disks (Harley y Prescott, 1999). The inhibition zone was measured taking the distance between the borders of the filter disks with the fungal extract to the nearest bacterial colony. The bacteriostatic effect was measured as an inhibition zone of 1 cm or more (Lee et al., 2004) around the impregnated disk, documented as susceptible.

3.6.3 Host specificity test

We determined the patogenicity of the fungi inoculated in the leaves by observing any change in the color of leaf tissue. In this way we establish the relationship between the fungi and the health of the leaf tissue of *T. testudinum*. To determine if the fungi inoculated is a pathogen we looked for damaged similar to those seen on the original tissue recuperated for the sample (chlorotic symtoms or necrosis) and the loss of vitality of the host. If no change occurs in the tissue, the fungi were considerate to be non-pathogenic to the host plant (Fernández, 2005).

4. RESULTS AND DISCUSION

4.1 Isolation of endophytic fungi

A total of 1404 fragments from leaves of *T. testudinum* were processed from each sampling. Half of the fragments (702) came from undamaged leaves and another half (702) from damaged leaves. Infection has occurred in 138 fragments of undamaged and damaged leaves, confirming the presence of endophytic fungi in the tissue of *T. testudinum* (Table 2).

The infection was greater during the second sampling (0.70) compared to the first sampling (0.30). This can be a result of the climatic change that occurred between sampling dates temperature (24˚C to 28˚C), which can influence the distribution of the organisms in the marine systems (Barnabe, 1996), or could be due to a natural difference in the abundance of the endophytic fungi in the plant tissue. However, the difference in pieces of leaves colonize by mycelia fungi was independent of the date of the sampling, as showed by the X^2 test ($p \geq 0.5$).

Another variable considered was the depth from where the samples were collected. Sample collected at depths of 101 cm or more showed an infection rate of 19% where samples collected at depth between 10-50 cm showed and infection rate of 42% (Figure 5). This may be due to the fact that the substrate is more accessible for the fungi in the edge of the beach. This factor permitted that the spore flow through in less time, arriving quickly, and colonizing in greater quantity in shallow areas. Also, the sunlight is

more effective providing the hypha the adequate stimulus to growth and extends apically inside the plant, like the circadian cycles suggested (Merrow et al., 1997).

Fungal distribution on the leaves was very similar for each section (Table 2), suggesting that there is no preference for a specific foliar area. In the fist sampling infection rate was higher on the center and for the second sampling the infection rate was higher on the base, with a relative frequency of 0.38.

An additional variable considered was the condition of the leaf (undamaged and damaged). The results demonstrated that both undamaged and damaged tissues were infected by fungal endophytes. Also, the same genera (*Aspergillus*, *Cladosporium*, *Culvularia*, *Penicillium*, and *Scopulariopsis*) were isolated from damaged and undamaged tissue (Figure 6 and 7). Interestingly, exudates producing genera (*Botrytis cinerea* and *Acremonium strictum*) were only isolated from damaged tissue. We found a significant difference between the genera of endophytic fungi recovered from damaged and undamaged leaves ($p \geq 0.5$) for the first sampling. The second sampling did not show any significant difference ($p \leq 0.5$).

Our results suggest two possibilities: (1) endophytic fungi affect the health of the plant in their struggle of surviving when substrate conditions are not favorable; or (2) endophytic fungi are not responsible for the damaged that we observe in the tissues of *T. testudinum* in the sample processed. To corroborate and establish the relationship between the fungi and the host plant we carried out host specificity test (Section 4.4).

Table 2. Relative frequency (RF) of infection in *T. testudinum* by leaf section.

	First sampling		Second sampling	
Section	No. of colonies	RF	No. de colonies	RF
Apex	15	0.33	34	0.33
Center	17	0.38	30	0.29
Base	13	0.29	39	0.38

Table 3. Relative frequency (RF) of infection damaged/undamaged tissue of T. *testudinum*.

	First sampling		Second sampling	
Type of tissue	No. of colonies	RF	No. de colonies	RF
Damaged	7	0.19	29	0.59
Undamaged	38	0.81	20	0.41

4.2 Identification of isolates

This study determined the diversity and distribution of endophytic fungi associated to damaged and undamaged leaf tissue of *T. testudinum*. We identified eight genera of mycelial endophytic fungi that produced exudates in pure cultures. *Cladosporium cladosporioides* (0.11), *Curvularia lunata* (0.08), *Penicillium janthinellum* (0.23), and *Scopulariopsis asperula* (0.05), were present in the two samplings. We also recovered *Colletotrichum spp* (0.01) and *Aspergillus niger* (0.01), and *P. crysogenum* (0.15) in the fist sample. *Aspergillus flavus* (0.1), *A. fumigatus* (0.06), *A. ustus* (0.01), *A. versicolor* (0.07), *Botrytis cinerea* (0.03), *Acremonium strictum* (0.06), and *Curvularia brachyspora* (0.03) were isolated from the second sampling. A more diverge assemblage of endophytic fungi was documented from undamaged leaves (0.53). For the first sampling the colonies were obtained particularly from undamaged leaves (0.81), while for the second sampling more colonies were recovered from damaged leaves (0.59).

In a previous study Malavé (2006) also documented the presence of *Aspergillus fumigatus, A. ustus, A. flavus, Botrytis* spp., *Cladosporium oxysporum*, and *Penicillium janthinellum*. In contrast to Wilson (1996) we found *Aspergillus niger* in undamaged leaf tissue. This is the first report of *A. niger*, *C. lunata*, *Colletotrichum* spp., *A. versicolor*, and *Acremonium strictum* as endophytes of *T. testudinum*.

Unfortunately, little is known about the specific nutrients and growth factors required by most of the marine microbes. As a result of these difficulties, less microbial organisms are cultivable under standard laboratory conditions. Presently, this condition, certainly limits the scope and ability to isolate and culture interesting and new microbes (Lipton, 2004). We have to become aware that many species of endophytes will not sporulate in commonly used agar cultures, and that some loose the ability to sporulate deteriorates rapidly with consecutive transfer (Redlin and Carris, 1996).

The distribution and presence of fungal endophytes are especially unknown in many marine plants and marine environments. It is regulated by factors such as the mode of transmission, the presence of other micro and macroherbivore species, plant age, nutrition, and the abiotic environment. In *T. testudinum* from Cabo Rojo, the assamblage of fungal endophytes is diverse, but include dominant species.. *Penicillium janthinellum* was the most abundant in the first sampling (0.42), while for the second sampling was *Aspergillus flavus* (0.23).

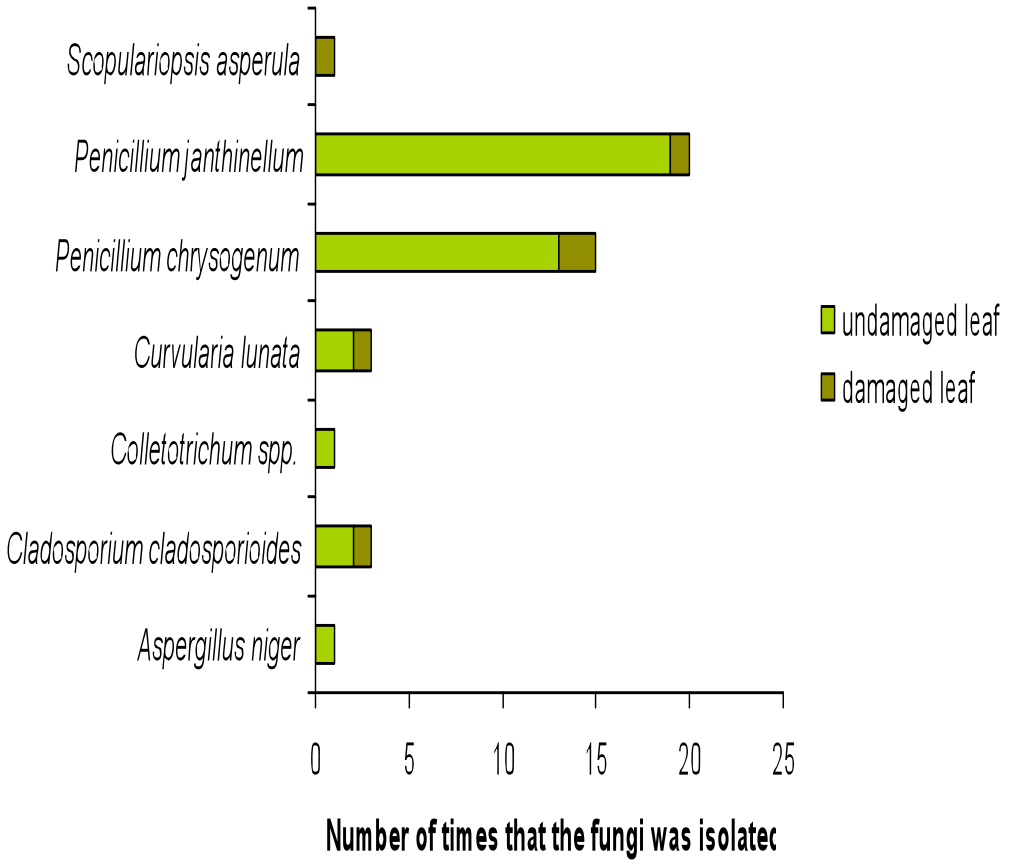

Figure 5. Isolated fungi from tissue of *T. testudinum* during the first sampling.

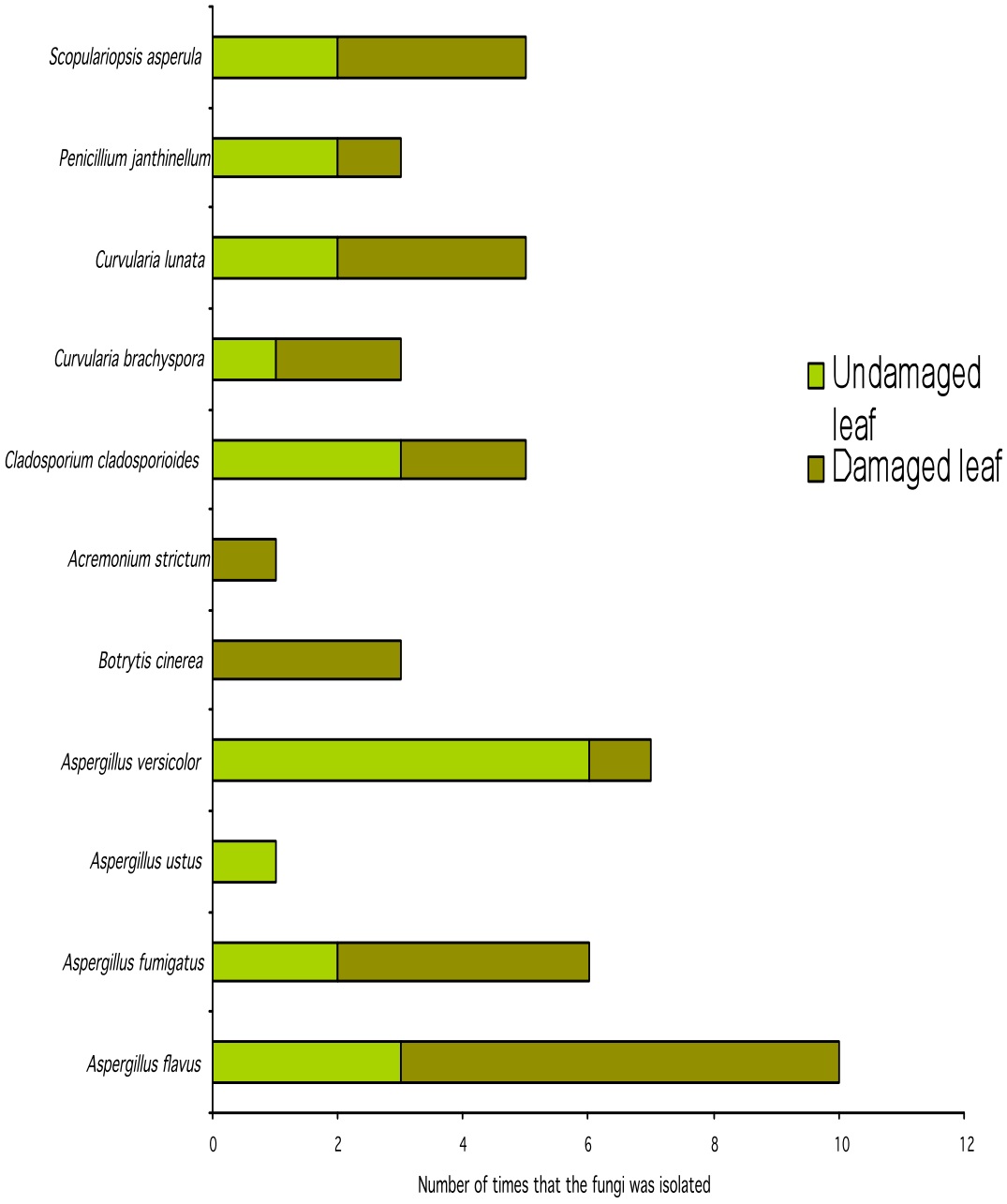

Figure 6. Isolated fungi from tissue of *T. testudinum* during the second sampling.

4.2.1 *Aspergillus* Micheli

Aspergillus was one of the most abundant genera isolated from the second sampling from undamaged and damaged leaves. Species identified included *A. flavus*, *A. fumigatus, A. niger, A. ustus,* and *A. versicolor*.

Aspergillus is a genus of intense biological, industrial, agricultural and medicinal importance. Micheli in 1729 was the first to distinguish stalks and spore heads, but it was not until the middle of the 19th century that these fungi began to be recognized as active agent in decay processes, as causes of human and animal disease, and as fermenting agents capable of producing valuable metabolic products (Raper and Fennel, 1965).

Aspergillus species are found widely distributed in nature from a great variety of substrate. Due to the facility of dispersion of their conidia through air and to their small size, these can remain suspended in the environment during a long period of time. *Aspergillus* spp. are generally regarded as opportunistic pathogens that require wounds or otherwise weakened hosts for colonization (Raper and Fennel, 1965). Each one of these species may have the capacity to produce bioactive compounds.

Aspergillus fumigatus Fresen. was first recognized as an endophytic fungus in the leaves of *Cynodon dactylon.* In spite of their patogenicity, the fungi has the capacity to produce two metabolites called asperfumoid and asperfumin, as a group with 6 composed bioactive compounds that include monomethylsulochrin, fumigaclavine C, fumitremorgin C, physcion, helvolic acid and 5alpha, 8alpha-epidioxy-ergosta-6, 22-diene-3beta-ol (Liu et al., 2004).

Pathogenicity attributes of *A. fumigatus* include a combination of factors secreted from growing mycelia and terminal hyphal cells, cell wall structural components, including hydrophobins, and pigments that confer resistance to phagocytic killing (Hogan et al., 1996). One set of putative *A. fumigatus* virulence factors are mycotoxins, secreted fungal secondary metabolites that are harmful to humans and animals (Nielsen and Smedsgaard, 2003); may act as toxins in the host cell include fumagillin, helvolic acid, fumitremorgins, phthioic acid, and gliotoxin among others Gliotoxin in particular has been fingered as likely virulence factor due to its cytotoxic (Grovel et al., 2002), enotoxic (Nieminen et al., 2002), and apoptosis stimulating properties (Kweon et al., 2003).

Aspergillus flavus, described by Link in 1809, is known by the production of aflatoxins (Desjardins and Hohn, 1997). The aflatoxins B1, B2, G1 and G2 are the major four toxins among at least 16 structurally related toxins. Aflatoxin B1 is particularly important, since it is the most toxic and potent hepatocarcinogenic natural compound ever characterized (Bennett & Klich, 2003). Different *A. flavus* strains may or may not produce either aflatoxin B1 and/or B2. Other toxic compounds produced by *A. flavus* are sterigmatocystin, cyclopiazonic acid, kojic acid, nitropropionic acid, aspertoxin, aflatrem, gliotoxin, and aspergillic acid. In addition, *A. flavus* may produce some other secondary metabolites such as dihydroxyaflavinine, indole, paspalinine and versicolorin A. Two sexually reproducing species in the *A. flavus* complex, *A. alliaceus* and *A. albertensis*, produce a high amount of ochratoxin A, and are considered to be responsible for ochratoxin A contamination of figs (Bayman et al., 2002).

Aspergillus niger Tiegh. (Figure 8) is a filamentous ascomycete ubiquitous in the environment and has been implicated in opportunistic infections in humans. *A. niger* is most widely known for its role as a citric acid producer (Magnuson and Lasure, 2004). With production of citric acid at over one million metric tons annually, *A. niger* citric acid production serves as a model fungal fermentation process. As a common member of the microbial communities found in soils, *A. niger* plays a significant role in the global carbon cycle. This organism is a soil saprobe with a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose. A variety of these enzymes are important in the biotechnology industry, and it also is an important ochratoxin A producer most frequently found in warmer, tropical regions of the world (Bennett and Klich, 2003).

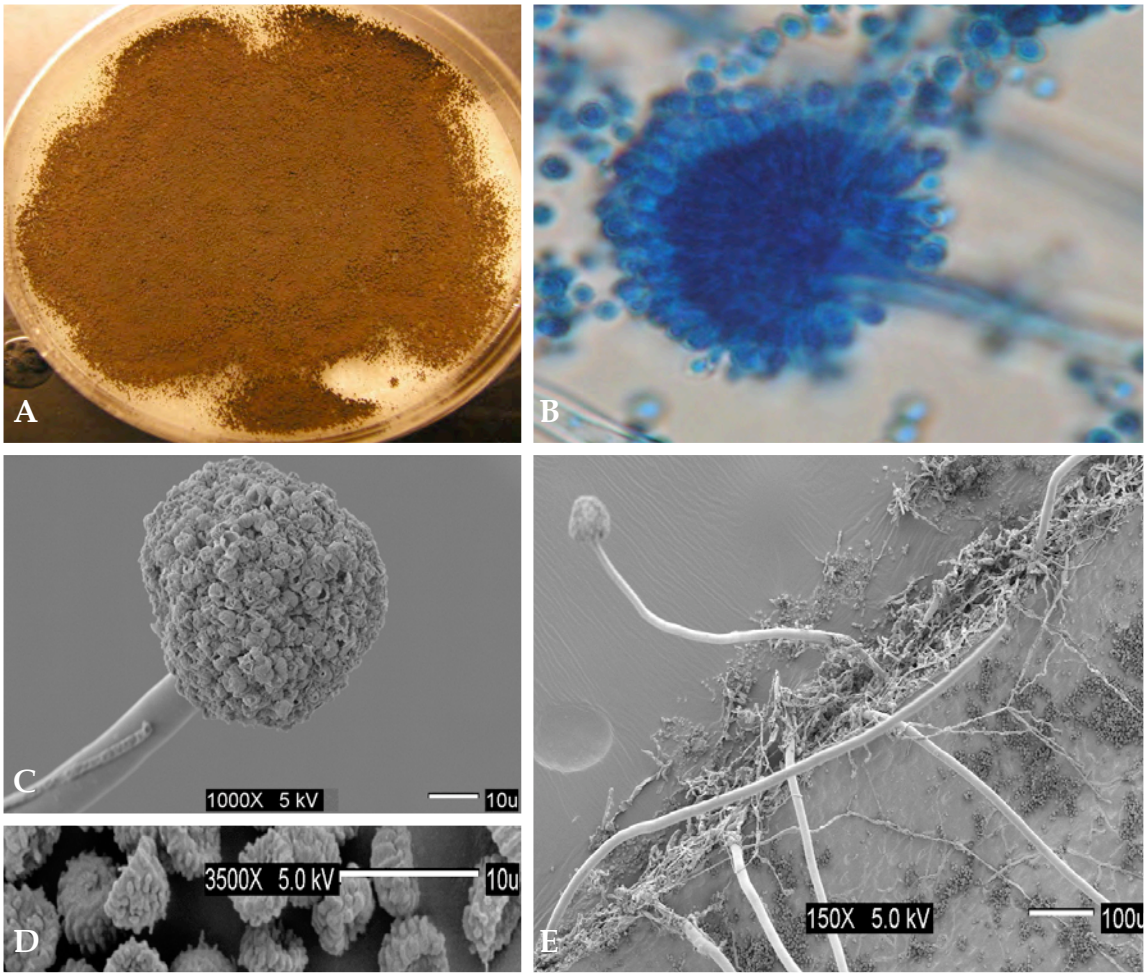

Figure 7. Colony of *Aspergillus niger* in MEA agar (A), bright field microscopy (B) and scanning electron microscopy (C) of conidiophores and conidia (D), and mycelium and conidiophores emerging from tissue of *Thalassia testudinum* (D).

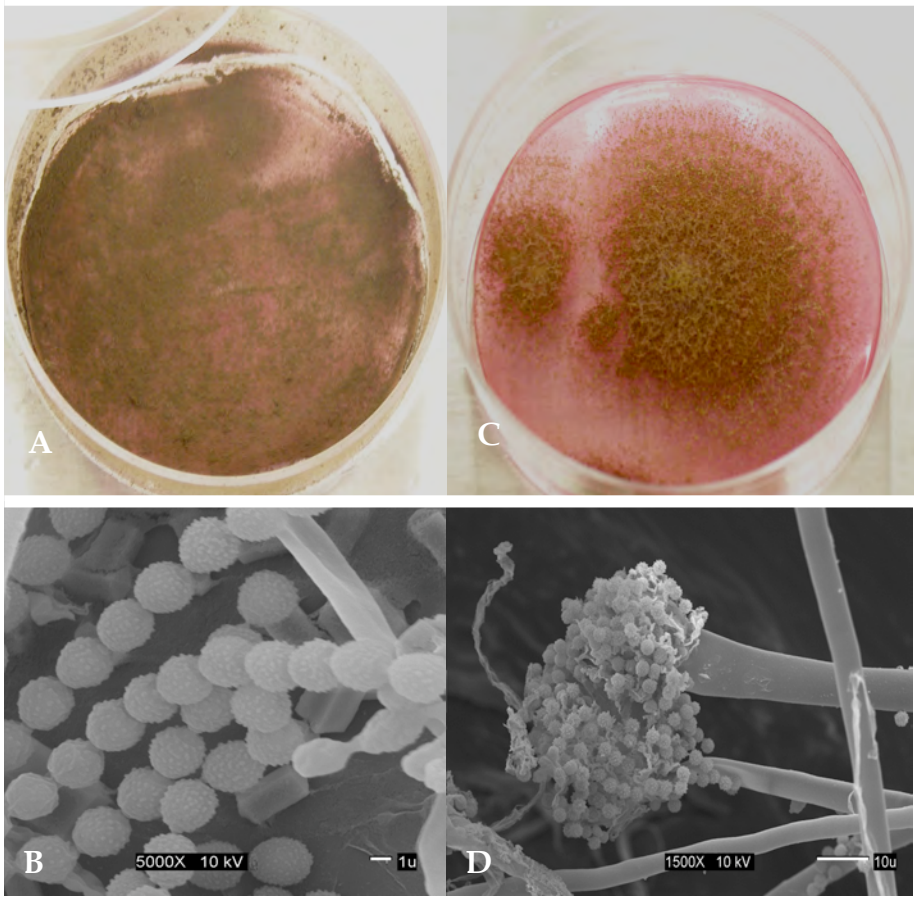

Figure 8. Colony of *Aspergillus fumigatus* in RBA agar (A) and scanning electron microscopy of the conidial parallel chains (B). Colony of *Aspergillus ustus* in RBA agar (C) and scanning electron microscopy of the conidiophores (D).

4.2.2 *Botritys* Micheli ex. Fries

Botrytis species belong to the most geographically widespread group of plant pathogens and saprophytes and cause serious losses to many commercial crops. We reported for the first time *Botrytis cinerea* as a fungal endophyte of *T. testudinum*. From all the *Botrytis* species, *B. cinerea* has the largest host range and includes leaves of rye (*Secale cereale* L.), and strawberry (*Fragaria*), flowers of apple (*Malus*), black currant (*Ribes americanum* L.), eggplant (*Solanum melongena* L.), grape (*Vitis*), pear (*Pyrus communis* L.), potato (*Solanum tuberosum* L.), raspberry (*Rubus*), rose (*Rosa*) and tomato, as well as other host tissue (Carris and Redlin, 2000). This fungi survive the latent period as inactive hyphae within host tissue (Jarvys, 1977).

We only isolated one colony of *B. cinerea* (0.01) from a damaged leaves during the second sampling. Previous studies of Espinosa-García and Langenheim (1992) suggested that certain species of endophytes may function as antagonist or stimulators to other fungi, such as *B. cinerea* and *Pestalotiopsis funerea*. This let us infer that his growth could be regulated by others microorganisms.

Collado et al. (1995) describe four secondary metabolites from a shake culture of *B. cinerea*. Some with phytotoxic potential, such as botcinolide, a highly substituted lactone and botrydial, a tricyclic sesquiterpene. Aleu et al., (2000) also identified several related compounds of botcinolide and botrydial.

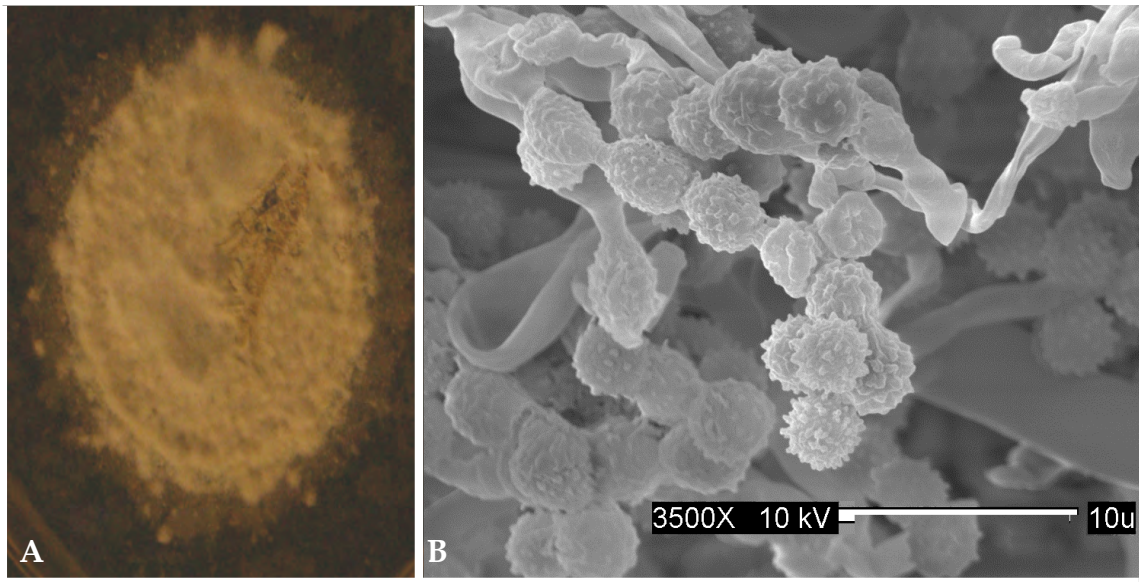

Figure 9. Colony of *Botrytis cinerea* in MEA agar (A) and scanning electron microscopy of conidiophores (B).

4.2.3 *Cladosporium* Link ex Gray de Hoog

Cladosporium was our third (0.12) most abundant genus isolated form *T. testudinum.* It is frequently found in outdoor air in temperate and tropical climates. It has been isolated from many different types of soil and is a major colonizer of plant litter (San-Martín et al., 1985). The ability to sporulate heavily, ease of dispersal, and buoyant spores give this fungus an ability to rapidly invade many different ecological niches, including marine habitats. Our isolate was identified as *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, also collected and purified from sponges and other marine organisms (molusks and algae) on the Chilean coast (San-Martín et al., 1985) and from *T. testudinum* from Buyé beach in Cabo Rojo by Malavé (2006).

From *C. cladosporioides* and *C. tenuissimum* lactones such as cladospolide A, B and C have been isolated. Cladospolide A and B were shown to be phytotoxic (Hirota et al., 1985). Other secondary metabolites found in *Cladosporium* species were cladosporin and emodin (Scott et al., 1971).

Two strains of the fungus *Cladosporium herbarum*, isolated from the sponges *Aplysina aerophoba* and *Callyspongia aerizusa*, respectively, yielded two new α-pyrones, herbarin A (4) and herbarin B (5), the known compound citreoviridin A (6), and the new phthalide herbaric acid (7). All structures were unambiguously established by 1D and 2D NMR and MS data (Jadulco et al., 2002).

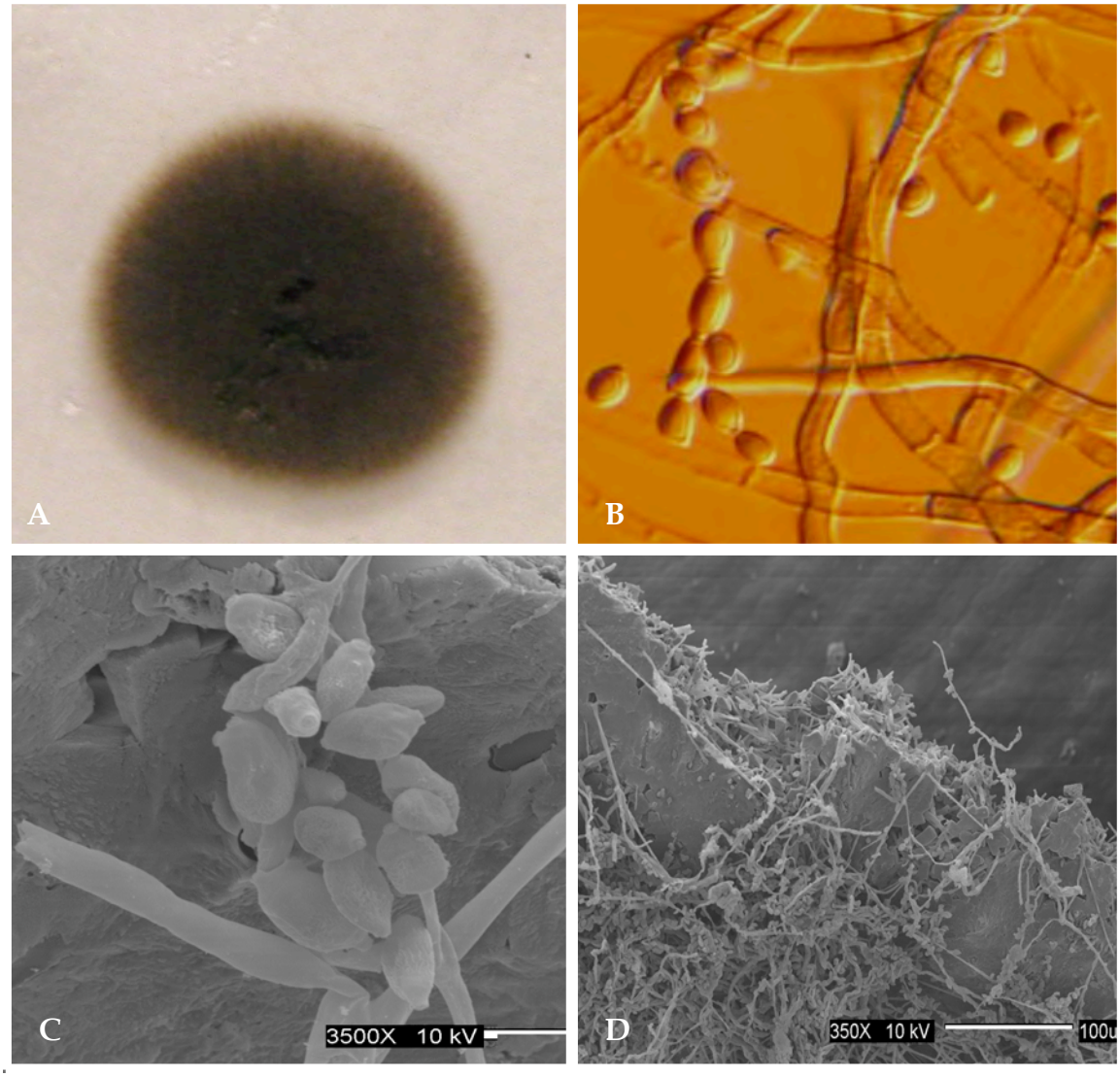

Figure 10. Colony of *Cladosporium cladosporioides in* MEA agar (A), Nomarsky microscopy (B) and scanning electron microscopy (C) of conidiophores, and mycelium of *C. cladosporioides* emerging from tissue of *Thalassia testudinum* (D).

4.2.4 *Curvularia* Boedijn

The genus *Curvularia* is caractherized as a facultative pathogen of many plant species and a saprophyte of soil. Most *Curvularia* species are found in tropical regions, although a few are found in temperate zones. *Curvularia* was defined by the type species *C. lunata* (Wakker) Boedijn. It was isolated from undamaged and damaged leaves (0.11) in both of our sampling.

C. lunata, isolated from the marine sponge *Niphates olemda*, yielded some new 1,3,8-trihydroxy-6-methoxyanthraquinone, named lunatin (1), the known modified bisanthraquinone cytoskyrin A (2), and the plant hormone (+)-abscisic acid (3). Both anthraquinones were found to be active against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* (Jadulco et al., 2002). Hobson et al., (1999) identified *C. lunata* as producing an anthraquinone biochromophore composed of over 70% cynodontin (1,4,5,8-tetrahydroxy-3-methylanthraquinone).

Márquez et al., (2007) has demonstrated the capacity of this genus to protect the host plant, *Dichanthelium lanuginosum*. He determined that *Curvularia* species confer the plant the capacity to survive at 50˚C in Yellowstone, condition in which other plants showed chlorotic or necrotic symptoms.

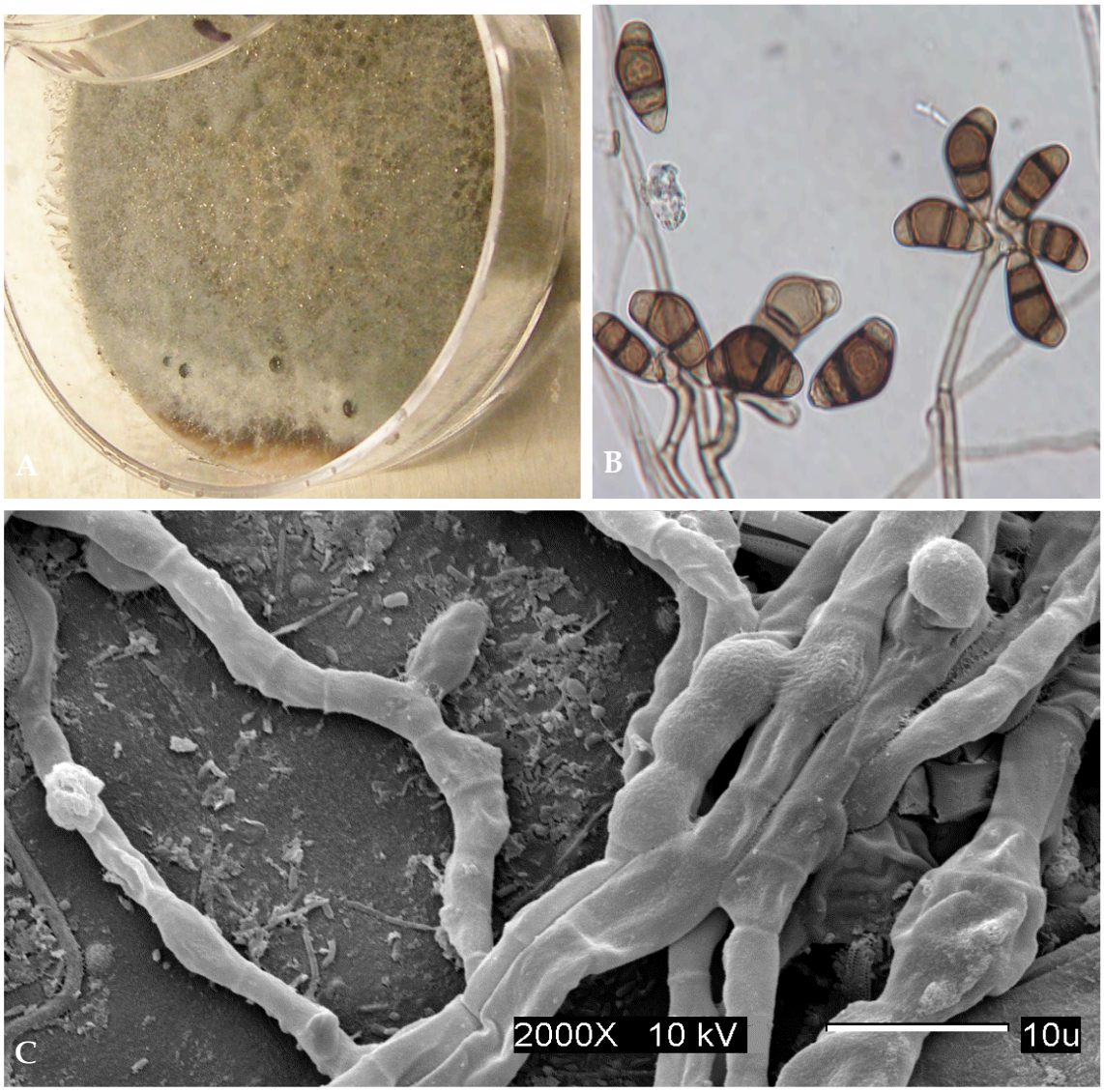

Figure 11. Colony of *Curvularia lunata* in MA agar (A), bright field microscopy (B), and scanning electron microscopy of conidiophores (C) .

4.2.5 *Acremonium* Link ex Fries

Acremonium endophytes show a highly decreased tendency to form stromata on plants, relying instead on seed transmission (White et al., 1991). Some of these endophytes (e.g. *A. coenophialum*) appear to exist entirely as endophytic in their hosts. Associations of the *Acremonium* group of endophytes are often considered as mutualistic, because of the benefial effects (increased hardiness) conferred to the hostess plants (Clay and Leuchtmann, 1989). *Acremonium strictum* W. Gams was isolated from damaged leaves on the first sampling (0.02) and from damaged and undamaged leaves in the second sampling (0.1).

Figure 12. Colony of *Acremonium strictum* in MA agar (A) and conidiophores and conidia in bright field microscopy (B).

4.2.6 *Penicillium* Link

Penicillium was the most abundant genus in our study (0.31). There are over 200 species of *Penicillium*, widely distributed in all environments. *Penicillium* species are important not only due to their widespread occurrence but also because of their ability to produce mycotoxins and other secondary metabolites. (Frisvad et al., 1998a)

Many species has been isolated as endophytes by culturable and non culturable methods (Vega and Posada, 2006**).** Chemotaxonomic studies are therefore often used as additional parameters for identification. Particular isolates may show strain-specific secondary metabolite profiles (Frisvad et al., 1998b). Like the genus *Aspergillus*, fungi with *Penicillium* anamorph states have historically yielded a wealth of biologically active fungal metabolites (Wang, 1995). Our purified colony with exudate production in PDA was identified as *Penicillium janthinellum* Biourge (Figure13A).

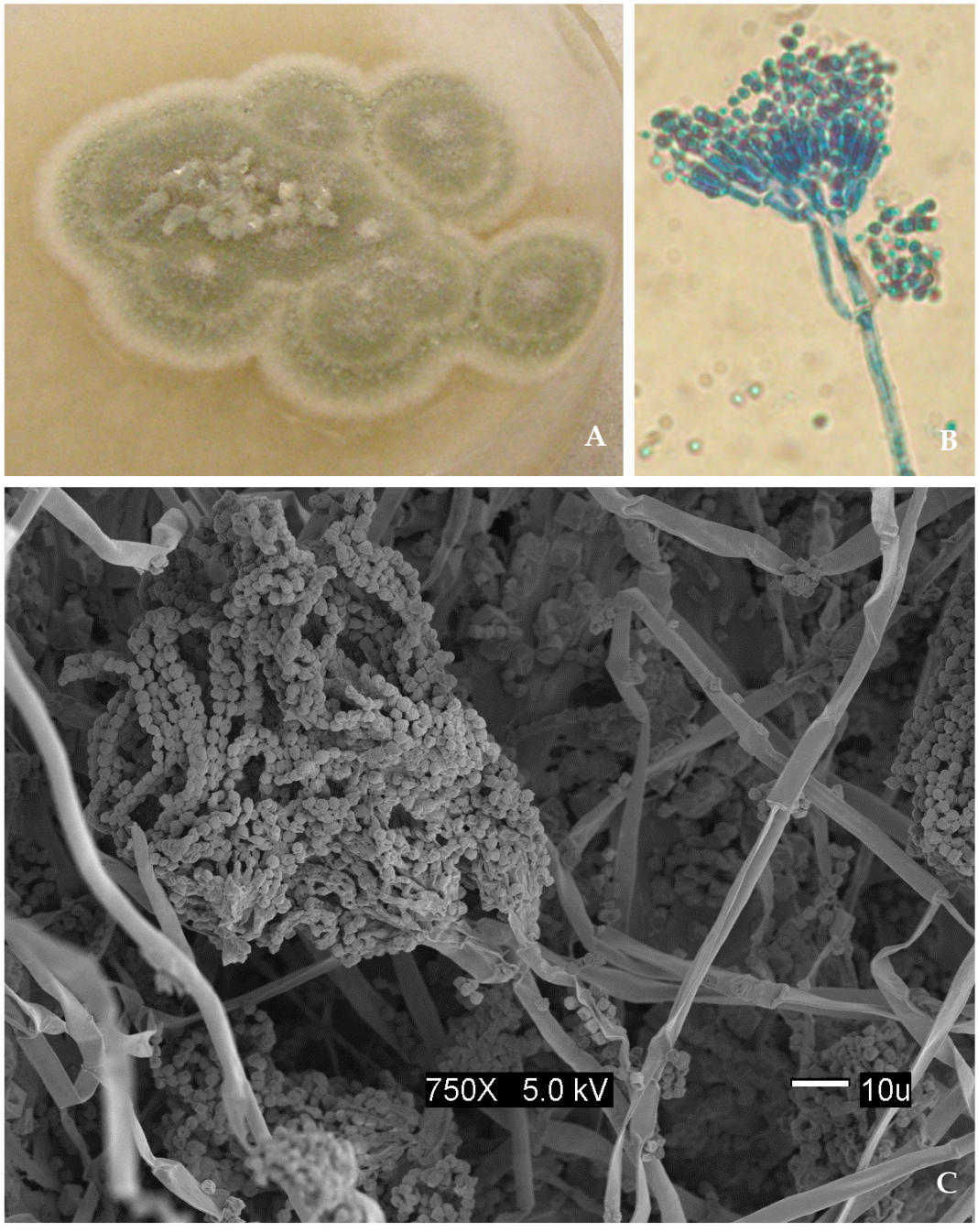

Figure 13. Colony of *Penicillium janthinellum* with exudates in MEA agar (A), bright field microscopy (B), and scanning electron microscopy of conidiophores (C).

4.2.7 *Scopulariopsis* Bainier

Scopulariopsis is a large genus comprised of a number of species commonly found in soil, decaying wood, and various other plant and animal products. *Scopulariopsis* species are among the most common nondermatophytic fungi that cause onychomycosis, and they are also responsible, although less frequently, for deep-tissue infections (Aguilar et al., 1999).

Species of *Scopulariopsis*, as endophytes, were also isolated from other substrates, like lichens (Petrini *et al*., 1990; Girlanda *et al*., 1997; Suryanarayanan *et al*., 2005) and other hosts (Kumaresan and Suryanarayanan, 2001, 2002; Kumar and Hyde, 2004). We isolated colonies of *Scopulariopsis asperula* (Sacc.) S. Hughes from damaged tissue in the first and second sampling and from undamaged tissue on the first sampling

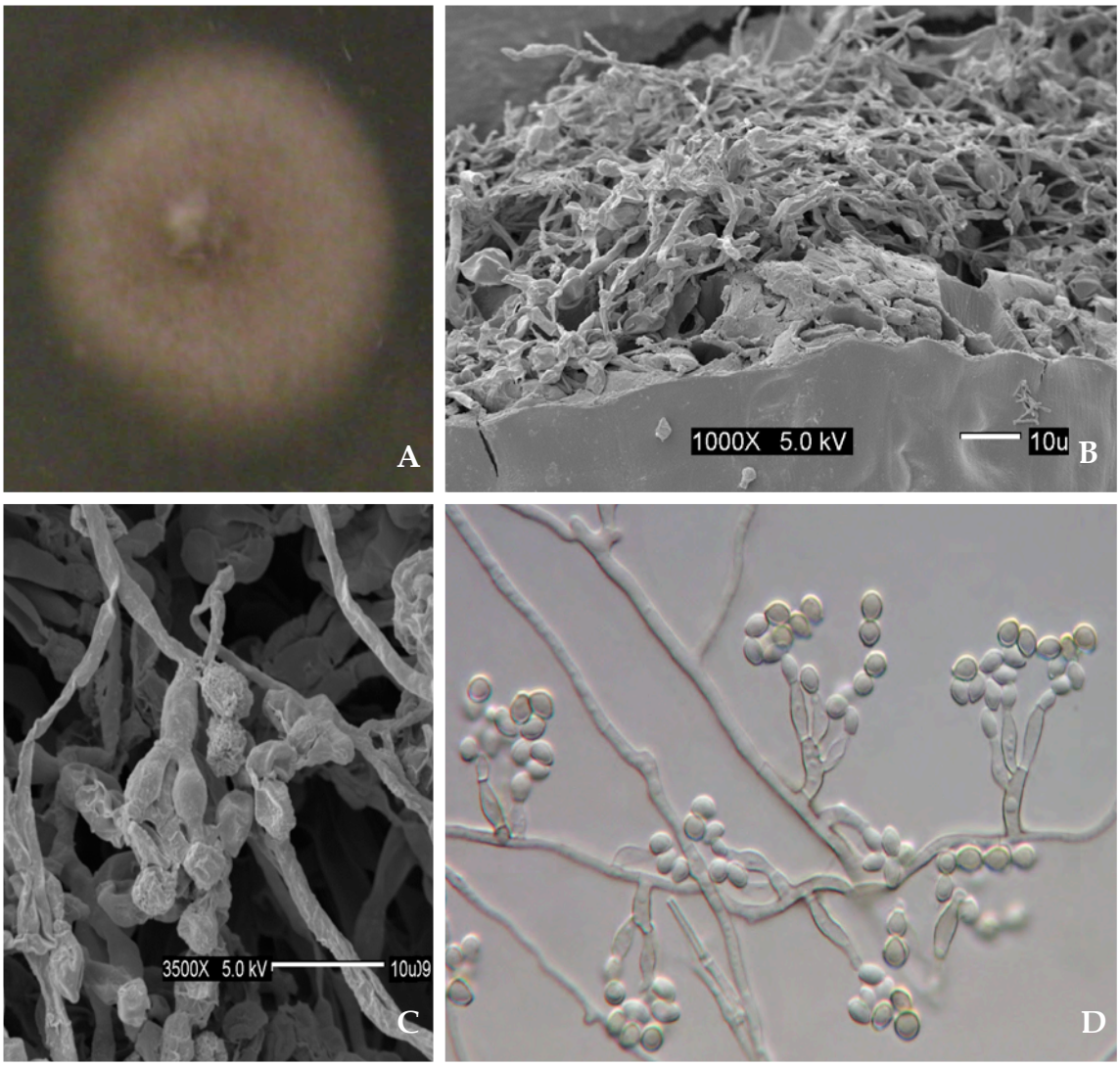

Figure 14. Colony of *Scopulariopsis asperula* in MEA agar (A), mycelium emerging from leaf tissue of *Thalassia testudinum* (B), scanning electron microscopy (C), and Nomarsky microscopy of conidiophores (D) .

4.3 Bioprospecting for bioactive compounds

A total of seven genera of exudate-producing fungi were identified, including *Aspergillus*, *Botrytis*, *Cladosporium*, *Acremonium*, *Colletotrichum*, *Curvularia*, and *Penicillium*. The exudates produced by these endophytic fungi were collected in sterile microtubes. Also, an extract of the liquid culture media was obtained, because the quantity of the fungal exudates recovered was small (>4 µl). We tested the effect of each fungal extract to inhibit the growth of a variety of opportunistic bacterial strains (Appendix 3). After the incubation period, none of the plant opportunistic bacterial strains presented significant inhibition zones (>1cm) around any of the filter disks impregnated with the extracts. Similar to the preliminary studies, only extracts from *Aspergillus fumigatus*, *Penicillium janthinellum*, and *Cladosporium cladsoporioides* showed antimicrobial activity against *E. coli* and *Pseudomonas aeruginosa* (Figure 15).

Two of the endophytic bacterial strains from the Solar Salterns showed inhibition zones when challenged with extracts of *Penicillium janthinellum* and *Curvularia lunata*. Other endophytic fungi showed some antimicrobial activity but with an inhibition zone of less than 1 cm. These results suggested that *P. janthinellum* and *C. lunata* are producing bioactive secondary metabolites that might have a prophylactic role inside the tissue of *T. testudinum,* regulating the microbial communities inside the host plant. Jadulco et al. (2002) tested bioactive compounds from *Curvularia lunata*, such as lunatin and cytoskyrin A, which proof to be active against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*.

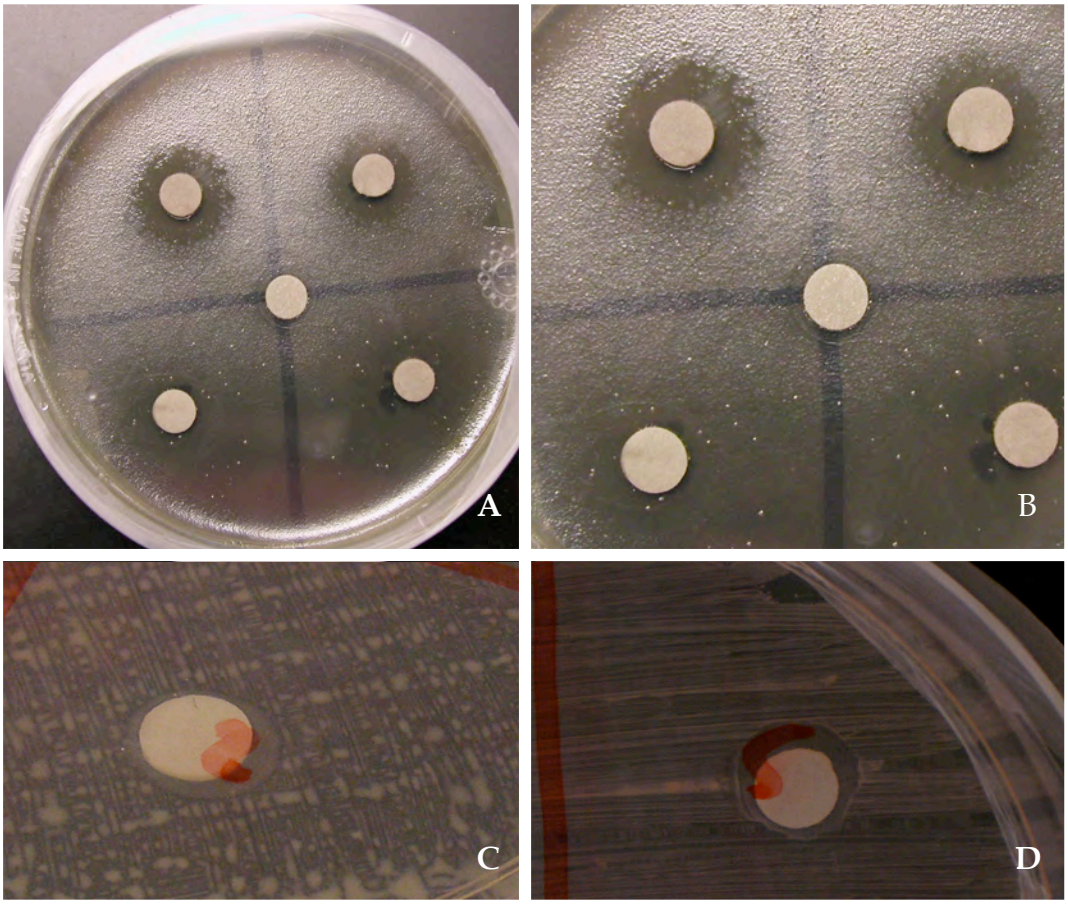

Figure 15. Antimicrobial effect observed in cultures of *Pseudomona aeruginosa* caused by *A. flavus* (A and B), *P. janthinellum* (C) and C. lunata (D).

4.4 Bioactive Compound Analysis

4.4.1 High Performance Liquid Chromatography

Results from the HPLC analysis indicated that there were two peaks at a time of about 38 minutes and 45 minutes with an intensity of 750 microvolts (Table 4). Neither of the fractions were active against bacteria. This lack of intensity in the peaks suggests that the concentration of the compounds tested that correspond to these peaks wasn't enough to have an effect on the bacterial strains evaluated. Because none of the samples showed activity against bacteria, no further continue these experiments

Table 4. High Performance Liquid Chromatography results.

Origin of fungal extracts	Absorbance	Percentage of acetonitrile	Number of peaks
Curvularia lunata	750 nicrovolts	30-40%	2
Penicillium janthinellum	Only noise	Only noise	Only noise

We failed to see any appreciable UV signals from the HPLC separations. *Curuvlaria lunata* did showed some evidence of two separable peaks that we isolated and checked for bioactivity. However, both of these failed to show bioactivity. The data that we have collected suggest that the concentration of the compounds responsible for the anti-bacterial activity may not be in great enough concentration in the fractions. Due to the low concentration, we were unsuccessful in isolating and characterizing the anti-bacterial compounds. The antibacterial activity may be confer by the relationship

between the endophytic fungi and the host plant. The multiple inoculation of the fungi in synthetic media could diminish the compound's activity. This could happen because the fungus is no longer in an environment where it needs to produce the antibacterial compounds to survive.

4.4.2 Mass Spectrometry

Mass spectrometry analysis (MSA) was performed on *Acremonium constrictum* (sample 8), *Aspergillis fumigatus* (sample 7), *Cladosporium cladosporioides* (sample 9), *Curuvlaria lunata* (sample 11) and *Penicillium janthinellum* (sample 12) extracts (Table 5). Initially, the mass spectral data for each of the extracts tested showed only junk peaks. The most prominent junk peak consistently appeared at around 354 amu. Additionally, the intensity of the total ion current (TIC) graph was very low. The TIC intensity only showed up in the range of 1.8e4 to 2.0e4 cps suggesting that either the compound of interest is not ionizable in the MSA or that the sample was not concentrated enough.

The Diode Array Detection (DAD) signal data showed a prominent peak between about 0.10 and 0.25 minutes, a good range for absorbance. A repeated TIC graph showed an intensity to the power of 5 cps for all of the samples that we tested, much higher than previously displayed. These new peaks help us to understand that the compound of interest was ionizable and it is being sufficiently detected in the mass spec.

Deleting the junk peaks make us able to detect two interesting peaks. These peaks consistently showed up at around 675 amu and at 837 amu. Either both or one of these peaks could possibly correspond to a compound produced by the endophytic fungi that is

limiting the bacterial growth. Unfortunately, as indicated by the TIC intensity and by the relatively taller junk and solvent peaks, the compounds appear to be present in relatively insignificant concentrations in the samples analyzed. We were unable to perform an anti-bacterial assay that would verify the activity of the compounds.

Table 4. MAS results.

Sample	Sample ionizable	Total ion current (TIC) intensity	Sample UV detectable	DAD	Possible masses of interest
7	Yes	5.0e5 cps	Yes	2.0 mAu	8.37.5 amu, 673.4 amu
8	Yes	4.8e5 cps	Yes	34.0 mAu	8.37.5 amu, 675.7 amu
9	Yes	4.3e5 cps	Yes	30.0 mAu	8.37.5 amu, 675.7 amu
11	Yes	3.9e5 cps	Yes	24.2 mAu	8.37.5 amu, 675.7 amu
12	Yes	3.9e5 cps	Yes	19.8 mAu	8.37.5 amu, 675.7 amu

4.5 Relationship between fungal isolates and the host plant

Aspergillus flavus, *Botrytis cinerea*, *Acremonium strictum*, *Cladosporium cladosporioides*, *Curvularia lunata*, and *Penicillium janthinellum* were tested *in vitro* host specificity test. None of these fungi caused damage in the infected tissue of *T. testudinum* that was on the containers under regulated environmental conditions (Figure 16). Only one *Cladosporium* species was recovered from a control leaf tissue. The procedure was repeated for this particular fungi and after one month no damaged was documented.

Individually infected leaves in a container presented multiple lesions (foliar stains and chlorotic and necrosis symptoms) (Figure 17). The necrosis appeared from the apex to the base and the chlorosis was near the center of all the leaves. Some of the lesions were similar to those observed from the leaves for the endophytes sampling.

None of the fungi inoculated were recovered from test tissue. This suggested that there was no virulence caused by the endophytic fungi inoculated. The damaged in the tissue of the individual leaves could be caused by lack of nature environmental conditions (the temperature constantly varies between 23˚C and 26˚C, and they lacked their root system).

Figure 16. Undamaged tissue of *Thalassia testudinum* under controlled environmental conditions after one month of the fungi inoculation.

Figure 17. Infected control (A) and experimental (B) leaves from containers at room temperature.

CONCLUSIONS

- Endophytic fungi were recuperated from undamaged and damaged tissue of *Thalassia testudinum*.

- A total of eight genera and twelve species were reported. These include the filamentous fungi such as *Aspergillus flavus, A. fumigatus, A. niger, A. ustus, A. versicolor, Botrytis cinerea, Acremonium strictum, Cladosporium cladosporioides, C. sphaerospermum, Colletotrichum* sp., *Curvularia lunata*, *Penicillium janthynellum,* and *Scopulariopsis asperula*.

- This is the first report of *A. niger, C. lunata*, *Colletotrichum* spp., *A. versicolor*, and *Acremonium strictum* as endophytes of *T. testudinum*.

- Some strains of endophytic fungi asociated to *T. testudinum* were exudate producing fungi, such as *A. niger, A. fumigatus, A. versicolor, Botrytis cinerea*, *Colletotrichum sp*., C. *lunata, C. cladosporioides, P. janthinellum,* and *Scopulariopsis asperula.*

- *Aspergillus fumigatus, Cladosporiun cladosporioides, and Penicillium janthinellum* showed some biological activity inhibiting the growth of *Pseudomonas aeruginosa* and *Escherichia coli*.

- Based on host specificity tests *Aspergillus fumigatus*, *Botrytis cinerea*, *Acremonium strictum*, *Cladosporium cladosporioides*, *Curvularia lunata*, and *Penicillium janthinellum* did not damaged the healthy tissue of *T. testudinum*, indicating a non-pathogenic role in the plant.

- This was the first attempt to study the interaction between endophytic fungi and endophytic bacteria from tissue of *Thalassia testudinum* performing bioassays.

- The HPLC and the MSA suggested the presence of possible bioactives compounds, which could not be identified due to the small amount collected from the pure cultures.

- The interaction between the exudate-producing endophytic fungi and the endophytic bacterial strains from tissue of *T. testudinum* suggest that the fungi could be having a prophylactic role in the host plant, inhibiting the growth of some other microorganisms.

RECOMMENDATIONS

- To extend the study to different populations of *Thalassia testudinum* to compare the community of endophytic fungi associated.

- To implement molecular systematic techniques for the corroboration of the genus and species identified by microscopic techniques.

- Perform RFLP analysis to provide more information about the role of the fungal endophytes associated to *T. testudinum* corroborating and supporting any bioassays realized.

- Development of new techniques for the analysis of non-culturable fungi form *T. testudinum*, because explorations for new bioactive metabolites would be limited to few groups of microorganisms, which are readily isolated and grown under standard conditions.

- Conduct cytotoxic assays to measure the ability of the fungal extract to inhibit the growth of cancer cells in vitro.

- Look for the effect of the fungal endophytes that coexist in the same leaves, inoculating them in a selective media, and identifying the possible bioactive compounds produced.

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Appendix A. Genus and species of endophytic fungi isolated from undamaged/damaged leaves during each sampling.

Isolated fungi	First sampling		Second sampling	
	Undamaged	**Damaged**	**Undamaged**	**Damaged**
Acremonium strictum	0	1	2	3
Aspergillus flavus	0	0	3	7
Aspergillus fumigatus	0	0	2	4
Aspergillus niger	1	0	0	0
Aspergillus ustus	0	0	0	1
Aspergillus versicolor	0	0	6	1
Botrytis cinerea	0	0	0	3
Cladosporium cladosporioides	5	1	3	2
Colletotrichum sp.	1	0	0	0
Curvularia brachyspora	0	0	1	2
Curvularia lunata	2	1	2	3
Penicillium janthinellum	19	1	2	1
Penicillium crysogenum	13	2	0	0
Scopulariopsis asperula	0	1	2	2
Total	41	7	23	29

Apendix B. Bioassays results, for the interaction between the endophytic fungi and opportunistic bacteria.

Bacteria specie	1	2	3	4	5	6
Human pathogen						
Escherichia coli	+	-	-	+	+	-
Pseudomonas aeruginosa	+	-	-	+	+	-
Phytopathogen						
Burkhodenia cepacia	-	-	-	-	-	-
Erwinia chrysanthemi	-	-	-	-	-	-
E. caratovora pv. Caratovora	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-
P. cichorii	-	-	-	-	-	-
Xanthomonas axonopodis	-	-	-	-	-	-
X. axonopodis pv. Translucens	-	-	-	-	-	-
X. campestris pv. Campestre -	-	-	-	-	-	-
X. campestris pv. phascali	-	-	-	-	-	-
X. campestris pv. Fuscans	-	-	-	-	-	-

1 = *Aspergillus fumigatus*
2 = *Botrytis cinerea*
3 = *Acremonium strictum*
4 = *Cladosporium cladosporioides*
5 = *Curvularia lunata*
6 = *Penicillium janthinellum*

+ inhibition zone of more than 1 cm
- inhibition zone of less than 1 cm

Apendix C. Bioassay results for the interaction between of the endophytic fungi and bacteria.

Bacteria site	1	2	3	4	5	6
Solar Saltern 1	-	-	-	-	-	-
Solar Saltern 2	-	-	-	-	-	-
Solar Saltern 3	-	-	-	-	-	-
Solar Saltern 4	-	-	-	-	-	-
Solar Saltern 5	-	-	-	-	-	-
Solar Saltern 6	-	-	-	-	-	-
Solar Saltern 7	-	-	-	-	-	-
Solar Saltern 8	-	-	-	-	-	+
Solar Saltern 9	-	-	-	-	+	+
Vieques 1	-	-	-	-	-	-
Vieques 2	-	-	-	-	-	-
Vieques 3	-	-	-	-	-	-
Vieques 4	-	-	-	-	-	-
Vieques 5	-	-	-	-	-	-
Vieuqes 6	-	-	-	-	-	-

1 = *Aspergillus fumigatus*
2 = *Botrytis cinerea*
3 = *Acremonium strictum*
4 = *Cladosporium cladosporioides*
5 = *Curvularia lunata*
6 = *Penicillium janthinellum*

- = less than 1 cm
+ = more than 1 cm

Apendix D. Frequencies: Endophytic fungi isolated

Absolute frequence

Sampling		Total
1	44	47.31
2	49	52.69
Total	93	100.00

Relative frequencies

Sampling		Total
1	0.47	0.30
2	0.53	0.70
Total	1.00	1.00

Stadistic	Value	gl	p___
Chi Cuadrado Pearson	7.68	1	0.0056
Chi Cuadrado MV-G2	7.91	1	0.0049
Coef.Conting.Cramer	0.40		
Coef.Conting.Pearson	0.37		_____

Appendix E. Frequencies: Isolated fungi per leaf section

Absolute frequencies

Sampling	apex	base	center	Total
1	15	13	17	45
2	34	39	30	103
Total	49	52	47	148

Relative frequencies

Sampling	apex	base	center	Total
1	0.10	0.09	0.11	0.30
2	0.23	0.27	0.20	0.70
Total	0.33	0.46	0.31	1.00

Stadistic	Value	gl	p
Chi Cuadrado Pearson	7.58	1	0.0062
Chi Cuadrado MV-G2	7.81	1	0.0053
Coef.Conting.Cramer	0.36		
Coef.Conting.Pearson	0.30		

Appendix F. Frequencies: Isolated fungi from each depth (cm)

Absolute frequencies

In columns:Fraction

Sampling	0-50	51-100	101-more	Total
1	42	14	10	66
2	51	22	9	82
Total	93	36	19	148

Frecuencias relativas al total

In columns:Fraction

Sampling	0-50	51-100	101-more	Total
1	0.28	0.09	0.07	0.44
2	0.35	0.15	0.06	0.56
Total	0.63	0.24	0.13	1.00

Stadistic	Value	gl	p
Chi Cuadrado Pearson	0.35	1	0.5528
Chi Cuadrado MV-G2	0.35	1	0.5521
Coef.Conting.Cramer	0.15		
Coef.Conting.Pearson	0.15		