UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

IUSA Instituto Universitario Sanidad Animal Seguridad Alimentaria

TESIS DOCTORAL

Interacción Molecular entre el Alga Roja *Grateloupia imbricata* y sus Algas Endófita *Microspongium tenuissimum* y Epífita *Ulvella leptochaete*

Miguel Ángel Navarro Ponce

2015

Las Palmas de Gran Canaria

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

IUSA Instituto Universitario Sanidad Animal Seguridad Alimentaria

Programa de Doctorado de Sanidad Animal

Bienio 2007 - 2009

Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

Título de la Tesis:

Interacción molecular entre el alga roja *Grateloupia imbricata* y sus algas endófita *Microspongium tenuissimum* y epífita *Ulvella leptochaete*

Tesis Doctoral presentada por D. Miguel Ángel Navarro Ponce

Dirigida por el Dr. Rafael Robaina Romero

El Director **El Doctorando**

Las Palmas de Gran Canaria, a 6 de Noviembre de 2015

A mis abuelos

Justo y Eulogia
José y Carmen

AGRADECIMIENTOS

En primer lugar quiero expresar mi más sincero agradecimiento al Dr. Rafael Robaina, director de esta tesis doctoral. Gracias Rafa por confiar en mí, por darme la oportunidad de formarme y trabajar a tu lado. Gracias por la dedicación, por la pasión y entusiasmo que transmites. A la Dra. Pilar García, por su ayuda en el trabajo diario en el laboratorio.

Quiero agradecer a mis compañeros/as de laboratorio Mario Vergara, Maite Zarranz y Claudia Pérez, y a Elizabeth Rivero Santana, por los buenos momentos compartidos. Al Departamento de Biología de la Universidad de Las Palmas de Gran Canaria y todo su personal. Al personal de conserjería y de la Biblioteca de Ciencias Básicas, y a todos aquellos que de una forma u otra han colaborado en la realización de esta tesis.

Una parte del trabajo presentado ha sido realizado en las instalaciones del Instituto Universitario de Sanidad Animal y Seguridad Alimentaria de la Universidad de Las Palmas de Gran Canaria (IUSA). En este sentido quiero manifestar un especial agradecimiento al Dr. Juan Manuel Afonso López y la Dra. María Jesús Zamorano. Gracias JuanMa por tu atención, amabilidad y

dedicación, sin tu ayuda este trabajo no hubiese sido posible. Gracias por darme la oportunidad de vivir una experiencia realmente enriquecedora. A la Dra. Ana Navarro y la Dra. Davinia Negrín, gracias por vuestra acogida, por vuestro compañerismo, por hacerme sentir "una" más, por los buenos momentos que me permitisteis vivir dentro y fuera del laboratorio. Dos frases quedarán marcadas para siempre ¡Tranquilo no te vas a mojar! y ¡el Southern es fácil eso en una semana lo tienes!

Quiero agradecer a la Dra. Magdalena Santana Casiano y al Dr. Melchor González Dávila, responsables del grupo de investigación QUIMA, del departamento de Química de la Universidad de las Palmas de Gran Canaria, el haberme permitido realizar parte de este trabajo en su laboratorio. En este punto quiero hacer una mención especial al Dr. Aridane González González, por su dedicación y por su ayuda incondicional. Aridane no es fácil expresar la admiración que siento por ti y menos aún mi gratitud, pero puedo simplificarlo en dos palabras ¡Gracias Amigo!

Sin lugar a dudas hay dos personas que sin ser científicas han "cargado" durante muchos años con el peso de esta tesis doctoral. Por su apoyo en los momentos difíciles, por estar cada día a mi lado aunque mi estado de ánimo lo desaconsejara, por habérmelo puesto

tan fácil que probablemente jamás pueda compensarles, quiero mostrar mi más profundo agradecimiento y admiración a mis padres.

Esta tesis doctoral elaborada por capítulos, forma ya parte de un capítulo de mi vida, capítulo que comenzó allá por el año 2001 cuando pisé por primera vez la facultad de Ciencias del Mar de la ULPGC. Sinceramente nunca fue ni mi objetivo ni mucho menos la ilusión de mi vida dedicarme a las ciencias marinas, pero ese paso que en un principio fue complicado me permitió conocer a la persona que cambiaría mi vida, Carolina. Comienza una nueva etapa en nuestras vidas y en ella ya no tendremos que escribir la tesis, aunque tendrás que soportar a un veterinario.

PRESENTACIÓN DE LA TESIS

La presente Tesis Doctoral titulada "**Interacción molecular entre el alga roja *Grateloupia imbricata* y sus algas endófita *Microspongium tenuissimum* y epífita *Ulvella leptochaete*** ha sido realizada en el Departamento de Biología de la Universidad de Las Palmas de Gran Canaria, dentro de la línea de investigación en Fisiología y Biotecnología Vegetal Marina perteneciente al Grupo de Investigación de Organismos, Poblaciones y Ecosistemas. La Tesis Doctoral ha sido desarrollada bajo el marco del proyecto BFU2006-06198 y su realización ha sido posible gracias a la beca FPU del Ministerio de Ciencia e Innovación con referencia AP2007-02302 y la beca de investigación de postgrado en temas de interés para la isla de Gran Canaria del Servicio de Educación del Cabildo de Gran Canaria.

El trabajo está estructurado en dos Capítulos elaborados en inglés a modo de artículos científicos para su inmediata publicación. Cada Capítulo consta de resumen, introducción, metodología, resultados, discusión y referencias. El Capítulo I estudia la incidencia de la infección por algas endófitas filamentosas en cultivos de *Grateloupia imbricata*, empleando técnicas de biología molecular para realizar su asignación taxonómica y determinar si el grado de interacción entre ambas se extiende a nivel de ADN. En el Capítulo II

se identifica la principal especie de alga epífita presente en *G. imbricata*, se lleva a cabo su asignación taxonómica mediante el empleo de marcadores moleculares y se realiza una aproximación al papel de las poliaminas en los mecanismos de defensa de *G. imbricata* frente a la infección.

A fin de cumplir los requisitos que establece el Reglamento de Elaboración, Tribunal, Defensa y Evaluación de Tesis Doctorales de la Universidad de Las Palmas de Gran Canaria, en la encuadernación se incluyen, escrito en castellano, los objetivos del trabajo, un resumen con la aportación y conclusiones, una introducción con el planteamiento general del trabajo y un amplio apartado de metodología.

ÍNDICE

OBJETIVOS

El principal objetivo de este trabajo fue evaluar el grado de interacción molecular entre el alga roja *G. imbricata* y sus principales algas endófitas y epífitas asociadas, evaluando la posibilidad de que esta interacción se extienda incluso a nivel de ADN y realizando una primera aproximación al papel de las poliaminas (PAs) en los mecanismos de defensa de *G. imbricata*.

Para alcanzar este objetivo general se establecieron los siguientes objetivos específicos:

1. Establecimiento del cultivo *in vitro* de explantos del alga roja *G. imbricata* para el aislamiento e identificación de sus principales algas endófitas y epífitas asociadas.
2. Asignación taxonómica de las algas endófitas y epífitas aisladas de *G. imbricata* mediante marcadores moleculares, *Internal Transcribed Spacer* (ITS1 o ITS2) combinados con ADNr.
3. Determinación del grado de interacción molecular entre el alga roja *G. imbricata* y su principal endófito asociado mediante técnicas moleculares de hibridación de ADN "Southern blot".

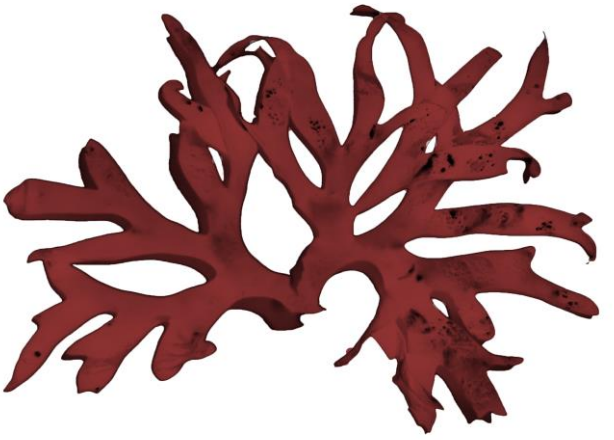
4. Determinar los niveles de PAs en talos de *G. imbricata* en los primeros estadios de la infección por algas epífitas filamentosas.

5. Evaluar la influencia del metil jasmonato (MeJA) sobre los niveles de PAs de *G. imbricata*.

6. Estudiar la influencia del peróxido de hidrógeno (H_2O_2) en los niveles de PAs del alga roja *G. imbricata*.

7. Determinar el potencial antioxidante de las poliaminas, mediante su capacidad de eliminar el H_2O_2 en disolución.

Resumen y Conclusiones Generales



RESUMEN Y CONCLUSIONES GENERALES

Durante el cultivo *in vitro* de explantos del alga roja *Grateloupia imbricata* (Holmes) recolectada en las poblaciones naturales al noreste de la isla de Gran Canaria se observó el crecimiento de pequeñas algas endófitas filamentosas pertenecientes a la división Phaeophyta.

Después de tres o cuatro semanas en cultivo es posible observar a simple vista los primeros filamentos de algas endófitas creciendo asociadas a *G. imbricata*. La liberación y el crecimiento del endófito se encuentra ligado a las zonas de corte de los explantos.

1. **Las observaciones en cultivo sobre un total de 7000 explantos de *G. imbricata* cultivados revelaron una incidencia de infección del 90 % en un periodo de cultivo no inferior a 6 meses**. Durante este periodo el endófito es capaz de colonizar completamente el explanto, y al ser examinados al microscopio se refleja su capacidad para penetrar y desarrollarse en el interior de los tejidos del hospedador

2. **Estas algas endófitas pigmentadas son fotosintéticamente activas, carbono-independientes, lo que permitió que se mantuvieran creciendo en cultivos unialgales para su**

identificación. Su simplicidad hace muy complicada la identificación atendiendo a sus características morfológicas por lo que la asignación taxonómica se llevo a cabo mediante el uso de marcadores moleculares.

3. **La amplificación mediante la reacción en cadena de la polimerasa (PCR) y posterior análisis filogenético de secuencias de la región (ITS1) combinada con el ADN ribosómico (5.8S) permitió la identificación del alga endófita como *Microspongium tenuissimum* (Hauck).** Los árboles filogenéticos se construyeron utilizando los métodos de inferencia filogenética "*Maximum parsimony*" y "*Maximum likelihood*". La secuencia de 370 pb fue depositada en la base de datos del GenBank con número de accesión KJ134990.

Respecto a la posibilidad de que la liberación del endófito se debiera a algún tipo de señal producida por el estrés generado durante el cultivo del explanto ya que el endófito se liberó a partir de material aparentemente libre de infección:

4. **Se pudo comprobar que talos aparentemente no infectados en cultivo sin cortar en explantos durante un periodo de 6 meses, no mostraron señal de infección, pero la PCR los determinó como positivos para la presencia de *M. tenuissimum*.**

5. **La prevalencia de la infección por *M. tenuissimum* en tres poblaciones naturales de *G. imbricata* en la isla de Gran Canaria fue estudiada por PCR, siendo ésta del 100% sobre un total de 50 ejemplares analizados, independientemente del estado de desarrollo de los talos.**

Para determinar el grado de interacción entre el endófito y su hospedador y estudiar una posible interacción molecular a nivel de ADN se llevaron a cabo ensayos de hibridación de ácidos nucleicos mediante Southern blot. Se sintetizó una sonda marcada con digoxigenina para la detección específica de la región ITS1-5.8S de *M. tenuissimum* en muestras de ADN de *G. imbricata* previamente digeridas con enzimas de restricción (*Eco*RI y *Pvu*II). Mediante análisis por Dot Blot se verifica la integridad, la sensibilidad y especificidad de la sonda para excluir la posibilidad de aparición de reacciones cruzadas.

6. **El patrón de bandas reveladas mediante el análisis por Southern blot indica la presencia de *M. tenuissimum* como endófito, revelando la existencia de algún tipo de interacción molecular a nivel de ADN entre ambas especies, de naturaleza desconocida y no descrita entre organismos eucariotas.**

7. ***Ulvella leptochaete* (Huber) fue aislada durante el cultivo *in vitro* del alga roja *G. imbricata*. Se realizó la correcta asignación taxonómica amplificando mediante PCR la región ITS2 combinada con el ADN ribosómico 5.8S y 28S y realizando análisis filogenéticos de las secuencias obtenidas** empleando los métodos "*Maximum parsimony*" y "*Maximum likelihood*". La secuencia de 211 pb fue depositada en la base de datos del GenBank con número de accesión KJ134991.

8. **Nuestras observaciones en cultivo indican que *U. leptochaete* se comporta como epi-endófito* cuando se asocia a *G. imbricata*. Inicialmente *U. leptochaete* se encuentra como epífito sobre los talos de *G. imbricata* pero durante su cultivo, en estados avanzados de infección, se observa la capacidad de penetrar en el interior de los tejidos del hospedador, comportándose como endófito.** Las técnicas para establecer cultivos asépticos de explantos de *G. imbricata* permiten mantener una incidencia para este epífito en cultivo realmente baja (aprox. 5%), pero esta incidencia eventualmente aumenta drásticamente, afectando prácticamente a la totalidad de los explantos en cultivo.

9. **Los niveles endógenos de poliaminas (PAs) en *G. imbricata* fueron examinados durante los estadios iniciales de la infección**

por *U. leptochaete* y reflejan un marcado aumento en la concentración de la diamina putrescina (Put) tanto en su forma libre como en su forma conjugada.

10. **El tratamiento de los talos de *G. imbricata* con metil jasmonato (MeJA) produce un incremento significativo en los niveles de putrescina libre y putrescina conjugada en los talos tratados, mostrando la capacidad del MeJA de modificar el metabolismo de las poliaminas** en el alga roja del mismo modo que lo hace en plantas superiores. Ésta podría ser una vía alternativa, mediante la cual las PAs pudiesen conferir resistencia frente a la infección por patógenos. Los jasmonatos tienen la capacidad de estimular la producción de metabolitos secundarios entre los que se incluyen las poliaminas, confiriendo protección local y sistémica frente a la infección en plantas superiores.

11. **Fue examinada la posibilidad de que el H_2O_2 tuviese efecto sobre los niveles de PAs en *G. imbricata* y se ha visto que a concentraciones suficientemente elevadas de H_2O_2 los tejidos responden con un incremento significativo de putrescina en su forma libre.**

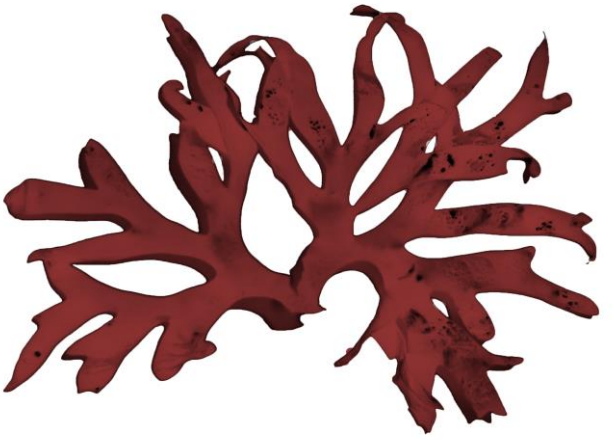
12. **En este estudio además se determinó el potencial antioxidante *in vitro* de la diamina putrescina, reflejando su**

capacidad de actuar directamente eliminando el H_2O_2 en disolución. De esta forma se refleja que las PAs y concretamente la diamina putrescina podrían jugar un doble papel en los mecanismos de defensa en el alga roja *G. imbricata*, por un lado su metabolismo podría proveer una fuente de peróxido de hidrógeno y por otro podrían actuar como moléculas con capacidad antioxidante disminuyendo el daño en los tejidos provocados por las especies de oxígeno reactivas y dificultando la infección por patógenos.

13. **El crecimiento de epífitos y endófitos representa una de las mayores amenazas para las cosechas de algas. Nuestros resultados representan una vía esperanzadora para su combate mediante la manipulación de los niveles y metabolismo de las poliaminas**.

(*) *Ulvella leptochaete* se presenta inicialmente como epífito en los talos de *Grateloupia imbricata*, sin embargo, durante el transcurso de la infección, *in vitro*, muestra la capacidad de comportarse como endófito, penetrando en el interior de los tejidos del hospedador y colonizándolo por completo. Por este hecho en el capítulo II nos referiremos a ésta como epi-endófita con el término "epi-endophyte"

Introducción General y Metodología



INTRODUCCIÓN GENERAL

Las macroalgas representan el hábitat de otros organismos marinos

Las macroalgas, como parte de las comunidades bentónicas marinas, constituyen el hábitat en el que se desarrollan una gran diversidad de organismos, que van desde virus, bacterias y cianobacterias (Dou *et al.* 1981; Wu *et al.* 1983; Apt & Gibor 1989; Müller *et al.* 1990; Müller 1991; Correa *et al.* 1993; Craigie & Correa 1996; Sawabe *et al.* 1998, 2000) hasta protozoos, hongos, (Kohlmeyer & Kohlmeyer 1979, Apt 1988a, Küpper & Müller 1999) e incluso otras algas (Andrews 1977; Goff 1983; Correa & McLachlan 1991).

Andrews (1976) y Dodds (1979) citan que las primeras observaciones de partículas víricas en algas marinas se realizaron en la década de los setenta. Gibbs *et al.* (1975) citaron la presencia de partículas víricas en el alga *Chara corallina*, mientras que Oliveira y Bisalputra (1978) demostraron que la infección vírica producía daños letales en las algas. Sin embargo, los estudios más detallados sobre las interacciones alga-virus han sido desarrollados en *Ectocarpus*

siliculosus y otras especies de ectocarpales (Müller *et al.* 1990; Müller 1991, 1992; Müller & Stache 1992; Müller & Parodi 1993).

Numerosos estudios han descrito la capacidad de las bacterias para causar infecciones secundarias en macroalgas (Correa *et al.* 1994; Correa & McLachlan 1994; Craigie & Correa 1996). En *Chondrus crispus* y *Mazzaella laminarioides*, estas infecciones secundarias aceleran los procesos de destrucción de los tejidos. Sin embargo, otros estudios citan la capacidad de las bacterias de actuar como agente infeccioso primario, sin la necesidad de la mediación de un agente facilitador, causando lesiones necróticas en los tejidos de las algas sobre las que se encuentran como epífitas (Andrews 1977; Weinberger *et al.* 1997; Craigie & Correa 1996). Un ejemplo de este tipo de interacción alga-bacteria lo constituye *Pseudoalteromonas bacteriolytica*, una bacteria marina patógena del alga parda *Laminaria japonica*.

Al igual que ocurre en las plantas terrestres, los hongos son potencialmente patógenos para las macroalgas marinas. Los síntomas más frecuentes causados por la infección incluyen la deformación y decoloración de los tejidos, formación de tumores y lesiones necróticas con pérdida de tejido (Kohlmeyer & Kohlmeyer 1979;

Kohlmeyer & Demoulin 1981; Goff 1983; Küpper & Müller 1999).

Si bien es cierto que la mayoría de los organismos asociados a las macroalgas se presentan como **epífitos**, colonizando la superficie de sus hospedadores (Wahl 1989), las macroalgas también hospedan una amplia variedad de organismos **endófitos** (Pueschel & van der Meer 1985; Apt & Gibor 1989; Correa *et al*. 1993; Correa *et al.* 1987). La distinción entre epífito y endófito no siempre es suficientemente clara entre los diferentes autores. En este trabajo nos ceñimos a los conceptos y terminología ofrecidos por Correa (1994): Los términos endófito y epífito indican únicamente una relación espacial entre las especies asociadas sin indicar el tipo de impacto que esta asociación puede generar.

Las asociaciones entre algas son un fenómeno muy común (Goff 1983). Se han citado numerosas especies de pequeñas algas filamentosas, de las divisiones Chlorophyta, Rhodophyta y Phaeophyta, viviendo en los tejidos de sus algas hospedadoras, íntimamente asociadas a éstas. Tal y como se describió anteriormente, en este tipo de interacción alga-alga, atendiendo a la relación espacial existente con el hospedador, el alga huésped puede ser clasificada como epífito o como endófito.

Las algas epífitas son una fuente de desventajas para sus hospedadores (D'Antonio 1985), éstas compiten por la luz y los nutrientes, disminuyendo su capacidad de crecimiento y regeneración, generan cambios anatómicos y químicos que alteran las propiedades del talo y pueden dar lugar a un herbivorismo accidental (D'Antonio 1985, Wahl 1989)

Las algas endófitas son generalmente algas multicelulares filamentosas con capacidad de crecer y reproducirse inmersas en los tejidos de sus algas hospedadores. Algunos de estas algas endófitas no realizan la fotosíntesis, obteniendo sus nutrientes a partir de sus hospedadores, por lo que son considerados como **parásitos** (Goff 1982). El término parásito indica dependencia fisiológica, sobre la base de la obtención de nutrientes (Hall 1974; Ahmadjian & Paracer 1986). Aproximadamente el 15% de todos los géneros de algas rojas conocidos se presentan únicamente como parásitos de otras algas rojas. Algunas de estas algas rojas parásitas (***adelfoparásitos***) presentan tales similitudes morfológicas con sus algas hospedadoras, que han hecho plantear la hipótesis de que estos parásitos pudieron surgir a partir de sus hospedadores (Setchell 1918), estando estrechamente relacionados con ellos, incluso más que con otros

géneros de algas parásitas (Goff 1982, 1991; Goff *et al.* 1996), o bien, que fuera un mero resultado de la convergencia evolutiva, debido a que ambos conviven en una asociación espacio temporal marcada, experimentando las mismas condiciones ambientales (Ronquist 1994), lo que en cierta medida recuerda a la teoría evolutiva de Lamarck, y no parece probable que ésta pueda ser la explicación a dichas similitudes. Aproximadamente el 90% de las algas rojas parásitas descritas pertenecen al grupo de adelfoparásitos. Sturch (1926) estudio las algas rojas parásitas que no son morfológicamente similares a sus hospedadores, los ***alloparásitos***, proponiendo que éstas podrían haber surgido a partir de algas rojas de vida libre que se convirtieron inicialmente en epífitas y posteriormente en endófitas dentro de los tejidos del hospedador y ocasionalmente en algas nutricionalmente dependientes.

Las macroalgas también hospedan algas **endófitas,** que son fotosintéticamente activas, carbono-independientes (Correa *et al.* 1987, 1988; Correa 1990). Al contrario que los endófitos parásitos descritos anteriormente, es incorrecto considerar a éstos simbiontes como parásitos, ya que la capacidad de establecerles en medios de cultivo unialgales, en los que se desarrollan con normalidad, muestran

su independencia nutricional (Correa & McLachlan 1992; Correa 1994).

A pesar de esta independencia nutricional cuando se mantienen en cultivo *in vitro*, en la naturaleza, muchos de éstos van a permanecer como **endófitos obligados**, debido a que presentan unos talos frágiles y ausencia de estructuras que les permitan mantenerse adheridos a un sustrato (Correa *et al.* 1988).

Debido a la capacidad de crecer aisladas de sus hospedadores bajo condiciones estándar de cultivo y a sus complejos ciclos de vida, inicialmente se consideró que las algas endófitas no producían efectos adversos sobre sus hospedadores. Sin embargo, producen alteraciones degenerativas a nivel celular durante el proceso de infección, debido principalmente a los mecanismos que emplean para penetrar en los tejidos de sus algas hospedadores, y que comprenden procesos mecánicos de compresión y perforación, y fenómenos de digestión de la matriz intercelular (Correa & McLachlan 1994; Correa *et al.* 1994). Entre los síntomas a los que pueden dar lugar destacan una reducción de la velocidad de crecimiento y regeneración, e importantes daños a nivel celular y tisular (hipertrofia, hiperplasia, vesículas y tumores) (Apt 1988 a, b, c; Correa & McLachlan 1992, 1994), además de

facilitar la aparición de infecciones secundarias, principalmente de origen bacteriano (Correa & McLachlan 1992).

Mecanismos de reconocimiento de agentes patógenos en macroalgas

Se han estudiado los mecanismos de reconocimiento del agente patógeno, la transducción de señal y la activación de rutas metabólicas involucradas en las reacciones de defensa en macroalgas (Bouarab *et al.*1999, 2001; Weinberger *et al.* 1999, 2001, 2002, 2005 a,b; Weinberger & Friedlander 2000 a,b; Bouarab *et al.* 2001,2004; Küpper *et al.* 2001, 2002, 2006; Potin *et al.* 2002; Kubanek *et al.* 2003; Hervé *et al.* 2006). Las interacciones con microorganismos y otras algas endófitas filamentosas son probablemente las mejor conocidas.

Los mecanismos de inmunidad natural de las macroalgas conservan algunos rasgos comunes con los de las plantas terrestres, en concreto, los mecanismos básicos de reconocimiento de agentes patógenos y señalización, lo que sugiere que esta maquinaria bioquímica esencial en las funciones celulares surgió muy pronto en la evolución (Fig 1).

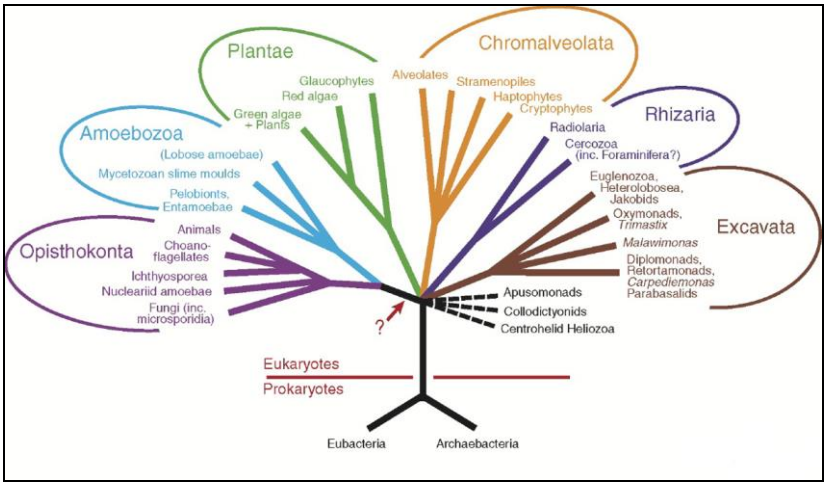


Fig 1 Árbol de diagrama que representa la organización de la mayoría de los eucariotas en seis grandes grupos (Simpson & Roger 2004)

Además poseen rasgos específicos basados en el aprovechamiento de sustancias químicas encontradas en el medio marino (Potin *et al.* 2002) entre las que se encuentran los compuestos halogenados.

El principio de toda respuesta defensiva eficaz es el reconocimiento de un agente patógeno que la induzca. Los oligosacáridos, las glucoproteínas, los glucopéptidos (Boller 1995) y diversos compuestos volátiles (Kessler 2001; Gaquerel *et al.* 2009) constituyen las principales moléculas inductoras de respuesta defensiva en plantas superiores.

El alga parda *Laminaria digitata* y el alga roja *Gracilaria conferta* constituyen los dos primeros ejemplos en los que se han

citado eventos de reconocimiento de agentes patógenos mediados por **inductores endógenos**.

El componente principal de la pared celular en Laminariales es el alginato, éste representa hasta el 60% de la pared celular de *L. digitata*. Durante la infección por bacterias patógenas con capacidad alginolítica se producen oligoalginatos. Kupper *et al.* (2001, 2002) demostraron la capacidad de estos oligoalginatos, concretamente el ácido α-1,4-L-gulurónico, para actuar como inductores endógenos, activando la emisión de peróxido de hidrógeno (H_2O_2). Este estallido oxidativo, es rápido y transitorio, no prolongándose más allá de 30 minutos. La respuesta oxidativa por parte de *L. digitata* ocurre únicamente cuando es inducida por el ácido gulurónico, otros oligoalginatos como el ácido manurónico no son eficaces.

La transducción de señal posterior a la inducción implica a las fosfolipasas A_2 (Kupper *et al.* 2001), enzimas que hidrolizan los enlaces éster presentes en los fosfolípidos, y tanto las oxilipinas como los ácidos grasos libres juegan un papel fundamental en la activación de los mecanismos de defensa (Kupper *et al.* 2006) de forma análoga a como ocurre en plantas superiores (Chandra *et al.* 1996).

Gracilaria conferta es capaz de reconocer los oligosacáridos procedentes de la descomposición del agar de su pared celular cuando es atacada por bacterias patógenas (Weinberger *et al.* 1997; 1999). El reconocimiento de oligosacáridos por parte de *G. conferta* se traduce en un aumento de la respiración y en una respuesta oxidativa mediante liberación de H_2O_2, como mecanismo de defensa, que es lo suficientemente fuerte como para provocar la muerte de las bacterias epífitas asociadas al alga. Concentraciones de 0.01-0.5 µM de agaro-oligosacáridos son suficientes para inducir la mitad de la respuesta máxima, si éstos tienen el tamaño óptimo. Esta liberación de H_2O_2 inducida por los oligosacáridos unida a la exposición a luz tienen como resultado la aparición de necrosis y talos blanqueados en *G. conferta*. El agente inductor puede ser eliminado con β-agarasa y el uso de antibióticos previene su acumulación, lo que indica que la bacteria es la responsable de la acumulación de los oligosacáridos que actúan como inductores de la liberación de especies de oxígeno reactivas (ROS) (Weinberger *et al.*1999; Weinberger & Friedlander 2000 a, b).

Las macroalgas no sólo son capaces de reconocer moléculas procedentes de la degradación de su pared celular. El alga roja *G. conferta* es capaz de responder a **inductores exógenos** generados por la presencia de al menos tres especies diferentes de bacterias epífitas, estas señales moleculares de origen exógeno, están constituidas por moléculas de bajo peso molecular (700-1500 Da) que se corresponden con péptidos de 4 a 20 aminoácidos que son excretados por bacterias patógenas (Weinberger & Friedlander, 2000a). La capacidad de los péptidos, proteínas y glucoproteínas de actuar como inductores exógenos había sido descrita previamente en plantas vasculares espermatofitas (Stekoll & West 1978; Wei *et al.* 1992; Nümberger *et al.* 1994; Scholtens-Toma & de Wit 1998).

Kupper *et al.* (2006) hacen referencia a la capacidad de los lipopolisacáridos bacterianos de inducir una fuerte reacción defensiva en el alga *L. digitata*, caracterizada por la producción de especies de óxigeno reactivas, la liberación de ácidos grasos saturados e insaturados y la acumulación de oxilipinas como el ácido 13-hidroxioctadecatrienoico y el ácido 15-hidroxieicosapentanoico. La formación de especies de oxígeno reactivas puede ser inhibida por

difenil iodonium (DPI), lo que sugiere que la fuente es una NAD(P)H oxidasa.

La interacción *Chondrus crispus* - *Ulvella operculata* (como *Acrochaete operculata*), que será estudiada en detalle en el aparatado correspondiente, representa el modelo de interacción directa alga-patógeno mejor estudiado (Bouarab *et al.* 1999). El alga verde *U. operculata* es endófita del esporofito de *C. crispus*, colonizando completamente el talo de su hospedador. Sin embargo, es incapaz de penetrar más allá de las capas más externas del gametofito. Esta susceptibilidad a la infección se debe a que únicamente el gametofito es capaz de reconocer al agente infectante, detectando moléculas exógenas, y activar una respuesta defensiva eficaz mediante la liberación de ROS.

Mecanismos de defensa de las algas frente a patógenos

En las plantas terrestres, la producción y acumulación de ROS es la primera respuesta tras el reconocimiento de un agente patógeno (Baker & Orlandi 1995, Lamb & Dixon 1997). Las ROS tienen efecto citotóxico directo sobre el patógeno (Mellersh *et al.* 2002) y actúan como señal induciendo otros mecanismos de defensa y mediando en la activación de los genes responsables de la defensa (Levine *et al.* 1994; Hancock *et al.* 2001; Neill *et al.* 2002).

En la mayoría de las interacciones planta-patógeno, la enzima NADPH sensible a difenil iodonium (DPI), localizada en la membrana plasmática, es la fuente de ROS (O'Donnell *et al.* 1993; Torres & Dangl 2005). La NADPH oxidasa fue descrita inicialmente en fagocitos de mamíferos. $Gp91^{phox}$ es la subunidad enzimática responsable de la transferencia de electrones de esta oxidasa en estas células (Lambeth 2004).

La emisión de H_2O_2 en macroalgas ha sido demostrada durante la infección de éstas por patógenos, entre los que se incluyen bacterias (Weinberger & Friedlander 2000b; Küpper *et al.* 2001, Weinberger *et al.* 2001) y algas endófitas filamentosas (Bouarab *et al.* 1999; Küpper *et al.* 2001, 2002). Esta emisión de ROS, a menudo inducida por el reconocimiento de moléculas en la membrana celular, es sensible a DPI en las macroalgas, tal y como ocurre en las plantas terrestres (Bouarab *et al.* 1999; Weinberger & Friedlander 2000b; Küpper *et al.* 2001, 2002).

El gametofito del alga roja *C. crispus* produce H_2O_2 en respuesta a extractos de *U. operculata* libres de células. Hervé *et al.* (2006) aislaron el gen *Ccrboh*, un homólogo a $Gp91^{phox}$. Este gen codifica un polipéptido de 825 aminoácidos, y su transcripción se ve

modificada durante la infección del gametofito de *C. crispus* por el endófito *U. operculata*.

La expresión del gen *Ccrboh* en macroalgas también se ve modificada por la acción los ácidos grasos libres y el metil jasmonato (MeJA).

Los ácidos grasos poliinsaturados (PUFAs) y derivados oxidados juegan un papel clave en la señalización como respuesta al estrés tanto en animales como en plantas (Farmer 1994; Farmer *et al.* 1998). En plantas terrestres, las infecciones y la exposición a cenobíticos inducen una cascada oxidativa de ácidos grasos libres (FFA) (Blechert *et al.* 1995). Los resultados obtenidos por Küpper *et al.* (2006) indican que las algas pardas y las algas rojas no son una excepción. El ácido araquidónico, el ácido linolénico y el MeJA inducen una fuerte respuesta oxidativa en *L. digitata*. Esta producción de ROS puede ser parcialmente inhibida por DPI, lo que indica que al menos de manera parcial, la NADPH oxidasa es la responsable de esta emisión.

El ácido araquidónico y el MeJA inducen resistencia a *L. digitata* frente a la infección tanto por filamentos epífitos como endófitos de *Laminaricolax tomentosoides* (Fig 2) (Küpper *et al.* 2009). Se especula con la posibilidad de que el ácido araquidónico, el

ácido linolénico y el MeJA estimulen la afinidad de la enzima Gp91^{phox} por su sustrato.

Muchos genes relacionados con la respuesta defensiva en plantas son regulados por moléculas de señalización tales como el ácido jasmónico (JA), el etileno y el H_2O_2 (Reymond & Farmer 1998; Orozco-Cárdenas & Ryan 1999).

En el alga roja *C. crispus* el MeJA silencia los genes relacionados con el metabolismo general y sobreexpresa aquellos genes relacionados con el estrés (Collén *et al.* 2006) y con los mecanismos de protección frente a patógenos (Gaquerel 2007) induciendo la síntesis de proteínas relacionadas con los mecanismos de defensa frente al alga endófita *U. operculata* lo que le confiere resistencia frente a la infección (Bouarab *et al.* 2004).

El papel de señalización del MeJA en *L. digitata* en combinación con los resultados obtenidos en el alga roja *C. cripus* sugieren que el MeJA actúa regulando los genes involucrados en los mecanismos de defensa de las algas, incluyendo la NADPH oxidasa (Collén *et al.* 2006).

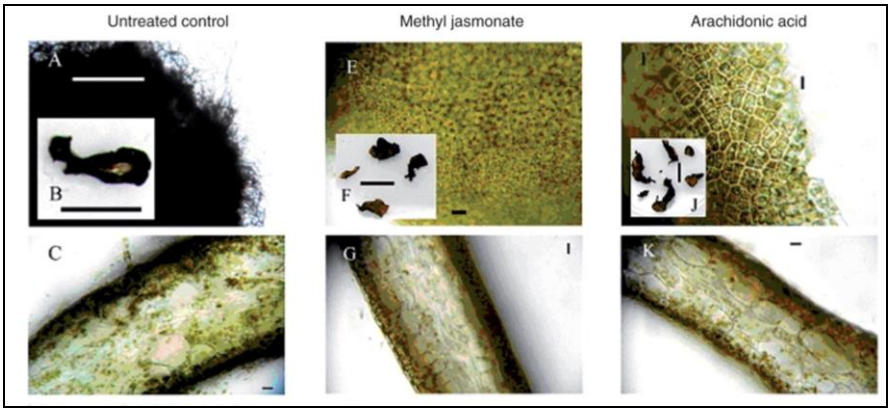


Fig 2 El ácido araquidónico y el MeJA inducen resistencia a *L. tomentosoides* en *L. digitata*. (A, E, I) Microfotografía de la superficie de talos de esporofitos de *L. digitata* (B, F, J) Macrofotografía de talos de esporofitos de *L. digitata*. (A, C) Control. Talos completamente cubiertos y mostrando la penetración de *L. tomentosoides* (E, G, I, K) Talos tratados con ácidos araquidónico o MeJA. Apenas son visibles filamentos epífitos o endófitos de *L. tomentosoides* (Küpper 2009)

Modelo bioquímico de un sistema patógeno-hospedador en macroalgas. *Chondrus crispus - Ulvella operculata*

Correa & McLachlan (1992) determinaron que el alga roja *C. crispus* se veía afectada negativamente por la infección tanto por el alga *Ulvella operculata* como por *U. heteroclada* (como *Acrochaete operculata* y *A. heteroclada*). Los efectos que estas infecciones

producen sobre el hospedador dependen de la especie implicada, de la fase del hospedador que sea atacada dentro de su ciclo de vida y del grado o densidad de la infección.

U. operculata es un alga estrictamente endófita incluso en condiciones de laboratorio (Correa *et al.* 1988) y presenta una marcada especificidad de hospedador, mostrando afinidad por establecerse en la fase esporofítica de *C. crispus* pero no así en la fase gametofítica (Correa & McLachlan 1991). *U. operculata* tiene la capacidad de invadir los talos de *C.crispus,* penetrando directamente en la pared celular externa del hospedador inmediatamente después del asentamiento de las zoosporas. Sin embargo, *U. heteroclada*, la cual se encuentra asociada de manera habitual a *C. crispus* (Correa 1988), requiere previamente asentarse como epífita antes de realizar la penetración. Ésta afecta de manera similar tanto a la fase esporofítica como gametofítica de *C. crispus* y antes de que tenga lugar la penetración del endófito, la alteración sobre las células del hospedador es prácticamente inapreciable (Correa 1990). Aunque en los primeros estudios los mecanismos de penetración eran por completo desconocidos, se observaba que conllevaban la digestión de los componentes de la pared celular del alga hospedadora (Correa & McLachlan 1994). Además, la penetración en profundidad en el tejido

de la fase esporofítica de *C. crispus* se realiza mediante digestión de la matriz intercelular y no por medios mecánicos.

El endófito *U. operculata* tiene la capacidad de colonizar completamente la fase esporofítica de *C. crispus*, sin embargo, no es capaz de penetrar más allá de las capas de células más externas de la fase gametofítica (Bouarab 2000) (Fig 3).

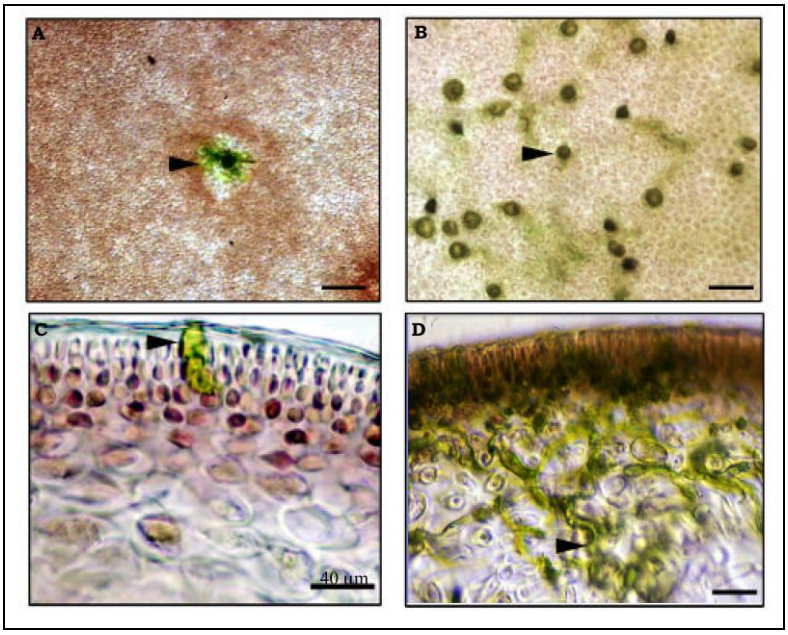


Fig 3 Interacción *C.crispus* - *U. operculata* (A, C) Gametofito muy poco infectado (B,D) Esporofito completamente invadido por el endófito (A, B) Vistas superficiales (C, D) Cortes transversales (Bouarab 2000)

Atendiendo al diferente patrón de sulfatación de la carragenina de la matriz extracelular que muestran ambas fases del

ciclo de vida, Bouarab *et al.* (1999) estudiaron la posibilidad de que estas diferentes fracciones de carragenina pudiesen modular la virulencia del patógeno. Se encontró que el oligosacárido λ-carragenina, presente en la fase esporofítica de *C. crispus*, induce la liberación de H_2O_2 por parte del endófito. Sin embargo el oligosacárido κ -carragenina, presente en la forma gametofítica de *C. crispus* no sólo no induce la liberación de H_2O_2 por parte del endófito, sino que favorece el reconocimiento de éste por parte del hospedador, lo que resulta en una disminución de la virulencia.

En este sistema en el que la fase gametofítica del hospedador es resistente a la acción del patógeno, mientras que la fase esporofítica es susceptible a la infección, los oligosacáridos actúan como señal en el reconocimiento celular, determinando no sólo la virulencia del agente patógeno sino la capacidad del hospedador de reconocer y limitar su penetración.

Los oligo-ι y λ-carragenina son capaces de inducir la liberación de ROS por el endófito (Bouarab 2000) (Fig 4), sin embargo, la producción de H_2O_2 es mucho más importante cuando el patógeno es incubado con oligo-λ-carragenina que con oligo-ι-carragenina. Los polímeros de carragenina son incapaces de inducir la emisón de ROS en *U. operculata*.

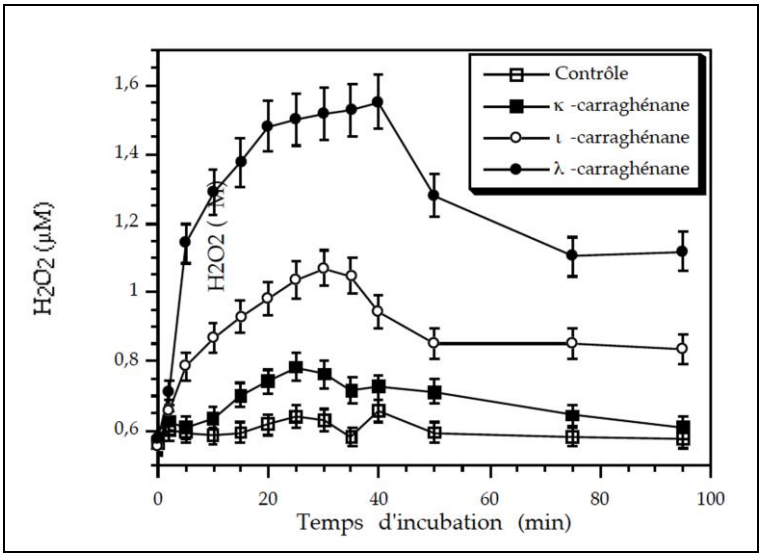


Fig 4 Emisión de H_2O_2 por *U. operculata* como respuesta a la inducción con oligocarragenina (Bouarab 2000)

Los oligo-λ-carragenina no sólo inducen la emisión de ROS por parte del endófito, además actúan estimulando la síntesis proteica, incrementando la actividad carragenolítica e induciendo la síntesis de polipéptidos específicos de la infección, lo que supone un marcado incremento de la virulencia. Los oligosacáridos son capaces de modular la virulencia del endófito hasta tal punto que las zoosporas de *U. operculata* previamente incubadas en oligo-λ-carragenina procedentes del esporofito invaden completamente al gametofito que de otro modo se muestra resistente a la infección (Bouarab 2000)

(Fig 5). Esta infección es comparable a la observada en el esporofito. Sin embargo, las zoosporas incubadas en oligo-κ-carragenina tienen muy baja capacidad infectiva y su capacidad de penetración se ve limitada a las capas más externas del hospedador (Bouarab 2000) (Fig 6).

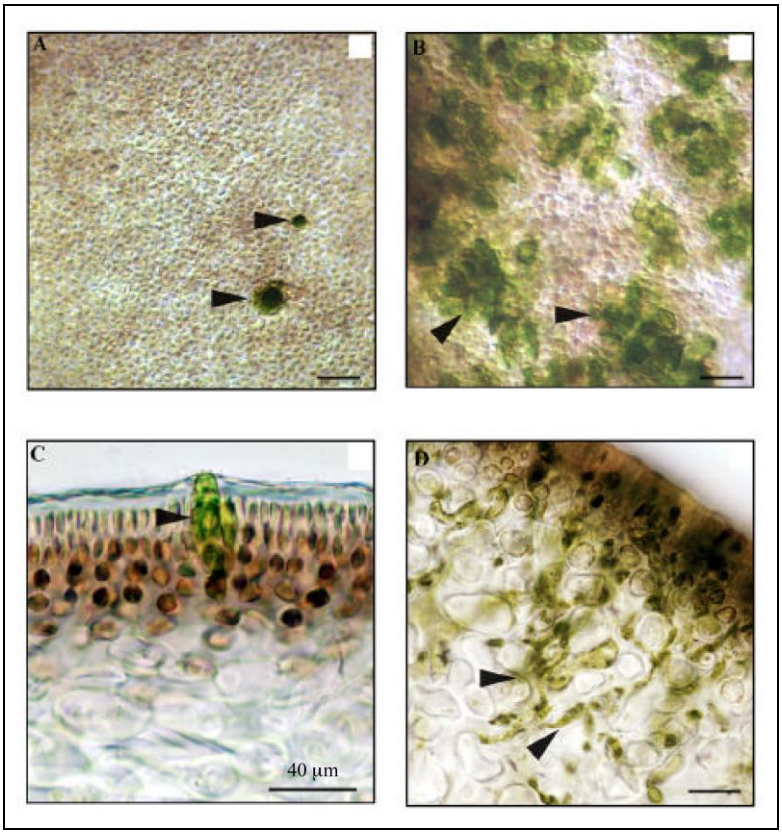


Fig 5 Efecto del pretratamiento de las zoosporas de *U. operculata* con oligo-λ-carragenina en la capacidad de infección de éstas sobre gametofitos de *C. crispus* (A, C) Gametofito infectado por patógenos no pretratados (B, D) Gametofito infectado por endófitos pretratados con oligo-λ-carragenina (A, B) Vista superficial (C, D) Corte transveral (Bouarab 2000)

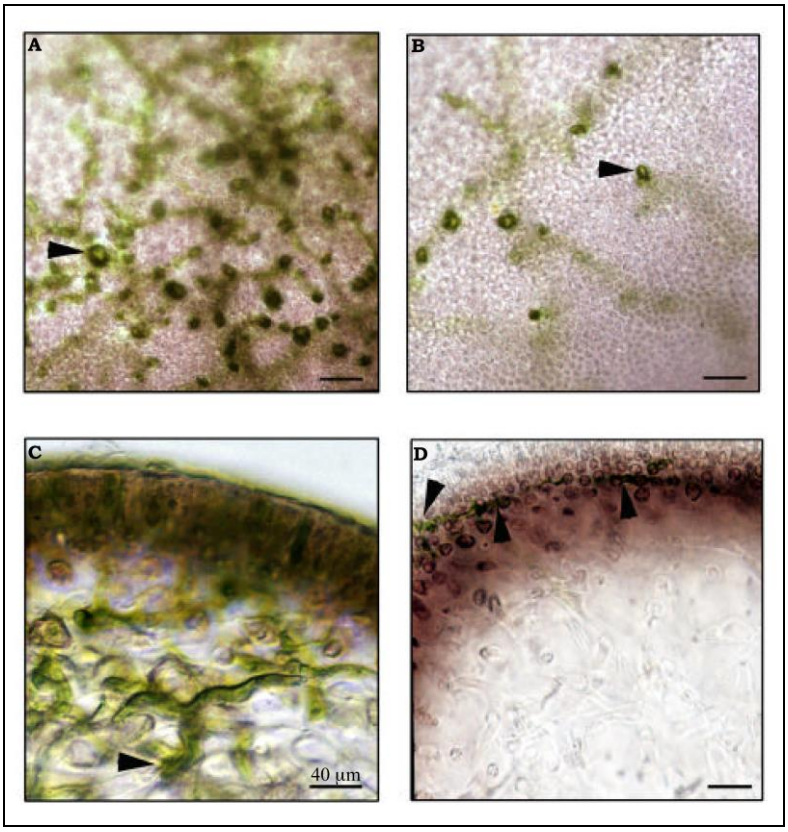


Fig 6 Efecto del pretratamiento de las zoosporas de *U. operculata* con oligo-κ-carragenina en la capacidad de infección de éstas sobre esporofitos de *C. crispus* (A, C) Esporofito infectado por patógenos no pretratados (B, D) Esporofito infectado por endófitos pretratados con oligo-κ-carragenina (A, B) Vista superficial (C, D) Corte transversal (Bouarab 2000)

Como se mencionó anteriormente los oligosacáridos no sólo modulan la virulencia del endófito, sino modifican su composición bioquímica, permitiendo al hospedador detectar al agente infectando y limitar su penetración. Los gametofitos de *C. crispus* producen

cantidades significativas de H_2O_2 al entrar en contacto con extractos de *U. operculata*, demostrando la existencia de alguna molécula que actúa como señal, permitiendo reconocer al endófito y desencadenando una respuesta oxidativa mediante la liberación de ROS. Esta liberación de H_2O_2 está al nivel de las respuestas oxidativas mostradas por plantas superiores. Sin embargo, la liberación de H_2O_2 por parte de los gametofitos que no han tomado contacto con el endófito es realmente baja (Bouarab 2000) (Fig 7).

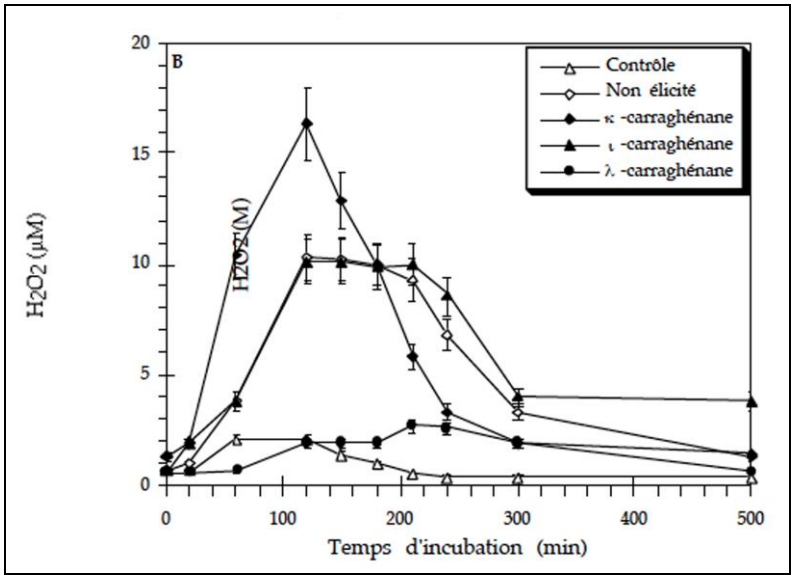


Fig 7 Respuesta oxidativa en gametofitos de *C. crispus* inducida por extractos de *U. operculata* previamente incubados en diferente oligosacáridos (Bouarab 2000)

Las PAs en los mecanismos de defensa en plantas superiores

Las poliaminas (PAs) son moléculas alifáticas de bajo peso molecular, de naturaleza policatiónica, compuestas por una cadena carbonada con grupos amino. Pueden ser clasificadas en dos grupos. En un grupo se encuentran las poliaminas comunes como la diamina putrescina (Put), la triamina espermidina (Spd) y la tetramina espermina (Spd), mientras que en el segundo grupo se incluyen las poliaminas poco comunes como la norespermina y la norespermidina entre otras.

Son moléculas ubicuas, comúnmente encontradas en todos los organismos (Tabor & Tabor 1984; Escribano & Legaz 1988; Evans & Malmberg 1989; Flores 1990; Lee & Chu 1992; Cohen 1998; Hosoya *et al.*2005), que a nivel celular se encuentran entre otras estructuras en la pared celular y la membrana plasmática (Ballas *et al.* 1983; Schubert *et al.* 1983; Slocum *et al.* 1984; Torrigiani *et al.* 1986; Schuber 1989; Bagni & Torrigiani 1992; Tiburcio *et al.* 1997). Pueden ser encontradas en su forma libre o en su forma conjugada, unidas a macromoléculas (Flores & Galston 1982; Geny *et al.* 1997).

Las PAs son esenciales en multitud de procesos fisiológicos en plantas como el crecimiento celular y la biosíntesis de macromoléculas, la división celular (Heimer *et al.* 1979; Berlin &

Forche 1981), estabilización de membranas celulares (Schubert *et al.* 1983; Roberts *et al.* 1986; Kaur-Sawhney & Applewhite 1993), protección del ADN y estimulador de su replicación, transcripción y traducción (Bagni & Torrigiani 1992; Wallace *et al.* 2003).

En plantas superiores están involucradas en diferentes procesos como la embriogénesis (Montague *et al.* 1978), el desarrollo de la raíz (Jarvis *et al.* 1985), la floración (Cabanne *et al*. 1981; Heimer & Mizrahi 1982), el desarrollo del fruto y el polen (Biasi *et al.* 1988; Bagni *et al.* 1981) y la senescencia (Altman *et al.* 1977; Kaur-Sawhney & Galston 1979; Kaur-Sawhney *et al.* 1982a,b), jugando además un papel importante en procesos relacionados con el estrés tanto biótico como abiótico (Groppa & Benavides 2008; Alcázar *et al.* 2006, 2010; Gill & Tuteja 2010; Hussain *et al.* 2011; Wimalasekera *et al.* 2011).

En algas se ha descrito la importancia de las PAs en procesos de estrés salino (Lee & Chen 1998), división celular (Cohen *et al.* 1984) y su implicación en procesos reproductivos (Guzmán-Urióstegui *et al.* 2002).

El catabolismo de las poliaminas se realiza mediante la acción de la diaminoxidasa (DAO) y la poliaminoxidasa (PAO). Estas enzimas se encuentran localizadas principalmente en la pared celular

(Angelini *et al.* 1993; Sebela *et al.* 2001). Los compuestos que resultan de la degradación oxidativa son anillos pirrólicos o derivados (Slocum *et al.* 1984). Como resultado de la actividad de la enzima DAO, la putrescina es oxidada a Δ'-pirrolina con liberación de amonio y peróxido de hidrógeno (H_2O_2). La oxidación de la espermina y la espermidina mediada por la enzima PAO da lugar entre otros productos de reacción a H_2O_2.

Las plantas han desarrollado sistemas de defensa específicos para protegerse del ataque de una amplia variedad de agentes patógenos (Staskawicz *et al.* 1995; Heath 2000). Tras la detección del patógeno se forma una lesión necrótica en el sitio de entrada del mismo (Stakman 1915), evitando de éste modo la propagación de la enfermedad. Este evento se conoce como respuesta hipersensible (HR). La HR se inicia tras detectar el ataque de un agente patógeno y se caracteriza por una rápida liberación de ROS que actúan directamente como tóxico sobre el patógeno. El catabolismo de las poliaminas actúa como fuente de H_2O_2 en la HR en plantas terrestres (Yoda *et al.* 2003, 2009).

Los niveles de PAs y la actividad de las enzimas DAO y PAO se ven incrementados durante el transcurso de la infección en plantas superiores evidenciando que las poliaminas, y el producto de su

degradación (H_2O_2), son elementos clave que inducen la HR en plantas (Yoda *et al*. 2003, 2006).

METODOLOGÍA

Establecimiento de cultivos de explantos asépticos de *G. imbricata*

La metodología que se ha seguido para establecer cultivos asépticos de *Grateloupia imbricata* fue la descrita por Robaina (1988) y Robaina *et al.* (1990a, b). Está basada en someter el material vegetal previamente a su cultivo a procedimientos de desinfección físicos y agentes desinfectantes químicos para eliminar los organismos asociados al talo.

Inicialmente se realiza un proceso de limpieza mecánica de la superficie de los talos empleando un cepillo o pincel, eliminando los restos de sustrato y epífitos. A continuación se extraen fragmentos de talo, en forma de discos de 3 mm de diámetro a lo largo de toda la superficie del mismo (explantos). Los explantos son sometidos a choque osmótico y limpieza por ultrasonidos, consistente en tres lavados en agua destilada estéril de 1 minuto y 30 segundos de duración, seguido de un lavado con agua destilada estéril, povidona yodada al 1 % (Betadine® al 10%) y una gota de detergente (Tween 80), que actúa como agente tensoactivo, durante 5 min. Finalmente se realiza un lavado en agua de mar estéril durante 1 min y 30 s. Los ultrasonidos generan ondas de alta frecuencia que permiten desprender de las paredes de los explantos los organismos epífitos. Los

ultrasonidos de baja intensidad empleados no afectan a la integridad de los explantos ni dañan a los organismos epífitos.

Los agentes químicos secundarios más comúnmente empleados en la desinfección de explantos en macroalgas son los antibióticos de amplio espectro combinados con antifúngicos y agentes antidiatomeas. Los explantos se incuban de tres en tres en 10 ml de una solución antimicrobiana durante tres días, bajo las mismas condiciones de luz y temperatura que tendrán posteriormente durante su cultivo. La solución antimicrobiana se prepara a partir de una solución concentrada cuya composición se muestra en la Tabla 1.

Antimicrobiano	Espectro de acción	mg / 100 ml
Penicilina	Bactericida Gram +	100
Ampicilina	Bactericida Gram + Gram-	25
Nistatina	Fungicida	25
GeO_2	Antidiatomeas	10
Agua de mar (Estéril)		100 ml
Añadir 1 ml de solución concentrada por cada 10 ml (volumen final) de solución antimicrobiana		

Tabla 1 Composición de la solución antimicrobiana concentrada y espectro de acción de cada uno de los componentes, empleada en la desinfección de explantos.

Composición y elaboración del medio de cultivo

Después del proceso de desinfección para lograr la asepsia de los explantos, éstos se transfieren a un medio de cultivo específico.

El medio de cultivo empleado fue una variación del medio PES (Provasoli Enriched Seawater, Provasoli 1968), con modificaciones que afectan a la composición y concentración de vitaminas, y a la composición de sales, aunque mantiene las mismas relaciones molares de los elementos originales del medio de Provasoli pero adaptado a las formas comerciales de los componentes por Robaina (1988).

El medio se prepara a partir de 3 soluciones stock que se conservan a 4ºC. Estas soluciones son una solución de micronutrientes (PII) (Tabla 2), una solución de EDTA- ferroso (EDTA-Fe) (Tabla 3) y una solución de vitaminas (Tabla 4 y Tabla 5).

Micronutriente	mg / 200 ml
$FeSO_4 \cdot 7H_2O$	50,34
H_3BO_3	1140
$MnSO_4 \cdot H_2O$	122,90
$ZnCl_2$	10,48 *
$CoCl_2$	4,03 *
Na-EDTA·$2H_2O$	1000
Agua doblemente destilada (DDW)	200 ml

* A partir de solución stock conteniendo 104,8 mg de Zn y 40,3 mg de Co en 10 ml de agua MiliQ estéril, conservada a – 20 ºC (añadir 1ml a 200 ml de solución P(II).

Tabla 2 Composición de la solución de micronutrientes (PII) para la elaboración del medio PES

Compuesto	mg / 50 ml
$FeSO_4 \cdot H_2O$	245
Na-EDTA·$2H_2O$	330
DDW	50 ml

Tabla 3 Composición de la solución de EDTA ferroso (EDTA-Fe) para la elaboración del medio PES

Vitamina	mg / 100 ml
Tiamina-HCl	100
Biotina	1 *
Piridoxina	1 *
B_{12}	0,2 *
DDW	100ml

* A partir de soluciones stock de Biotina, Piridoxina y B_{12}. Preparar y añadir en su totalidad.

Tabla 4 Composición de la solución de vitaminas para la elaboración del medio PES

Vitamina	mg / ml (en gua MiliQ estéril)
Biotina	10 mg / 10 ml
Piridoxina	10 mg / 10 ml
B_{12}	1 mg / 20 ml

Tabla 5 Composición de la solución de vitaminas para la elaboración del medio PES (Stock)

La composición final del medio PES se muestra en la Tabla 6. El agua de mar utilizada en la elaboración de los medios de cultivo es recogida, filtrada y tratada con UV en las instalaciones para cultivos marinos del Instituto Canario de Ciencias Marinas (ICCM).

La preparación del medio finaliza ajustando su pH a 7,8 y esterilizándolo en autoclave durante 20 min a 120 ºC y 1 $kgcm^{-2}$ de presión. Este procedimiento de esterilización incrementa de manera constante el pH del medio en 0,5 unidades.

$NaNO_3$	70 mg
Na_2-glicerol fosfato	10 mg
Solución PII	1 ml
Solución EDTA-Fe	0,5 ml
Solución de viminas	1 ml
Agua de mar	1 litro
pH	7,8

Tabla 6 Composición final del medio PES (Provasoli, 1968)

Cultivo de *G. imbricata*

El cultivo de explantos asépticos de *Grateloupia imbricata* en medio PES líquido se realizó en placas de Petri estériles de 45 mm, con 15 ml de medio de cultivo. La siembra de explantos y dispensación del medio de cultivo se llevó a cabo en el interior de una cámara de flujo laminar horizontal Glatt Labortecnic. El material de laboratorio usado en la siembra de explantos (pinzas, asas de siembra, etc.) fue esterilizado en autoclave durante 20 min a 120 ºC y flameado con alcohol en el interior de la cámara de flujo laminar después de cada uso.

Los explantos fueron incubados durante la esterilización y cultivados bajo condiciones de temperatura y fotoperiodo controladas

en una cámara de cultivo Koxka, manteniendo una temperatura de 18 ± 2 ºC y un fotoperiodo de 16:8 (horas de luz: horas de oscuridad) a 30 µmoles de fotones m^2s^{-1} a nivel de las placas de petri. Los cultivos se mantuvieron bajo estas condiciones con cambios semanales de medio de cultivo durante un periodo de al menos 6 meses, en función del experimento realizado.

Condiciones de cultivo *in vitro* de algas epífitas y endófitas

Los filamentos de las algas endófitas o epífitas que se desarrollaban asociadas a los talos de *Grateloupia imbricata* durante el cultivo *in vitro* de ésta, se aislaron y mantuvieron en cultivos unialgales.

Los filamentos que surgen en los explantos de *G. imbricata* fueron inicialmente cultivados en placas de Petri de 45 mm con 15 ml de medio PES líquido a una temperatura de 18 ± 2 ºC y un fotoperiodo de 16:8 con una intensidad de luz de 5 µmoles de fotones m^2s^{-1}, esta irradiación fue adaptada a partir de experimentos previos, al observarse como la menos perjudicial (crecimiento, no fotoinhibición, etc.) y posteriormente se transfieren a Erlenmeyer con 75 ml de medio de cultivo y de éstos a botellas de cultivo con 450 o 750 ml de medio PES (Fig 8).

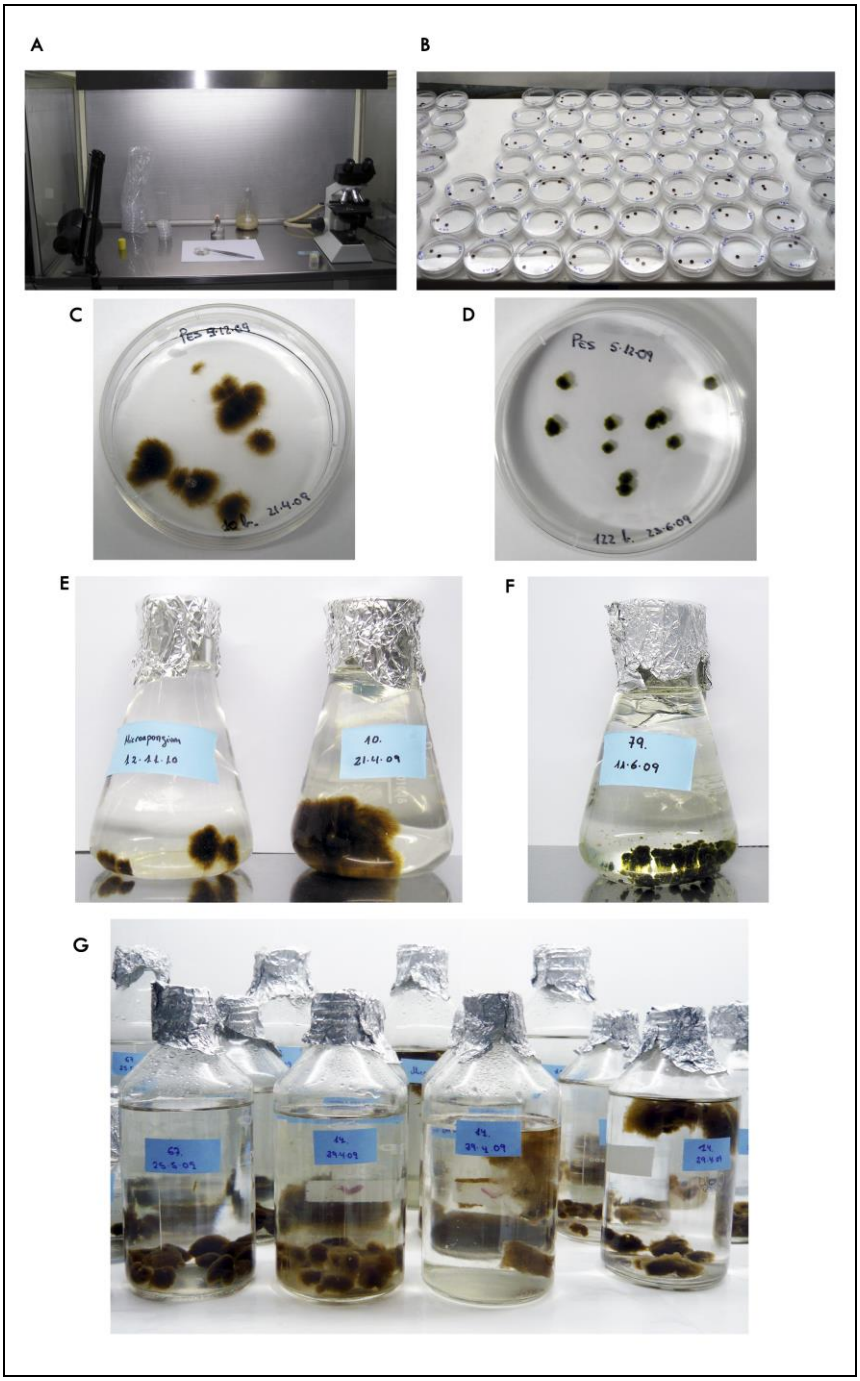

A
B
C
D
E
F
G

Fig 8 (A) Cámara de flujo laminar Glatt Labortecnic; (B) Cultivo de explantos de *G. imbricata* en placas de Petri de 45 mm, 15 ml de medio PES; (C) Aislamiento de *M. tenuissimum* y (D) Aislamiento de *U. leptochaete* creciendo en cultivo en placas de Petri de 45 mm, 15 ml de medio PES. (E) Cultivo de *M. tenuissimum* y (F) *U. leptochaete* en Erlenmeyer, 75 ml de medio PES; (G) *M. tenuissimum* en botellas de cultivo, 450 y 750 ml de medio PES.

Extracción de ADN genómico de *G. imbricata* y sus algas huéspedes

El ADN genómico de macroalgas se extrajo a partir de 100 mg de tejido procedente del talo de *Grateloupia imbricata* o a partir de 50 mg de filamentos de algas epífitas o endófitas, que previamente habían sido almacenadas a - 80 ºC, siguiendo el protocolo Hexadecyltrimethylammonium bromide (CTAB) de Murray & Thompson (1980), adaptado para la extracción de ADN genómico en *Grateloupia imbricata* en nuestro laboratorio (García-Maroto & Robaina, no publicado).

El procedimiento es el siguiente:

1. Triturar el tejido con nitrógeno líquido en morteros previamente conservados a -20 ºC. El tejido finamente dividido se vierte en tubos eppendorf de 2 ml.

2. Añadir 1 ml de tampón de extracción por cada 100 mg de tejido. La composición del tampón de extracción se muestra en la Tabla 7.

3. Incubar 1 hora a 65 ºC mezclando cada cierto tiempo suavemente, invirtiendo el tubo.

4. Añadir 1 volumen de cloroformo:isoamílico (24:1) mezclando suavemente por inversión del tubo hasta conseguir formar una emulsión.

5. Centrifugar durante 10 min a 3000 g y 4ºC.

6. Transferir la fase acuosa (fase superior) a un tubo eppendorf de 2 ml. Repetir los pasos 4 y 5 dos veces más.

7. Añadir dos tercios del volumen recuperado en la etapa anterior de isopropanol y mezclar suavemente invirtiendo el tubo hasta formar un precipitado de ADN.

8. Centrifugar durante 15 min a 10000 g y 4ºC. Se formará un pellet en el borde inferior del tubo que debe ser manejado con cuidado.

9. Drenar el sedimento lavando con etanol al 80% durante unos minutos y dejar secar. El sobrenadante puede ser eliminado con micropipeta con precaución para no arrastrar el pellet.

10. Resuspender el ADN en 50 ml de tampón TE. La composición del tampón TE se muestra en la Tabla 7.

Tampón de extracción CTAB	100 mM TrisHCL pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% CTAB, 0.1 % PVP (polyvinylpyrrolidone), 0,1 % SDS, 2% β-mercaptoetanol (añadir justo en el momento de usar).
Tampon TE	10 mM TrisHCl pH 7.5, 1 mM EDTA pH 8.0.

Tabla 7 Composición química del tampón de extracción CTAB y el tampón TE

El material de laboratorio empleado para realizar la extracción de ADN (tubos eppendorf y puntas de micropipeta) así como los tampones de extracción CTAB y tampón TE y las soluciones previas para su elaboración deben ser esterilizadas con un pase en autoclave de 2 h a 120 ºC.

La concentración y calidad del ADN fue determinada por espectrofotometría usando un espectrofotómetro Nanodrop 1000 v.3.7 (ThermoFisher Scientific, Wilmington, SA).

La cuantificación de ácidos nucleicos se basa en el método espectrofotométrico descrito por Sambrook *et al.* (1989), basado en la absorción a 260 nm de los ácidos nucleicos. El rendimiento por término medio fue de 150 a 300 ng/µl. Para determinar una posible

contaminación por carbohidratos se calcula la relación entre absorbancias 260/230 nm. Del mismo modo, para determinar si la muestra se encuentra libre de contaminación por proteínas y compuestos fenólicos se determina la relación 260/280 nm. De esta forma se realiza una estimación del grado de pureza de los ácidos nucleicos. Una relación Abs. 260/280 comprendido entre 1,8 y 2 es un indicador de pureza del ADN, mientras que un valor inferior a 1,8 indica contaminación por proteínas o por fenol. En este trabajo se seleccionaron las muestras con valores de 1.8 o superiores.

Purificación de muestras de ADN genómico y productos de PCR

Las muestras de ADN genómico fueron purificadas empleando el kit de purificación GenElute PCR Clean-Up (Sigma). El procedimiento realizado es el siguiente:

1. Montar la columna y acondicionarla añadiendo 0,5 ml de la solución "Column preparation solution" y centrifugando a 12000 g durante 30 s. Desechar el eluyente.

2. Añadir la solución "Binding Solution" en una relación 5 a 1 respecto al volumen solución de ADN a purificar. Transferir el producto a la columna acondicionada y centrifugar a 12000 - 16000 g durante 1 min. Descartar el eluyente.

3. Montar la columna en un tubo limpio y añadir 0,5 ml de la solución "Wash solution", centrifugando a 12000- 16000 g durante 1 minuto.

4. Colocar la columna en un tubo limpio y centrifugar a 16000 g durante dos minutos para eliminar los restos de etanol.

5. Transferir la columna a un tubo eppendorf de 1,5 ml y añadir 50 µl de "Elution solution" o agua ultrapura a la columna. Incubar a temperatura ambiente durante 1 min.

6. Centrifugar la columna a 16000 g durante 1 minuto.

Los productos de amplificación de PCR fueron purificados empleando el kit comercial Wizard® SV Gel and PCR Clean-Up System (Promega), el protocolo de purificación es el siguiente:

7. Añadir un volumen de “Membrane Binding Solution” al producto de amplificación de la PCR.

8. Colocar la columna “SV Minicolumn” en un tubo recolector.

9. Transferir el producto de PCR preparado a la columna "SV Minicolumn" e incubar durante 1 minuto a temperatura ambiente.

10. Centrifugar la columna a 16000 g durante 1 minuto. Retirar la columna del tubo recolector, descartar el contenido del mismo y volver a colocar la columna.

11. Lavar la columna añadiendo 700 µl de "Membrane Wash Solution", previamente diluido con etanol al 95%. Centrifugar la columna ensamblada en el tubo recolector durante 1 minuto a 16000 g. Vaciar el tubo recolector y volver a montar la columna. Repetir el lavado con 500 µl de "Membrane Wash Solution", centrifugando 5 minutos a 16000 g.

12. Descartar los residuos del lavado contenidos en el tubo recolector y centrifugar la columna durante 1 minuto para eliminar los restos de etanol.

13. Transferir cuidadosamente la columna a un tubo eppendorf de 1,5 ml, añadir 50 µl de agua ultrapura directamente en el centro de la columna, incubar a temperatura ambiente durante 1 minuto y centrifugar durante 1 minuto a 16000 g.

14. Desechar la columna y conservar el ADN entre 4º y -20 ºC.

Visualización de ADN en geles de agarosa

Las electroforesis de muestras de ADN se llevaron a cabo en geles de agarosa al 1,0% en tampón TAE. Las electroforesis se realizaron a un voltaje entre 60-100 V en tampón TAE.

Las muestras para electroforesis se preparan mezclando previamente la muestra con un tampón de carga 1X. Como marcadores de peso molecular se utilizaron fragmentos de ADN del fago lambda comerciales.

Para visualizar el ADN tras la realización de la electroforesis se realizó una tinción de los geles por inmersión en bromuro de etidio y se fotografió el gel expuesto a luz UV (260 nm) en un UV transluminator ChemiDoc™ XRS (Bio Rad). La composición del tampón TAE y el tampón de carga se muestra la Tabla 8.

Tampón TAE 50 X (1 litro)	TrisBase (Fw=121.14) 57,1 ml ácido acetico glacial y 100 ml de solución 0,5 M EDTA pH 8.0*. Ajustar a 1 l con agua MiliQ esteril. pH 8.5.
Tampón de carga 6 X	50% p/v sacarosa, 0,3% p/v azul de bromofenol, en agua MiliQ estéril.

* Solución EDTA (ethylenediamine tetraacetic acid) 0,5M. Para 500 ml de solución stock: 93,05 g EDTA disodium salt (FW=372,2). Disolver en 400 ml de agua MiliQ estéril, ajustar a pH 8.0 con NaOH y llevar a volumen final

Tabla 8 Composición química del tampón TAE y el tampón de carga

Hibridación de ADN "Southern blot"

El análisis mediante Southern blot (Southern 1975) permite la identificación específica de fragmentos de ADN previamente separados en geles de agarosa por electroforesis. La detección consiste en la hibridación de fragmentos de ADN utilizando como sondas fragmentos complementarios marcados. En el presente trabajo las sondas fueron marcadas con digoxigenina-dUTP.

Para la realización del Southern blot, el ADN genómico a hibridar debe ser digerido con enzimas de restricción que no corten el fragmento de ADN que se utiliza como sonda. Las enzimas de

restricción que se seleccionaron para realizar el presente estudio son *Eco*RI y *Pvu*II (Roche). Se digirieron 4 µg de ADN genómico con cada una de las enzimas. La composición de la reacción de digestión para cada enzima por µg de ADN genómico se muestra en la Tabla 9. El tampón es característico de cada enzima y viene indicado para lograr una actividad óptima, de acuerdo con las condiciones del fabricante.

	***Eco*RI**	***Pvu*II**
DNA	1 µg	1 µg
10 x SuRe/Cut Buffer	5 µl	2,5 µl
Agua Purificada hasta volumen total de:	50 µl	25 µl
Enzima de restriction	1 unidad	1 unidad

Tabla 9 Composición de las reacciones de digestión de ADN genómico por µg de ADN

El procedimiento a seguir para realizar la digestión del ADN es el siguiente:

1. Incubar el ADN genómico (1 µg) a 95 ºC durante 5 minutos para desnaturalizarlo.
2. Mantener las muestras en hielo durante 5 minutos.

3. Someter las muestras a una centrifugación corta (5 segundos)
4. Añadir el tampón específico de la enzima, 1 unidad de enzima de restricción y completar con H_2O purificada hasta volumen final de reacción (Ver tabla 9).
5. Centrifugar las muestras (5 segundos) e incubar 12 horas a 37 ºC.

El ADN producto de 4 reacciones de digestión (4 µg) se precipita con nitrato de sodio y etanol, y se resuspende en 10 µl de agua ultrapura.

Transferencia a membrana de Nylon.

El ADN producto de las reacciones de digestión con las enzimas de restricción *Eco*RI y *Pvu*II (10 µl a una concentración de 0,4 µg/µl) se carga en un gel de agarosa al 1,2 % (p/v). Los geles tienen unas dimensiones de 10 x 10 cm para los estudios que se presentan. La separación de las muestras se llevó a cabo en tampón TAE 1X a un voltaje constante (100V) durante 2 horas, en un sistema de electroforesis Power-Pac 300 (Bio-Rad).

El gel se tiñó con bromuro de etidio y se fotografió en un sistema de documentación de geles GelDoc XR (Bio-Rad) que consta de un transiluminador de 25 x 26 cm, una cámara oscura y cámara CCD de 1.4 Mpixels controlado por el programa Quantity One.

El gel se lavó durante 15 minutos en agua MiliQ para eliminar en la medida de lo posible el bromuro de etidio. Como paso previo a la transferencia y para favorecer el paso de los fragmentos de ADN de mayor tamaño a la membrana, el ADN se desnaturalizó siguiendo los siguientes pasos:

1. Baño del gel en una solución de desnaturalización durante 45 min.
2. Baño en agua MiliQ estéril durante unos segundos.
3. Baño en solución de neutralización por 30 minutos.
4. Baño en solución de neutralización durante 15 minutos.

La composición de las soluciones de desnaturalización y neutralización se muestran en la Tabla 10.

La transferencia de ADN a la membrana de Nylon Hybond-N^+ cargada positivamente (Amersham Pharmacia Biotech) se realiza por capilaridad de abajo hacia arriba. Para que esto tenga lugar se realizó el siguiente montaje.

En el recipiente de vidrio en el que se realizó la transferencia se colocó un soporte y sobre él se situó un cristal con las dimensiones del gel de electroforesis. El interior del recipiente para la transferencia se vertió solución de transferencia 20X SSC (Tabla 10) hasta alcanzar

el borde superior del soporte sobre el que se ha situado el cristal. Sobre esta superficie de cristal se colocó un papel Whatman 3M humedecido previamente en solución de transferencia 20X SSC, a modo de puente contactando con el recipiente que contiene la solución de transferencia.

Solución de desnaturalización Composición por litro	
	g/l
NaOH	20
NaCl	87,66
Agua MiliQ estéril	Hasta volumen final
Solución neutralizante **Composición por litro**	
	g/l
TRIS	60,6
NaCl	175,4
Agua MiliQ estéril	Hasta volumen final pH 7,5
Solución de transferencia 20X SSC **Composición por litro**	
	g/l
Citrato sódico	88,23
NaCl	175.32
Agua MiliQ estéril	Hasta volumen final pH 7,0

Tabla 10 Composición de las soluciones de desnaturalización, neutralizante y de transferencia 20X SSC

Las dimensiones del papel Whatman 3 M deben ser el mismo largo que el vidrio que soporte al gel y aproximadamente 10 cm más ancho para que absorba la solución de transferencia y facilite la migración de las moléculas de ADN por capilaridad.

A continuación el gel de electroforesis se situó sobre el papel Whatman controlando que no se formaran burbujas de aire. Sobre el gel se colocó la membrana de Nylon para la transferencia, previamente hidratada en agua estéril y empapada en solución 20X SSC. Es conveniente manejar la membrana con pinzas específicas que no dañen su superficie y evitar la formación de burbujas entre el gel y la membrana, eliminándolas en todo caso ya que evitarían una correcta transferencia de las moléculas de ADN. Sobre la membrana se colocó un papel Whatman del mismo tamaño que ésta, sin empapar, y a continuación una torre de papel, un vidrio y un peso. El montaje se mantuvo aproximadamente 12 horas para que tuviese lugar la transferencia. Una vez finalizada se desmontó el sistema, se marcó la posición de los pocillos en la membrana y se dejó secar al aire.

El ADN se fijó a la membrana con radiación ultravioleta a 120 J/cm en un Ultraviolet Crosslinkers CL-1000 (Fig 9).

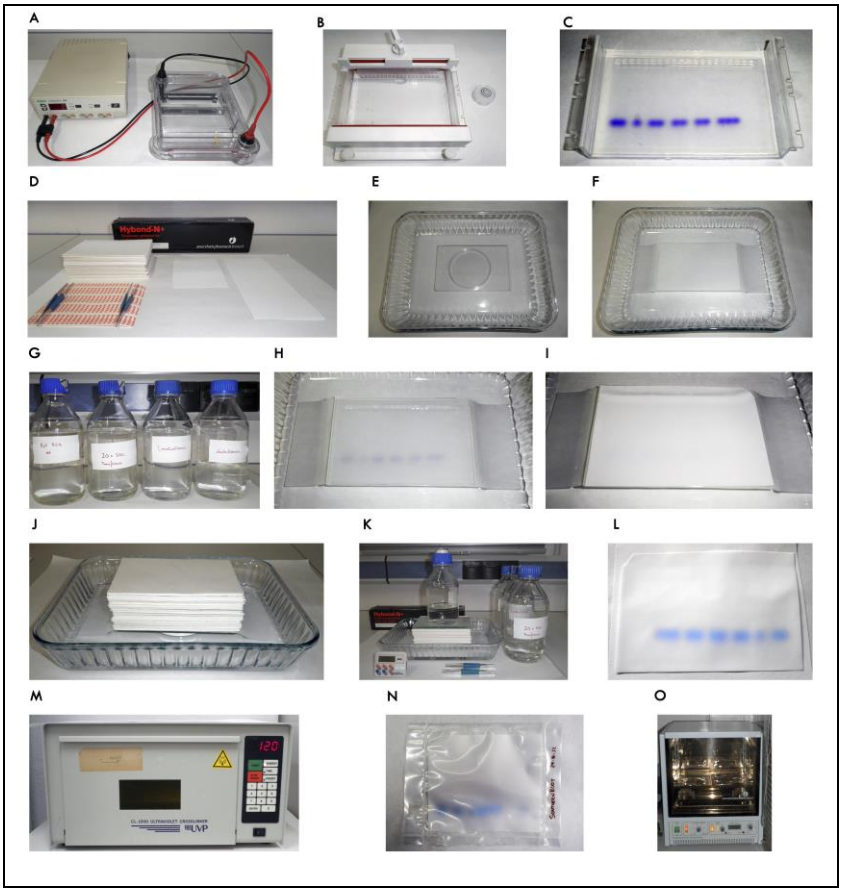


Fig 9 Hibridación de ADN mediante Southern blot (A-C) Sistema de electroforesis y gel de agarosa (D) Membrana de Nylon, papel Whatman 3M y pinzas (E-F) Cubetas de vidrio para transferencia (G) Soluciones de desnaturalización, neutralización y transferencia (H-K) Transferencia de ADN desde el gel de electroforesis a la membrana de Nylon (L-M) Fijación del ADN a la membrana de Nylon mediante radiación ultravioleta (N) Bolsa de prehibridación e hibridación (O) Incubador con agitación

Síntesis de la sonda marcada con digoxigenina

La sonda marcada con digoxigenina (DIG) se sintetizó usando el Kit DIG DNA Labeling Mix, 10X (Roche). Éste permite obtener una alta sensibilidad en la reacción de detección ya que incorpora un residuo DIG-dUTP cada 20 a 25 nucleótidos en el ADN recién sintetizado.

La cantidad de sonda de ADN marcada que se sintetiza depende directamente de la cantidad de ADN presente en la reacción y del periodo de incubación, de esta forma, con 3 µg de ADN de partida y tras una incubación de 20 h se obtienen 890 ng de sonda marcada con digoxigenina.

El procedimiento seguido para la síntesis es el siguiente:

1. Añadir 3 µg de ADN y completar con H_2O doblemente destilada estéril hasta un volumen de 10 µl.
2. Desnaturalizar el ADN durante 10 minutos a 95 ºC y enfriar posteriormente en hielo durante 5 minutos.
3. Añadir: 2 µl de Hexanucleotide Mix, 10X.
   2 µl de DIG DNA Labeling mix, 10X.
   1 µl de enzima Klenow
4. Mezclar y centrifugar brevemente.

5. Incubar a 37 ºC durante 20 horas.
6. Parar la reacción añadiendo 2 µl de EDTA 0,2 M (pH 8).

Prehibridación e hibridación

El procedimiento a seguir para realizar la prehibridación y la hibridación es el siguiente:

1. Humedecer la membrana en una solución 6X SSC
2. Introducir la membrana en una bolsa de hibridación, con el ADN colocado hacia arriba y realizar la prehibridación durante dos horas con 2,5 ml de solución de hibridación por cada 100 cm^2 de membrana de Nylon. El proceso se lleva a cabo en una cámara de incubación a una temperatura de 42 ºC (Fig 9). La solución de prehibridación está compuesta en un 50 % de formamida (Tabla 11), lo que equivale a realizar la prehibridación a 68 ºC.
3. La sonda debe ser desnaturalizada previamente a su uso, calentándola hasta los 95 ºC durante 5 min e inmediatamente debe ser mantenida en hielo hasta el momento en el que se realice la hibridación.
4. La solución de hibridación contenida en la bolsa de hibridación se desecha y se añade un volumen igual de

solución de hibridación nueva a la que se añade 25 ng de sonda marcada con digoxigenina por cada 100 cm^2 de membrana de Nylon.

5. La membrana hibrida durante 12 horas a un equivalente de 68 ºC de temperatura.
6. Después de este periodo se desecha la solución de hibridación y se procede a realizar una limpieza de la membrana de Nylon con el fin de eliminar los posibles enlaces inespecíficos que la sonda haya podido formar.
    - Lavar 2 veces (5 min) a temperatura ambiente con 50 ml de solución 2X SSC; SDS 0,1 % (p/v) por cada 100 cm^2 de membrana de Nylon.
    - Lavar 2 veces (15 min) a 68 ºC con 50 ml de solución 0,1X SSC; SDS 0,1 % (p/v) por cada 100 cm^2 de membrana de Nylon.

Solución de Hibridación (para 10 ml)	5 ml de formamina al 100 % 3 ml solución 2 x SSC 2 ml H_2O doblemente destilada estéril Blocking reagent 0,1 g
Composición final: 50 % formamida, 6x SSC, Blocking reagent 1 %	

Tabla 11 Composición de la solución de hibridación

Detección inmunológica

La detección inmunológica de ácidos nucleicos marcados con digoxigenina se llevó a cabo empleando el kit DIG Nucleic Acid Detection (Roche). La sonda marcada con digoxigenina es detectada después de su unión específica a la secuencia diana mediante inmunoensayo usando un anticuerpo anti-DIG-AP. Posteriormente una reacción de color con 5-bromo-4-cloro-3-indolfosfato (BCIP) y azul de nitro-tetrazolio (NBT) produce un precipitado azul insoluble que permite visualizar las moléculas hibridadas.

El procedimiento a seguir para llevar a cabo la detección inmunológica es el siguiente:

1. Después de la hibridación y los lavados empapar la membrana de Nylon durante unos segundos en solución **Washing buffer**.
2. Incubar durante 30 min en 100 ml de **Blocking solution.**
3. Incubar durante 30 min en 20 ml de **Antibody solution.**
4. Lavar dos veces durante 15 min en 100 ml de **Washing buffer.**
5. Equilibar incubando de 2-5 min en 20 ml de **Detection buffer.**
6. Incubar la membrana en 10 ml de **Color substrate solution** en oscuridad. La reacción de color comienza a los pocos minutos de la incubación y se completa después de 16 h. En los ensayos de hibridación realizados en este trabajo se llevó a cabo la detección inmunológica por periodos de entre 30 minutos y 1 hora.
7. Cuando la intensidad de la bandas reveladas en la membrana fue la adecuada la reacción se detuvo añadiendo 50 ml de agua destilada estéril.

La composición de las soluciones empleadas en la detección inmunológica se muestra en la Tabla 12.

Solución	Composición	Estabilidad
Washing buffer	0,1 M ácido malico; 0,15 M NaCl; pH 7,5; 0,3 % (v/v) Tween 20	15-25 ºC
Maleic acid buffer	0,1 M ácido málico; 0,15M NaCl; pH 7,5	15-25 ºC
Detection buffer	0,1 M Tris-HCl; 0,1 M NaCl; pH 9,5	15-25 ºC
Blocking solution	Diluir 1:10 la solución Blocking reagent*	No almacenar
Blocking reagent*	Disolver el agente bloqueante a una concentración final del 10 % (p/v) y autoclavar la solución	2-8 ºC
Antibody solution	Diluir el anti-digoxigenin-AP 1:5000 en Blocking solution	12 h a 2-8 ºC
Color substrate solution	Añadir 200 µl de la solución stock de NBT/BCIP a 10 ml de Detection buffer	No almacenar

Tabla 12 Composición de las soluciones de la reacción de detección inmunológica

Determinación de poliaminas mediante HPLC

Las poliaminas (PAs) a nivel celular pueden encontrarse en tres fracciones diferenciadas: solubles libres, solubles conjugadas e insolubles. Para su análisis por cromatografía líquida de alta resolución (HPLC) es necesario realizar previamente un proceso de extracción y su posterior derivatización.

La metodología que se ha seguido para realizar la extracción de PAs de *Grateloupia imbricata* ha sido empleada previamente con éxito en vegetales marinos en nuestro laboratorio (Marián *et al.* 2000a,b; Guzmán-Urióstegui *et al* 2002; Sacramento *et al*. 2004; Sacramento *et al.* 2007; Zarranz *et al.* 2012). La extracción se realizó en frío, para minimizar la actividad enzimática, y en medio ácido diluido.

Las muestras (600 mg de tejido) se dividieron finamente con nitrógeno líquido en morteros de porcelana a -20 ºC. La extracción se realizó añadiendo ácido perclórico (PCA) al 5% en una proporción de 1,5 ml (PCA): 600 mg de peso fresco. La muestra se maceró durante 10 minutos, formándose un extracto que se recogió en tubos eppendorf y se centrifugó a 9000g durante 20 minutos a 6ºC. El sobrenadante obtenido por centrifugación contiene las fracciones de poliaminas solubles en ácido (solubles libres y solubles conjugadas).

Las fracciones insolubles que permanecen en el sedimento no fueron objeto de estudio en el presente trabajo.

El sobrenadante debe ser separado en alícuotas. Así, 260 µl se mantuvieron a -80 ºC para su posterior derivatización, constituyendo el análisis de la fracción de poliaminas solubles libres. La fracción de poliaminas solubles conjugadas, antes de la derivatización, son sometidas a un proceso de hidrólisis ácida. Para ello 300 µl del sobrenadante se hidrolizaron con un volumen igual de ácido clorhídrico (HCl) en viales de vidrio cerrados con llama durante 12 horas a una temperatura de 100 ºC. Posteriormente el contenido del vial se recogió en tubos eppendorf y se evaporó el HCl. El material resultante se redisolvió en 260 µl de PCA al 5%, constituyendo la fracción de poliaminas solubles conjugadas, y se mantuvieron a -80 ºC hasta el momento de su derivatización.

Para una correcta cuantificación de las PAs se llevó a cabo la adición de un estándar interno previamente a la reacción de derivatización. El estándar interno es una sustancia de síntesis química no presente de forma natural en las muestras y del que se añade una cantidad conocida, lo que nos permitió corregir las pérdidas de PAs derivadas de la eficiencia del proceso de derivatización.

La cuantificación de las PAs está basada en la determinación del factor de respuesta relativo (F). Este parámetro relaciona el comportamiento de la sustancia problema (X) con el estándar interno (EI). Analizando soluciones compuestas de muestras estándar y estándar interno se obtienen los tiempos de retención de cada una de poliaminas que formarán parte de la muestra problema y el factor de respuesta (Tabla 13).

El factor de respuesta puede ser determinado mediante la siguiente ecuación:

$$F = A_X\,[EI] / A_{EI}\,[X]$$, donde A es el área de cada pico

Al añadir el estándar interno a cada una de las muestras problema, el factor de respuesta nos permite conocer la concentración de cada una de las PAs que forman parte de la muestra, utilizando la siguiente ecuación:

$$[X] = A_X\,[EI] / F\,A_{EI}$$

	Put	Spd	Spm	HTD
T_R	7,197	8,469	9,781	7,781
F	0,659	1,237	1,444	---

Tabla 13 Tiempo de retención y factor de respuesta relativo de las poliaminas (Put, Spd, Spm) respecto al estándar interno HTD .

Para su detección por HPLC las poliaminas deben ser sometidas a un proceso de derivatización que incorpore a su molécula una sustancia química capaz de ser detectada mediante fluorescencia. El cloruro de dancilo es uno de los reactivos que mejores resultados ofrece y más ampliamente empleados en la derivatización y posterior análisis de poliaminas en vegetales marinos (Marián et al. 2000 a,b; Sacramento *et al.* 2004; Sacramento *et al.* 2007; Zarranz *et al.* 2012) y se basa en la metodología descrita inicialmente por Mercé *et al.* (1995).

A cada muestra, 260 µl de extracto, se le añadieron 40 µl de una solución 0,05 mM del estándar interno 1,7 diaminoheptano (HTD) (Sigma-Aldrich) a una concentración final de 6,66 µM. Se añadieron posteriormente 200 µl de una solución saturada de Na_2CO_3 y 400 µl de una solución de cloruro de dancilo en acetona (5 mg/ml de acetona grado HPLC) y se incubarón a temperatura ambiente durante 12 h en

oscuridad. Para eliminar el exceso de cloruro de dancilo se añadieron 100 µl de una solución acuosa de prolina (100 mg/ml de H_2O grado HPLC) y se incubaron durante 30 minutos en oscuridad.

Cuando la reacción se completó, para extraer las PAs derivatizadas se añadieron 500 µl de tolueno grado HPLC (Panreac), se sometieron las muestras a vortex durante unos segundos y se centrifugaron a 9000 g durante 7 min. Posteriormente se recogieron 400 µl del sobrenadante que contiene las PAs derivatizadas. Las muestras se evaporaron durante 12 horas en una campana de vacío a temperatura ambiente, en tubos eppendorf de 1,5 ml cubiertos con lana de vidrio. El sedimento se resuspendió en 800 µl de acetonitrilo grado HPLC y se filtró a través de membranas de 0,45 µm (Millex-Millipore).

La separación de las PAs se realió en un columna SphereClone C-18 5 µm ODS (250 x 4,6 mm) en condiciones de gradiente de fase móvil acetonitrilo-agua (Tabla 14), con un flujo constante de 1 ml/min.

Tiempo (min)	Agua:Acetonitrilo
0	30:70
3	30:70
4	0:100
11	0:100
12	30:70
20	30:70

Tabla 14 Programa de separación de PAs en el HPLC, utilizando elución en gradiente.

El análisis cromatográfico se realizó con un detector de fluorescencia (Varian Prostar) a una longitud de onda de excitación de 365 nm y emisión de 510 nm.

La figura 10 muestra un cromatograma de estándares de poliaminas y estándar interno.

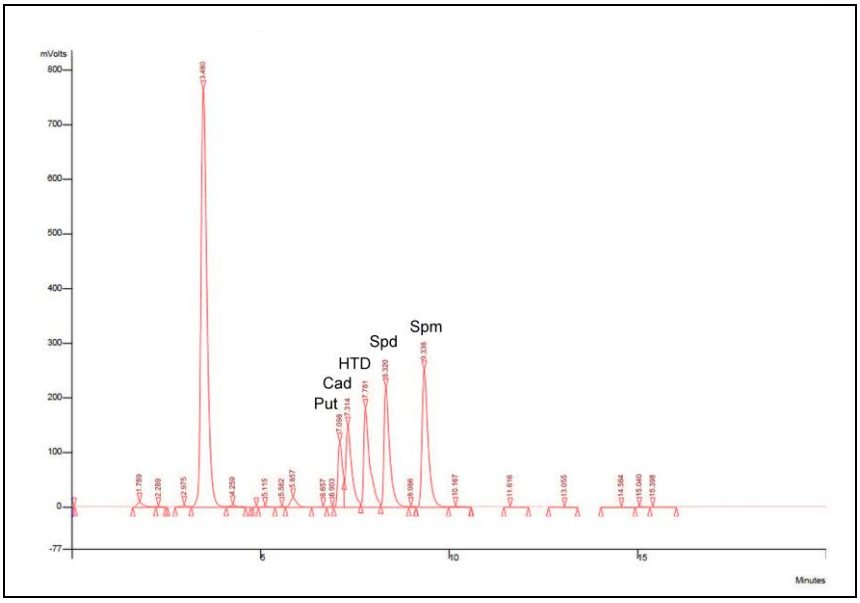


Fig 10 Cromatograma obtenido para los diferentes patrones de PAs. Put = putrescina, HTD = diamino heptano, Spd= espermina, Spm = espermidina

Determinación de peróxido de hidrógeno (H_2O_2) en agua de mar

La concentración de H_2O_2 presente en las muestras de agua de mar analizadas se determinó mediante la utilización de un sistema de flujo continuo (FeLume, Waterville Analytical, USA) desarrollado por King *et al.*(2007).

REFERENCIAS

Ahmadjian V, Paracer S (1986) Symbiosis. An introduction to biological associations. University Press of New England, Hanover.

Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Tiburcio AF (2010) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta, 231: 1237-1249.

Alcázar R, Marco F, Cuevas JC, Patron M, Ferrando A, Carrasco P, Altabella T (2006) Involvement of polyamines in plant response to abiotic stress. Biotechnology letters, 28: 1867-1876.

Altman A, Kaur-Sawhney R, Galston AW (1977) Stabilization of oat leaf protoplasts through polyamine-mediated inhibition of senescence. Plant physiology, 60: 570-574.

Andrews JH (1979) Pathology of seaweeds: current status and future prospects. Cellular and Molecular Life Sciences, 35: 429-429.

Andrews JH (1977) Observations on the pathology of seaweeds in the Pacific Northwest. Canadian journal of botany, 55: 1019-1027.

Andrews JH (1976) The pathology of marine algae. Biological review, 51: 211-253.

Angelini R, Bragaloni M, Federico R, Infantino A, Porta-Puglia A (1993) Involvement of polyamines, diamine oxidase and peroxidase in resistance of ckick-pea to *Ascochyta rabiei*. Journal of Plant Physiology, 142: 704-709.

Apt KE (1988a) Morphology and development of hyperplasia on *Cystoseira osmundacea* (Phaeophyta) associated with *Haloguignardia irritans* (Ascomycotina). American journal of botany, 979-984

Apt KE (1988b) Etiology and development of hyperplasia induced by *Streblonema sp*. (Phaeophyta) on members of the L*aminariales* (Phaeophyta). Journal of phycology, 24: 28-34

Apt KE (1988c) Galls and tumor-like growths on marine macroalgae. Diseases Aquatic Organisms, 4: 211-217

Apt KE, Gibor A (1989) Development and induction of bacteria-associated galls on *Prionitis lanceolata* (Rhodophyta). Diseases of aquatic organisms, 6: 151-156.

Bagni N, Torrigiani P (1992) Polyamines: A new class of growth substances. Progress in Plant Growth Regulation.

C.M.Karssen, L.C. van Loon and D. Vreugdenhill (eds.) Kluwer Academic Publishers: 264-275.

Bagni N, Adamo P, Serafini-Fracassini D, Villanueva VR (1981) RNA, proteins and polyamines during tube growth in germinating apple pollen. Plant physiology, 68: 727-730.

Baker CJ, Orlandi EW (1995) Active oxygen in plant pathogenesis. Annual Review of Phytopathology, 33:299-321.

Ballas SK, Mohandas N, Marton LJ, Shohet SB (1983) Stabilization of erythrocyte membranes by polyamines. Proceedings of the National Academy of Sciences of the United States of America, 80: 1942–1946.

Berlin J, Forche E (1981) DL-α-Difluoromethyl ornithine causes enlargement of cultured tobacco cells. Zeitschrift für Pflanzenphysiologie, 101: 277-282.

Biasi R, Bagni N, Costa G (1988) Endogenous polyamines in apple and their relationship to fruit set and fruit growth. Physiologia Plantarum, 73: 201-205.

Blechert S, Brodschelm W, Holder S, Kammerer L, Kutchan T, Mueller M (1995) The ocadecanoid pathway: signal molecules for the regulation of secondary pathways.

Proceedings of the National Academy of Science of the USA, 92: 4099-4105.

Boller T (1995) Chemoperception of microbial signals in plant cells. Annual review of plant biology, 46: 189-214.

Bouarab (2000) Bases biochimiques et cellulaires des interactions plante-pathogène dans le système *Chondrus crispus Acrochaete operculata*. These de doctorat. L'Universite Paris. 107 pp.

Bouarab K, Adas F, Gaquerel E, Kloareg B, Salaun JP, Potin P (2004) The innate immunity of a marine red alga involves oxylipins from both the eicosanoid and octadecanoid pathways. Plant Physiology, 135: 1838-1848.

Bouarab K, Kloareg B, Potin P, Correa JA (2001) Ecological and biochemical aspects in algal infectious diseases. Cahier de Biologie Marine, 42: 91-100.

Bouarab K, Potin P, Correa JA, Kloareg B (1999) Sulfated oligosaccharides mediate the interaction between a marine red alga and its green algal pathogenic endophyte. The Plant Cell, 11: 1635-1650.

Cabanne F, Dalebroux MA, Martin-Tanguy J, Martin C (1981) Hydroxycinnamic acid amides and ripening to flower of

Nicotiana tabacum var. xanthi nc. Physiologia Plantarum, 53: 399-404.

Chandra S, Heinstein PF, Low PS (1996) Activation of phospholipase A by plant defense elicitors. Plant Physiology, 110: 979-986.

Cohen E, Shoshana A, Heimer YH, Mizrahi Y (198) Polyamine biosynthetic enzymes in the cell cycle of Chlorella. Plant Physiology 74:385-88.

Cohen SS (1998) A guide to the polyamines. Oxford: Oxford University Press.

Collén J, Hervé C, Guisle-Marsollier I, Léger JJ, Boyen C (2006) Expression profiling of *Chondrus crispus* (Rhodophyta) after exposure to methyl jasmonate. Journal of Experimental Botany, 57: 3869-3881.

Correa JA (1990) Pigmented algal endophytes of *Chondrus crispus* Stackhouse: host-specificity, fine structure and effects on host performance in infections by *Acrochaete operculata* Correa & Nielsen and *A. heteroclada* Correa & Nielsen, Canada: Dalhousie University. PhD dissertation.

Correa JA (1994) Infections by pigmented algal endophytes: misuse of concepts and terminology. Revista Chilena de Historia Natural, 67: 4-8.

Correa JA, Flores V, Garrido J (1994) Green patch disease in *Iridaea laminarioides* (Rhodophyta), caused by *Endophyton* sp. (Chlorophyta). Diseases of Aquatic Organisms, 19; 203-213.

Correa JA, McLachlan JL (1994) Endophytic algae of *Chondrus crispus* (Rhodophyta). V. Fine structure of the infection by *Acrochaete operculata* (Chlorophyta). European Journal of Phycology, 29: 33-47

Correa JA, Flores V, Sánchez P (1993) Deformative disease in *Iridaea laminarioides* (Rhodophyta): Gall development associated with an endophytic cyanobacterium. Journal of Phycology, 29: 853-860.

Correa JA, McLachlan JL (1992) Endophytic algae of *Chondrus crispus* (Rhodophyta). IV. Effects on the host following infections by *Acrochaete operculata* and *A. heteroclada* (Chlorophyta). Marine Ecology Progress Series, 81: 73-87.

Correa JA, McLachlan JL (1991) Endophytic algae of *Chondrus crispus* (Rhodophyta). III. Host specificity. Journal of Phycology, 27: 448-459.

Correa JA, Nielsen R, Grund DW (1988) Endophytic algae of *Chondrus crispus* (Rhodohyta). II. A*crochaete heteroclada* sp. nov., *A. operculata* sp. nov., and *Phaeophila dendroides* (Chlorophyta). Journal of phycology, 24: 528-539

Correa JA, Nielsen R, Grund DW, McLachlan J (1987) Endophytic algae of Irish moss (*Chondrus crispus* Stackh.). In Twelfth International Seaweed Symposium. Springer Netherlands: 223-228.

Craig JS, Correa JA (1996) Etiology of infectious diseases in cultivated *Chondrus crispus* (Gigartiales, Rhodophyta). Hidrobiologia, 326/327: 97-104.

D'Antonio C (1985) Epiphytes on the rocky intertidal red alga *RhodomelaLarix* (Turner) C. Agardh: Negative effects on the host and food for herbivores?.Journal of Experimental Marine Biology and Ecology, 86: 197-218.

Dodds JA (1979) Viruses of marine algae. Experientia, 35: 440-442.

Dou C, Guangheng L, Shize S (1981) Studies on alginic acid decomposing bacteria II. Rot disease of *Laminaria* summer

sporelings caused by alginic acid decomposing bacteria. Oceanologia Et Limnologia Sinica, 2, 005.

Escribano MI, Legaz ME (1988) High performance liquid chromatography of the dansyl derivatives of putrescine, spermidine, and spermine. Plant physiology, 87: 519-522.

Evans PT, Malmberg RL (1989) Do polyamines have roles in plant development?. Annual review of plant biology, 40: 235-269.

Farmer EE (1994) Fatty acids signalling in plants and their associated microorganisms. Plant Molecular Biology, 26: 1423-1437.

Farmer EE, Weber H, Vollenweider S (1998) Fatty acid signaling in *Arabidopsis.* Planta 206: 167-174.

Flores HE (1990) Polyamines and plant stress. En: Flores HE (Ed) Stress Responses in Plants: Adaptation and acclimatatation mechanism. Whiley-Liss Inc. New York: 217-239.

Flores HE, Galston AW (1982) Analysis of polyamines in higher plants by high performance liquid chromatography. Plant physiology, 69: 701-706.

Gaquerel E, Weinhold A, Baldwin IT (2009) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphigidae) and its natural host *Nicotiana attenuata*. VIII. An

unbiased GCxGC-ToFMS analysis of the plant's elicited volatile emissions. Plant Physiology, 149: 1408-1423.

Gaquerel E, Hervé C, Labrière C, Boyen C, Potin P, Salaün J-P (2007) Evidence for oxilipin synthesis and induction of a new polyunsaturated fatty acid hydroxylase activity in *Chondrus crispus* in response to methyl jasmonate. Biochimica et Biophysica Acta. 1771: 565-575.

Geny L, Broquedis M, Martin-Tanguy J, Bouard J (1997) Free, conjugated, and wall-bound polyamines in various organs of fruiting cuttings of Vitis vinifera L. cv. Cabernet Sauvignon. American journal of enology and viticulture, 48: 80-84.

Gibbs A, Skotnicki AH, Gardiner JE, Walker ES, Hollings M (1975) A tobamovirus of a green alga. Virology, 64: 571-574.

Gill SS, Tuteja N (2010) Polyamines and abiotic stress tolerance in plants. Plant signaling & behavior, 5: 26-33.

Goff LJ (1991) Symbiosis, interspecific gene transfer, and the evolution of new species: A case study in the parasitic red algae. Symbiosis as a source of evolutionary innovation, speciation and morphogenesis. Eds. L. Margulis and R. Fester, London: The MIT press, 341-363.

Goff LJ (1983) Marine algal interactions: epibiosis endobiosis, parasitism and disease. In Proceedings of the Joint China-U.S. Phycology Symposium (Tseng, CK., ed.). Science Press, Beijing. 221-274

Goff LJ (1982) The biology of parasitic red algae. In: Progress in phycological research, Volume 1. Round, F.E. and Chapman D.J., eds. Elsevier, NY: 289-369.

Goff LJ, Moon DA, Nyvall P, Stache B, Mangin K, Zuccarello G (1996) The evolution of parasitism in the red algae: molecular comparisons of adelphoparasites and their hosts. Journal of Phycology, 32: 297-312.

Groppa MD, Benavides MP (2008) Polyamines and abiotic stress: recent advances. Amino acids, 34(1), 35-45.

Guzmán-Urióstegui A, García-Jiménez P, Marián F, Robledo D, Robaina RR (2002) Polyamines influence maturation in reproductive structures of *Gracilaria cornea* Gracilariales, Rhodophyta). Journal of Phycology, 38: 1169–1175.

Hall R (1974) Pathogenism and parasitism as concepts of symbiotic relationships. Phytopathology, 64: 576-577.

Hancock JT, Desikan R, Neill SJ (2001) Role of reactive oxygen species in cell signalling pathways. Biochemical Society Transactions, 29: 345-350.

Heath MC (2000) Hypersensitive response-related death. In Programmed Cell Death in Higher Plants. Springer Netherlands. pp. 77-90

Heimer YM, Mizrahi Y (1982) Characterization of ornithine decarboxylase of tobacco cells and tomato ovaries. Biochemical Journal, 201: 373-376.

Heimer YM, Mizrahi Y, Bachrach U (1979) Ornithine decarboxylase activity in rapidly proliferating plant cells. FEBS letters, 104: 146-148.

Hervé C, Tonon T, Collèn J, Corre E, Boyen C (2006) NADPH oxidases in Eukaryotes: red algae provide new hints! Current Genetics, 49: 190-204.

Hosoya R, Hamana K, Isobe M, Yokota A (2005) Polyamine distribution profiles within cyanobacteria. Microbiology and Culture Collections, 21: 3-8.

Hussain SS, Ali M, Ahmad M, Siddique KH (2011) Polyamines: natural and engineered abiotic and biotic stress tolerance in plants.Biotechnology advances, 29: 300-311.

Jarvis BC, Yasmin S, Coleman MT (1985) RNA and protein metabolism during adventitious root formation in stem cuttings of Phaseolus aureus. Physiologia Plantarum, 64: 53-59.

Kaur-Sawhney R, Applewhite PB (1993) Endogenous protein-bound polyamines: correlation with regions of cell division in tobacco leaves, internodes and ovaries. Plant Growth Regulation, 12: 223-227.

Kaur-Sawhney R, Galston AW (1979) Interaction of polyamines and light on biochemical processes involved in leaf senescence. Plant, Cell & Environment, 2(2), 189-196.

Kaur-Sawhney R, Shih LM, Cegielska T, Galston AW (1982a) Inhibition of protease activity by polyamines: Relevance for control of leaf senescence. FEBS letters, 145: 345-349.

Kaur-Sawhney R, Shih LM, Flores HE, Galston AW (1982b) Relation of polyamine synthesis and titer to aging and senescence in oat leaves. Plant physiology, 69: 405-410.

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science, 291: 2141-2144.

Kohlmeyer J, Demoulin V (1981) Parasitic and symbitic fungi on marine algae. Botanica Marina, 24: 9-18.

Kohlmeyer J, Kohlmeyer E (1979) Marine mycology. Academic Press. New York. 690 pp.

Kubanek J, Jensen PR, Keifer PA, Sullards MC, Collins DO, Fenical W (2003) Seaweed resistance to microbial attack: a targeted chemical defense against marine fungi. Proceedings of the National Academy of Sciences of the USA, 100: 6916-6921.

Küpper FC, Gaquerel E, Cosse A, Adas F, Peters AF, Müller DG, Kloareg B, Salaün J-P, Potin P (2009) Free fatty acids and methyl jasmonate trigger defense reactions in *Laminaria digitata*. Plant & Cell Physiology, 50: 789-800.

Küpper FC, Gaquerel E, Boneberg EM, Morath S, Salaün JP, Potin P (2006) Early events in the perception of lipopolysaccharides in the brown alga *Laminaria digitata* include an oxidative burst and activation of fatty acid oxidation cascades. Journal of experimental botany, 57: 1991-1999.

Küpper FC, Müller DG, Peters AF, Kloareg B, Potin P (2002) Oligoalginate recognition and oxidative burst play a key role

in natural and induced resistance of sporophytes of Laminariales. Journal of chemical ecology, 28: 2057-2081.

Küpper FC, Kloareg B, Guern J, Potin P (2001) Oligoguluronates elicit an oxidative burst in the brown algal kelp *Laminaria digitata*. Plant physiology, 125: 278-291.

Küpper FC, Müller DG (1999) Massive occurrence of the heterokont and fungal parasites *Anisolpidium*, *Eurychasma* and *Chytridium* in *Pylaiella littoralis*(Ectocarpales, Phaeophyceae). Nova Hedwigia, 69: 381-389.

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annual Review of Plant Physiology and Plant Molecular Biology, 48: 251-275.

Lambeth (2004) Nox enzymes and the biology of reactive oxygen. Nature Reviews Immunolgy, 4: 181-189.

Lee T-S, Chen M-H (1998) Hyposaline effect on polyamine accumulation in *Ulva fasciata* (Ulvales, Chlorophyta). Botanical Bulletin of Academia Sinica 39: 167-174.

Lee TM, Chu C (1992) Ethylene-induced polyamine accumulation in rice (Oryza sativa L.) coleoptiles. Plant physiology, 100: 238-245.

Levine A, Tenhaken R, Dixon RA, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell, 79: 583-593.

Marián FD, Garcia-Jimenez P, Robaina RR (2000a) Polyamine levels in the seagrass Cymodocea nodosa. Aquatic Botany, 68: 179–184.

Marián FD, García-Jiménez P, Robaina RR (2000b) Polyamines in marine macroalgae: Levels of putrescine, spermidine and spermine in the thalli and changes in their concentration during glycerol-induced cell growth in vitro. Physiologia Plantarum, 110: 530-534.

Mellersh DG, Foulds IV, Higgins VJ, Heath MC (2002) H_2O_2 plays different roles in determining penetration failure in three diverse plant-fungal interactions. The plant Journal, 29: 257-268.

Montague MJ, Koppenbrink JW, Jaworski EG (1978) Polyamine metabolism in embryogenic cells of Daucus carota I. Changes in intracellular content and rates of synthesis. Plant Physiology, 62: 430-433.

Müller DG (1992) Intergeneric transmission of a marine plant DNA virus. Naturwissenschaften, 79: 37-39.

Müller DG (1991) Mendelian segregation of a virus genome during host meiosis in the marine brown alga *Ectocarpus siliculosus*. Journal of plant physiology, 137: 739-743.

Müller DG, Parodi E (1993) Transfer of a marine DNA virus from *Ectocarpus* to *Feldmannia* (Ectocarpales, Phaeophyceae): aberrant symptoms and restitution of host. Protoplasma, 175: 121-125.

Müller DG, Stache B (1992) Worldwide occurrence of virus infections in filamentous marine brown algae. Helgoländer Wissenchaftliche Meeresuntersuchungen, 46: 1-8.

Müller DG, Kawai H, Stache B, Lanka S (1990) A virus infection in the marine brown alga *Ectocarpus siliculosus* (Phaeophyceae). Botanica Acta, 103: 72-82.

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8: 4321-4325.

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. Journal of Experimental Botany, 53: 1237-1247.

Nürnberger T, Nennstiel D, Jabs T, Sacks WR, Hahlbrock K, Scheel D (1994) High affinity binding of a fungal

oligopeptide elicitor to parsley plasma membranes triggers multiple defence responses. Cell 78: 449–460.

O'Donell BV, Tew DG, Jones OT, England PJ (1993) Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. Biochemical Journal, 290: 41-49.

Oliveira L, Bisalputra T (1978) A virus infection in the brown alga *Sorocarpus uvaeformis* (Lyngbye) Pringsheim (Phaeophyta, Ectocarpales). Annals of Botany, New Series, 43: 439-445.

Orozco-Cardenes M, Ryan CA (1999) Hydrogen peroxide is generated systematically in plant leaves by wounding and systemin via the octadecanoid pathway. Proceedings of the National Academy of Sciences of USA, 96: 6553-6557.

Potin P, Bouarab K, Salaün JP, Pohnert G,Kloareg B (2002) Biotic interactions of marine algae. Current opinion in plant biology, 5: 308-317.

Provasoli L (1968) Media and prospects for the cultivation of marine algae. In: Watanabe A, Hattori A, editors. Cultures and collections of algae. Japanaese Society of Plant Physiology. Hakone. 63-75

Pueschel CM, Meer JVD (1985) Ultrastructure of the fungus *Petersenia palmariae* (Oomycetes) parasitic on the alga *Palmaria mollis* (Rhodophyceae). Canadian journal of botany, 63: 409-418

Reymond R, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. Current Opinion in Plant Biology, 1: 404-411.

Robaina RR (1988) Biotecnología del cultivo in vitro de algas rojas (Rhodophyta) de interes industrial. Tesis doctoral. Universidad de Las Palmas de Gran Canaria: 237 pp.

Robaina RR, García P, García-Reina G , Luque A (1990a) Morphogenetic effect of glycerol on tissue of the red seaweed *Grateloupia doryphora*. Journal of Applied Phycology 2: 137-143.

Robaina RR, García-Reina G, Luque A (1990b) The effects of the physical characteristics of the culture medium on the development of red seaweeds in tissue culture. Hydrobiologia 204/205: 137-142.

Roberts DR, Dumbroff EF, Thompson JE (1986) Exogenous polyamines after membrane fluidity in bean leaves. A basis

for potential misinterpretation of their true physiological role. Planta, 167: 395-401.

Ronquist F (1994) Evolution of parasitism among closely related species: phylogenetic relationships and the origin of inquilinism in gall wasps (Hymenoptera, Cynipidae). Evolution, 241-266.

Sacramento AT, García-Jiménez, P, Robaina RR (2007) The polyamine spermine induces cystocarp development in the seaweed *Grateloupia* (Rhodophyta). Plant Growth Regulation, 53:147–154.

Sacramento AT, García-Jiménez P, Alcázar R, Tiburcio AF, Robaina RR (2004) Influence of polyamines on the sporulation of G*rateloupia* (halymeniaceae, rhodophyta). Journal of Phycology, 40: 887–894.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. New York: Cold spring harbor laboratory press.

Sawabe T, Tanaka R, Iqbal MM, Tajima K, Ezura Y, Ivanova EP, Christen R (2000) Assignment of *Alteromonas elyakovii* KMM 162(T) and five strains isolated from spot-wounded fronds of *Laminaria japonica* to *Pseudoalteromonas elyakovii*

comb. nov and the extended description of the species. International Journal of Systematic and Evolutionary Microbiology, 50: 265-271.

Sawabe T, Makino H, Tatsumi M, Nakano K, Tajima K, Iqbal MM, Yumoto I, Ezura Y, Christen R (1998) *Pseudoalteromonas bacteriolytica* sp. nov., a marine bacterium that is the causative agent of red spot disease of *Laminaria japonica*. International Journal of Systematic Bacteriology, 48: 769-774.

Scholtens-Toma IM, DeWit PJGM (1988) Purification and primary structure of a necrosis-inducing peptide from the apoplastic fluids of tomato infected with *Cladosporium fulvum* (syn. *Fulvia fulva*). Physiological and Molecular Plant Pathology . 33: 59–67.

Schubert F (1989) Influence of polyamines on membrane functions. Biochemical Journal, 260: 1-10.

Schubert F, Hong K, Düzgüenes N, Papahadjopoulos D (1983) Polyamines as modulators of membrane fusion: aggregation and fusion liposomes. Biochemistry, 22: 6134-6140.

Sebela M, Radova A, Angelini R, Tavladoraki P, Frébort I, Peè P (2001) FAD-containing polyamine oxidase: a timely challange

for researchers in biochemistry and physiology of plants. Plant Science, 160: 197-207.

Setchell WA (1918) Parasitism among the red algae. Proceedings of the American Philosophical Society, 155-172.

Slocum RD, Kaur-Sawhney R, Galston AW (1984) The physiology and biochemistry of polyamines in plants. Archives of Biochemistry and Biophysics, 235: 283-303.

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology, 98: 503-517

Stakman EC (1915) Relation between Puccinia graminis and plants highly resistant to its attack. Journal of Agricultural Research, 4: 193-199.

Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG, Jones JD (1995) Molecular genetics of plant disease resistance. SCIENCE NEW YORK THEN WASHINGTON, 661-661.

Stekoll M, West CA (1978) Purification and properties of an elicitorof castor bean phytoalexin from culture filtrates of the fungus *Rhizopus stolonifer*. Plant Physiology. 61: 38–45.

Sturch HH (1926) *Choreocolax polysiphoniae*, Reinsch. Annals of Botany, 585-605.

Tabor CW, Tabor H (1984) Polyamines. Annual review of biochemistry, 53: 749-790.

Tiburcio AF, Altabella T, Borrell A, Masgrau C (1997) Polyamine metabolism and its regulation. Physiologia Plantarum, 100: 664-674.

Torres MA, Dangl JL (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Current Opinion in Plant Biology, 8: 397-403.

Torrigiani P, Serafini-Fracassini D, Biondi S, Bagni N (1986) Evidence for the subcellular localization of polyamines and their biosynthetic enzymes in plant cells. Journal of PlantPhysiology, 124: 23-29.

Wahl M (1989) Marine epibiosis. I. Fouling and antifouling: some basic aspects. Marine Ecology Progress Series, 58: 175-189.

Wallace HM, Fraser A, Hughes A (2003) A perspective of polyamine metabolism. Biochernisty Journal, 376: 1 - 14.

Wei Z-M, Laby R, Zumoff CH, Bauer DW, He SY, Collmer ZM, Beer SV (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257: 85–88.

Weinberger F, Leonardi P, Miravalles A, Correa JA, Lion U, Kloareg B and Potin P (2005a) Dissection of two distinct defense-related responses to agar oligosaccharides in *Gracilaria chilensis* (Rhodophyta) and *Gracilaria conferta* (Rhodophyta). Journal of Phycology, 41, 863-873.

Weinberger F, Pohnert G, Berndt ML, Bouarab K, Kloareg B, Potin P (2005b) Apoplastic oxidation of L-asparagine is involved in the control of the green algal endophyte *Acrochaete operculata* Correa & Nielsen by the red seaweed *Chondrus crispus* Stackhouse. Journal of Experimental Botany, 56: 1317-1326.

Weinberger F, Pohnert G, Kloareg B, Potin P (2002) A signal released by an endophytic attacker acts as a substrate for a rapid defensive reaction of the red alga *Chondrus crispus*. European Journal of Chemical Biology, Synthetic Biology, and Bio-Nanotechnology, 3: 1260-1263.

Weinberger F, Richard C, Kloareg B, Kashman Y, Hoppe H-G, Friedlander M (2001) Structure-activity relationship of oligoagar elicitors toxard *Gracilaria conferta* (Rhodophyta). Journal of Phycology, 37: 418-426.

Weinberger F, Friedlander M (2000a) Endogenous and exogenous elicitors of a hypersensitive response in *Gracilaria conferta* (Rhodophyta). Journal of applied phycology, 12: 139-145.

Weinberger F, Friedlander M (2000b) Response of *Gracilaria conferta* (Rhodophyta) to oligoagars results in defense against aga-degrading epiphytes. Journal of Phycology, 36: 1079-1086.

Weinberger F, Friedlander M, Hoppe HG (1999) Oligoagars elicit a physiological response in *Gracilaria conferta* (Rhodophyta). Journal of Phycology, 35(4), 747-755.

Weinberger F, Hoppe HG, Friedlander M (1997) Bacterial induction and inhibition of a fast mecrotic response in *Gracilaria conferta* (Rhodophyta).Journal of applied phycology, 9: 277-285.

Wimalasekera R, Tebartz F, Scherer GF (2011) Polyamines, polyamine oxidases and nitric oxide in development, abiotic and biotic stresses. Plant Science, 181: 593-603.

Wu CY, Chen D, Li J (1983) On the diseases of cultivated *Laminaria japonica*. In C.K. Tseng (ed.), Proceedings of the Joint China-U.S. Phycology Symposium. Science Press, Beijing, China: 211–220.

Yoda H, Fujimura K, Takahashi H, Munemura I, Uchimiya H, Sano H (2009) Polyamines as a common source of hydrogen peroxide in host-and nonhost hypersensitive response during pathogen infection. Plant molecular biology, 70: 103-112.

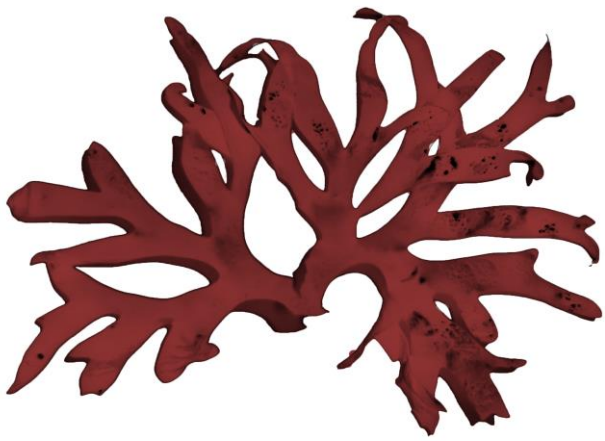
Yoda H, Hiroi Y, Sano H (2006) Polyamine oxidase is one of the key elements for oxidative burst to induce programmed cell death in tobacco cultured cells. Plant Physiology, 142:193–206.

Yoda H, Yamaguchi Y, Sano H (2003) Induction of hypersensitive cell death by hydrogen peroxide produced through polyamine degradation in tobacco plants. Plant Physiology, 132:1973–1981.

Zarranz-Elso M, García-Jiménez P, Robaina RR (2012) Endogenous polyamine content and photosynthetic performance under hypo-osmotic conditions reveal *Cymodocea nodosa* as an obligate halophyte. Aquatic Biology, 17: 7-17.

CAPÍTULO I

DNA-DNA interaction between the endophytic alga *Microspongium tenuissimum* and its host the red alga *Grateloupia imbricata*



Navarro-Ponce M.A., Afonso López J.M., R.R. Robaina (2015) DNA-DNA interaction between the endophytic alga *Microspongium tenuissimum* and its host the red alga *Grateloupia imbricata*. Journal of Phycology. (Submitted)

Abstract

Microspongium tenuissimum (Hauck) A.F. Peters (Chordariaceae, Phaeophyta) was isolated from the macroalgae *Grateloupia imbricata*. Ribosomal DNA internal transcribed spacer non-coding region (ITS1) combined with 5.8S rDNA were used to conduct the taxonomic assignment of the isolated material. Observations *in vitro* culture, in total 7000 explants, showed that the incidence of infection by these endophytic algae was near 90 % as confirmed by PCR. In most infected thalli, the infection was not evident neither macroscopic nor microscopically and we attribute the endophyte release to stress generated in culture. The present work reports the development to a specific DIG-labeled probe targeting rDNA (ITS1) to detect the endophyte in asymptomatic health tissues of *G. imbricata* through a specific signal generated by Southern blotting hybridization. This assay showed evidences of a close relationship existing between *M. tenuissimum* and its host *G. imbricata* according to the pattern of fragments generated in the hybridization against the digested genomic DNA of both suggesting the posibility of a molecular interaction occurs at the DNA level which would open up a field of possibilities for genetic engineering.

INTRODUCTION

The macroalgae are host to a range of endophytic organisms including different species of multicelular filamentous pigmented algae (Correa *et al.* 1987, 1988, 1994; Ellertsdóttir & Peters 1997; Plumb 1999; Peters 2003; Gauna & Parodi 2008; Gauna *et al*. 2009; Deng *et al.* 2011). Structural intimate association among the participating algae is quite common (Goff 1983), thus small filamentous green, brown, and red algae epiphytes and endophytes species have been reportedly found living deeply rooted within the tissues of larger algal hosts.

Endophytic algae are photosynthetically active pigmented algae, which are usually filamentous species of the Rhodophyta, Phaeophyta and Chlorophyta. They may retain the ability to grow separately from their hosts in standard culture medium (Nielsen 1979; Correa *et al* 1988; Correa & McLachlan *et al.* 1991, 1994). In *Chondrus crispus*, up to 95% of host plants are infected with algal endophytes (Correa *et al.* 1987; Plumb 1999) in the medulla or inner cortex, showing that they are capable of nutritional independence, and endophytic brown algal filaments were found in 85% of the examined thalli of *Laminaria* spp. 59% of which had no visible morphological changes (Ellertsdóttir & Peters 1997). Infection prevalence by

Endophyton ramosum in populations of the red algal host *Mazzaella laminarioides* has been recorded at up to 75% (Correa *et al*. 1997). Whilst such evidences exist of these strong structural interaction between host algae and its endophytes although the exact nature of this relationship host/endophyte is not well known. Modern taxonomy of filamentous endophytic algae should not only be based on morphological observations but must be supported with phylogenetic analysis using molecular markers due to the limited number of morphological characteristics (Nielsen *et. al.* 2013, 2014).

The occurrence of endophytic algae within *Grateloupia spp.* has been reported (Peter 2003; Kim *et al.* 2014). The filamentous algae known as *M. tenuissimum* (Peters 2003) has been seen to sprout from axenic cultures of the marine macroalgae *G. imbricata* (as *Grateloupia doryphora)* in experiments to establish *in vitro* cultures (Robaina *et al.* 1990a,b). Their incidence *in vitro* was assumed to be related to the stress caused to the explants in the initial stages of culture, but their appearance in the oldest cultures showing no signs of infection has remained a mystery.

Research has not been carried out at the molecular level to determine whether DNA-DNA level interactions take place. Although the capacity of parasitic red algae to establish pit connections between

cells of hosts and parasites allowing transfer organelles is known (Goff & Coleman 1984, 1985).

In this work, the main endophyte specie present *in vitro* cultures of *G. imbricata* (Rhodophyta) were isolated in culture and taxonomically assigned using molecular markers. We also used a molecular approach to evaluate the prevelence of endophytes in the populations using PCR as well as developing a specific DIG-labeled probe for specific detection of *M. tenuissimum* which allows follow the extent of DNA-DNA interaction between the endophyte and its host *G. imbricata* through Southern blotting.

METHODS

Culture of *G. imbricata* and isolation of endophytic algae

Grateloupia imbricata Holmes is an intertidal red algae that grows along the northeast coast of Gran Canaria, Canary Islands, Spain (García-Jiménez *et al.* 2008). Samples of fresh thalli of *G. imbricata* were collected from 2007 to 2012, transferred to the laboratory and cut into explant discs with a diameter of 3 mm, which were disinfected and tested for sterility using previously described methods to ensure that explants were aseptic (Robaina *et al.* 1990a,b). Sterile disc fragments (up to 7000) were then cultured in Provasoli enriched seawater medium, PES (Provasoli 1968) for at least three months until endophytes were observed growing from the thallus; samples were isolated to obtain clonal unialgal cultures. The culture conditions were 30 µmol photon m^2s^{-1} at 18 ± 2ºC on 16:8 h light:dark cycle, in Petri dishes containing 15 ml of medium. The endophytes were cultured under the same conditions described above but at a concentration of 5 µmol photon m^2s^{-1}. They were transferred to fresh medium on a weekly basis.

Taxonomic assignment using molecular markers

The morphological simplicity of endophytes makes their taxonomy particularly complex. Thus, molecular taxonomic

assignment and phylogeny should be inferred from molecular markers such as nuclear-encoded ribosomal DNA (nrDNA) together with the internal transcribed spacer (ITS) regions. Moreover, the molecular marker selected, ITS1, varies considerably among different genera of brow algae (Saunders & Druehl 1992; Peters 1998; Burkhardt & Peters 1998; Peters & Burkhardt 1998; Coyer *et al.* 2001; Peters & Ramírez 2001; Draisma *et al.* 2003).

DNA extraction was performed according to the standard Hexadecyltrimethylammonium bromide (CTAB) procedure (Murray & Thompson 1980) from 100 mg fresh weight of algal tissue powdered in liquid nitrogen. Then the DNA samples were cleaned using GenElute^{TM} PCR Clean-Up Kit (Sigma), according to the manufacturer's protocol. The DNA was quantified and qualified by UV-spectrophotometry at 260/280 nm using the Nanodrop 1000 spectrophotometer v3.7 (ThermoFisher Scientific) and simultaneously verified by electrophoresis in a 1.0% agarose gel.

PCR amplifications were performed in a GeneAmp 2400 thermal cycler (PerkinElmer Inc.). The reaction volume was 50 µl, and comprised 25 ng of genomic DNA, 0.4 µM of each primer, 0.5 µl of Taq polymerase (2.5 U/µl), 4 µl of dNTP Mixture (2.5 mM each) and 10 µl of 5X Buffer (PrimeSTAR® HS DNA Polymerase, Takara).

Primer pairs that were used to amplify the ITS1 were 5'-AAACTGCGCGAAAGAATCTC -3' (forward) and 5'-CAACAGACACTCCGACAAGC -3' (reverse). The temperature profile was an initial 3-min denaturation step at 94 ºC, followed by 33 cycles of denaturation, annealing, and extension: 95 ºC for 1 min, 55 ºC for 30 sec, 72 ºC for 1.5 min, and then a final 5-min extension period at 72 ºC (Peters 2003). As a result a fragment of 370 bp comprising 100% of ITS1 region and 115 bp of the 5.8S rDNA gene sequence was obtained from the amplified samples. The Wizard® SV Gel and PCR Clean-Up System (Promega) was used to purify PCR products directly from a PCR amplification. The sequence was deposited in Genebank under access number KJ134990.

Analyses of molecular data were performed using MEGA v5.03 (Tamura *et al.* 2011). Maximum parsimony (MP) and maximum likelihood (ML) analyses were used to estimate phylogenetic relationships. Related sequences were downloaded from GenBank according to the BLAST results shown in (Table I.1). Sequence alignments were performed using ClustalW (Thompson *et al.* 1994). The MP tree was obtained using the Min-mini heuristic algorithm with a search factor of 3. The bootstrap consensus tree was inferred from 1000 replicates. To construct an MP tree, only sites at which there

were at least two different kinds of nucleotides, each represented at least twice, were used (parsimony-informative sites). The maximum likelihood method was based on the Tamura-Nei model. The bootstrap consensus tree was inferred from 1000 replicates. The tree was drawn to scale, with branch lengths measured based in the number of substitutions per site.

Species name	GenBank Accession number
I115*Gi*	KJ134990
Microspongium tenuissimum	KJ439849
Chordaria flagelliformis	AB066052
Chordaria flagelliformis	AJ229129
Elachista mollis	AB050974
Elachista tenuis	AB052214
Laminarcolax tomentosoides	Z98566
Laminariocolax aecidioides	AJ439850
Ectocarpus siliculosus	FN564466

Table I.1 GenBank accession number of the ITS sequences used in present study for the taxonomic assignment of filamentous endophytic algae associated a *G. imbricata*

Prevalence of endophytes revealed by PCR

Conventional PCR was used to detect infection in tissue of *G. imbricata*. Genomic DNA was extracted from 50 thalli collected from three different natural populations in Gran Canaria (Spain) at different locations. The material was checked by stereomicroscopy and appeared to be free of endophytes and were disinfected following the protocol established by Robaina *et al.* (1990a,b). PCR was performed under the conditions described above. Three positive samples were selected and the product of amplification were sequenced and compared to the sequence of the endophyte using MEGA v5.03 following the above procedure.

Previous observations have shown that when complete thalli are cultivated, endophytes are not released in culture; the latter only seems to occur when the host is cultivated as an explant. To test whether apparently clean thalli still harbour endophytes although they do not sprout in the medium, 4 full intact *G. imbricata* thalli were maintained in culture for a set period of 6 months with apparent absence of infection. The presence/absence of the endophyte in the thalli was verified using conventional PCR as described above.

Molecular interaction revealed by Southern blot analysis

Southern blot analysis was performed to determine whether a DNA level interaction between the endophyte and its host occurs which could indicate presence of foreign DNA (from *M. tenuissimum*) in the genome of *G. imbricata*. To analyse whether the positive signals were generated by integration of the marker gene into the host genome, samples were subjected to Southern blot analysis with a DIG-labelled probe, made from our KJ134990 sequence of *M. tenuissimum*. The experiments were performed under the most restrictive conditions to avoid cross-hybridisation. Genemic DNA was digested with restriction enzymes that do not cut the target region analysis specifically. (Fig I.1). Two Samples of *G. imbricata* genomic DNA (4 µg) were digested with *Eco*RI, and *Pvu*II (Roche Applied Science, Germany), respectively, following the manufacturer's protocol. This was also performed using two samples of *M. tenuissimum* genomic DNA.

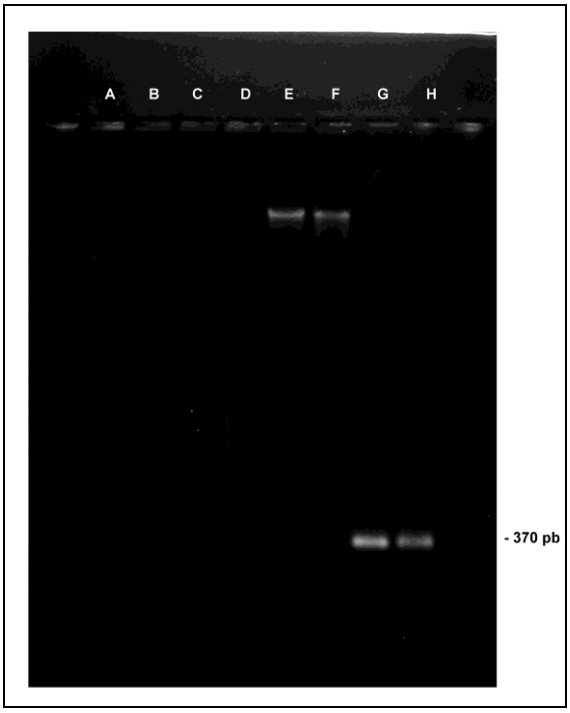


Fig I.1 Agarose gel electrophoresis of digested genomic DNA. (A, B) Genomic DNA from *M. tenuissimum* digested with the restriction enzymes *Eco*RI and *Pvu*II respectively. (C, D) Genomic DNA from *G. imbricata* digested with the restriction enzymes EcoRI and *Pvu*II respectively. (E) Undigested genomic DNA from *M. tenuissimum*. (F) Undigested genomic DNA from *G. imbricata*. (G, H) PCR of *M. tenuissimum* internal transcribed spacer region 1 (ITS1) isolated from *G. imbricata* after digestion with the restriction enzymes *Eco*RI and *Pvu*II respectively.

Samples were separated on a 1.2 % (w/v) agarose gel with 1X TAE buffer solution at 100 V. The gel was stained with ethidium bromide, photographed in a UV Transilluminator 2000 (Bio-Rad), and washed in sterile water for 15 min. The DNA was transferred to a Hybond-N^+ nylon membrane (Amershan Pharmacia Biotech)

overnight using 20X SSC as the transfer buffer. The blotted filter was cross-linked in an ultraviolet cross-linker LC-1000 (UVP, Upland, CA, USA) at 120 J/cm.

Digoxigenin-labelled DNA probes were prepared from the KJ134990 sequence of *M. tenuissimum* (see Fig I.2) and labelled using DIG DNA Labelling Mix (Roche Applied Science), according to the manufacturer's instructions.

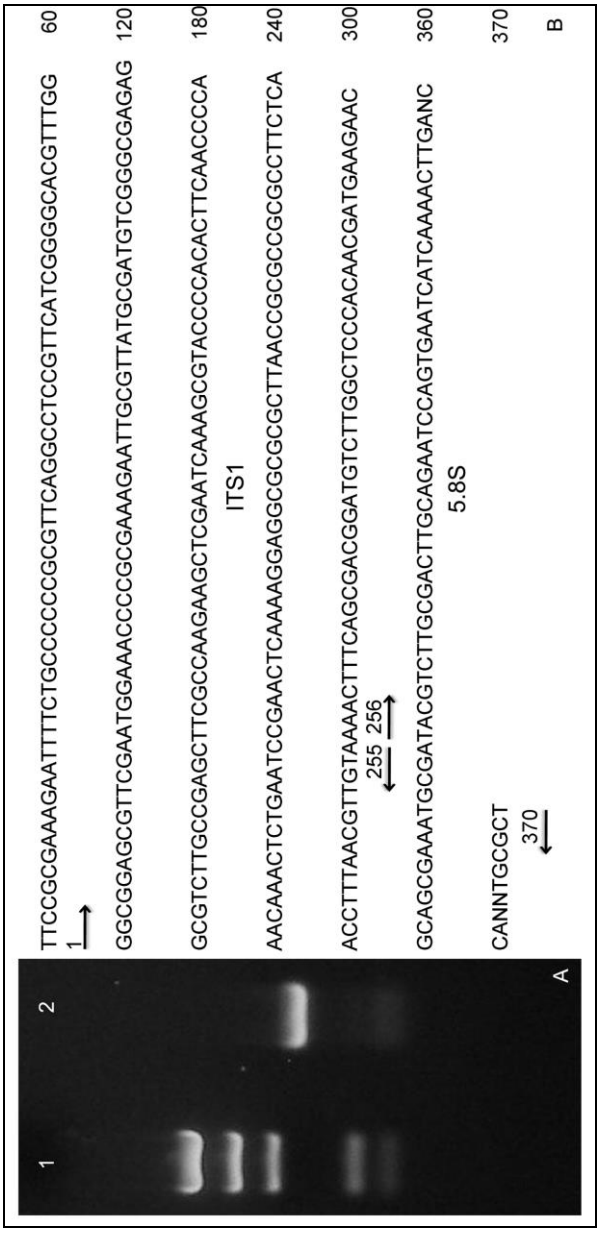


Fig I.2 (A) Agarose gel electrophoresis of 1. Molecular weight marker; 2. ITS1 region complete sequence and 5.8S rDNA partial sequence. (B) Nucleotides sequence of the *M. tenuissimum* ITS1-5.8S rDNA analyzed fragment.

From 890 ng of template, the amount of probe labelled with digoxigenin-dUTP synthesised after 20 h at 37 ºC was 3000 ng. The probe (370 bp) comprises 100 % of ITS1 (255 bp) and 115 bp of the 5.8S rDNA gene region. ITS1 region and 5.8S rDNA amplified from *M. tenuissimum* were introduced as positive control. Since it was also not possible to ensure the presence of endophyte-free material because all our samples tested positive by PCR, the experiments were devoid of negative controls. However we verified that the probe does not produce cross-hybridization with *Ulvella leptochaete* (epi-endophyte of *G. imbricata*) by Dot blot.

The membrane was pre-hybridised for 2 h and later hybridised in a hybridisation bag with buffer containing 50 % formamide, 6X SSC and blocking reagent containing 1% labelled probe at 42 ºC for 12 h. After hybridisation, the DNA blot was washed under extremely restricted conditions of washing twice at room temperature using 2X SSC with 0.1 % SDS for 5 min, followed by washing twice at 68 ºC with 0.1X SSC and 0.1 % SDS for 15 min. Signal generation of the DIG-labelled DNA probe was performed using the DIG Nucleic Acid Detection Kit (Roche Applied Science), following the manufacturer's instructions. Using Dot blot technique, a simple variant of Southern blot analysis, we verify the integrity, specificity (absence of cross-

hybridization) and sensitivity of the DIG-labeled probe.

RESULTS

Isolation of endophytes and culture observations

Within three or four weeks in culture, endophytes were growing in association with host species, and were visible to the naked eye. Light microscopic examination revealed that filaments of brown algae covered most of the surfaces and inner space of the tissue. Endophytic filaments were pigmented cells and were distinguishable from the interior of explants, particularly when they had deteriorated (bleaching) due to the infective load. Mainly brown morphotypes comprising uniseriate and filamentous species, resembling ectocarpale life forms algae sprouted from approximately 90 % of the cultivated samples (n=7000) after six months in culture. The endophytes isolated from *G. imbricata* grew well when isolated in PES medium (Fig I.3).

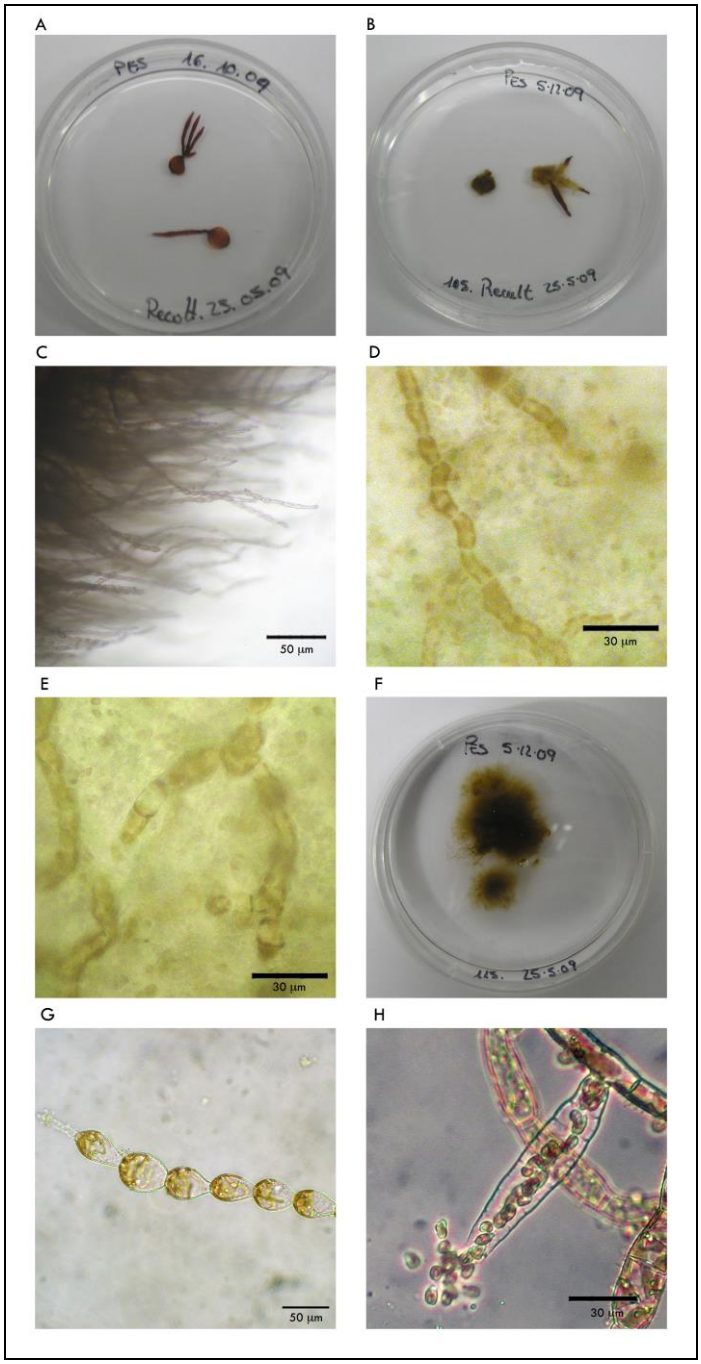

A
B
C
D
50 µm
30 µm
E
F
30 µm
G
H
50 µm
30 µm

Fig I.3 Overview of the *G. imbricata* culture in the PES medium and its endophyte *M. tenuissimum*. (A) Healthy *G. imbricata* explants (B) Infected *G. imbricata* explant. Filamentous endophytes growing on host surface (C) Detail filaments endophytes growing on the explant of *G. imbricata*. (D, E) Endophytes filaments growing inside *G. imbricata*. Bleached explants. (F) *M. tenuissimum* free-living culture isolated from *G. imbricata.* (G) Detail of *M. tenuissimum.* (H) Sporagium releasing mature spores

Taxonomic assignment using molecular markers

The partial 5.8S rDNA sequence and the ITS 1 region of the isolated material were deposited into GenBank with accesion number KJ134990. The amplified region included 255 pb from the rDNA ITS1 and 115 pb of the 5.8S rDNA.

A total of 9 sequences were analyzed, including our isolation and eight sequences obtained from GenBank. Our sequence KJ134990 grouped with a branch containing the sequence AJ439849 of *M. tenuissimum* in a tree based on ML/MP analysis (Fig I.4). Which was supported by 99 % (ML tree) and 100 % (MP tree) bootstrap values. The differences between our sequence and the previously published in GenBank was approximately 1%.

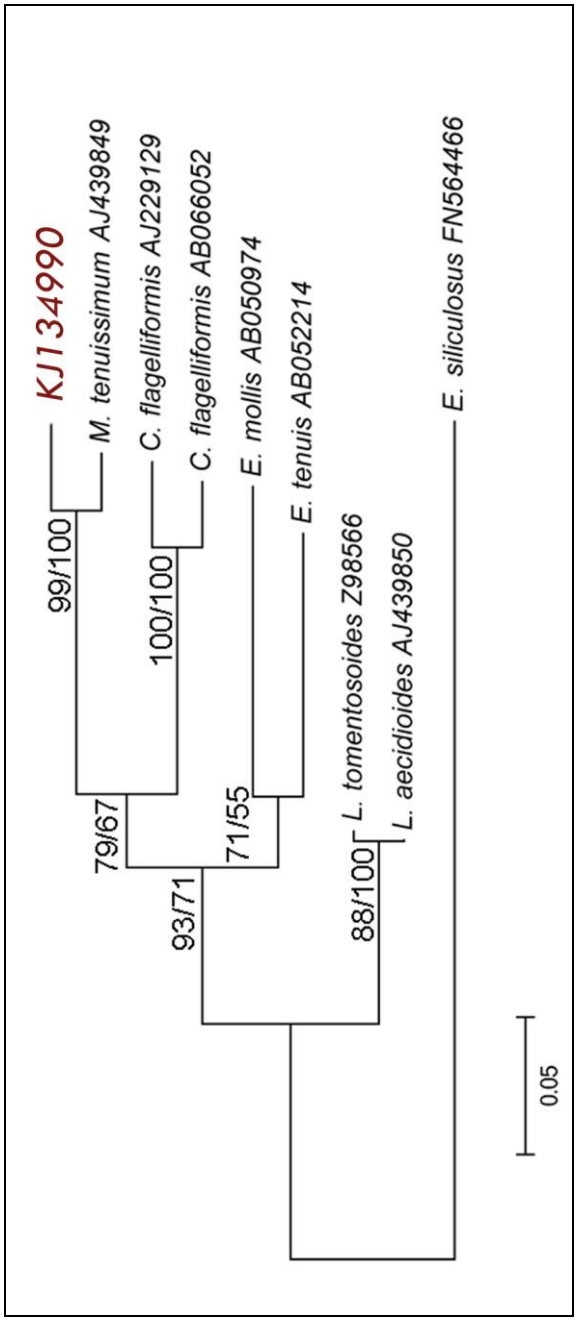


Fig I.4 Phylogenetic analysis. Strict consensus tree based on ITS1 and partial 5.8S rDNA sequence of *M. tenuissimum* using ML/MP methods

Presence or absence of endophyte in health tissue using PCR

PCR amplification of DNA extracted from health tissue of *G. imbricata* showed that endophytism was present in the 50 samples collected from the three populations of *G. imbricata* along the northeast coast of Gran Canaria (Fig I.5).

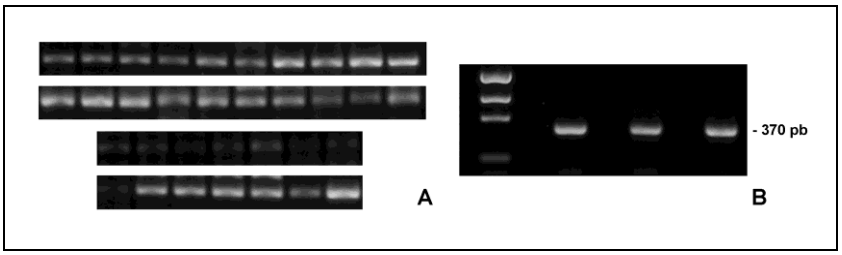


Fig I.5 (A) Ethidium bromide-stained agarose gel of PCR amplification products from target endophyte strains amplified from *G. imbricata* DNA. (B) Three purified PCR products randomly selected and used in the taxonomic assignment confirming the presence of *M. tenuissimum.* Result of a PCR screen using *G. imbricata* DNA but specific primers for IT1 region and 5.8S rDNA of *M. tenuissimum.*

PCR products were purified from three randomly selected samples and the phylogenetic analysis of the sequences confirmed the presence of infection by *M. tenuissimum*.

The intact thalli grown for 6 months without obvious signs of infection also tested positive by PCR for the presence of the fragment although they showed no evidence filaments growing inside.

The DNA concentration is important for the ability of the PCR to detect the endophyte. Also, the resolution of the electrophoresis is a

bottleneck in detecting a positive sign because when the amount of amplified product added to the mix is below 1 µg, only a faint band or even no band is observed, generating false negatives. In the following section we will solve this problem by Southern blotting analysis.

Southern blot analysis to determine DNA-DNA interactions

ITS probe development for specific detection of *M. tenuissimum* based on hybridization in Southern blotting (Fig I.6) was checked for integrity, specificity and sensitivity through dot blots.

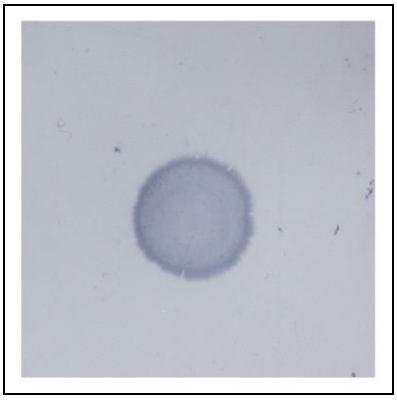


Fig I.6 Dot Blot analysis that revealed DIG-labeled probe membrane bound using NBT/BCIP solution for developing.

Dot blot hybridization intensity with DIG-labelled probe against the purified PRC product corresponding to ITS1-5.8S rDNA of *M. tenuissimum* is proportional to the concentration of fragment fixed to the membrane. The technique is able to detect concentrations

of 0.1 ng after a developing process of not more than 30 minutes (Fig I.7).

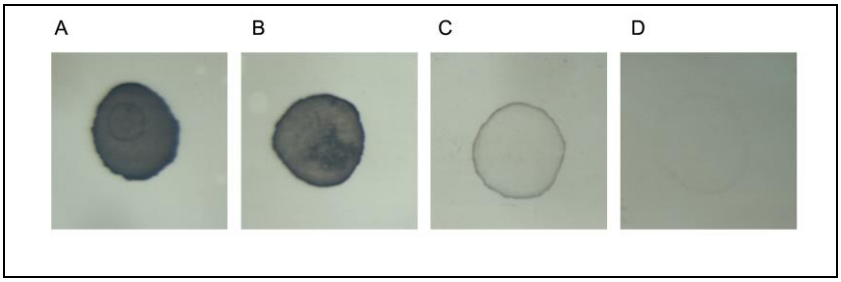


Fig I.7 Dot blot hybridization intensity classes with DIG-labeled probe against different concentrations of PCR products ITS1-5.8S fragment of *M. tenuissimum.* (A) 10 ng (B) 5 ng (C) 1ng (D) 0.1 ng

Dot blot analysis excludes cross-hybridization probe against region ITS1-5.8S rDNA of *G. imbricata.* The intensity of the signal generated by hybridization of the probe against 125 genomic DNA digest with restriction enzymes (*Eco*RI) is several times higher than the signal generated by the hibridization against 250 ng of DNA *G. imbricta.* The infection in the sample used was confirmed by PCR. These two factors confirmed that the probe hybridizes selectively to *M. tenuissimum* but not to *G. imbricata* (Fig I.8).

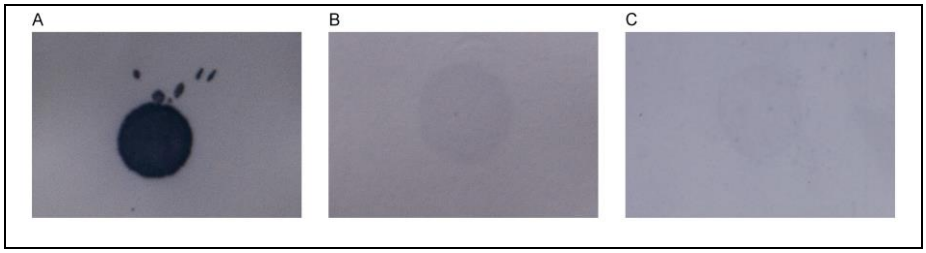


Fig I.8 Dot blot hybridization with DIG-labeled probe against: (A) ITS1-5.8S purified PCR products from *M. tenuissimum* (125 ng) (B) Genomic DNA (125 ng) from *M. tenuissimum* digested with *Eco*RI. (C). Genomic DNA from *G. imbricata* (250 ng) digest with *Eco*RI.

As shown in Fig I.9, hybridisation signals conducted by Southern blotting were detected in the two samples of *G. imbricata* that were analyzed. Hibridization of the specific probe to the genomic DNA of *G. imbricata* digested with *Eco*RI generated a single band with an approximate size of 8750 pb. (Fig I.9 - line 2). When the probe is targeted to DNA of *M. tenuissimum* digest with *Eco*RI revealed one fragment of aproximately 3670 pb (Fig I.9 - line 4). The samples of *G. imbricata* were tested as positive for *M. tenuissimum* using PCR. Thus the presence of a single band discarded cross hybridization. This result indicates the possible existence of molecular interaction at DNA level between *G. imbricata* and ist endophyte *M.* tenuissimum. When genomic DNA of *G. imbricata* was digested with *Pvu*II (Fig I.9 - line1), the probe revealed a single fragment of approximately 1350 pb. In contrast, the probe targeted to DNA of *M.*

tenuissimum digested with *Pvu*II detects a fragment with approximately 1120 pb (Fig I.9 - line 3).

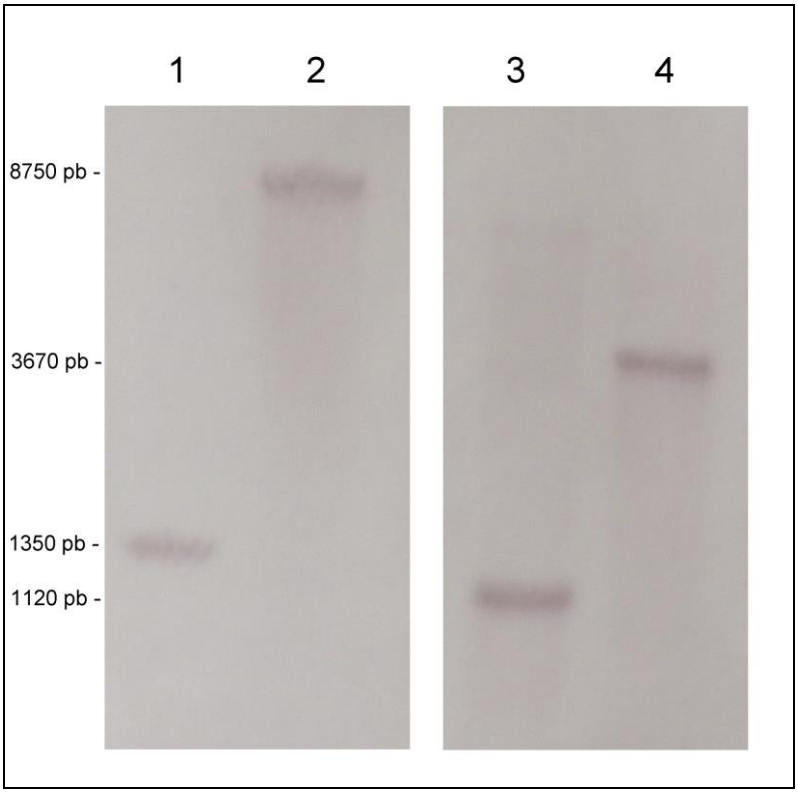


Fig I.9 Southern blotting hybridization using the digoxigenin-labeled probe from the PCR product of ITS1-5.8S rDNA of *M. tenuissimum* isolated from *G. imbricata*. (1) Genomic DNA of *G. imbricata* digested with *Pvu*II. Probe reveals a single fragment of 1350 pb (2) Genomic DNA of *G. imbricata* digested with *Eco*RI revealed a single fragment of 8750 pb (3) Probe targeted to genomic DNA of *M. tenuissimum* digested with *Pvu*II reveals a single fragment of 1120 pb (4) Genomic DNA of *M. tenuissimum* digested with *Eco*RI. Probe reveals a single band of 3670 pb.

The PCR is shown as an effective method to detect infection with *M. tenuissimum* in the tissues of its host. However, if the amount of amplified fragments is low, the resolution of the electrophoresis and the limitations of the human eye not detect the infection, leading to false negatives. In this sense the specific DNA probes targeting to rDNA internal transcribed spacer showed great sensitivy to detection of *M. tenuissimum* closely associated with *G. imbricata*.

As shown in (Fig I.10) southern blot signal can be detected in PCR products when the concentrations of DNA in the PCR reaction are as low as 2.5 ng (Line I), 0.25 ng (Line J) and 0.025 ng (Line K) even in absence of any visible ethidium bromide stained product on the gel.

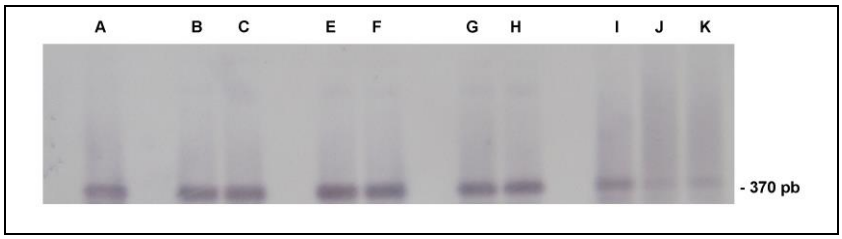


Fig I.10 Southern blotting hybridization using the digoxigenin-labeled probe from the PCR product of ITS1-5.8S rDNA of *M. tenuissimum* isolated from *G. imbricata* (A) Positive Control. PCR products of ITS1-5.8S rDNA from *M. tenuissimum* (4 ng). (B-H) PCR products (4 ng) of ITS1-5.8S rDNA of *M. tenuissimum* amplified from DNA of *G. imbricata* (I-J) Probe revealing positive signals against PCR products of ITS1-5.8S of *M. tenuissium* amplified from low concentrations of DNA of *G. imbricata* (2.5 ng, 0.25 ng and 0.025 ng) respectively

The technique of southern blot showed positive signal in six PCR producs in which the ITS1-5.8S rDNA of *M. tenuissimum* was amplified from DNA samples of *G. imbricata*, when the DNA concentration transferred to the membrane was 4 ng, in absence of visible signal by electrophoresis.

DISCUSSION

Infections by filamentous microscopic epi or endophytic algae are common in macroalgae (Correa *et al.* 1994; Peters & Ellertsdóttir 1996; Peters & Schaffelke 1996; Ellertsdóttir & Peters 1997; Peters & Burkhardt 1998; Peters 2003, Kim *et al.* 2014). Correa *et al*. (1987) reported that the frequency of infected mature fronds of *C. crispus* could exceed 80% of the fronds, and might even reach 90%. Using microscopic examination, Elleertsdóttir & Peters (1997) showed an 85% prevalence of infection by endophytic brown algae in *Laminaria* spp. and that this infection was present in the medulla or inner cortex. Other studies show that up to 95% of host plants within *C. crispus* populations are infected with algal endophytes (Correa *et al.* 1987; Plumb 1999).

The observation of filamentous brown alga morphotypes was more than incidental when performing *in vitro* cultures of the red seaweed *G. imbricata*. Even when the explants were apparently free of other algal, bacterial or fungus growth for months, brown filaments sometimes sprout from some of them, thus the phenomena was related to a kind of stress (Robaina *et al*. 1990a,b). In this study, when we left the explants to age in culture, 90 % (n= 7000 explants), showed an endophyte infection, apparently sharing the same morphotype, thus

confirming our interpretation that the endophyte was already inside the specimens.

Furthermore, all samples of this filamentous brown morphotype isolated from all samples of *G. imbricata* were identified as *M. tenuissimum,* as revealed by phylogenetic analysis performed using ITS. The high prevalence of infection in natural populations sampled was confirmed by PCR. Although this technique allows us to detect to *M. tenuissimum* living as an endophyte algae tissues, but does not establish the type of relationship between the endophyte and its host, much less at the molecular level.

Our results agreed with the preliminary ones of Peters (2003) who cited the presence of *M. tenuissimum* from *G. imbricata* (under name *G.* doryphora) in the Canary Islands. The results thus reveal the existence of a strong association between a red algal species and a brown species, the latter living inside the former as an endophyte. Whether this association generates mutual benefits requires further investigation, but our experience *in vitro* would indicate the existence of an actual infection, with structural and molecular consequences as discussed below.

This type of strong association between two algae has previously been reported, although the exact nature of the host-

endophyte relationship is not yet properly understood (Correa & McLachlan 1994). Sometimes, the authors focus on the biochemical basis of the interaction, such as the case of the red *C. crispus-Ulvella operculata* (under name *Acrochaete operculata*) in which the oligosaccharide signals involved in cell-cell recognition and the role of H_2O_2 in the interaction between the red alga and its green algal endophyte have been shown (Bouarab *et al*. 1999).

Goff & Coleman (1984) showed that during the infection process, the parasitic marine red alga *Choreocolax* transferred its nuclei via secondary pit connections to its red algal host *Polysiphonia.* These nuclei are able to remain viable for weeks, modulating the host responses to infection, showing what could be a regulatory mechanism of parasitism. The main characteristic of the parasitic red algae is its ability to transfer its nuclei into the host organism cytoplasm. These nuclei in the cytoplasm are able to replicate and invade adjacent host cells, where they are packaged as spores and may infect other thalli (Goff & Coleman 1995). Secondary pit connections enable the transfer of parasite genetic information; the parasite nuclei transferred to the cytoplasm of host cells can control and direct the physiology of the host (Goff & Coleman 1985).

Our results of Southern blotting reveal the apparent existence of DNA-DNA interaction between the endophyte *M. tenuissimum* and its host *G. imbricata*, at least in the region corresponding to the rRNA cistron. The results in Fig I.9 show that there is apparently a single copy of the analysed fragment (KJ134990) in the genome of the endophyte *M. tenuissimum*, as well as in its strongly associated host *G. imbricata*, although in a different genomic position, as determined by the different length of the fragments to which the probe hybridised. It should be noted that if this was the result of cross-hybridisation of the probe with the sample of *G. imbricata*, at least two bands would appear, with one band corresponding to the probe-host DNA cross-hybridisation and the other to the endophyte, that must match the lenght of the hybridization band in the endophyte DNA. On the other hand, the conditions used for the southern blot were extremely restrictive to avoid cross-hybridisation. The presence of a single copy in each DNA also suggests that the analysed fragment is apparently integrated into the host genome, and that its location is not extrachromosomal (ie. A separate nucleus as described above for other algae). Although this hypothesis should be confirmed with additional tests here we show the first evidence of the close relationship a molecular level the this pathosystem present.

Gene transfer of functional genes is accepted as the theoretical foundation of the endosymbiotic origin of cellular organelles. This is usually seen between prokaryotes (Jain *et al.* 2002), from prokaryotes to other organisms as has been reported from endosymbiotic bacteria into insect host cell nuclei (Kondo *et al.* 2002), and is described between both mitochondria (Palmer *et al.* 2000) and chloroplasts (Martin *et al.* 2002), and the cell nucleus. Nevertheless, the advances in our knowledge of the genome of eukaryotes has brought to light the important role also played by horizontal gene transfer (HGT) in the evolution of eukaryotes where it often plays a role in functional processes (Keeling & Palmer 2008). Recent research shows that the frequency with which this phenomenon occurs is much higher than expected, that it contributes to the content of eukaryotic genomes and confers adaptive advantages (Keeling 2009). Horizontal gene transference seems to occur during parasitism as reported in parasitic protozoa (Richards *et al.* 2003), as well as in pathogenenic relationships, as occurs in some species of fungi. The presence of interspecific virulence genes has been described as a possible mechanism for the appearance of new diseases, a prominent example of which is he virulence gene Tox A. This (11kb) gene encoding a critical virulence factor was transferred from one species of fungal

pathogen to another, thus conferring virulence on an avirulent fungus (Friesen *et al.* 2006; Giovannetti *et al.* 1999). Descriptions of gene transference from host plants to a pathogenic fungus are rare, despite the fact that this process is very important in the creation of a relation between the host and the pathogen. Interkingdom fungus-plant horizontal transference in *Pyrenophora* species has been recorded, and study of the transferred genes has shown that they codify extracell proteins the function of which is to facilitate the infection of plants by phytopathogenic fungi, interfering with plant defence mechanisms or bringing about degradation in plan cell membranes. There is also evidence that other transferred genes are involved when the host's carbohydrates are used by the parasite (Sun *et al.* 2013) .Other examples include *Metarhizium robertsii*, which is capable of acquiring the sterol carrier gene Mr-npc2a from its insect host by means of HGT (Zhao *et al.* 2014).

Studies on *Parasitella parasitica*, a facultative parasite of many mucorales, for example *Absidia glauca*, have shown that infection includes the formation of a plasmatic continuum between the host and the parasite. This phenomenon apparently allows the host to be invaded by nuclei of the parasite. Infection is accompanied by the

fusion of both mycelia and is associated with the transfer of genes (Kellner *et al*. 1993).

Horizontal gene transfer is a mechanism of adaptation to unfavorable environmental conditions. In terms of adaptation to extreme conditions, it is known that diatoms and other algae survive and develop in sea ice. To survive this event, sea ice diatoms produce ice-binding proteins, however it seems that the genes encoding these ice-binding proteins are not typical of diatoms, suggesting that they are acquired by horizontal transfer from ice-associated bacteria (Raymond and Kim 2012).

Gene transfer has even been described among plants. Parasitic plants characterized by their reduced photosynthetic ability have suffered gene loss from chloroplast. *Cistanche deserticola* is a parasitic plant (Holoparasite) typical of desert areas, but has undergone fewer losses and studies suggest that this occurs in part because genes acquired from its host *Haloxylon ammodendron* by means of horizontal gene transfer (Li *et al*. 2013).

Several studies have previously reported some cases of transfer of rRNA genes between prokaryotes (Mylvaganan & Dennis 1992; Van Berkum *et al.* 2003). Transfer of ribosomal DNA internal transcribe spacer (ITS1 and ITS2) genes have been cited between

fungi, such as *Thanatephorus cucumeris* and *Ceratobasidium oryzae-sativae* (Xie *et al*. 2008). The 18S rRNA gene of a perkinsid alveolate was detected in *Ciliophrys infusionum* (foraminifera) (Yabuki *et al*. 2014). Davis and Wurdack (2004) have shown examples of HGT of rRNA between flowering plants.

In the present study, we have provided evidence of a high prevalence of the brown endophyte *M. tenuissimum* in its host, *G. imbricata*, to the extent that they interact at the DNA level with apparent endophyte-host gene transference of rRNA coding regions. Further work is required to explore the functional consequences of such transference, to determine whether more genes are being transferred and how endophyte and host molecular crosstalk triggers or prevent their separation, among other interesting phenomena. From a biotechnological standpoint, the implementation of gene transference techniques constitutes the current bottleneck in the development of macroalgal biotechnology; the potential utilization of HGT as shown in this work therefore deserves further exploration.

REFERENCES

Bouarab K, Potin P, Correa JA, Kloareg B (1999) Sulfated oligosaccharides mediate the interaction between a marine red algae and its green algal pathogenic endophyte. The Plant Cell 11: 1635-1650.

Burkhardt E, Peters AF (1998) Molecular evidence from nrDNA ITS sequences that *Laminariocolax* (Phaeophyceae, Ectocarpales sensu lato) is a worldwide clade of closely related kelp endophytes. Journal of Phycology 34: 682-691.

Correa JA, Buschmann A, Retamales C, Beltrán J (1997) Infectious diseases of *Mazzaella laminarioides* (Rhodophyta): Changes in infection prevalence and disease expression associated with season, locality, and within-site location. Journal of Phycology 33: 344-352.

Correa JA, Flores V, Garrido J (1994) Green patch disease in *Iridaea laminarioides* (Rhodophyta) caused by *Endophyton* sp.(Chlorophyta). Diseases of aquatic organisms, 19: 203-203.

Correa JA, McLachlan JL (1994) Endophytic algae of Chondrus cripus (Rhodophyta). V. Fine structure of the infection by

Acrochaete operculata (Chlorophyta). European Journal of Phycology 29: 33-47.

Correa JA, McLachlan JL (1991) Endophytic algae of C*hondrus crispus* (rhodophyta). III. Host specificity. Journal of Phycology, 27: 448-459.

Correa JA, Nielsen R, Grund DW (1988) Endophytic algae of *Chondrus crispus* (rhodohyta). II. *Acrochaete heteroclada* sp. nov., *A. operculata* sp. nov., and *Phaeophila dendroides* (Chlorophyta). Journal of phycology, 24: 528-539.

Correa JA, Nielsen R, Grund DW, McLachlan JL (1987) Endophytic algae of Iris moss (*Chondrus cripus* Stackh.). Hydrobiologia 151/152: 223-228.

Coyer JA, Smith GJ, Andersen RA (2001) Evolution of *Macrocystis* spp. (Phaeophyceae) as determined by ITS1 and ITS2 sequences. Journal of Phycology 37: 574-585.

Davis CC, Wurdack KJ (2004) Host-to-Parasite gene transfer in flowering plants: Phylogenetic evidence from Malpighiales. Science AAAS 305: 676-678.

Draisma SGA, Peters AF, Fletcher RL (2003) Evolution and taxonomy in the Phaeophyceae: effects of the molecular age

on brown algal systematic. European Journal of Phycology 39:87-102.

Deng Y, Tang X, Ding L, Lian S (2011) A new record from China of epiphytic marine algae, *Acrochaete leptochaete* (Chaetophoraceae, Chlorophyta) with its primary experimental biology. Chinese Journal of Oceanology and Limnology, 29: 350-355.

Ellertsdóttir E, Peters AF (1997) High prevalence of infection by endophytic brown algae in populations of *Laminaria* spp. (Phaeophyceae). Marine Ecology Progress Series 146: 135-143.

Friesen TL, Stukenbrock EH, Liu Z, Meinhardt S, Ling H, Faris JD, Rasmussen JB, Solomon PS, McDonald BA, Oliver RP (2006) Emergence of a new disease as a result of interspecific virulence gene transfer. Nature Genetics 38:953-956.

García-Jiménez P, Geraldino PJL, Boo SM, Robaina RR (2008) The red alga *Grateloupia imbricata* a species introduced into the Canary Island. Phycological Research 56:166-171.

Gauna MC, Parodi ER (2008) Green epi-endophytes in *Hymenena falklandica* (Rhodophyta) from the Patagonian coasts of

Argentina: Preliminary observations. Phycological research, 56: 172-182.

Gauna MC, Parodi ER, Cáceres EJ (2009) Epi-endophytic symbiosis between *Laminariocolax aecidioides* (Ectocarpales, Phaeophyceae) and *Undaria pinnatifida* (Laminariales, Phaeophyceae) growing on Argentinian coasts. Journal of applied phycology, 21: 11-18.

Giovannetti M, Azzolini D, Citernesi AS (1999) Anastomosis Formation and Nuclear and Protoplasmic Exchange in Arbuscular Mycorrhizal Fungi. Applied and Environmental Microbiology 65: 5571-5575.

Goff LJ (1983) Marine algal interactions: epibiosis, endobiosis, parasitism and disease. In: Tseng CK, editor. Proceedings of the Joint China-U.S. Phycology Symposium 221- 274.

Goff LJ, Coleman AW (1995) Fate of parasite and host organelle dna during cellular trasnformation of red algae by their parasites. The Plant Cell 7: 1899-1911.

Goff LJ, Coleman AW (1985) The role of secondary pit connections in red algal parasitism. Journal of Phycology 21: 483-508.

Goff LJ, Coleman AW (1984) Transfer of nuclei from a parasite to its host. Proccedings of the National Academy of Sciences of United States of America 81: 5420-5424.

Jain R, Rivera MC, Moore JE, Lake JA (2002) Horizontal gene transfer in microbial genome evolution. Theorical population biology 61: 489-495.

Keeling PJ (2009) Functional and ecological impacts of horizontal gene transfer in eukaryotes. Current opinion in genetics & development 19: 613-619.

Keeling PJ, Palmer JD (2008) Horizontal gene transfer in eukaryotic evolution. Nature Reviews Genetics 9: 605-618.

Kellner M, Burmester A, Wostemeyer A, Wostemeyer J (1993) Transfer of genetic information from the mycoparasite Parasitella parasitica to its host Absidia glauca. Current genetics 23: 334-337.

Kondo N, Nikoh N, Ijichi N, Shimada M, Fukatsu T (2002) Genome fragment of *Wolbachia* endosymbiont transferred to X chromosome of host insect. Proccedings of the National Academy of Sciences of United States of America 99: 14280-14285

Kim C, Kim YS, Choi HG, Nam KW (2014) New records of three endophytic green algae from *Grateloupia* spp. (Rhodophyta) in Korea. Algae, 29: 127-136.

Li X, Zhang T-C, Qiao Q, Ren Z, Zhao J, Yonezawa T, Hasegawa M, Crabbe MJC, Li J, Zhong Y (2013) Complete chloroplast genome sequence of holoparasite Cistanche deserticola (Orobanchaceae) reveals gene loss and horizontal gene transfer from its host Haloxylon ammodendron (Chenopodiaceae). PLoS ONE 8(3): e58747.

Martin W, Rujan T, Richly E, Hansen A, Cornelsen S, Lins T, Leister D, Stoebe B, Hasegawa M, Penny D (2002) Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. Proccedings of the National Academy of Sciences of United States of America 99: 12246-12251.

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8: 4321-4325.

Mylvaganan S, Dennis PP (1992) Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic

archaebacterium Haloarcula marismortui. Genetics 130: 399-410.

Nielsen R (1979) Culture studies on the type species of *Acrochaete*, *Bolbocoleon* and *Entocladia* (Chaetophoraceae, Chlorophyceae). Botaniska Notiser, 132: 441-449.

Nielsen R, Gunnarsson K, Daugbjerg N, Petersen G (2014) Description of *Ulvella elegans* sp. nov. and U. *islandica* sp. nov.(Ulvellaceae, Ulvophyceae) from Iceland–a study based on morphology of species in culture and tuf A gene sequences. European Journal of Phycology, 49: 60-67.

Nielsen R, Petersen G, Seberg O, Daugbjerg N, O'Kelly CJ, Wysor B (2013) Revision of the genus *Ulvella* (Ulvellaceae, Ulvophyceae) based on morphology and tufA gene sequences of species in culture, with *Acrochaete* and *Pringsheimiella* placed in synonymy. Phycologia, 52: 37-56.

Palmer JD, Adams KL, Cho Y, Parkinson CL, Qiu Y-L, Song K (2000) Dynamic evolution of plant mitochondrial genomes: mobile genes and introns and highly variable mutation rates. Proceedings of the National Academy of Sciences of United States of America 97: 6960-6966.

Peters AF (2003) Molecular identifications, distribution and taxonomy of brown algal endophytes, with emphasis on species from Antarctica. In. Chapman ARO, Anderson RJ, Vreeland VJ, Davison IR editors. Proceedings of the 17th International Seaweed Symposium 293-301.

Peters AF (1998) Ribosomal DNA sequences support taxonomic separation of the two species of Chorda: reinstatement of *Halosiphon tomentosus* (Lyngbye) Jaasund (Phaeophyceae, Laminariales). European . Journal of Phycology 33: 65-71.

Peters AF, Ramírez ME (2001) Molecular phylogeny of small brown algae, with special reference to the systematic position of *Caepidium antarcticum* (Adenocystaceae, Ectocarpales). Cryptogamie Algologie 22:187-200.

Peters AF, Burkhardt E (1998) Systematic position of the kelp endophyte *Laminarionema elsbetiae* (Ectocarpales sensu lato, Phaeophyceae) inferred from nuclear ribosomal DNA sequences. Phycologia 37:114-120.

Peters AF, Ellertsdóttir E (1996) New record of the kelp endophyte *Laminarionema* elsbetiae (Phaeophyceae, Ectocarpales) at Helgoland and its life history in culture. Nova Hedwigia 62: 341-349.

Peters AF, Schaffelke B (1996) *Streblonema* (Ectocarpales, Phaeophyceae) infection in the kelp *Laminaria saccharina* (Laminariales, Phaephyceae) in the western Baltic. Hydrobiologia 326/327: 111-116.

Plumb J (1999) Population dynamics and endophytic flora of *Chondrus crispus* (Rhodophyta): a temporal study. Phd thesis, University of the West of England, Bristol, UK.

Provasoli L (1968) Media and prospects for the cultivation of marine algae. In: Watanabe A, Hattori A, editors. Cultures and collections of algae. Japanaese Society of Plant Physiology. Hakone. 63-75.

Raymond JA, Kim HJ (2012) Possible role of horizontal gene transfer in the colonization of sea ice by algae. PLoS One 7(5): e35968.

Richards TA, Hirt RP, Williams BA, Embley TM (2003) Horizontal gene transfer and the evolution of parasitic protozoa. Protist 154:17-32.

Robaina RR, García P, García-Reina G, Luque A (1990a) Morphogenetic effect of glycerol on tissue of the red seaweed *Grateloupia doryphora*. Journal of Applied Phycology 2: 137-143.

Robaina RR, García-Reina G, Luque A (1990b) The effects of the physical characteristics of the culture medium on the development of red seaweeds in tissue culture. Hydrobiologia 204/205: 137-142.

Saunders GW, Druehl LD (1992) Nucleotide sequences of the internal transcribed spacers and 5.8S rRNA genes from *Alaria marginata* and *Postelsia palmaeformis* (Pheophyta: Laminariales). Marine Biology. 115: 347-352.

Sun B-F, Xiao J-H, He S, Liu L, Murphy RW, Huang D-W (2013) Multiple interkingdom horizontal gene transfers in Pyrenophora and closely related species and their contributions to phytopathogenic lifestyles. PLoS ONE 8(3): e60029.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28: 2731–2739.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap

penalties and weight matrix choice. Nucleic Acids Research 22: 4673-4680.

Van Berkum P, Terefework Z, Paulin L, Suomalainen S, Lindström K, Eardly BD (2003) Discordant phylogenies within the rrn loci of rhizobia. Journal of bacteriology 185: 2988-2998.

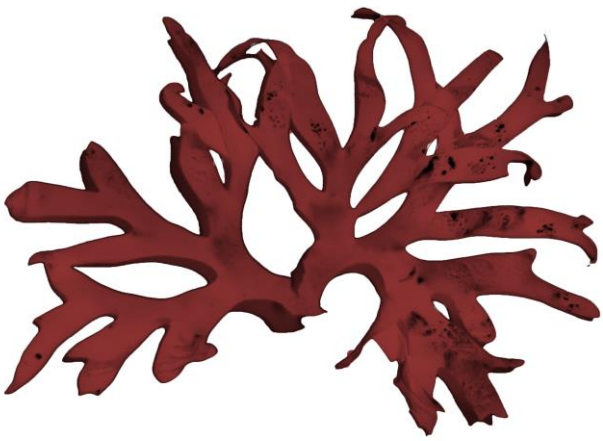
Xie J, Fu Y, Jiang D, Li G, Huang J, Li B, Hsiang T, Peng Y (2008) Intergeneric transfer of ribosomal genes between two fungi. BMC Evolutionary Biology 8: 87

Yabuki A, Toyofuky T, Takishita K (2014). Lateral transfer of eukaryotic ribosomal RNA genes: An emerging concern for molecular ecology of microbial eukaryotes. ISME Journal 8: 1544-1547.

Zhao H, Xu C, Lu H-L, Chen X, Leger RJSt, Fang W (2014) Host-to-Pathogen gene transfer facilited infection of insects by a pathogenic fungus. PLoS One 10(4): e1004009.

CAPÍTULO II

Polyamines in the host/pathogen interaction of *Grateloupia imbricata* and *Ulvella leptochaete*



Navarro-Ponce M.A., A.G. González, R.R.Robaina (2015) Polyamines in the host/pathogen interaction of *Grateloupia imbricata* and *Ulvella leptochaete.* (In preparation)

Abstract

An epi-endophytic filamentous green algae was isolated during the *in vitro* cultivation of *Grateloupia imbricata*. The phylogenic analyses, using the ITS2 region as a molecular marker combined with the 5.8S and 28S ribosomal DNA allowed to identify it as *Ulvella leptochate* (Huber) Nielsen. Our *in vitro* observations indicate that it was inicially observed as an epiphyte, but in advanced stages of infection, it has the capacity to penetrate the interior of the host tissues and behave as an endophyte. In this work, chemical signalling during infection was investigated, with focus of molecules implicated in defense mechanisms in higher plants, such as polyamines, methyl jasmonate (MeJA) and H_2O_2. The polyamine levels (PAs) were examined in the initial stages of infection and showed a marked increase in the concentration of putrecine in both, its free and conjugated forms. The polyamines are a common source of hydrogen peroxide (H_2O_2) in response to the infection by pathogens in terrestrial plants and could play a similar role during the infection of *G. imbricata* by the epi-endophyte *U. leptochaete*. The treatment of the thalli of *G. imbricata* with methyl jasmonate (MeJA) (500 µM), a well know chemical agent in the plant defense produces a significant increase in the levels of putrescine, in both the free and conjugated

forms, twelve hours after treatment, showing an increase of 4 and 2 times respectively compared to baseline levels. Thus, MeJA could modify the metabolism of the polyamines and contribute to the development of a more efficient defensive response in the same way as in the higher plants. The reactive oxygen species (ROS) also mediate the pathogen-host interaction in red algae, in a similar way as occurs in vascular plants. The possiblity that H_2O_2 had an effect on the PAs levels in *G. imbricata* was examined. It has been found that with sufficiently elevated levels of H_2O_2, tissues respond with an increase in the concentration of putrescine in its free form approximately two times that in respect to the control samples. In excessively high concentrations of H_2O_2 (50 mM), the thalli of *G. imbricata* are bleached and produce tissue death, drastically lowering the levels of putrescine in both studied forms.

In this study, it was also determined that the potential antioxidant diamine putrescine, showed its capacity to act directly on H_2O_2 as a radical scavenger. In this sense, in *in vitro* experiments, putrescine is capable of decomposing-eliminating 50% of the peroxide in the solution immediately when it is found in concentrations from three to six orders of magnitude above the concentrations of H_2O_2. Thus, this reflects that the PAs and specifically diamine putrescine

could play a double role in the defence mechanisms in the red algae *G. imbricata*. On the one hand, its metabolism could provide a source of hydrogen peroxide and on the other it could act as a molecule with antioxidant capacities, diminishing the damage in the tissues provoked by the ROS and preventing infections by pathogens.

INTRODUCTION

Epi or endophytic green algae constitute one the most diverse and primitive groups of green algae which are considered as ubiquitous (Bast *et al.* 2014). The epiphytic or endophytic filamentous green algae of the genus *Ulvella* (Chaetophoraceae, Chlorophyta) was first established by Pringsheim (1862) under the name *Acrochaete* (it was submitted for review by Nielsen *et. al.* 2013). The morphological characteristics of these filamentous algae hinder its proper taxonomic assignment, so that classification in this taxa should be commonly based on molecular markers, such as the plastid *tuf*A gene and the internal trasncribed spacer (ITS) regions of the rRNA (Sussmann *et al.* 1999; Rinkel *et al.* 2012; Nielsen *et al.* 2013).

Ulvella leptochaete (Huber) R.Nielsen (as *Endoderma leptochaete*) has been cited growing epiphytically on the species of the genus *Chaetomorpha*, *Cladophora*, *Ceramium* and recently in the genus *Chaetomorpha* as *Acrochaete leptochaete* (Deng *et al.* 2011, 2012). Infection for *Ulvella leptochaete has* also been reported in *Grateloupia lanceolata* (Kim *et al.* 2014).

The damage that filamentous algae cause on the host is determined by its nature and the degree of infection. Epiphytes or endophytes may be a source of disadvantages for their host: decreasing the growth rate and its reproductive capacity, altering the properties of the thallus (D'Antony 1985), or even competing for nutrients and light (Sand-Jensen & Revsbech 1987; Sand-Jensen 1997).

Species of the genus *Ulvella* have been identified as the cause of massive cell and tissue destruction in the red alga *Chondrus crispus*. *Ulvella operculata* and *Ulvella heteroclada* have been described as directly producing poor physiological functioning in its host or acting as an agent that facilitates secundary infections by other pathogens, thereby aggravating the primary negative impact of the endophyte (Correa & McLachlan 1992, 1994).

As sesile organisms living attached to the rocks or sandy substrates, marine macroalgae must deploy mechanisms to resist the attack by pathogens. The defense mechanisms against the attack of epi or endophytes are poorly known in macroalgae in respect to terrestrial plants, even though, there are several consensuses that similar processes are used (Potin *et al.* 2002).

In terrestrial plants, such defense reactions in the relationship between pathogen and host are initiated by the host, following the recognition of an extracellular signal mediated by molecules known as elicitors that trigger a cascade of defence responses in the host (Wei *et al.* 1992; Wojtaszek 1997; Yang *et al.* 1997).

The production of hydrogen peroxide (H_2O_2) and other (ROS), is a typical rapid response for the development of defense against invading organisms in terrestrial plant. ROS are toxic to the pathogen and act as secondary messengers in the host defence (Levine *et al.* 1994; Mehdy 1994; Baker & Orlandi 1995; Lamb & Dixon 1997; Mellersh *et al*. 2002). A common mechanism activated by the plant-pathogen interaction is the generation of superoxide radicals by a diphenylene-iodiniym (DPI)-sensitive (O'Donnell *et al*. 1993) and a receptor-actived NADPH-oxidase (Levine *et al.* 1994; Doke & Miura 1995; Lamb & Dixon 1997).

The pathogens also induce a defensive response in macroalgae apparently similar to vascular plants (Weinberger 2007). Weinberger *et al*. (1999) and Weinberger and Friedlander (2000) also described an oxidative burst in *Gracilaria conferta*, as a result of exposure to certain elicitors. This production of hydrogen peroxide acts as a

defensive response inducing the elimination of epiphytic bacteria (Weinberger & Friedlander 2000).

The interaction between the red alga *C. crispus* and its endophyte *U. operculata* is the best known reference of a pathosystem in the host-pathogen association between algae. Carrageenan oligosaccharides mediate the interaction between the algae endophytes and its host, thus determining the virulence of the infecting agent. The pathogen is able to colonize the sporophyte, due to the recognition of molecules of λ-carrageenan oligosaccharides, which are present only in the sporophyte and induce the release of H_2O_2 and increase carragenolytic activity by the pathogen. However, the gametophytes are more resistant than tetrasporophytes to infection, due to the fact that they are capable of producing an oxidative burst and a superoxide radical in response to the L-asparagine amino acid released by the pathogen. (Bouarab *et al.* 1999, 2001; Weinberger *et al.* 2005). As it occurs in higher plants, NADPH oxidase is apparently a key enzyme and catalyzes the generation of an oxidative burst in response to the pathogen attack (Hervé *et al.* 2006).

Polyamines (PAs) are organic molecules with two or more amino groups that can be bound to cell membranes and a variety of macromolecules or can be found in a free state inside the cell. They

are implicated in the modulation of a wide array of fundamental processes such as the stabilization and regulation of physical and chemical properties of cell membranes (Schubert *et al.* 1983; Roberts *et al.* 1986; Kaur-Sawhney & Applewhite 1993), cell division and differentiation, nucleic acids structure and DNA replications and the modulation of enzyme activities (Evans & Malmberg 1989, Galston & Kaur-Sawhney 1990). In terrestrial plants they are involved in the regulation of plant growth, affect the pollen maturation, grain germination and flower develpment (Smith 1985; Torrigiani *et al.* 1987; Gerats *et al.* 1988; Evans & Malmberg 1989; Harkess *et al.* 1992; Chibi *et al.* 1994; Rey *et al.* 1994; Das *et al.* 1995). Moreover, PAs play a key role in the development of plant adaptation and response toward abitic and biotic stresses (Alcázar *et al.* 2006; Groppa & Benavides 2008; Alcázar *et al.* 2010; Hussain *et al.* 2011)

Polyamines (PAs) may be used as a source of H_2O_2 during the infection by pathogens (Yoda *et al.* 2009). In higher plants, an increase in the levels of free and bound PAs has been described after the pathogen inoculation. These PAs are degraded by the polyamine oxidase (PAO) and the diamine oxidase (DAO) producing, in this process, a liberation of H_2O_2. (Yamakawa *et al.* 1998; Walters 2000; Cowley & Walters 2002; Walters 2003a,b; Yoda *et al.* 2009). On the

contrary, antioxidant properties of PAs have been noted (Drolet *et al.* 1986; Bors *et al.* 1988) to be effective as scavengers of free radicals, such as H_2O_2 among others (Kafy *et al.* 1986; Ha *et al.* 1998; Das & Misra 2004; Fujisawa & Kadoma 2005) and protect against the toxic effects of these (Velikova *et al.* 2000; Tkachenko *et al.* 2001; Rider *et al.* 2007).

The hormone methyl jasmonate (MeJA) play a important role as signaling molecule in biotic and abiotic stress, including defense mechanisms against pathogens in higher plants (Enyedi *et al.* 1992; Creelman & Mullet 1997; Santino *et al.* 2013). MeJA produces an increase in the endogenous levels of diamines and polyamines (Biondi *et al.* 2000; Haggag & Abd-El-Kareem 2009; Horbowicz *et al.* 2011) increasing the plant resistance against pathogens. The genes involved in these defense mechanisms are strongly influenced by the action of MeJA in the same way that they are influenced by ethylene. (Xu *et al.* 1994). In red algae *C. crispus* MeJA induces the expression of genes related to stress and silent genes related to the general metabolism (Collen *et al.* 2006).

Therefore, according to the literature, PAs may play two different roles as a protective barrier against host attack by:

1) Liberation of H_2O_2 through the catabolims of PAs

2) Scavenging of H_2O_2 produced by the pathogen

In this work we used the pathosystem defined by *G. imbricata* and its epi-endophyte *U. leptochaete* to I. determine how PAs levels are influenced during the early stages of infection in *G. imbricata* by the epi-endophyte *U. leptochaete*, II. determine if the PAs show the capacity to produce scavenging of H_2O_2 *in vitro*, III: determine how the PAs levels respond in *G. imbricata* when they are cultivated in the presence of MeJA, IV: study if the H_2O_2 liberated by the pathogen may act by stimulating the production of PAs.

METHODS

Material plant culture conditions and epi-endophyte isolation

Grateloupia imbricata Holmes (Rhodophyta) was collected on the rocky shores along the Northeast coast of Gran Canaria, Canary Islands, Spain. Explants of fresh thalli of *G. imbricata* were cultured in a Provasoli enriched seawater medium, PES (Provasoli 1968). The culture conditions were 30 µmol photon m^2s^{-1} at 18 ± 2ºC on 16:8 h light:dark cycle, in Petri dishes containing 15 ml of medium. Filamentous green algae associated to *G. imbricata* were isolated and maintained in an unialgal culture in Petri dishes with PES medium at 5 µmol photon m^2s^{-1} and at 18 ± 2ºC on 16:8 h light:dark cycle.

Molecular analysis of the green epi-endophytes

To conduct the taxonomic assignment of epi-endophytic filamentous green algae, genomic DNA extraction was performed according to the standard hexadecyltrimethyl ammonium bromide (CTAB) procedure (Murray & Thomson 1980) from 100 mg fresh weight of isolated material powdered in liquid nitrogen. DNA preparations were cleaned using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich), according to the manufacturer's protocol and quantified by UV-spectrophotometry at 260/280 nm using the Nanodrop 1000 spectrophotometer v3.7 (ThermoFisher Scientific).

PCR amplifications were performed in a GeneAmp 2400 thermal cycler (PerkinElmer Inc.) Approximately 210 bp of the rDNA ITS2 region was amplified using the primer (5' CAYRYCTGCCTCAGCGTCGG 3') which is specific for the 5.8S rDNA in green algal endophytes (Bown *et al.* 2003) and (5' TCCTCCGCTTATTGATATGC 3') (White *et al*. 1990). The reaction volume was 50µl, and comprised 25 ng of genomic DNA, 0.4µM of each primer, 0.5 µl of Taq polymerase (2.5U/µl), 4 µl of dNTP Mixture (2.5mM each) and 10µl of 5X Buffer (PrimeSTAR® HS DNA Polymerase, Takara). PCR temperature profile was as follows: An initial 5-min denaturation at 95 ºC, 30 cycles of danaturation at 94 ºC for 1 min; anneling at 50 ºC for 1 min; extension at 72 ºC for 2 min and a final extension cycle of 72 ºC for 5 min. The sequence was deposited in Genebank under access number KJ134991 (Fig II.1).

Phylogenetic analyses were performed using MEGA v5.03 (Tamura *et al*. 2011). Sequences were downloaded from GenBank according to the BLAST (Table II.1) and aligned using Clustal W (Thompson *et al*. 1994). Maximum parsimony (MP) and maximum likelihood (ML) analyses were used to estimate phylogenetic relationships on data sets of 12 nucleotide sequences and 156 informative positions. For the MP analyses, the bootstrap consensus

tree inferred from 1000 tree replicates. The MP tree was obtained using the Min-mini heuristic algorithm with a search factor of 3, only parsimony-informative sites are used. ML method was based on the Tamura-Nei model (Tamura *et al*. 2011).

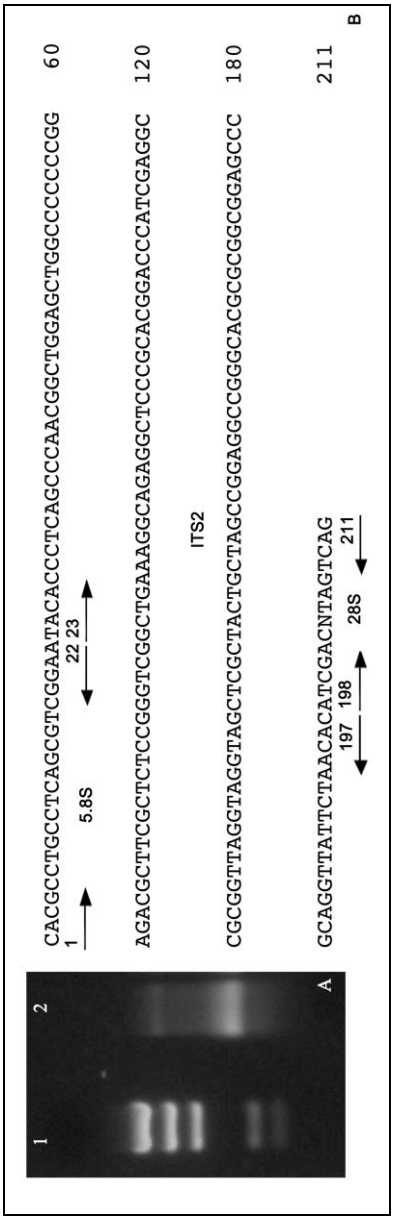


Fig II. 1 (A) Agarose gel electrophoresis of 1. Molecular weight marker; 2. 5.8S rDNA partial sequence, ITS2 region complete sequence and 28S rDNA partial sequence. (B) Nucleotide sequence of the putative *U. leptochaete* isolated from culture ITS1-5.8S rDNA used in phylogenetic analysis

Species name	GenBank Accession number
I82Gi	KJ134991
Ulvella leptochaete	EF595464
Ulvella leptochaete	EF595355
Ulvella repens	EF595449
Ulvella repens	EF595450
Ulvella viridis	EF595456
Ulvella viridis	EF595462
Ulvella heteroclada	EF595447
Ulvella heteroclada	EF595446
Ulvella operculata	AJ437663
Ulvella operculata	AJ437662
Blidingia minima	EF595512

Table II.1 GenBank accession number of algal sequences used in phylogenetic analysis

Determination of polyamines

Putrescine (Put), spermidine (Spd) and spermine (Spm) in the extracts were quantified in the free acid soluble and conjugated acid soluble amines. The method for extraction was those previously used in algae in our laboratory (Sacramento *et al.* 2007; Sacramento *et al.* 2004; Guzmán-Urióstegui *et al*. 2002; Marián *et al*. 2000a,b; Zarranz-Elso *et al*. 2012).

In short, tissue samples (600 mg) were powdered in a small mortar with liquid nitrogen. Polyamines were extracted with 5% perchloric acid (PCA) in a proportion of 1.5 ml (PCA) : 600 mg of material (fresh weight). The supernatants, obtained by centrifugation (20 min, 9000g at 6 ºC) of said extract, contain acid-soluble fractions (free and conjugated). This supernatant of 260 µl were reserved (free fraction) for derivatization and 300 µl (bound fraction) were hydrolyzed in flame sealed glass vial with an equal volume of hydrochloric acid 12M (HCl) at 100 ºC overnight. HCl was evaporated and the residue was resuspended in 260 µl of PCA. Dansylation prior to analysis by High-Performance Liquid Chromatography (HPLC) was performed according to the method described below. In the dansylation of PAs for HPLC, each sample was supplemented with 40 µl of internal standard, 1,7 diaminoheptane

(HTD) (Sigma-Aldrich) 0.05 mM at 6.66 µM final concentration to correct for the loss of polyamines during derivatization. 200 µl of a saturated solution of Na_2CO_3 and 400 µl of an acetone solution of dansyl chloride (5 mg/ml acetone) were added to each sample. The dansylation reaction proceeded overnight at room temperature. To remove the excess of dansyl chloride, 100 µl of an aqueous solution of proline (100 mg:ml) was added. When the reaction had been completed, after 30 minutes, samples were mixed with 500 µl of toluene, vortex was applied and organic and aqueous phases were separated by centrifugation (7 min, 9000 g). Finally 400 µl of supernatants were evaporated and resuspended in 800 µl of acetonitrile, for HPLC analysis.

The dansyl-PAs were quantified by HPLC, using the method described by Marcé *et al.* (1995). PAs were analysed in a 5µm reverse phase column (Varian C-18) and were detected through a detector (Varian ProsStar) by fluorescence at 365 nm (excitation) and 510 nm (emission). To determine the retention time and the response factor, several standard solutions were prepared of the three PAs (Put, Spd and Spm) together with HTD to calculate the response factor.

Statisical analysis

The number of replicates in which the results are based are shown in each experiment. The data were statistically analyzed (SPSS V.19, Chicago, IL). The means ± standard deviation (SE) were calculated and subjected to a Student´s *t*-test (unpaired samples), a non parametric Mann-Whitney (Wilcoxon) U test or 1-way ANOVA test and Tukey´s post hoc test. Statistical analysis were used to determine significant differences in PAs content in the different experiments; $p < 0.05$.

Effect of infection by *U. leptochaete* on endogenous polyamines levels of *G. imbricata*

The objective is to determine whether free and bound PAs levels change significantly during the course of infection in tissues of *G. imbricata* colonized by *U. leptochaete.*To do this, thalli of *G. imbricata* were collected from a natural bed located at the northeast coast of Gran Canaria, Canary Islands, Spain. Thalli, without visible epiphytes or endophytes, neither reproductive structures and showing no damage from herbivore's attacks, were selected under the stereomicroscope as the control group. Infected thalli with filamentous green algae growing as epiphytes (which grew isolated in a culture

and identified as *U. leptochaete*) were selected as the infected group. The experiments consisted of 7-8 replicates.

Both plant materials were cleaned and sonicated with ultrasond three times in sterile seawater to eliminate other epiphytes. The samples were disinfected by immersing them in a 7 % iodine solution as described (Robaina et al. 1990a,b). Algae were acchimatized to laboratory conditions and cultured in sterile seawater for 24 h to reduce variability between individual thalli before they were used for the experiments

Effect of MeJA on polymines levels in tissue of *G. imbricata*

Entire thalli of *G. imbricata*, 0.6 g fresh weight (FW), were cultived for 12 h in 50 ml of sterile natural seawater with 30 µmol photon m^2s^{-1} at 18 ± 2ºC, and concentrations of 100 and 500 µM of methyl jasmonate (MeJA) (Sigma-Aldrich). Inmediately after this period, samples were frozen in liquid nitrogen and stored at -80 ºC until extraction of PAs.

Methyl jasmonate (Sigma-Aldrich) was dissolved in ethanol and diluted later with sterile double distilled seawater. Control samples were incubated with a final ethanol concentration equal to

that provided by the stock of MeJA (0.002 % final concentration in the medium). The experiments consisted of 4-5 replicates.

Effect of H_2O_2 on endogenous polyamine levels of *G. imbricata*

Thalli of *G. imbricata*, 0.6 g FW, 5-6 replicates for each treatment, without visible epiphytes nor endophytes or herbivore beats attack, and without sexual structures, were incubed 12 h in 50 ml of sterile natural seawater at 30 µmol photon m^2s^{-1}, and 18 ± 2ºC 50 ml sterilized seawater with 50 µM, 5 mM and 50 mM H_2O_2. After 12 h, they were immediately frozen in liquid nitrogen and stored at -80 ºC. Algae were acchimatized to laboratory conditions and cultured in sterile seawater 24 h to reduce variability between individual thalli before they were used for the experiments.

Determination of H_2O_2 in seawater

The concentration of H_2O_2 was measured in seawater with a continuous flow injection analysis instrument (FeLume, Waterville Analytical, USA) using the chemiluminescense reaction of 10-methyl-9-(ρ-formylphenyl) acridiniuym carboxylate trifluoromethane-sulfonate (acridinium ester, AE) with H_2O_2 (King *et al*. 2007). Concentrations of H_2O_2 were determined by a calibration curve. Each measurements were done by triplicate.

AE reagent (1µM) was prepared in MQ water (18MΩ). AE solvent was buffered to pH 3 with 1 mM phophate buffer. MQ water was treated with 3 mgL^{-1} catalase (Sigma) for at least 30 min to avoid the H_2O_2 traces. 0.1 M Na_2CO_3 (Sigma-Aldrich) buffer was prepared in MQ water at pH 11.3. All the reagents were trace metal grade.

In vitro H_2O_2 scavenging activity of the polyamines was studied by addition of different concentrations of putrescine (ranging 10^{-6} M to 10^{-1} M) in 50 ml of sea water containing H_2O_2. H_2O_2 was added to the sea water to final concentrations of 50, 100, 500 and 1000 nM. Measurements were performed in triplicate, immediately after homogenization the solution. Kinectics studies were performed for two concentration of putrescine ($5x10^{-3}$ M and 10^{-4} M) in seawater containing H_2O_2 levels at concentrations of 500 and 1000 nM. H_2O_2 concentration in the medium was measured every 20 minutes for two hours.

RESULTS

Isolation of epi-endophytes in *G. imbricata*

Epi-endophytes growing in association with G. *imbricata* were isolated in PES medium (Fig II.2). Algae sprouted from the explants shown to have a low incidence in culture. Eventually produce a drastic increase of infection affecting practically all the explants.

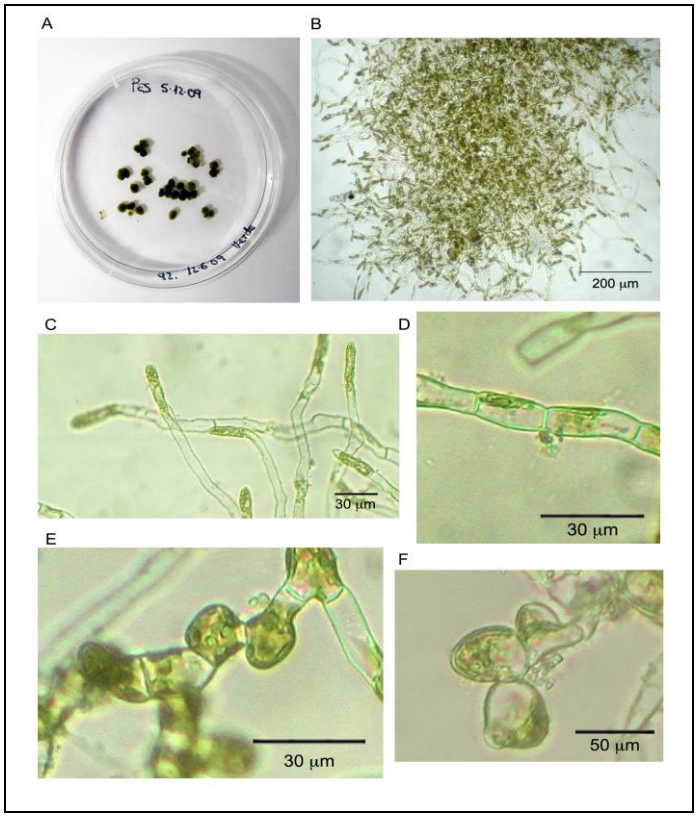


Fig II. 2 (A) *U. leptochaete* free-living culture isolated from *G. imbricata* (B) Plant in culture, showing general morphology (C) Fragment from larger thallus showing continued growth (D) Vegetative cells showing the parietal chloroplast (E) Cells showing pyrenoids (F) Sporangium

Taxonomic assignment of green epi-endophytes

A highly variable ITS2 region and adjacent 5.8S and 28S rDNA gene were obtained by PCR amplification of a 211 pb fragment. The determined sequence was deposited into GenBank with accession No. KJ134991. Three isolates were randomly selected and three identical sequences were obtained.

The sequence KJ134991 was grouped into a branch containing two *U. leptochaete* sequences (EF595464 and EF595355) in a tree based on ML/MP analysis. The ML and MP trees showed that the two sequences of the *U. leptochaete* form a single monophyletic clade with our sequences, which was supported by 100 % (ML tree) and 100 % (MP tree) bootstrap values (Fig II.3).

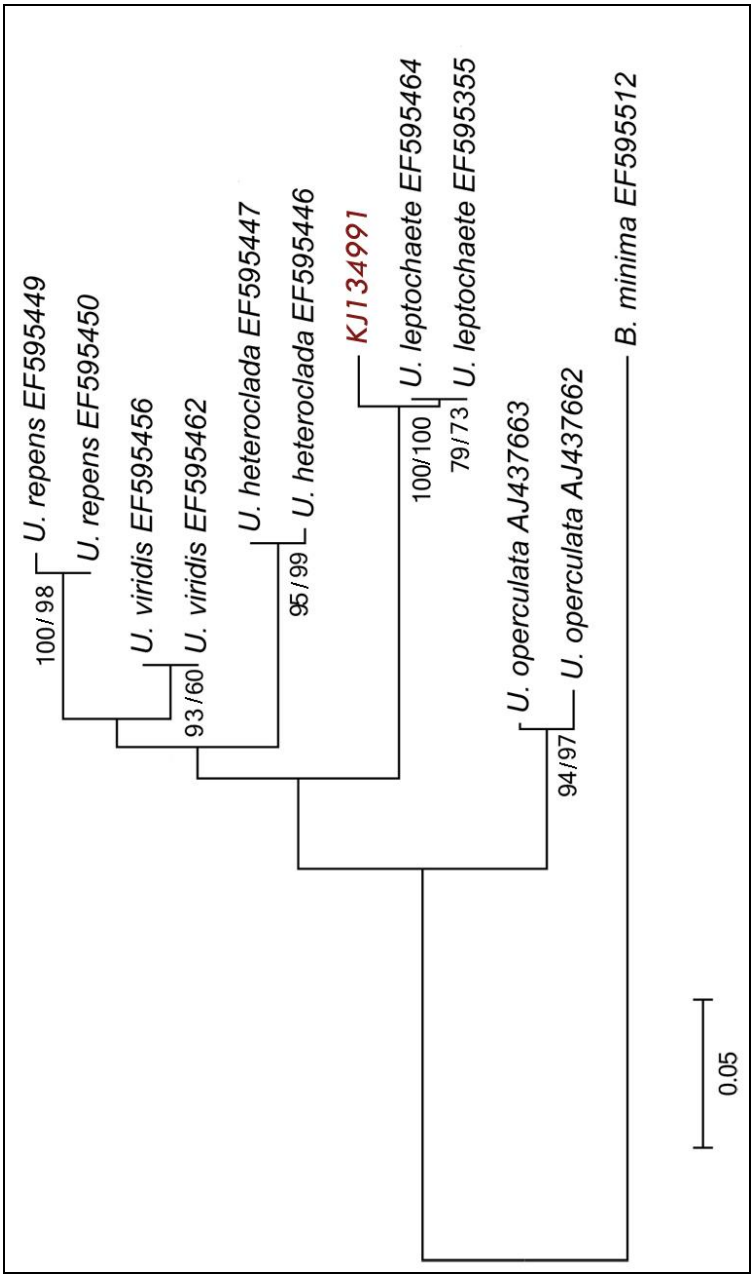


Fig II. 3 Tree phylogenetic from ML/MP analysis infered from partial 5.8S rDNA gene, ITS2 region and the partial 28S rDNA gene sequence, showing the relationship between species isolated from *G. imbricata* with species representatives of the genus *Ulvella*

Effect of the presence of *U. leptochaete* on polyamine levels of *G. imbricata*

The effect of the epi-endophytic alga *U. leptochaete* on the production and accumulation of PAs in the host *G. imbricata* was studied. The levels of PAs in the algal samples are shown in (Fig II.4)

PAs analysis indicates that total PAs concentration significantly increased in the thalli of the specimens subjected to the effect of the infection. Thereby, the concentration of total polyamines in uninfected control samples was (0.0153 ± 0.0006 µmol g^{-1} fresh wt) whereas those levels increased to (0.06 ± 0.01 µmol g^{-1} fresh wt) in samples where *U. leptochaete* was growing epiphytically, $p < 0.05$.

The Putrescine is the amine that is present in higher concentrations in both the control and infected samples. In addition, the concentration of putrescine was significantly affected by the presence of infection. Levels of total putrescine increased close to 2-fold in infected samples compared to that in the control ($p < 0.05$) (Fig II.4A). Different type of PAs were also measured as free and bound-soluble fractions (Fig II.4B and 4C). The results demonstrated that the presence of infection in *G. imbricata* significantly increased ($p < 0.05$) the concentration of both fractions of PAs. The bound-soluble PAs were more abundant than free soluble PAs for all the studied

cases. This increasing was a factor of 3 for free-putrescine and 2 for bound-soluble putrescine, in respect to those in the control experiments, where *G. imbricata* grew without any epiphytes. However, no significant differences were found in the levels of spermine and spermidine.

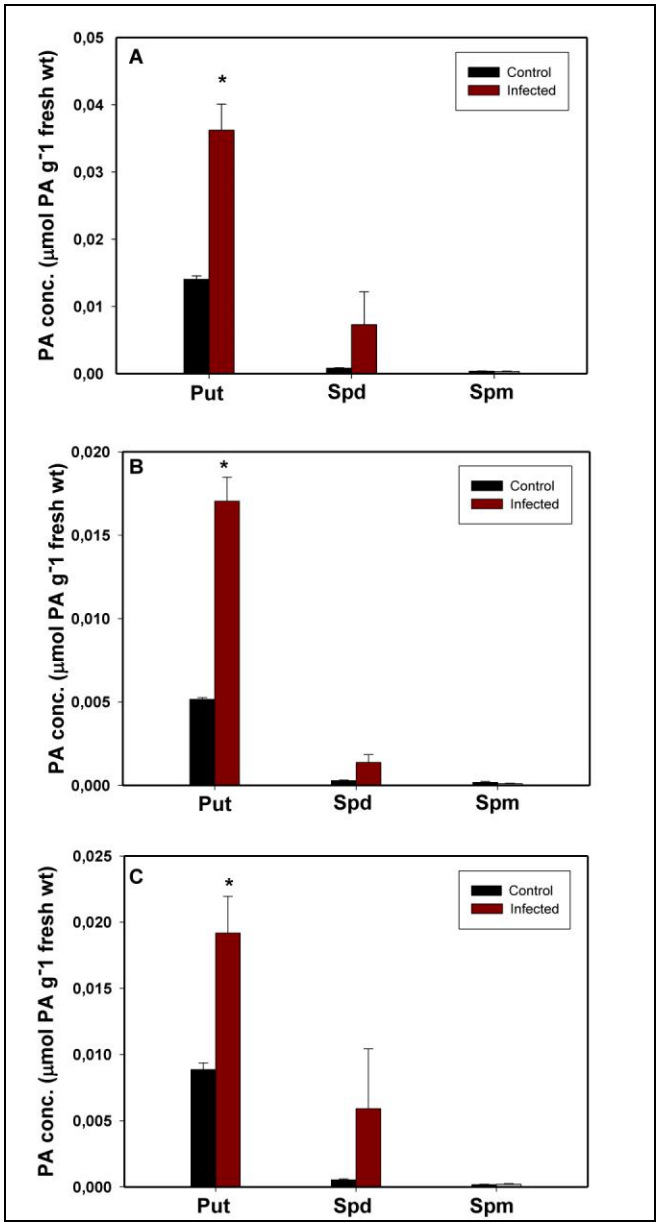


Fig II.4 Endogenous polyamines concentration (Mean ± SE) in control samples free of epiphytes and infected samples. (A) Total endogenous PAs content; (B) Free PAs fraction; (C) Bound-soluble PAs fraction. (*) Show significant differences from Student´s t-test ($p < 0.05$)

Effect of MeJA on polyamines levels of *G. imbricata*

The adition of 100 µM MeJA to thalli of *G. imbricata* did not produce any significant effect on levels of free endogenous PAs (Fig II.5.A). In contrast, the concentration of bound-soluble putrescine showed over-accumulation of approximately twice the baseline level after 12 hours of incubation.

MeJA induced over-acumulation of free and bound putrescine when the concentration increased to 500 µM of MeJA afters 12 hours of incubation. These results showed close to a 4 fold increase) (p<0.05) in the levels of free putrescine in respect to the control experiments (Fig II.6A). The level of bound-soluble putrescine also increased 2-fold compared to the control samples (Fig II.6B).

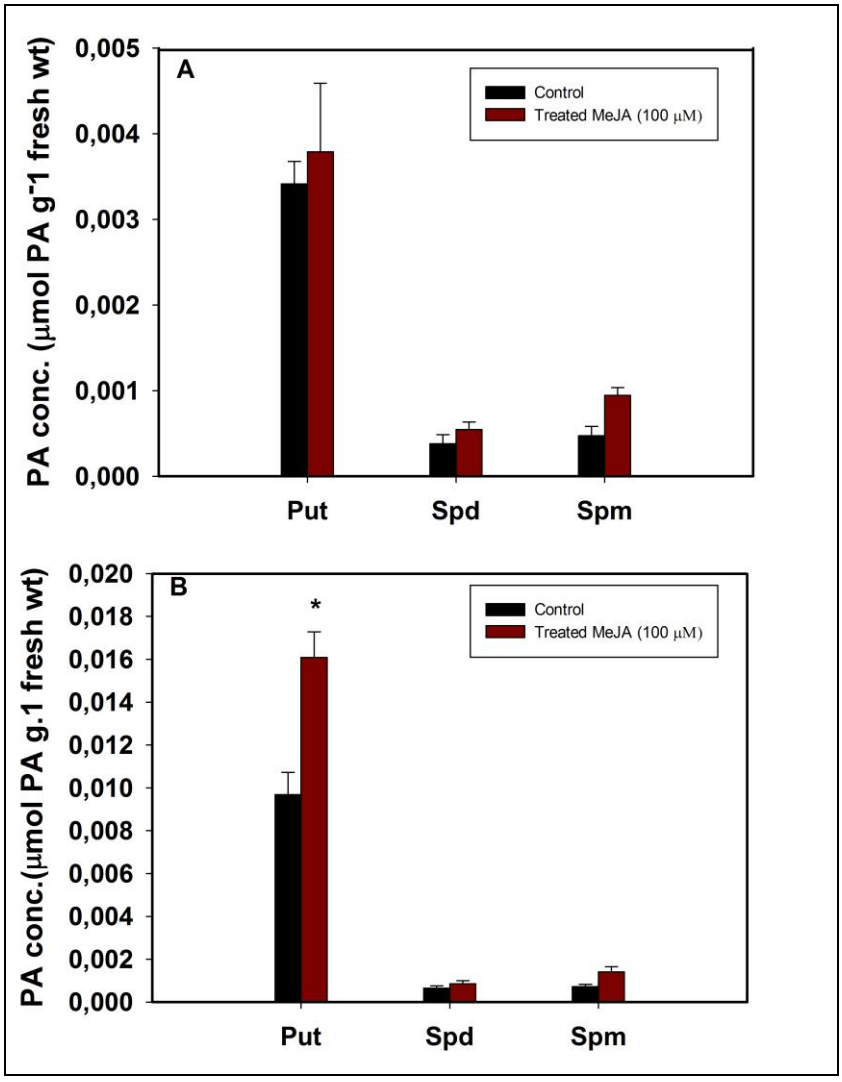


Fig II.5 Effects of treatment for 12 h with 100 µM MeJA on the endogenous polyamines concentration (Mean ± SE) of *G. imbricata*. (A) Free PAs fraction; (B) Bound-soluble PAs fraction. (*) Show significant differences from Student´s t-test ($p < 0.05$).

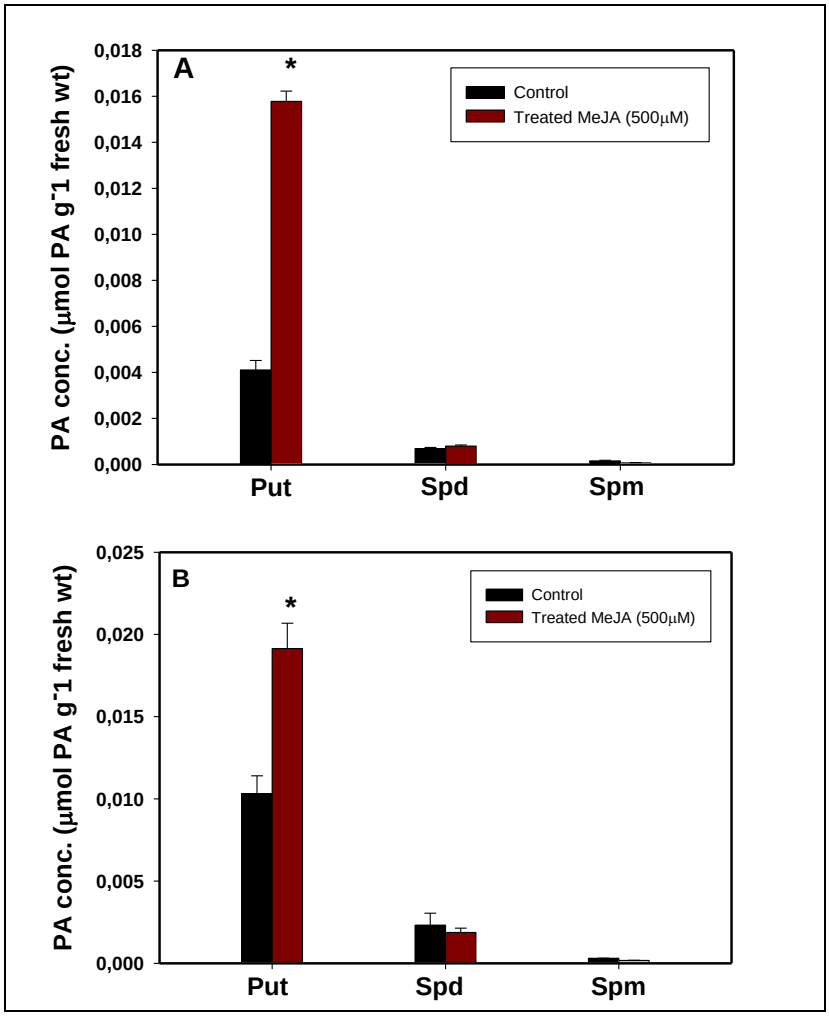


Fig II.6 Effects of treatment for 12 h with 500 µM MeJA on the endogenous polyamines concentration (Mean ± SE) of *G. imbricata*. (A) Free PAs fraction; (B) Bound-soluble PAs fraction. (*) Show significant differences from Student´s t-test ($p < 0.05$).

Effect of H_2O_2 on the production of polyamines by *G. imbricata*

When 50 µM of H_2O_2 was added to the cultures, no significant variations were seen in the endogenous levels of free polyamines (Fig II.7A) or in bound-soluble polyamines (Fig II.7B) in *G. imbricata* compared to untreated samples.

Concentrations of 5 mM of H_2O_2 increased the levels of free soluble putrescine ($p < 0.05$) in thalli of *G. imbricata*. No significant diferences were obtained in the concentrations of spermidine and spermine in respect to the controlled experiments where thalli was not treated (Fig II.7A). There were no significant changes observed in the levels of polyamines conjugated at the concentrations of 5 mM H_2O_2 (Fig II.7B).

When thalli were exposed to higher levels of H_2O_2 (50 mM), the concentration of free soluble putrescine, spermidine and bound-soluble putrescine showed a marked decrease.(Fig IV.4B). Due to these high levels of H_2O_2, the thalli were bleached and clearly dying.

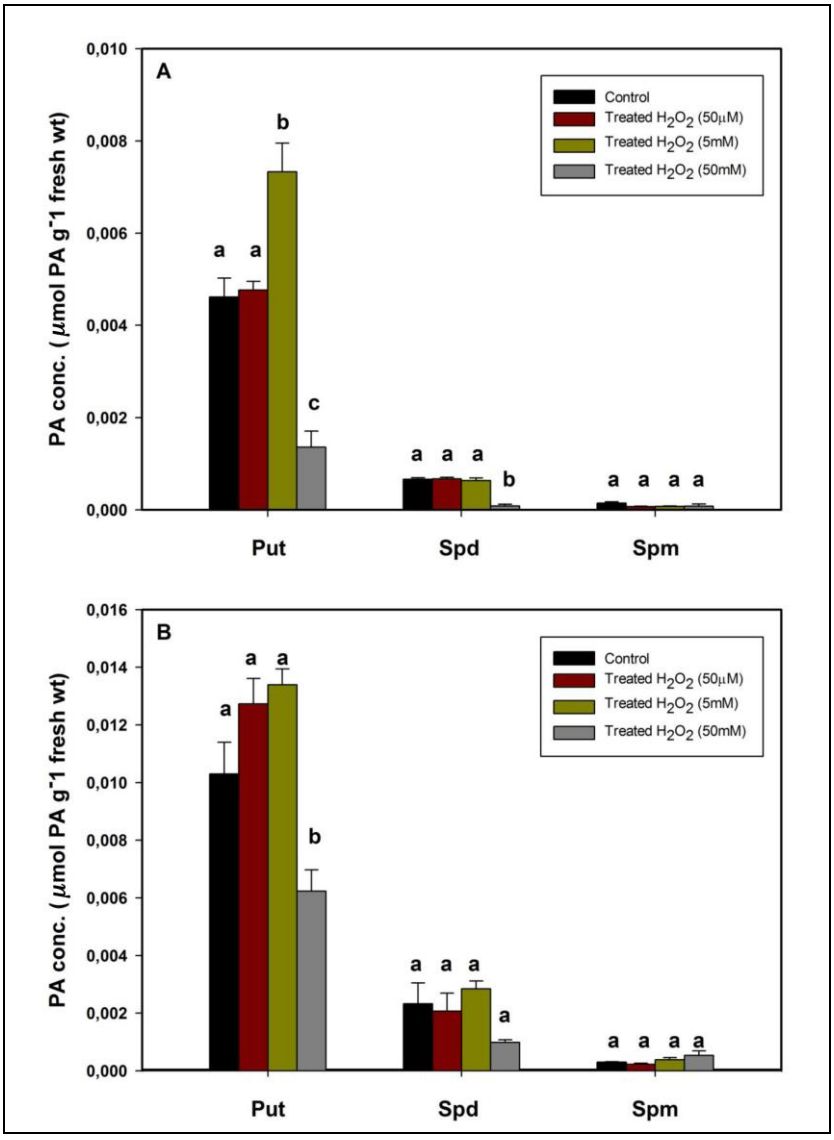


Fig II.7 Effects of treatement for 12 h with H_2O_2 on the endogenous polyamine concentration (Mean ± SE) of *G. imbricata*. (A) Free PAs fraction; (B) Bound-soluble PAs fraction. (a,b,c) Show significant differences from ANOVA and Tukey´s post hoc tests ($p < 0.05$)

Putrescine as H_2O_2 radical scavengers

In order to demonstrate if PAs can act as scavengers for H_2O_2 in a solution, putrescine was selected as a reference PAs to measure the concentration of H_2O_2 in the presence of putrescine (0 - 10^{-1} M) at pH 8.0 ± 0.1 and at a constant temperature (25 ± 0.05 ºC) (Fig. II.8).

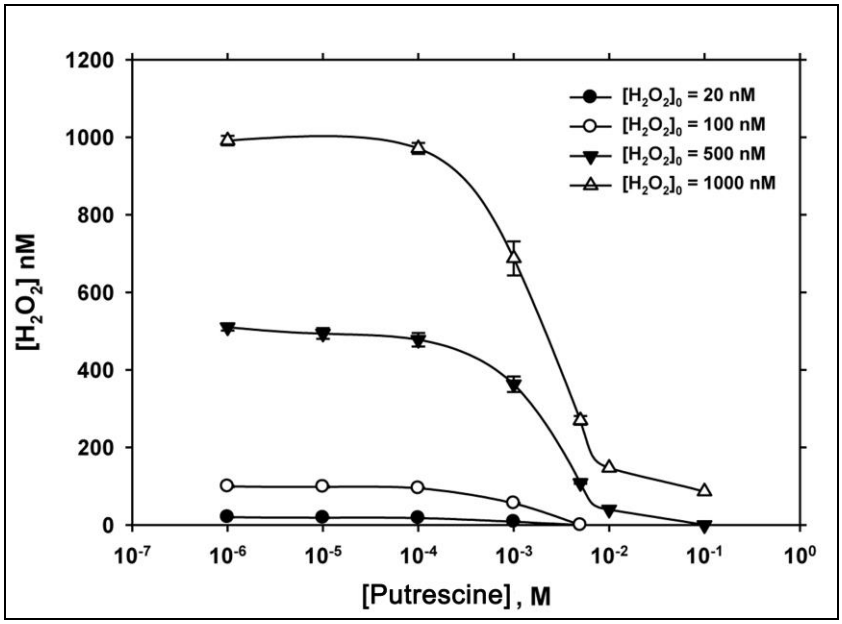


Fig II.8 Effect of the putrescine on H_2O_2 concentration in samples of seawater. Putrescine at indicated concentrations (raging 10^{-6} to 10^{-1} M) were added to 50 ml of seawater containing H_20_2 ranging 20 to 1000 nM

The concentration of H_2O_2 decreased in the solution as an initial concentration of putrescine also increased. However, half-descomposition of H_2O_2 is only achieved when the concentration of putrescine is from 3 to 6 orders of magnitude above the concentration of H_2O_2. In this sense, when the concentration of H2O2 was 20 nM, 0.91mM of putrescine was needed to reduce the concentration of H_2O_2 50%. In addition, 1.3, 2.29 and 2.34 mM of putrescine was enough to achieve the 50% descomposition of H_2O_2 when it was at 100, 500 and 1000 nM. (Fig II.9).

The reaction of scavenging for H_2O_2 by putrescine is instantaneous and after this reaction, the H_2O_2 levels were stable over time. Kinectics studies were performed for two initial concentrations of putrescine from 0 to 5 mM.

These studies help to determine the scavenging of H_2O_2 when the concentration of diamine putrescine is found just above or just below the concentrations that produce the elimination of 50% of the peroxide present in the medium according to Fig II.9. These results demonstrated the huge rate of the reaction between H_2O_2 and putrescine in seawater.

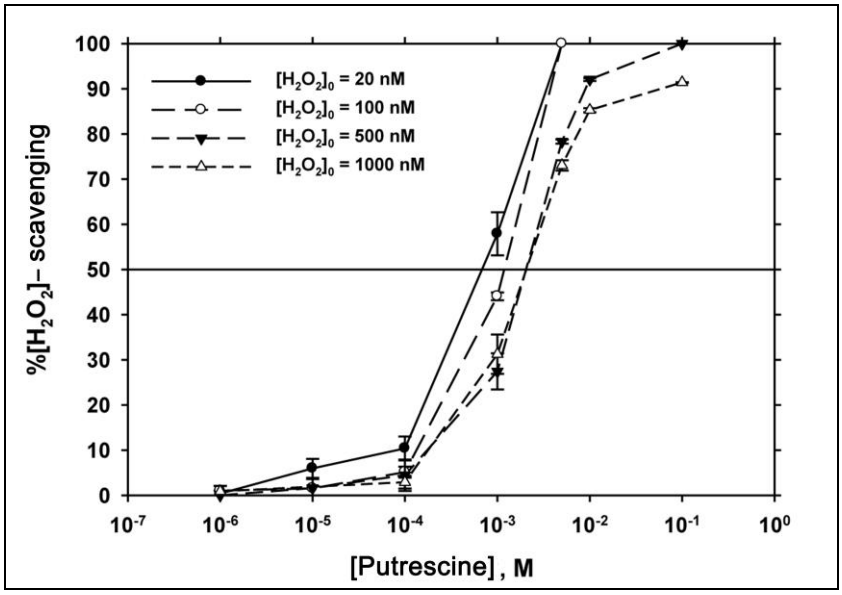


Fig II.9 Percent of scavenging of H_2O_2 by the effect of the addition of different concentrations of putrescine (ranging 10^{-6} to 10^{-1} M) in seawater. The initial concentrations of H_2O_2 were 20, 100, 500 and 1000 nM.

When H_2O_2 was 562 ± 13 nM, the addition of 0.1 mM of putrescine, produced that H_2O_2 decreased 4% (539 ± 9 nM). In addition 5 mM of putrescine decreases H_2O_2 by 76% in respect to the control experiments (Fig II.10).

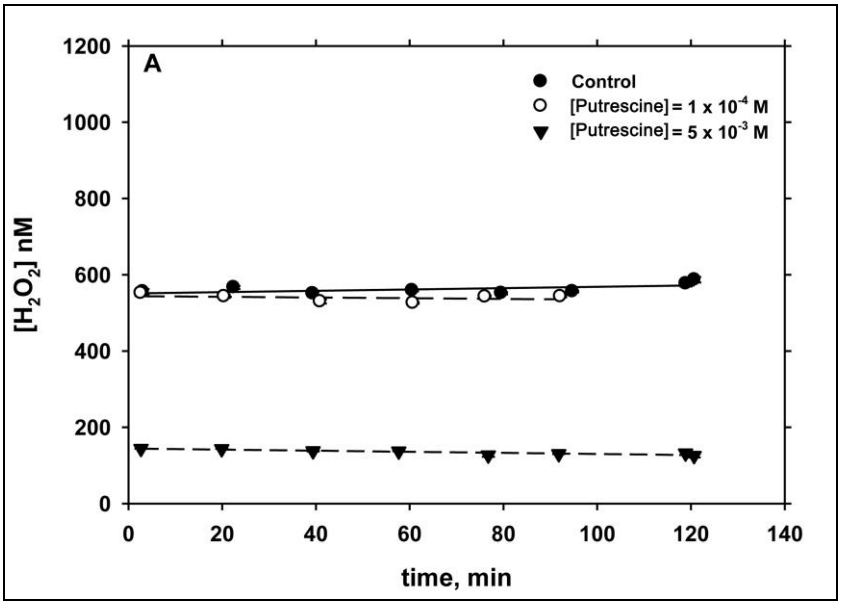


Fig II.10 Kinetics of the radical scavenging activity of putrescine on H_2O_2 in seawater. Effect of the addition of putrescine (10^{-4} and 5 x 10^{-3} M) on the initial concentration of H_2O_2 (562 ± 13 nM).

Considering an initial concentration of H_2O_2 970 ± 22 nM, the addition of 0.1 and 5 mM of putrescine generates a descomposition of H_2O_2 which makes the concentrations decrease by 7% and 76% respectively (Fig II.11).

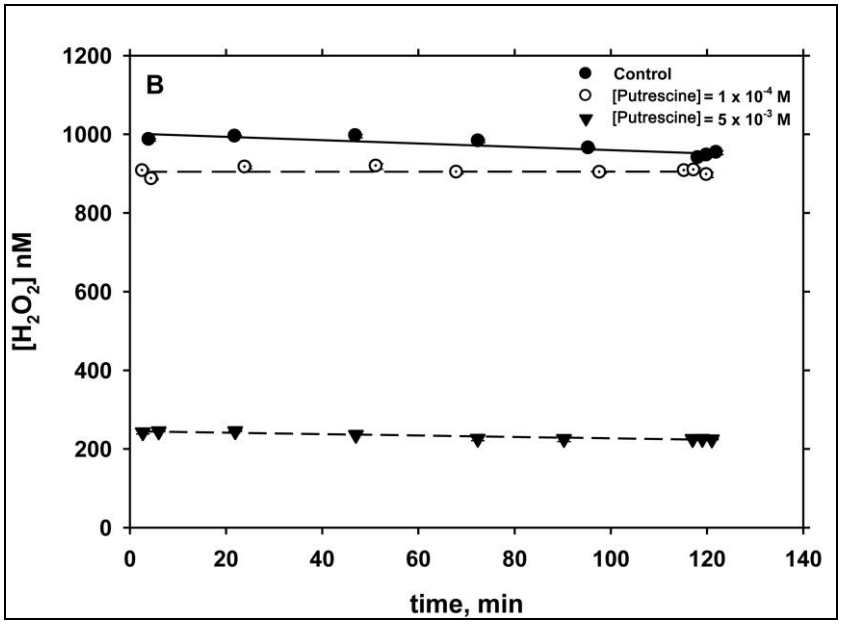


Fig II.11 Kinetics of the radical scavenging activity of putrescine on H_2O_2 in seawater. Effect of the addition of putrescine (10^{-4} and 5 x 10^{-3} M) on the initial concentration of H_2O_2 (970 ± 22 nM)

DISCUSSION

U. leptochaete an ubiquitous epiphyte that become an infectious endophytic algae

The present study has shown the presence of the *U. leptochaete* (Huber) Nielsen, a species of *Chaetophoraceae* (Chlorophyta) living as an epi-endophyte associated with *G. imbricata*. During our laboratory *in vitro* culture of the macroalgae *G. imbricata*, it was possible to isolate a green filamentous algae which were maintained in an unialgal culture under controlled conditions of photoperiod and temperature.

Molecular phylogeny analyses were based on the internal transcribed spacer (ITS2) region. The suitability of using ITS molecular markers to identify filamentous algae epiphytes and endophytes has been amply demonstrated in previous studies (Burkhardt & Peters 1998; Peters & Burkhardt 1998; Peters & Ramírez 2001; Bown *et al*. 2003; Deng *et al*. 2012; Rinkel *et al*. 2012). In a tree using Maximum Likelihood and Maximum Parsimony methods, confirmed that the isolated filamentous green algae belong to the species *U. leptochaete*. Epiphytic and endophytic green algae comprise one of the most diverse and phylogenetically primitive

groups of green algae and are considered ubiquitous in the world's oceans (Bast *et al.* 2014).

Genus *Ulvella* consists of some of the most ubiquitous epi or endophytic green algae found growing endophytically in another plant), epi-endophytically (wholly or partially with other plants), endozoically (in animals) (Nielsen & McLachlan 1986; O'Kelly *et al*. 2004a,b). It can be seen on a range of seaweed hosts, including green, brown and red algae where it commonly occurs as endophytes or epiphytes on other algae hosts (Nielsen *et al.* 2013; Rinkel *et al.* 2012). The review of host associations indicates that some *Ulvella* species are host generalist (*U. leptochaete*, *Ulvella viridis*), and others are more host specific (*U. operculata* with *C. crispus*) (Correa & McLachlan 1991).

U. leptochaete (Huber) Nielsen is an epi-endophyte belonging to this genus, with previous reports decribing it living as an epiphyte on species of the genera *Chaetomorpha*, *Cladophora* and *Ceramium* as described by Huber (1982) under the name *Endoderma leptochaete*. Deng *et al*. (2012) cited *U. leptochaete* as an endophyte on the macroalgae of the genus *Chaetomorpha* under the name *A. leptochaete*. Kim *et al.* 2014 cited infection by *U. leptochaete* growing as an endophyte in the field populations of *Grateloupia* lanceolata,

Grateloupia ellíptica and *Grateloupia. turuturu* reporting high frequency of infections at around 85%.

Our observations in culture show that *U. leptochaete* initially behaves as an epiphyte but with endophytic filaments growing inside the host tissues during late *in vitro* culture. *U. leptochaete* has shown to have a low incidence of infection in explants of *G. imbricata* when these are appropriately treated to ensure its asepsis but eventually produce a drastic increase of infection affecting practically all the explants belonging to this lot. *U. leptochaete* requires an initial epiphytic stage in *G. imbricata* and then it is able to invade the inside of the host as an endophyte, similarly described for *U. heteroclada* (Correa 1990).

Most of the knowledge we have about the effects of endofitism in macroalgae has been obtained using endophytes belonging to clorophyta division and specifically of the genus *Ulvella*. Although many of these relationships have no impact on the host during early stages, in more advanced stages of infection, they cause severe changes in the host tissues such as deformation or necrosis (Correa *et al.* 1987,1988; del Campo *et al.* 1988; Correa & McLachlan 1992; Craige & Correa 1996; Bouarab 2001).

Ulvella geniculata under the name *Acrochaete geniculata* is associated with the presence of destructive disease in *Ulva rigida*, characterized by green spots and perforations of the host thalli (Del Campo *et al*. 1998). *U. operculata* and *U. heteroclada* are pathogens of *C. crispus*, and their effects on the host can be the primary cause of disfunctions due to invasion of the cortical tissue (Correa 1990) which eventually results in the demise of infected fronds or as agents facilitating secundary infections by other pathogens (Correa & McLachlan 1992).

U. operculata is a primary invasive organism of *C. crispus*, with direct penetration through the host's structurally intact outer cell wall inmediately after zoospore settlement. It seems that the mechanism of host penetration involves digestion of the host cell-wall components (Correa & McLachlan 1994).

From epiphytic settlement to the infection: The chemical battle polyaminas and H_2O_2

Associations between red algae and endophytic pathogens provide examples of host recognition through perception of cell wall galactans. The endophytic green algae *Endophyton ramosun*, the causative agent of green patch disease in the red algae *Mazzarella laminarioides*, discriminates between agarophytes and

carrageenophytes and is unable to penetrate or develop normally on agarophytic algae (Sánchez & Correa 1996). Even more specifically, *U. operculata*, infects carragenophytes containing λ-carrageenan more successfully than carrageenophytes containing κ-carrageenan (Correa & McLachlan 1991). κ and λ-type carrageenans were shown to control endophyte penetration increasing or reducing the endophyte virulence (Bouarab 1999).

There are various studies which indicate that the red algae are able to perceive potentially pathogenic organisms and their activity and to respond to them. Pathogens activate or induce macroalgal defense mechanisms. When algae are infected by pathogens they respond by activating mechanisms to decrease the potencial damage created by the pathogens. Defence reactions involves an oxidative burst by releasing reactive oxygen species such as H_2O_2 (Küpper *et al.* 2002; Weinberger & Friedlander 2000; Bouarab *et al.* 1999, 2001).

It has been shown that red algae of the genus *Gracilaria* have the capacity to perceive agar oligosaccharides that are released during enzymatic attacks by opportunistic pathogens upon their cell wall matrix (Weinberger et al 1999). The physiological response of *Gracilaria* resulted in an elimination of associated epiphytic bacteria, and in particular of agar degraders (Weinberger and Friedlander

2000). An incrasing number of works have shown the emission of ROS following inducer recognition mediates host-pathogen interactions in algae. *C. crispus* cell wall matrix polysaccharides include either κ or λ-type carrageenans, in gametophytes and tetrasporophytes respectively. The different fractions of polysaccharides modulate the endophyte penetration and determine the virulence of the endophyte *U. operculta* on each of the different forms of the host, with λ-carrageenans increasing and κ-carrageenans reducing the endophyte virulence. Thus the virulence of the green algal pathogens *U. operculata* is mediated by the recognition of carrageenan oligosaccharides released from its red algal host (Bouarab *et al*. 1999, 2001). λ-carrageenan oligosaccharides induced the release of H_2O_2, stimulated protein synthesis, increased carragenolytic activity, and induced specific polypeptides in the pathogen, resulting in a marked increase in pathogenicity. However, κ-carrageen oligosaccharides did not induce the release of H_2O_2 from *U. operculata* but hindered amino acid uptake and enhanced their recognition by the host, hence its less virulent. Gametophytes exhibits a large burst of H_2O_2, while only low levels were released from the sporophytes (Bouarab *et al.* 1999).

Hydrogen peroxide is shown to be a key molecule in the host/pathogen interaction in macroalgae. It is interesting to know the possible role of polyamines in the relationship between *G. imbricata* and *U.leptochaete* and their possible interaction with H_2O_2.

Our work shows a significant increase in the diamine putrescine (both in free form and in its conjugated form) during the initial stage of infection in *G. imbricata* by the epi-endophyte *U. leptochaete*. The stress caused by damage to host tissues during the penetration of the pathogen alters the levels of polyamines in the host and this phenomenon could play an important role in the defense mechanisms against infection. Diamine putrescine could be oxidatively deaminated by the action of diamine oxidase (DAO) releasing hydrogen peroxide as well as a poliamine oxidase (PAO) action on spermidine and spermine, contributing to hinder the penetration of the infecting agent.

Polyamines are a common source of hydrogen peroxide in host- and non-host hypersensitive responses during pathogen infection in terrestrial plants (Yoda *et al.* 2009). Plants have developed specific defense systems which are effective against attacks from a wide range of pathogens (Staskawicz *et al.* 1995; Heath 2000). The hypersensitive response (HR) is a powerful resistance system against these attacks.

Stakman (1915) described the hypersensitivity as a rapid, localized necrosis of host cells at the infecction site. It is characterized by the rapid death of a limited number cells in the vicinity of the invading pathogen (Heath 2000). The HR is initiated by a recognition of the pathogen attack and is also associated with a rapid production and accumulation of reactive oxygen species (Low & Merida 1996) like H_2O_2. There two types of HR, one in which a plant recognizes avirulent proteins derived from pathogens. This type of HR is called host HR (Hammond-Kosack & Jones 1997). Another type of HR is non-specific to particular pathogens (Heath 2000). Yoda *et al.* (2009) suggested that polyamines are commonly utilized as the source of H_2O_2 in both defense mechanisms, confirming that polyamines were accumulated in the apoplast of *Arabidopsis thaliana* infected with *Pseudomonas syringae*, and of rice infected with *Magnaporthe grisea*.

Torrigiani *et al.* (1997) investigated the possible involvement of polyamines in the tobacco virus (TMV) induced by a hypersensitive reaction (HR) in *Nicotiana tabacum* which resulted in increased concentrations of free and conjugated putrescine and spermidine, as well as the activities of their biosynthetic enzymes activities, particularly in the necrotic lesions. Using the same system, Marini *et al.* (2001) confirmed the results above and determines that the

hypersensitive response is accompanied by an increase in enzyme activity of the DAO.

Negrel *et al.* (1984) showed that the activity of ornithine decarboxylase (ODC) is increased 20 fold in leaves of *Nicotiana tabacum* following infection with the tobacco mosaic virus. In contrast, no altered levels of polyamines were found. Cowley & Walters (2002) examined polyamine levels and activities of enzymes of polyamine biosynthesis and catabolism during the hypersensitive reaction of the barley to the powdery mildew fungus *Blumeria graminitis* f. sp. hordei. Levels of free putrescine and spermine and conjugated forms of putrescine, spermine and spermidine were greatly increased after inoculation. These increased levels of polyamines are accompanied by elevated activities of polyamine biosynthetic enzymes and the polyamine catabolic enzymes, diamine oxidase (DAO) and polyamine oxidase (PAO).

In this sense, Angelini *et al.* (1993) investigated diamine oxidase (DAO) activities and polyamine levels in chickpeas inoculated with conidia *Ascochyta rabiei* showing that the putrescine level and DAO activities increased markedly after infection. There was a greater enhancement of both in the resistant form of chickpeas regarding the form susceptible to infection. Thus, it is explained as a

possible role for polyamines and polyamine catabolism in resistance to pathogens.

Jasmonates and polyamines

Jasmonates are important signalling molecules in higher plants, including methyl jasmonate (MeJA) and jasmonic acid (JA). They are involved in many processes during plant development such as germination, root growth, fertility, fruit ripening and senescense (Sembdner & Parthier 1993; Creelman and Mullet 1995, 1997).

Plant responses to many abiotic stresses are orchestrated by jasmonates which play a important role in the response to water deficit and high or ultraviolet light (Creelman & Muller 1997, Weber 2002; Cheong & Choi 2003). Also, jasmonates play a dual role in plants. Several works have implicated jasmonates in the signalling pathway mediating induced defenses in pathogen attacked plants (Farmer & Ryan 1992; Creelman and Mullet 1995, 1997) by altering the expression of genes, they may be implicated in the signal transduction and elicitor induced responses (Blechert *et al*. 1995).

In the red alga *C. crispus,* exposure to MeJA up-regulated stress related genes and down regulated genes involved in general metabolism and energy conversion. MeJA or a related compound has a physiological role as a stress hormone in red algae (Collen *et al*.

2006). These results agree with other reports by (Gaquerel 2005) and (Bouarab *et al.* 2004) which suggest that MeJA triggers the oxidation of polyunsatured fatty acids, induces the activities of the two defence-related enzymes and confers resistance of *C. crispus* against the filamentous green algal endophyte *U. operculata.* In this sense, MeJA induces an oxidative burst in *Laminaria digitata,* which supposes an advantage, that favors the resistance against infection by the brown algal endophyte, *Laminariocolax* tomentosoides (Küpper *et al*. 2009)

The present study demonstrates that incubation with high concentration of MeJA produces accumulation of free and conjugated putrescine in *G. imbricata*. We hypothesized that this could be an alternative means by which the MeJA confers resistance to infection by filamentous algae such as those belonging to the genus *Ulvella*, according to what we have described above. Jasmonates have been shown to stimulate the production of secondary metabolites (Gundlach *et al.* 1992), including hydroxicinnamic acid amides (HCAs) (Lee *et al.*1997; Mader 1999; Biondi *et al.*2000). HCAs are formed from covalent binding of polyamines to hydroxycinnamic acids. In plants, the HCAs constitute the bulk of the acid-soluble polyamine pool (Flores & Martin-Tanguy 1991). It has been shown that after the attack of a pathogen agent, the polyamine levels, in its free and

conjugated forms, suffer severe changes in the infected tissues. Walters (2000) shows this phenomenon in leaves infected with pathogenic fungi. Jasmonates have the capacity to confer local and systematic protection in tomato and potato plants against *Phytophthora infestants* and *Pythium ultimum* (Cohen *et al.* 1993). Biondi *et. al.* (2001) show that MeJA up-regulated the expression of the genes related to the biosynthesis and the degradation of polyamines in tabacco plants. Similar results were presented by Mitchell and Walters (2003) who demonstrated that MeJA confers systematic protection to barley plants affected by pathogenic fungi, altering the metabolism of the polyamines in the treated barley leaves, which gives them protection (Walters *et al.* 2002).

H_2O_2 scavenging and PAs production

The possibility that H_2O_2 had an effect on PAs levels in *G. imbricata* has been studied and it has been shown that sufficiently elevated concentrations of H_2O_2, tissues respond with an increase in the concentration of putrescine in its free form approximately two times that in respect to the control samples. In excessively high concentrations of H_2O_2 (50 mM), the thalli of *G. imbricata* are bleached and produce tissue death, drastically lowering the levels of putrescine in both studied forms. In addition, based on *in vitro* studies,

it has been demonstrated that the functions of diamine putrescine act directly as a free radical scavenger of H_2O_2, which could have an important role in minimizing tissue damages caused by H_2O_2 released by the pathogens during penetration. Concentrations of putrescine at mM levels produce the instant decomposition-elimination of H_2O_2 *in vitro*, when it is at concentrations as high as 1000 nM of H_2O_2. Concentraciones ranging from 10^{-6} at 10^{-3} M are usually employed *in vitro* experiments to determine the physiological effects of polyamines in species belonging to the genus *Grateloupia* (García-Jimenez et al. 1998; Sacramento et al. 2004).

Polyamines at very high concentration levels are known to scavenge superoxide radicals *in vitro* (Kafy et al. 1986) although the high concentrations used, make it difficult to give a biological interpretation of the results. Das & Misra (2004) did not find scavergers of superoxide radicals, however, and cited polyamines (eg. putrescine, spermidine, spermine and cadaverine) to be potent scavengers of hydroxyl radicals at physiological concentrations. It has been shown that spermine and spermidine act effectively on singlet oxygen. Putrescine provides defenses against oxidavite stress in *Escherichia coli* cell cultures exposed to H_2O_2, increasing the cell survival (Tkachenko *et al.* 2001). The addition of polyamines protects

genetically modified *E. coli* mutant cells from the toxic effect of oxygen when they have lost the ability to biosynthesize it (Chattopadhyay *et al.* 2003). In the same way yeast strains that have a reduced content of polyamines are more sensitive to oxidative damage (Chattopadhyay *et al.* 2006). Hernández *et al.* (2006) have shown the polyamines (spermine and spermidine) to function as protectors of membrane lipoperoxidation induced by H_2O_2 in *Trypanosoma cruzi*.

Investigations in free cell systems have demostrated that polyamines protect DNA from damage caused by reactive oxygen species. In this sense, spermine and spermidine protect plasmid DNA against single-strand breaks induced by singlet oxygen (Khan *et al.* 1992). The mechanism by which polyamines provide protection include direct scavenging of oxidant agents (Fujisawa & Kadoma 2005).

The results obtained in the pathogenic system *U. leptochaete* and *G. imbricada* also show an opposite scenery in which the potencial production of H_2O_2 by the pathogenic endophyte itself (or as a results of the infection) may be counteracted by the scavenging activity of polyamines, particularly putrescine that increased significantly in the infected algae. The kinetic studies suggest that the scavenging activity, although dependent of relative high

concentrations, may be nevertheless exerted very topical by concentrating the production or the accumulation of polyamines in the spots of infection.

In conclusion, *U. leptochaete* an apparently inoffensive epiphyte with low incidence in *G. imbricata* (as compared with *M. tenuissimum*), may became an infectious endophyte with time. The chemical combat trigger after infection may depend in the production of polyamines, which may counteract infection by producing H_2O_2 by its oxidation. MeJA may contribute also to enhance polyamines production. As opposite, the potential oxidative burst and H_2O_2 production caused by the endophye itself, or as a result of the infection process may be counteracted also by the H_2O_2-scavenging of polyamines. Epiphyte settlement and endophyte growth is one of the main devastating situation in macroalgal aquaculture to date, thus the possibility to manipulate polyamine (or related substances) production in the host algae as a treatment should be considered as a promising field for further studies in "phycopathology".

REFERENCES

Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Tiburcio AF (2010). Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta, 231:1237-1249.

Alcázar R, Marco F, Cuevas JC, Patron M, Ferrando A, Carrasco P, Altabella T (2006) Involvement of polyamines in plant response to abiotic stress. Biotechnology letters, 28: 1867-1876.

Angelini R, Bragaloni M, Federico R, Infantino A, Porta-Pugua A (1993) Involvement of polyamines, diamine oxidase and peroxidase in resistance of chickpea to Ascochyta rabiei. Journal of Plant Physiology, 142: 704-709.

Baker CJ, Orlandi EW (1995) Active oxygen in plant pathogenesis. Annual Review of Phytopathology, 33: 299-321.

Bast F, Bhushan S, John AA (2014) DNA barcoding of a new record of epi-endophytic green algae *Ulvella leptochaete* (Ulvellaceae, Chlorophyta) in India. Journal of biosciences, 39: 711-716.

Biondi S, Scaramagli S, Capitani F, Altamura MM, Torrigiani P (2001) Methyl jasmonate upregulates biosynthetic gene expression, oxidation and conjugation of polyamines, and inhibits shoot formation in tobacco thin layers. Journal of Experimental Botany, 52: 231-242.

Biondi S, Fornalé S, Oksman-Caldentey K-M, Eeva M, Agostani S, Bagni N (2000) Jasmonates induce over-accumulation of methyl putrescine and conjugated polyamines in *Hyoscyamus muticus* L. root cultures. Plant Cell Reports, 19: 691-697.

Blechert S, Brodschelm W, Hölder S, Kammerer L, Kutchan TM, Mueller MJ, Zenk MH (1995) The octadecanoic pathway: signal molecules for the regulation of secondary pathways. Proceedings of the National Academy of Sciences, 92: 4099-4105.

Bouarab K, Adas F, Gaquerel E, Kloareg B, Salaün JP, Potin P (2004) The innate immunity of a marine red alga involves oxylipins from both the eicosanoid and octadecanoid pathways. Plant Physiology, 135: 1838-1848.

Bouarab K, Potin P, Weinberger F, Correa J, Kloareg B (2001) The *Chondrus crispus-Acrochaete operculata* host-pathogen

association, a novel model in glycobiology and applied phycopatholgy. Journal of Applied Phycology, 13: 185-193.

Bouarab K, Potin P, Correa J, Kloareg B (1999) Sulfated oligosaccharides mediate the interaction between a marine red alga and its green algal pathogenic endophyte. The Plant Cell, 11: 1635-1650.

Bown P, Plumb J, Sanchez-Barcaldo P, Hayes PK, Brodie J (2003) Sequence heterogeneity of green (Chlorophyta) endophytic algae associated with a population of *Chondrus crispus* (Gigartinaceae, Rhodophyta). European Journal of phycology, 38: 153-163.

Burkhardt E, Peters AF (1998) Molecular evidence from nrDNA ITS sequences that *Laminariocolax* (Phaeophyceae, Ectocarpales sensu lato) is a worldwide clade of closely related kelp endophytes. Journal of Phycology, 34: 682-691.

Chattopadhyay MK, Tabor CW, Tabor H (2006) Polyamine deficiency leads to accumulation of reactive oxygen species in a spe2Δ mutant of *Saccharomyces cerevisiae*. Yeast, 23: 751-761.

Chattopadhyay MK, Tabor CW, Tabor H (2003) Polyamines protect *Escherichia coli* cells from the toxic effect of

oxygen. Proceedings of the National Academy of Sciences, 100: 2261-2265.

Cheong JJ, Do Choi Y (2003) Methyl jasmonate as a vital substance in plants. TRENDS in Genetics, 19: 409-413.

Chibi F, Matilla AJ, Angosto T, Garrido D, Bornman CH (1994) Changes in polyamine synthesis during anther development and pollen germination in tobacco (*Nicotiana tabacum*). Physiologia Plantarum, 92: 61-68.

Cohen Y, Gisi U, Niderman T (1993) Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic methyl ester. Phytopathology, 83: 1054-1062.

Collén J, Hervé C, Guisle-Marsollier I, Léger JJ, Boyen C (2006) Expression profiling of *Chondrus crispus* (Rhodophyta) after exposure to methyl jasmonate. Journal of experimental botany, 57: 3869-3881.

Correa JA (1990) Pigmented algal endophytes of *Chondrus crispus* Stackhouse: Host specificity, fine structure, and effects on host performance in infections by *A. operculata* Correa & Nielsen and *A. heteroclada* Correa & Nielsen. PhD Thesis, Dalhousie University, Nova Scotia: 272 pp.

Correa JA, McLachlan JL (1994) Endophytic algae of *Chondrus crispus* (Rhodophyta). V. Fine structure of the infection by *Acrochaete operculata* (Chlorophyta). European Journal of Phycology, 29: 33-47.

Correa JA, McLachlan JL (1992) Endophytic algae of *Chondrus crispus* (Rhodophyta). IV. Effects on the host following infections by *Acrochaete operculata* and *A. heteroclada* (Chlorophyta). Marine Ecology Progress Series, 81: 73-87.

Correa JA, McLachlan JL (1991) Endophytic algae of *Chondrus crispus* (Rhodophyta). III. Host specificity. Journal of Phycology, 27: 448-459.

Correa JA, Nielsen RN, Grund DW (1988) Endophytic algae of Chondrus crispus (Rhodophyta). II. *Acrochaete heteroclada sp.* nov. *A. operculata sp.* nov., and *Phaeophila dendroides* (Chlorophyta). Journal of Phycology, 24: 528-539.

Correa JA, Nielsen RN, Grund DW, McLachlan J (1987) Endophytic algae of Irish moss (*Chondrus crispus* Stackh.). Hydrobiology, 41: 223-228.

Cowley T, Walters DR (2002) Polyamine metabolism in barley reacting hypersensitive to the powdery mildew fungus

Blumeria graminis f. sp. *hordei*. Plant Cell Environ, 25: 461-468.

Craigie JS, Correa JA (1996) Etiology of infectious diseases in cultivated *Chondrus crispus* (Gigartinales, Rhodophyta). In Fifteenth International Seaweed Symposium. Springer Netherlands. pp 97-104.

Creelman RA, Mullet JE (1997) Biosynthesis and action of jasmonates in plants. Annual review of plant biology, 48: 355-381.

Creelman RA, Mullet JE (1995) Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proceedings of the National Academy of Sciences, 92: 4114-4119.

Das KC, Misra HP (2004) Hydroxyl radical scavenging and singlet oxygen quenching properties of polyamines. Molecular and cellular biochemistry, 262: 127-133.

Das S, Bose A, Ghosh B (1995) Effect of salt stress on polyamine metabolism in Brassica campestris. Phytochemistry, 39: 283-285.

Del Campo E (1998) Patología de macroalgas marinas: Endófitos, virus y metales pesados como agentes causales. Efectos fisio-

patológicos y epidemiología. Tesis Doctoral. Universidad de Las Palmas de Gran Canaria: 111 pp.

Deng Y, Tang X, Huang B, Teng L, Ding L (2012) Molecular identification and culture observation on *Acrochaete leptochaete* (Chaetophoraceae, Chlorophyta) from China. Chinese Journal of Oceanology and Limnology, 30: 476-484.

Deng Y, Tang X, Ding L, Lian S (2011) A new record from China of epiphytic marine algae, *Acrochaete leptochaete* (Chaetophoraceae, Chlorophyta) with its primary experimental biolgoy. Chinese Journal of Oceanology and Limnology, 29: 350-355.

Doke N, Miura Y (1995) *In vitro* activation of NADPH-dependent O_2-generationg system in a plasma membrane-rich fraction of potato tuber tissues by treatment with an elicitor form *Phytophthora infestans* or with digitonin. Physiological and Molecular Plant Pathology, 46: 17-28.

Drolet G, Dumbroff EB, Legge RL, Thompson JE (1986) Radical scavenging properties of polyamines. Phytochemistry, 25: 367-371.

Enyedi AJ, Yalpani N, Silverman P, Raskin I (1992) Signal molecules in systemic plant resistance to pathogens and pests. Cell, 70: 879–886

Evans PT, Malmberg RL (1989) Do polyamines have roles in plant development?. Annual review of plant biology, 40: 235-269.

Farmer EE, Ryan CA (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. The Plant Cell, 4: 129-134.

Flores HE, Martin-Tanguy J (1991) Polyamines and plant secondary metabolites. Biochemistry and physiology of polyamines in plants, 57-76.

Fujisawa S, Kadoma Y (2005) Kinetic evaluation of polyamines as radical scavengers. Anticancer research, 25: 965-969.

Gaquerel, E (2005) Bases biochimiques de l'immunité innée de l'algue rouge *Chondrus crispus*: caractérisation et rôle des oxylipines dans les mécanismes précoces de défense. PhD Thesis, Brest, 2005.

Galston AW, Sawhney RK (1990) Polyamines in plant physiology. Plant physiology, 94: 406-410.

Garcia-Jimenez P, Rodrigo M, Robaina RR (1998) Influence of plant growth regulators, polyamines and glycerol interaction

on growth and morphogenesis of carposporelings of *Grateloupia* cultured in vitro. Journal of Applied Phycology, 10: 95-100.

Gerats AG, Kaye C, Collins C, Malmberg RL (1988) Polyamine levels in Petunia genotypes with normal and abnormal floral morphologies. Plant physiology, 86: 390-393.

Groppa MD, Benavides MP (2008) Polyamines and abiotic stress: recent advances. Amino acids, 34: 35-45.

Gundlach H, Müller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proceedings of the National Academy of Sciences, 89: 2389-2393.

Guzmán-Urióstegui A, García-Jiménez P, Marián F, Robledo D, Robaina RR (2002) Polyamines influence maturation in reproductive structures of *Gracilaria cornea* Gracilariales, Rhodophyta). Journal of Phycology, 38: 1169–1175.

Ha HC, Sirisoma NS, Kuppusamy P, Zweier JL, Woster PM, Casero RA (1998) The natural polyamine spermine functions directly as a free radical scavenger. Proceedings of the National Academy of Sciences, 95: 11140-11145.

Haggag WM, Abd-El-Kareem F (2009) Methyl jasmonate stimulates polyamines biosynthesisand resistance against leaf rust in wheat plants. Archives Of Phytopathology And Plant Protection, 42:16-31.

Hammond-Kosack KE, Jones JD (1997) Plant disease resistance genes. Annual review of plant biology, 48: 575-607.

Harkess RL, Lyons RE, Kushad, MM (1992) Floral morphogenesis in Rudbeckia hirta in relation to polyamine concentration. Physiologia Plantarum, 86: 575-582.

Heath MC (2000) Hypersensitive response-related death. In Programmed Cell Death in Higher Plants. Springer Netherlands. pp. 77-90.

Hernández SM, Sánchez MS, de Tarlovsky MNS (2006) Polyamines as a defense mechanism against lipoperoxidation in *Trypanosoma cruzi*. Acta tropica, 98: 94-102.

Hervé C, Tonon T, Collén J, Corre E, Boyen C (2006) NADPH oxidases in Eukaryotes: red algae provide new hints! Current Genetics, 49: 190-204.

Horbowicz M, Kosson R, Wiczkowski W, Koczkodaj D, Mitrus J (2011) The effect of methyl jasmonate on accumulation of 2-phenylethylamine and putrescine in seedlings of common

buckwheat (Fagopyrum esculentum). Acta Physiologiae Plantarum, 33: 897–903

Huber J (1892) Contributions à la connaissance des Chaetophorées epiphytes et endophytes et de leurs effinités. Annalesdes Sciences Naturelles, Botanique, sér, 16: 265-359.

Hussain SS, Ali M, Ahmad M, Siddique KH (2011) Polyamines: natural and engineered abiotic and biotic stress tolerance in plants.Biotechnology advances, 29: 300-311.

Kafy AML, Haigh CG, Lewis DA (1986) In vitro interactions between endogenous polyamines and superoxide anion. Agents and actions, 18: 555-559.

Kaur-Sawhney R, Applewhite PB (1993) Endogenous protein-bound polyamines: correlation with regions of cell division in tobacco leaves, internodes and ovaries. Plant growth regulation, 12: 223-227.

Khan AU, Di Mascio P, Medeiros MH, Wilson T (1992) Spermine and spermidine protection of plasmid DNA against single-strand breaks induced by singlet oxygen. Proceedings of the National Academy of Sciences, 89: 11428-11430.

Kim C, Kim YS, Choi HG, Nam KW (2014) New records of three endophytic green algae from *Grateloupia* spp.(Rhodophyta) in Korea. Algae, 29: 127-136.

King DW, Cooper WJ, Rusak SA, Peake BM, Kiddle JJ, O'Sullivan DW, Theberge SM (2007) Flow injection analysis of H2O2 in natural waters using acridinium ester chemiluminescence: Method development and optimization using a kinetic model. Analytical Chemistry, 79: 4169-4176.

Küpper FC, Gaquerel E, Cosse A, Adas F, Peters AF, Müller DG, Potin P (2009) Free fatty acids and methyl jasmonate trigger defense reactions in *Laminaria digitata*. Plant and cell physiology, 50: 789-800.

Küpper FC, Müller DG, Potin, P (2002) Oligoalginate recognition and oxidative burst play a key role in natural and induced resistance of sporophytes of *Laminariales*. Journal of Chemical Ecology, 28: 2057-2081.

Lamb C, Dixon RA (1997) Oxidative burst in plant disease resistance. Annual Review of Plant Physiology and Plant Molecular Biology, 48: 251-275.

Levine A, Tenhaken R, Dixon RA, Lamb C (1994) H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell, 79: 583-593.

Low PS, Merida JR (1996) The oxidative burst in plant defense: function and signal transduction. Physiologia Plantarum, 96: 533-542.

Mader JC (1999) Effects of Jasmonic Acid, Silver Nitrate and L AOPP on the Distribution of Free and Conjugated Polyamines in Roots and Shoots of *Solanum tuberosum* in vitro. Journal of plant physiology, 154: 79-88.

Marcé M, Brown DS, Capell T, Figueras X, Tiburcio AF (1995) Rapid high performance liquid chromatographic method for the quantitation of polyamines as their dansyl derivatives: application to plant and animal tissues. Journal of Chromatography B, 666: 329-335.

Marián FD, Garcia-Jimenez P, Robaina RR (2000a) Polyamine levels in the seagrass Cymodocea nodosa. Aquatic Botany, 68: 179–184.

Marián FD, García-Jiménez P, Robaina RR (2000b) Polyamines in marine macroalgae: Levels of putrescine, spermidine and spermine in the thalli and changes in their concentration

during glycerol-induced cell growth in vitro. Physiologia Plantarum, 110: 530-534.

Marini F, Betti L, Scaramagli S, Biondi S, Torrigiani P (2001) Polyamine metabolism is upregulated in response to tobacco mosaic virus in hypersensitive, but not in susceptible, tobacco. New phytologist, 149(2), 301-309.

Mehdy M (1994) Active oxygen species in plant defence against pathogens. Plant Physiology, 105: 467-472.

Mellersh DG, Foulds IV, Higgings VJ, Heath MC (2002) H_2O_2 plays different roles in determining penetration failure in three diverse plant-fungi interactions. The Plant Journal, 29: 257-268.

Mitchell AF, Walters DR (1995) Systemic protection in barley against powdery mildew infection using methyl jasmonate. Aspects of Applied Biology, 42: 323-326.

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic acids research, 8: 4321-4326.

Negrel J, Vallée JC, Martin C (1984) Ornithine decarboxylase activity and the hypersensitive reaction to tobacco mosaic virus in *Nicotiana tabacum*. Phytochemistry, 23: 2747-2751.

Nielsen R, Petersen G, Seber O, Daugbjerg N, O'Kelly CJ, Wysor B (2013) Revision of the genus *Ulvella* (Ulvellaceae, Ulvophyceae) based on morphology and tufA gene sequences of species in culture, with *Acrochaete* and *Pringsheimiella* placed in synonymy. Phycologia, 52: 37–56

Nielsen R, McLachlan J (1986) Investigations of the marine algae of Nova Scotia. XVI. The occurrence of small green algae. Canadian journal of botany,64: 808-814.

O'Donnell BV, Tew DG, Jones OT, England PJ (1993) Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. Biochemical Journal, 290: 41-49.

O'Kelly CJ, Bellows WK, Wysor B (2004a) Phylogenetic position of *Bolbocoleon piliferum* (Ulvophyceae, Cholorophyta): Evidence from reproduction, zoospore and gamete ultrastructure, and small subunit rRNA gene sequences. Journal of Phycology. 40:209–22.

O'Kelly CJ, Wysor B, Bellows WK (2004b) Gene sequence diversity and the phylogenetic position of algae assigned to the genera *Phaeophila* and *Ochlochaete* (Ulvophyceae, Chlorophyta). Journal of Phycology. 40:789–99.

Peters AF, Ramírez ME (2001) Molecular phylogeny of small brown algae, with special reference to the systematic position of *Caepidium antarcticum* (Adenocystaceae, Ectocarpales). Cryptogamie Algologie, 22: 187-200.

Peters AF, Burkhardt E (1998) Systematic position of the kelp endophyte *Laminarionema elsbetiae* (Phaeophyceae, Ectocarpales *sensu lato*) inferred from nuclear ribosomal DNA sequences. Phycologia, 37: 114-120.

Potin P, Bouarab K, Salaün JP, Pohnert G, Kloareg B (2002) Biotic interactions of marine algae. Current opinion in plant biology, 5: 308-317.

Pringsheim N (1862) Beiträge zur Morphologie der Meeres-Algen. Physikalische Abhandlungen der Königlichen Akademie der Wissenschaften zu Berlin, 1-37.

Provasoli L (1968) Media and prospects for the cultivation of marine algae. In: Watanabe A, Hattori A, editors. Cultures and collections of algae. Japanaese Society of Plant Physiology. Hakone, 63-75.

Rey M, Díaz-Sala C, Rodríguez R (1994) Exogenous polyamines improve rooting of hazel microshoots. Plant cell, tissue and organ culture, 36: 303-308.

Rider JE, Hacker A, Mackintosh CA, Pegg AE, Woster PM, Casero Jr RA (2007) Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide. Amino Acids, 33: 231-240.

Rinkel BE, Hayes P, Gueidan C, Brodie J (2012) A molecular phylogeny of *Acrochaete* and other endophytic green algae (Ulvales, Chlorophyta). Journal of phycology. 48: 1020-1027.

Robaina RR, García-Reina G, Luque A (1990a) The effects of the physical characteristics of the culture medium on the development of red seaweeds in tissue culture. Hydrobiologia, 204/205:137–142

Robaina RR, García-Jiménez P, Brito I, Luque A (1990b) Morphogenetic effect of glycerol on tissue culture of the red seaweed *Grateloupia doryphora*. Journal of Applied Phycology, 2: 137–143

Roberts DR, Dumbroff EB, Thompson, JE (1986) Exogenous polyamines alter membrane fluidity in bean leaves a basis for potential misinterpretation of their true physiological role. Planta, 167: 395-401.

Sacramento AT, García-Jiménez, P, Robaina RR (2007) The polyamine spermine induces cystocarp development in the

seaweed *Grateloupia* (Rhodophyta). Plant Growth Regulation, 53:147–154.

Sacramento AT, García-Jiménez P, Alcázar R, Tiburcio AF, Robaina RR (2004) Influence of polyamines on the sporulation of G*rateloupia* (halymeniaceae, rhodophyta). Journal of Phycology, 40: 887–894.

Santino A, Taurino M, De Domenico S, Bonsegna S, Poltronieri P, Pastor V, Flores V (2013) Jasmonate signaling in plant development and defense response to multiple abiotic stresses. Plant Cell Reports, 32:1085–1098.

Schuber F, Hong K, Duzgunes N, Papahadjopoulos D (1983) Polyamines as modulators of membrane fusion: aggregation and fusion of liposomes. Biochemistry, 22: 6134-6140.

Sembdner GAPB, Parthier B (1993) The biochemistry and the physiological and molecular actions of jasmonates. Annual review of plant biology, 44: 569-589.

Smith TA (1985) Polyamines. Annual Review of Plant Physiology, 36: 117-43.

Stakman EC (1915) Relation between *Puccinia graminis* and plants highly resistant to its attack. Journal of Agricultural Research, 4: 193-199.

Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG, Jones JD (1995) Molecular genetics of plant disease resistance. SCIENCE NEW YORK THEN WASHINGTON, 661-661.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA 5: Molecular Evolutionary Genetics Analysis. Center for Evolutionary Functional Genomics The Biodesign Institute, 85287-5301.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.Nucleic acids research, 22: 4673-4680.

Tkachenko A, Nesterova L, Pshenichnov M (2001) The role of the natural polyamine putrescine in defense against oxidative stress in *Escherichia coli*. Archives of microbiology, 176: 155-157.

Torrigiani P, Rabiti AL, Bortolotti C, Betti L, Marani F, Canova A, Bagni N (1997) Polyamine synthesis and accumulation in the hypersensitive response to TMV in *Nicotiana tabacum*. New Phytologist, 135(3), 467-473.

Torrigiani P, Altamura MM, Pasqua G, Monacelli B, Serafini-Fracassini D, Bagni N (1987) Free and conjugated polyamines during de novo floral and vegetative bud formation in thin cell layers of tobacco. Physiologia plantarum, 70: 453-460.

Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. Plant Science, 151: 59-66.

Walters DR (2003a) Resistence to plant pathogens: possible roles for free polyamines and polyamine catabolism. New Phytologist, 159: 109-115.

Walters DR (2003b) Polyamines and plant disease. Phytochemestry, 64: 97-107.

Walters DR (2000) Polyamines in plant-microbe interactions. Physiological and Molecular Plant Pathology, 57: 137-146.

Walters DR, Cowley T, Mitchell Ann (2002) Methyl jasmonate alters polyamine metabolism and induces systemic protection against powdery mildew infection in barley seedlings. Journal of experimental botany, 53: 747-756.

Weber H (2002) Fatty acid-derived signals in plants. Trends in plant science,7: 217-224.

Wei ZM, Laby RJ, Zumoff CH, Bauer DW, He SY, Collmer, A, Beer SV (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science, 257: 85-88.

Weinberger F (2007) Pathogen-Induced defence and innate immunity in macroalgae. The Biology Bulletin, 213: 290-302.

Weinberger F, Pohnert G, Berndt ML, Bouarab K (2005) Apoplastic oxidation of L-asparagine is involved in the control of the green algal endophyte *Acrochaete operculata* Correa & Nielsen by the red seaweed *Chondrus crispus* Stackhouse. Journal of Experimental Botany, 56: 1317–1326.

Weinberger F, Friedlander M (2000) Response of *Gracilaria conferta* (Rhodophyta) to oligoagars results in defence against agar degrading epiphytes. Journal of Phycology, 36: 1079-1086.

Weinberger F, Friedlander M, Hoppe H-G (1999) Oligoagars elicit a physiological response in *Gracilaria conferta* (Rhodophyta). Journal of Phycology, 35: 747-755.

White TJ, Bruns SL, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* (Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J., editors) 315-322. Academic Press, San Diego.

Wojtaszek P (1997). Oxidative burst: an early plant response to pathogen infection. Biochemical Journal, 322: 681–692.

Xu YI, Chang PFL, Liu D, Narasimhan ML, Raghothama KG, Hasegawa PM, Bressan RA (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. The Plant Cell Online, 6: 1077-1085.

Yamakawa H, Kamada H, Satoh M, Ohashi Y (1998) Spermine is a salicylate-independent endogenous inducer for both tobacco acidic pathogenesis-related proteins and resistance against tobacco mosaic virus infection. Plant Physiology 118: 1213–1222.

Yang Y, Shah J, Klessig D (1997) Signal perception and transduction in plant defence responses. Genes & Development, 11: 1621-1639.

Yoda H, Fujimura K, Takahashi H, Munemura I, Uchimiya H, Sano H (2009) Polyamines as a common source of hydrogen

peroxide in host- and nonhost hypersensitive response during pathogen infection. Plant Molecular Biology, 70: 103-112.

Zarranz-Elso M, García-Jiménez P, Robaina RR (2012) Endogenous polyamine content and photosynthetic performance under hypo-osmotic conditions reveal *Cymodocea nodosa* as an obligate halophyte. Aquatic Biology, 17: 7-17.