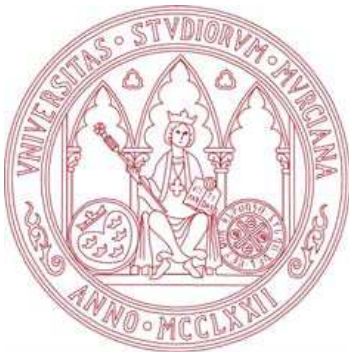


UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

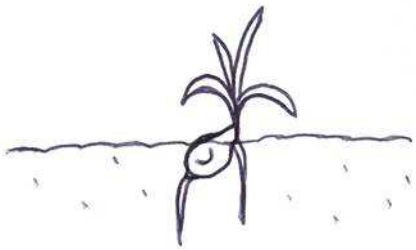
Photosynthetic activity and bacterial association in seeds of the seagrass *Posidonia oceanica* (L.) Delile

Actividad fotosintética y asociación bacteriana en semillas de la fanerógama marina *Posidonia oceanica* (L.) Delile

D. Manuel David Celdrán Sabater

2012

Para mis padres, abuelos y hermanos



Agradecimientos

Durante los años que he pasado en la construcción de mis experimentos, trabajo de campo, análisis de datos, redacción de artículos, estancias en el extranjero y redacción de mi tesis no puedo sino sentirme enormemente feliz y en deuda con la vida porque me haya regalado la oportunidad de hacer lo que realmente me gusta.

Quisiera comenzar agradeciendo a mi familia todo el apoyo que me han brindado durante estos años de trabajo.

A mi madre quiero agradecerle toda la preocupación porque mis estudios fueran bien, y porque saliera del agua pronto los días de buceo. Mamá, has sido un apoyo indispensable para mí, no solo por lo que eres, sino por el cariño que me has dado y la dedicación que has tenido constante en mí. A mi padre quiero agradecerle especialmente el apoyo incondicional en todo lo que he emprendido en mi vida. Eres un referente de trabajo y esfuerzo para mí. Siempre has estado ahí, abandonando todo por mí. Sin tus ánimos y sin todos los esfuerzos que has hecho para que yo pudiera hacer lo que más me gustaba, simplemente no podría haber llegado hasta aquí. A mi hermano Miguel, por haberse convertido un referente de honestidad y libertad para mí. Quiero que sepas que no podría tener un hermano mejor en el mundo. A mi hermana Mayte por haberse convertido en mi confidente, mi apoyo en momentos difíciles, alguien demasiado importante para mi, eres luz para mi camino pequeñaja.

A mi abuelo Paco y mi abuela Isabel. Sois los pilares de mi vida, gracias por estar siempre tan atentos, por vuestros consejos, por vuestro desbordado cariño y por todo lo que me habéis dado. Quiero que sepáis que lo que soy ahora mismo es gracias a vosotros.

A mi abuelo Leoncio y mi abuela Adela, porque siempre me habéis apoyado en mis estudios y trabajos, porque pese a todo el trabajo que tenéis siempre estáis preguntando por mí y cómo me va por Murcia. Gracias por vuestro cariño.

Y quiero agradecer a todos mis tíos, padrinos, primos etc.,(Paco, Maribel, Isidoro, Ana, Lola, Adeli, Santiago Jose, Paqui, Leo, Javi, Ana Isabel, Eva, Pedro, Fran, Raquel, Jose, Alejandro, Alberto, Paula, Daniela, Daniel, Isaac, Fina, Carmen, Loli..) y un largo etcétera y a todos los que ya no están, gracias por toda vuestra atención, porque me hayáis alentado para seguir adelante y por todo vuestro apoyo y cariño.

También quiero agradecer a Conchi y a sus hijas el cariño que me tienen y sus ánimos en cualquier asunto, sabéis que sois de mi familia.

A mi director de tesis Arnaldo Marín, quiero agradecerle especialmente todo lo que ha hecho por mí durante estos cuatro años. Han sido muchas las horas que hemos compartido trabajando, a veces bajo duras condiciones de frío bajo el agua, otras hasta arriba de correcciones de artículos. Gracias por todo el trabajo que has dedicado en mi formación. También quiero agradecerte su preocupación porque nunca me faltara material de ningún tipo y estuviera económicamente cubierto en todo momento, por el apoyo en mis estancias en el extranjero, congresos etc. Pero sin duda me quedo con algo que aprecio especialmente de ti, y es su inagotable buen humor y tranquilidad. Han sido un regalo para mí, todos los buenos momentos, las risas y el buen ambiente que creaste, gracias Arnaldo.

A todo el equipo de Ecología Acuática: Carlos, Pedro, David Sánchez, Piedad, Maridol, Tano, Félix, Dani, Jose Antonio, Susana, Simone, Natalia, Nuria y a los que han pasado que han formado inequívocamente parte de mi estancia aquí como Lázaro y Pancho. A Óscar y Paula quiero agradecerle todos los buenos momentos y risas que hemos tenido durante los años de carrera y ahora durante la tesis doctoral, ha sido todo un privilegio compartir estos años con vosotros, tanto académicamente como en el terreno del compañerismo. Especialmente es mi agradecimiento para Javier Lloret por haber sido prácticamente mi cotutor, por su interés en mi formación y por la inmensa ayuda que siempre me ha dado.

Así mismo agradezco al resto de compañeros del departamento de Ecología los buenos momentos vivido juntos: Viqui, Isa, Victor, Paqui, Pablo, Jorge, Carlos, Marta, Fabi, Gabi, María del Mar, Marisa, Viqui, Rosa, Javi 'el alemán', Jesús, Jhony, y muchos más.

Quiero permitirme un párrafo para dar las gracias de manera muy especial a Pepa Velasco y Andrés Millán. Sabéis que en este departamento entré gracias a vosotros. Para mi habéis sido, cotutores, compañeros, pero sobre todo confidentes, en quien podía depositar mis preocupaciones y sobre todo en quién confiar. Os debo mucho. Gracias por vuestro interés y por adoptarme como si hubiera sido uno más de vuestros "zagales". Sois parte de lo que soy ahora, muchas gracias.

A todos los profesores y personal del Departamento de Ecología e Hidrología, en especial a Pepa y Juan por su ayuda y su disposición. También al personal del SACE y el CAID por su ayuda en laboratorio y su profesionalidad. Gracias Almudena y María del Mar, Juana, Manolo, Jose, etc. Por toda la ayuda que me han brindado siempre y sin reservas.

A los compañeros de Fisiología animal, a Borja, Carolina, Natalia, Ana, Rodrigo, Ander etc.. También a David Verdiell, Antono Sanchez Amat y a los compañeros de los departamentos de Fisiología vegetal, a Maria Ángeles por su dedicación en la nueva línea que comenzaré tras la tesis. Por supuesto no me olvido de mis compis de máster de los cuales ya he nombrado a la mayoría y también a Rosa, Miriam, Suni etc..

Quiero agradecer a todos los tutores de Universidades extranjeras que me acogieron en sus equipos en cada una de las estancias que realicé durante mi doctorado:

A Gerard Pergent y Christine, por toda su hospitalidad, por enseñarme a la perfección cómo se realiza el análisis Lepidocronológico y a todo su equipo de Pasquale Paoli University en Córcega, Francia.

A Kun Seop Lee por recibirme en su equipo como uno más a pesar de las limitaciones culturales y del idioma. Por proporcionarme absolutamente todo aquello que necesité y por ser una de las mejores personas que he conocido en mi vida. Asimismo agradecer a su equipo de Biología marina ("a todos los Kim") de Pusan Nacional Univeristy en Corea del Sur. Fue una experiencia muy enriquecedora profesional y personalmente.

Por último agradecer de manera especial a Jennifer Verduin y Mike Van Keulen del equipo de ciencias ambientales y biología de la Universidad de Murdoch, Perth, (Australia) todo lo que hicieron por mí. Jennifer, gracias por alojarme en tu casa, por todo el papeleo, por adoptarme casi como a un hijo, sin ti, simplemente no hubiese realizado la estancia en Western Australia. Y también por proporcionarme las esperanzas de una postdoctoral analizando el resto de especies de Posidonia en vuestro equipo, es algo que no puedo agradecer solo con palabras.

A mis amigos de Cartagena que me acompañan desde la infancia, Alex, Ingrid, Juanjo, Fran, Ana, Marta, Ian, Jesús, Paco, Toni, Jose Manuel, Carlos, Fernando y María, etc... Vosotros habéis compartido conmigo mi amor por la naturaleza y la ecología, os llevo conmigo siempre.

A Isa R.S., por haberme ayudado con la fotosíntesis y pasarme mucha documentación sobre el PAM. Pero sobre todo por haberme regalado ilusión y amor esté último año de mi tesis, por todo lo que hemos hecho juntos, por haber significado tanto para mí, y por haberme enseñado cosas que no se aprenden leyendo. Has sido y serás alguien muy importante para mí.

Y a mi grupo de Isla Plana, Paco, Rosa, Luzma, Pablo, Conchi, Agustín, Ángel, Carol, Emilio, Choni, Gustavo, etc. Por vuestra amistad, por todos los momentos divertidos que pasamos juntos, nuestras paellas y barbacoas, fiestas y excursiones. Os quiero. Especialmente quiero agradecer a mi amigo Paco y a su familia lo que han significado para mi, antes, durante y después de mi tesis. Paco, eres mi apoyo y mi guía, mi compañero de penas y alegrías, no puedo imaginar nadie mejor que tu para acompañarme en este viaje que es la vida.

Y por último agradecer a todos los lectores de esta tesis su interés y curiosidad por los contenidos de la misma. Espero que disfrutéis tanto como lo he hecho yo durante estos cuatro años.

Muchas gracias a todos.

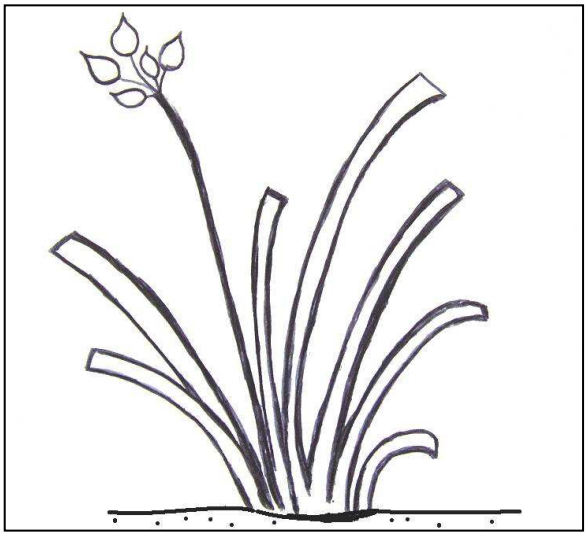
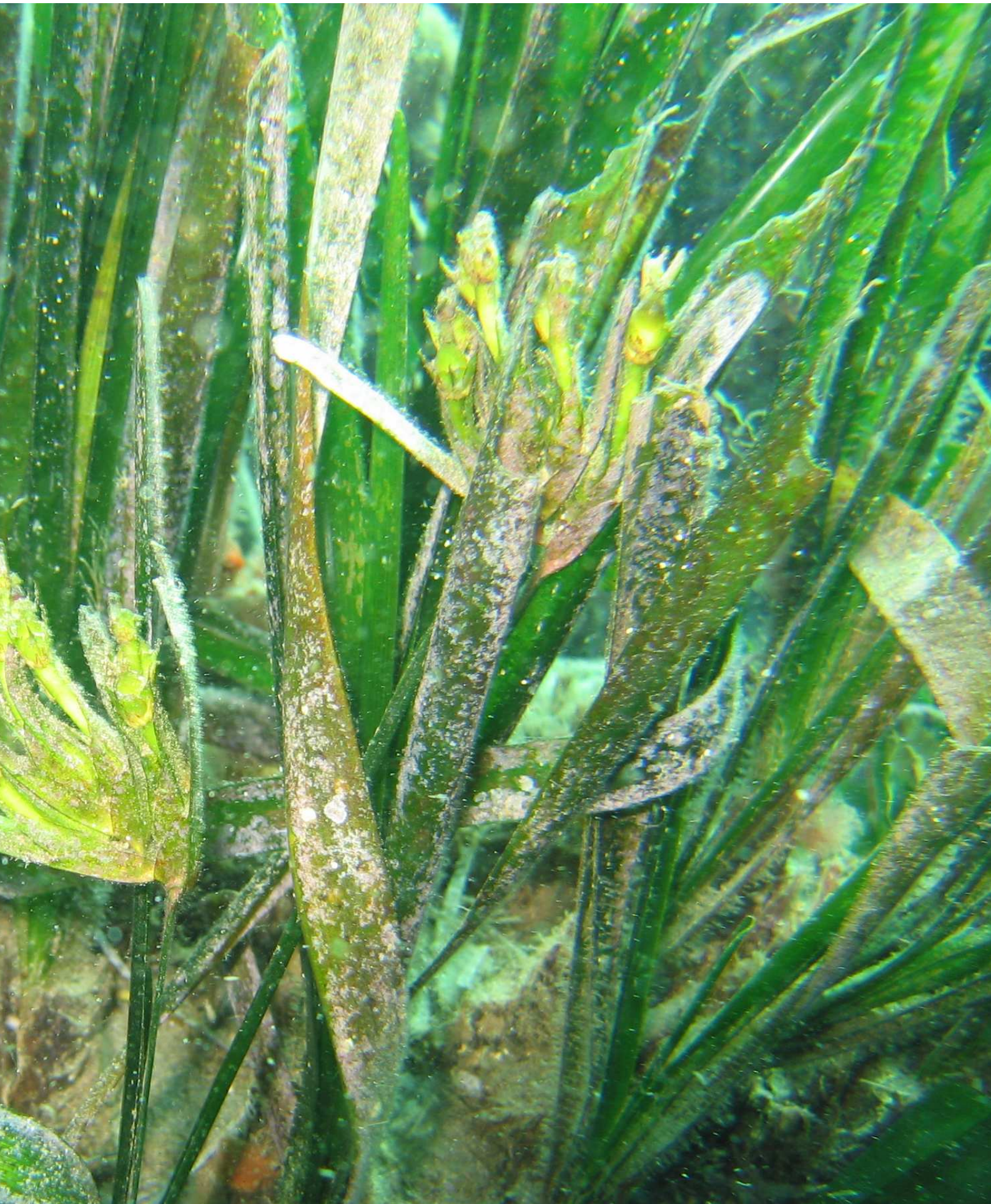


Table of contents

Resumen



Resumen

Introducción General

Posidonia oceanica (L,) Delile, es la fanerógama endémica más abundante del Mar Mediterráneo. Forma extensas praderas que se encuentran desde la superficie hasta los 50 metros de profundidad. *P. oceanica* juega un importante rol ecológico y es responsable de la construcción de "barreras de Posidonia" que semejantes a las barreras de coral, protegen las costas de la erosión y la acción de las olas. Dichas barreras están formadas por rizomas ortotropos y plagiotropos junto a una red de raíces y sedimento. Como los bosques en el medio terrestre, las praderas de *P. oceanica* son la comunidad climax y su presencia atestigua un medio ambiente estable.

Las fanerógamas marinas aportan una gran cantidad de servicios al ecosistema entre los que se encuentran: su utilización como materiales de construcción, comida, protección de la costa, control de la erosión, purificación del agua, mantenimiento de pesquerías, secuestro de dióxido de carbono, aliciente para el turismo, investigación médica etcétera.

Concretamente *P. oceanica* ha sido tradicionalmente usada como "cama" para el ganado, también para el relleno de colchones. Asimismo se utilizaba como material aislante de tejados.

Esta fanerógama también genera la deposición y sedimentación de partículas suspendidas, previniendo asimismo la resuspensión de dichos materiales.

La estructura que supone sus hojas y rizomas representan un importante "microhábitat guardería" para los alevines de peces y larvas de crustáceos y otros animales. Asimismo realiza una labor de secuestro de carbón en forma de CO2, no solo por la utilización del C para sus estructuras sino también por la absorción de carbono que realiza la comunidad de epífitos que habita sobre sus hojas.

Esta planta marina también genera un beneficio económico directo para empresas de turismo costero que llevan a turistas de todo el mundo a realizar buceos en las praderas.

Por último, *P. oceanica* está siendo investigada para algunas aplicaciones médicas. Gokce y Haznedaroglu llevaron a cabo un estudio en el que se concluyó que la administración de extracto de *P. oceanica* durante 15 días en ratas genera ciertos beneficios para la diabetes. Asimismo las sustancias fenólicas que genera *P. oceanica* están siendo investigadas para su utilización anticancerígena.

A pesar de la importancia que tiene *P. oceanica* para el medio ambiente y para la sociedad, esta planta marina se ha visto afectada por diversos impactos. Sus poblaciones se han visto reducidas en los últimos tiempos a causa de la pesca de arrastre, desarrollo costero y eutrofización. Asimismo la aparición de especies exóticas y el calentamiento global están incidiendo negativamente en su conservación.

Un método para la reforestación marina de áreas impactadas (una vez cesa la causa del impacto) es la replantación con plántulas de *P. oceanica*. Algunos experimentos realizados por nuestro grupo han obtenido éxitos de supervivencia tras la reforestación con semillas de *P. oceanica* de un 80 % un año tras el trasplante, similares a los obtenidos por Balestri et al., (1998) como éxito natural de reclutamiento.

Existen numerosos estudios sobre aspectos biológicos y ecológicos en fanerógamas marinas tanto desde el punto de vista de la restauración como el puramente biológico. Concretamente sobre *P. oceanica* existe una amplia literatura sobre la descripción de aspectos fisiológicos y ecológicos entre otros. Sin embargo existen lagunas en la información con respecto a algunos aspectos de la semilla de *P. oceanica* tales como el incremento en su desarrollo auspiciado por asociaciones con microorganismos y la presencia de actividad fotosintética en su epidermis.

La asociación entre microoganismos y semillas juega un importante rol en la promoción del crecimiento vegetal y como control biológico de patógenos. Limura y Hosono determinaron ya en 1998 la actividad antifúngica desarrollada por una bacteria fermentativa-oxidativa Gram-negativa y otra bacteria Gram-positiva contra un hongo que atacaba semillas de trigo. Asimismo Puente et al., (2009), describieron la asociación entre el cactus *Pachycereus pringlei* y una bacteria epífita la cual ayudaba a los propágulos vegetales a el crecimiento sobre sustratos rocosos.

La fotosíntesis en semillas, sin embargo, es un hecho prácticamente desconocido. Clásicamente se ha pensado que la actividad fotosintética es llevada a cabo en los cloroplastos de tejidos foliares, (hojas y primordios foliares). Tschiersch et al., (2011) describió fotosíntesis en semillas inmaduras de guisante y barley caryopsis durante algunas etapas del desarrollo de la semillas, mas concretamente, durante la embiogénesis.

Todas las semillas maduras de las especies del género Posidonia son de color verde. Este color denota la presencia de pigmentos fotosintéticos (clorofila “a” y “b”) y posiblemente actividad fotosintética.

Hipótesis y objetivos principales

La presente tesis doctoral realiza un estudio sobre ciertos aspectos de la semilla de *P. oceanica* como respuesta al incremento en la supervivencia y crecimiento de las plántulas. Dichos aspectos se desarrollan en el marco de un medio ambiente oligotrófico, el Mar Mediterráneo, donde hay un bajo contenido en nutrientes y los organismos se ven forzados a desarrollar habilidades especiales para sobrevivir. El estudio se ha centrado en las habilidades que ha desarrollado la semilla para la asociación con microorganismos y para llevara a cabo actividad fotosintética.

En este contesto, la hipótesis principal se basa en la idea de que la semilla de *P. oceanica* es capaz de desarrollar ciertos mecanismos especiales para aumentar el crecimiento de la plántula y las posibilidades de supervivencia. Uno de esos mecanismos está relacionado con la asociación microbiana con Marinomonas, con la finalidad de evaluar si generan un efecto positivo en el crecimiento de la plántula. El segundo mecanismo se basa en la capacidad de desarrollar actividad fotosintética por parte de la epidermis de la semilla madura sin hojas y que garantizaría el éxito en la supervivencia de las plántulas.

De acuerdo con estos mecanismos, los objetivos principales de esta tesis doctoral son:

i) Estimar el efecto de dos bacterias; *Marinomonas posidonica* y *Marinomonas mediterranea (*las cuales viven de manera natural epífitamente en la planta de *P. oceanica)*, sobre el crecimiento de las futuras plántulas.

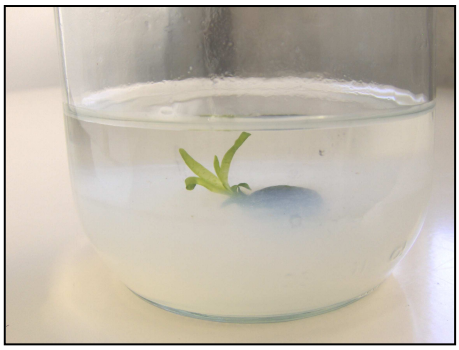
ii) Testar la actividad fotosintética en la epidermis de la semilla madura de *P. oceanica* y aumentar el conocimiento sobre este mecanismo en las primeras etapas de la germinación, determinando si es funcional o no.

iii) Comparar la actividad fotosintética en otras especies del género Posidonia y determinar el rol evolutivo que ha jugado esta habilidad y sus posibles consecuencias ecológicas.

Capítulo 1. Efecto microbiano de Marinomonas mediterranea y Marinomonas posidonica, en el crecimiento foliar y en la comunidad de algas epífitas asociadas a Posidonia oceanica.

Resumen

Los efectos beneficiosos de microorganismos asociados a plantas en la productividad vegetal son ampliamente conocidos en ecosistemas terrestres, pero aun no han sido descritos en el medio ambiente marino.



En este capítulo se testó la hipótesis basada en si dos bacterias: *Marinomonas posidonica* y *Marinomonas. mediterranea*, las cuales han sido descritas como microbiota asociada a *P. oceanica*, influyen en el crecimiento foliar y en la comunidad de epífitos de las plántulas. Se llevaron a cabo cuatro tratamientos: (1) plántulas inoculadas con *Marinomonas mediterranea*, (2) plántulas inoculadas con *Marinomonas posidonica*, (3) plántulas no inoculadas con bacterias y cultivadas en agua estéril y (4) plántulas no inoculadas pero cultivadas en agua de mar natural.

Se realizaron dos experimentos; uno en el que se evaluó el crecimiento foliar en laboratorio bajo condiciones controladas durante tres meses. El segundo se llevó a cabo en el medio natural y se evaluó la estructura de la comunidad de epífitos durante otros tres meses.

El experimento en laboratorio mostró un aumento en el área foliar de las plántulas que fueron inoculadas con *Marinomonas posidonica*. Por el contrario, las plántulas que fueron inoculadas con *Marinomonas mediterranea* no mostraron ningún crecimiento significativo. Asimismo, *Marinomonas posidonica* estimuló cambios en el crecimiento de la comunidad de epífitos por lo que se sugiere que tiene una influencia

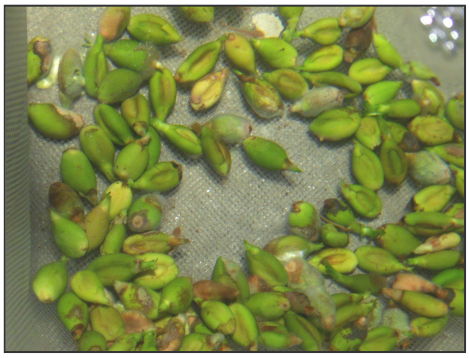
no solo en el crecimiento foliar sino también en la estructura de la comunidad de macroepífitos asociada.

El capítulo 1 fue publicado en 2012:

Celdrán, D; Espinosa E; Sánchez-Amat A; and Marín, A. 2012 Effects of the epibiotic bacteria, *Marinomonas mediterranea* and *Marinomonas posidonica*, on leaf growth and epiphyte communities of the seagrass *Posidonia oceanica*. Marine Ecology Progress Series 456, 21-27.

Capítulo 2. *Actividad fotosintética de la semilla de Posidonia oceanica.*

Resumen



En este capítulo se estudió la actividad fotosintética realizada por la semilla de *P. oceanica* en relación a la evaluación de los efectos de la luz en el éxito de la germinación.

Las imágenes obtenidas al microscopio electrónico de transmisión mostraron la presencia de cloroplastos en células de la epidermis de la semilla, cerca del núcleo, en la periferia del citoplasma. Las membranas tilacoidales se observaron bien desarrolladas y conteniendo gránulos de almidón lo que indica que eran fotosintéticamente activas. Se analizó la relación entre fotosíntesis versus irradiancia en la semilla de *P. oceanica* incubada a 15 y 21 ºC .

La fotosíntesis neta de la semilla resultó positiva y compensó la respiración a 90 µmol quanta m−2 s−1 (PAR) para ambas temperaturas. La fotosíntesis neta resultó negativa para el resto de valores de irradiancia. Para testar los efectos de la luz en el éxito de la germinación, se ubicaron semillas a la luz y a la oscuridad resultando un significativo mayor éxito en la germinación para el tratamiento de semillas expuestas a la luz.

La habilidad de realizar fotosíntesis por parte de la semilla de *P. oceanica* podría corresponder a un mecanismo que garantice la supervivencia de las plántulas en aguas templadas demostrando la especialización a la que ha llegado esta especie.

El capítulo 2 fue publicado en 2011:

Celdran, D. & Marin, A. Photosynthetic activity of the non-dormant *Posidonia oceanica* seed. Marine Biology. 158, 853–858 (2011).

Capítulo 3. *Fotosínesis en la semilla de Posidonia oceanica. (L.) Delile, incrementa el crecimiento de la plántula.*

Resumen

La semilla de *P. oceanica* tiene actividad fotosintética durante el desarrollo de la plántula lo cual sugiere que la fotosíntesis realizada por epidermis de la semilla puede contribuir al crecimiento foliar.

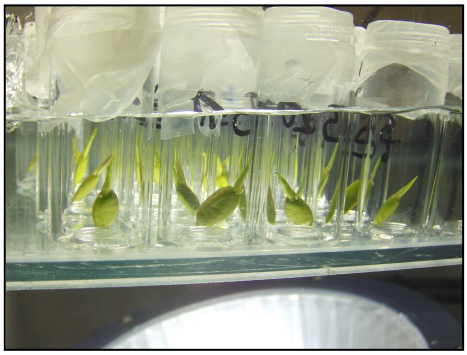
El objetivo de este trabajo fue examinar la contribución de la fotosíntesis de la semilla para el crecimiento foliar y la movilización de reservas y nutrientes durante los primeros tres meses del desarrollo de la plántula de *P. oceanica*. Para ello, re realizó el análisis del crecimiento foliar, concentración de almidón y azúcares libres, C, N y P, en la semilla de las plántulas sometidas a diferentes tratamientos de luz-oscuridad.

Los resultados probaron que la actividad fotosintética de la semilla de incrementa significativamente el área foliar. El contenido en almidón y en azúcares libres no se vio alterado por la exposición a la luz de las semillas, lo cual sugiere que la contribución de la semilla fue invertida totalmente en producción foliar y no en un incremento en la reservas de nutrientes. Los resultados también demostraron que la contribución fotosintética fue en la misma medida que la producción fotosintética llevada a cabo por las hojas por lo cual, dicha contribución es enormemente importante y funcional para la plántula.

Capítulo 4*. Actividad fotosintética en la semilla de Posidonia Australianas y Mediterránea antes de la separación de los continentes.*

Resumen

La existencia de especies de Posidonia en dos partes tan distantes del globo, nos hace preguntarnos si dos de las especies más abundantes de Posidonias australianas (*P .australis* y *P. sinuosa*) comparten la habilidad fotosintética que presenta la especie Meditarranea (*P. oceanica*).



Mediante análisis fluorométricos mediante PAM y análisis de producción de oxígeno se compararon ciertos parámetros fotosintéticos de las tres especies: la eficiencia máxima del fotosistema II (Y), el ratio de transporte electrónico (ETR), el cual fue representado como (ETR) versus Irradiancia, el "quenching" fotoquímico (PQ), el "quenching" no fotoquímico (NPQ) y los parámetros de producción de oxigeno tales como: la respiración (R), producción bruta (GP) y producción neta (NP).

Tras análisis estadísticos de dichos parámetros, los resultados revelaron que las tres especies presentan actividad fotosintética y que dicha actividad debió ser una habilidad adquirida antes de su separación geográfica, antes del Eoceno Tardío heredada de un antecesor común. Asimismo, dentro de cada especie, la (NP) compensó la (R) lo cual sugiere que la actividad fotosintética en la semilla del género Posidonia es un mecanismo de compensación a la respiración. Dicho mecanismo se presenta probablemente para contrarrestar la falta de O2 que acontece en el interior de la semilla como resultado de la germinación y el desarrollo de la plántula. Finalmente, la relativa alta producción de oxígeno y valores de ETR en la semilla de *P. oceanica* respecto de las dos especies australianas revela que esta especie ha debido adaptarse a un medio mucho más oligotrófico donde los nutrientes están en muy baja concentración, tal como es el Mar Mediterráneo.

Discusión General

La floración de *P. oceanica* es un evento poco frecuente e irregular. Asimismo el reclutamiento de semillas de *P. oceanica* es un hecho excepcional debido a la alta tasa de abortos y prelación por parte de herbívoros. Además cuando *P. oceanica* florece, sus semillas se liberan solo durante cuatro meses. Si a todo esto le añadimos que los pocos frutos que se generan están altamente dispersos en la pradera o en las playas una vez arrancados, se entiende que la disposición de semillas sea muy baja y por tanto su estudio muy excepcional.

Los pocos estudios que existen sobre la semilla de *P. oceanica* se han centrado en la germinación "in vitro" y en la estructura histológica y morfológica de la semilla además de su contenido en reservas y nutrientes. Así pues existen grandes lagunas de información sobre aspectos tales como la asociación con microorganismos o la realización de actividad fotosintética por parte de su epidermis. Los resultados aportados en esta tesis, sin embargo arrojan un poco de luz sobre algunas características de la semilla de *P. oceanica*, algunas de las cuales suponen un nuevo paradigma para la biología.

En los experimentos llevados a cabo con semilla de *P. oceanica* se ha corroborado que ésta, establece una relación con algunos microorganismos, más concretamente con *Marinomonas posidonica* para favorecer el crecimiento foliar y determinar la comunidad de epífitos asociada. Estos dos aspectos hacen confirmar que las relaciones simbióticas entre microorganismos y plantas no solo se dan en el medio terrestre sino también en el marino.

La descripción de actividad fotosintética por parte de la semilla de *P. oceanica*, ha sido sin duda el mayor descubrimiento durante este trabajo de tesis. Esta afirmación supone un cambio en la creencia de que la actividad fotosintética se realiza solo en los cloroplastos de las hojas, dándose también en la epidermis de la semilla de esta fanerógama. Sin embargo, la fotosíntesis en semilla de *P. oceanica* ha reportado mucha información respecto de la ecología y funcionamiento de esta planta.

El contenido de clorofila (relacion"a/b") y los parámetros de fotosíntesis neta y respiración obtenidos en el primer capítulo nos permite asegurar que la semilla puede ser considerada una planta de "sombra" (mientras que la planta adulta tiene una relación de clorofila "a/b" mayor) y que el rango de profundidad óptimo donde la semilla puede realizar la fotosíntesis máxima es de 5 a 25 metros, también distinguiéndose de su progenitor que puede habitar desde casi la superficie hasta prácticamente los 50 metros en aguas claras. Asimismo se comprobó que la fotosíntesis realizada por la semilla es mayor a 15ºC que a 21ºC. Esto supone que las semillas liberadas al principio de primavera tienen una producción neta mayor que las liberadas en los últimos meses de primavera. Dicho mecanismo puede deberse a suplir las deficiencias de radiación solar y fotoperiodo que a principios de estación son menores que a finales.

La fotosíntesis en semilla de *P. oceanica* también influye positivamente en su germinación y continua siendo fotosintéticamente activa al menos tres meses tras ella. La contribución fotosintética que aporta la semilla a la plántula es similar a la aportada por las hojas lo que confirma su funcionalidad.

En el segundo y cuarto capítulo se midió la producción neta. Dicho parámetro da una idea de la producción instantánea, es decir referente al metabolismo diario de la semilla. Los resultados del tercer capítulo de producción foliar dan una idea del balance metabólico durante los tres meses en los que se midió. Sin embargo todos los resultados coinciden en que la contribución de la fotosíntesis desarrollada por la semilla de *P. oceanica* es invertida para compensar la respiración como producto del crecimiento de las plántulas.

Así pues, la fotosíntesis de la semilla parece ser un mecanismo de compensación de la respiración para evitar el bajo contenido de oxígeno que se genera en el interior de la semilla durante su desarrollo pero también para compensar el consumo de oxígeno para el crecimiento de la plántula.

Asimismo, los experimentos de los capítulos tercero y cuarto también coinciden en que el almidón, azúcares libres y nutrientes de la semilla se movilizan independiente de la presencia de luz.

La actividad fotosintética fue testada también en semillas de Posidonias australianas, concretamente en *P. australis* y *P. sinuosa*. Ambas especies presentaron actividad fotosintética lo que asegura que dicha habilidad fue adquirida de un antecesor

común con *P. oceanica* y que habitó durante del Eoceno tardío hace unos 37.2-33.9 millones de años. Las tres especies mostraron valores fotosintéticos similares (Y), (QP) y (NPQ) lo cual sugiere que tienen similares eficiencias fotosintéticas y similares flujos de energía invertidos en la fotoquímica de la planta y disipados en forma de calor en mecanismos fotoprotectores respectivamente.

Sin embargo, la especie Mediterránea y las especies australianas no son demasiado cercanas genéticamente encontrándose algunas divergencias entre ellas.
P. oceanica mostró un significativo significativo mayor (ETR) que *P. australis* y *P. sinuosa*, y mayor peso de semilla que sus parientes australianas. Estas diferencias se deben a que *P. oceanica* ha tenido que evolucionar en un ambiente muy pobre en nutrientes, el Mar Mediterráneo.

Las especies australianas, sin embargo, han evolucionado en un amplio gradiente latitudinal permitiendo a las distintas especies moverse gradualmente según las distintas condiciones ambientales. Esto ha permitido que se adapten a una mayor variedad de hábitats lo que explica su variabilidad en tamaño, forma contenido en nutrientes y actividad fotsintética.

Conclusiones

1. La inoculación con la bacteria *Marinomonas posidonica* en plántulas de *P. oceanica* estimula el crecimiento foliar, lo cual confirma que los microorganismos también pueden ejercer un efecto positivo en la productividad de plantas en ambientes marinos. Asimismo, la comunidad de macroepífios que crecen sobre plántulas de *P. oceanica* se ve alterada por la inoculación de *Marinomonas posidonica*. Los cambios principales se debieron a la abundancia en la comunidad de epífitos de: las algas incrustantes rojas, filamentosas, y corticadas rojas, todas ellas más abundantes en plántulas tratadas con *Marinomonas posidonica*. Sin embargo *Marinomonas mediterranea* no produjo ningún efecto estimulatorio en el crecimiento de plántulas de *P. oceanica* ni generó cambio alguno en la estructura de la comunidad de epífitos.

2. El análisis de clorofilas demuestra la existencia de pigmentos fotosintéticamente activos en la semilla de *P. oceanica*. El contenido de clorofila es de 0,26 ± 0.06 mg Chl a·gdw^{-1} y 0.16 ± 0.04 mg Chl b·gdw^{-1}. La mayor producción neta de la semilla es obtenida a 90 µmol quanta m-2 s-1 para incubaciones a 15 y 21ºC (0.013 ± 0.06 mg O2·g dw^{-1}·h^{-1} a 15ºC y 0.006 ± 0,005 mg O2·g dw^{-1}·h^{-1} a 21ºC). A este valor de irradiancia, la NP compensa R. Finalmente el éxito en la germinación es significativamente mayor en semillas iluminadas respecto a semillas no iluminadas.

3. Plántulas con semillas expuestas a la luz muestran una mayor superficie foliar que plántulas con semillas a la oscuridad. La fotosíntesis en semilla de *P. oceanica* es funcional, contribuyendo al crecimiento de la plántula. La actividad fotosintética de la semilla continúa al menos tres meses tras la germinación y su contribución fotosintética es similar a la contribución aportada por las hojas lo cual remarca la importancia de la de la contribución por parte de la semilla.

4. La reserva de carbohidratos (almidón y azúcares libres) y nutrientes (C, N y P) en semillas iluminadas no muestra diferencias significativas respecto de semillas no iluminadas. La movilización de reservas y nutrientes en la semilla es independiente a la fotosíntesis. Asimismo, hay un crecimiento progresivo de las plántulas de Posidonia independientemente de la luz lo cual confirma que la iluminación no es un detonante para la germinación en la semilla de *P. oceanica*.

5. Semillas de *P. australis* y *P. sinuosa* presentan también actividad fotosintética mediante mediciones con fluorímetro (PAM). La presencia de actividad fotosintética en especies australianas y en la especie mediterránea indica que la capacidad fotosintética fue una habilidad adquirida de un antecesor común durante el Eoceno tardío (entre 37.2 y 33.9 millones de años)

6. Las especies australianas y la especie mediterránea de Posidonia presentan similares eficiencias fotosintéticas, "quenching" fotoquímicos (flujos de energía invertidos en la fotoquímica de la planta) y "quenching" no fotoquímicos (flujos de energía disipados en forma de calor como método fotoprotector). Sin embargo, *P. oceanica* muestra significativo mayor ETR y un significativo mayor tamaño que sus parientes australianas.

General Introduction

Sapere aude
(Oracio)

General Introduction

Importance of the seagrass P. oceanica in the Mediterranean Sea

Posidonia oceanica (L.) Delile is the most important and abundant seagrass in the Mediterranean Sea. This endemism forms extensive meadows from the surface down to 40 m water depth and plays a major ecological role, being able to build a ‘‘matte’’, a monumental construction resulting from horizontal and vertical growth of rhizomes with entangled roots and entrapped sediment (Figure 1). As with forests in the terrestrial environment, *P. oceanica* meadows are the climax community and their presence attests to a relatively stable environment.

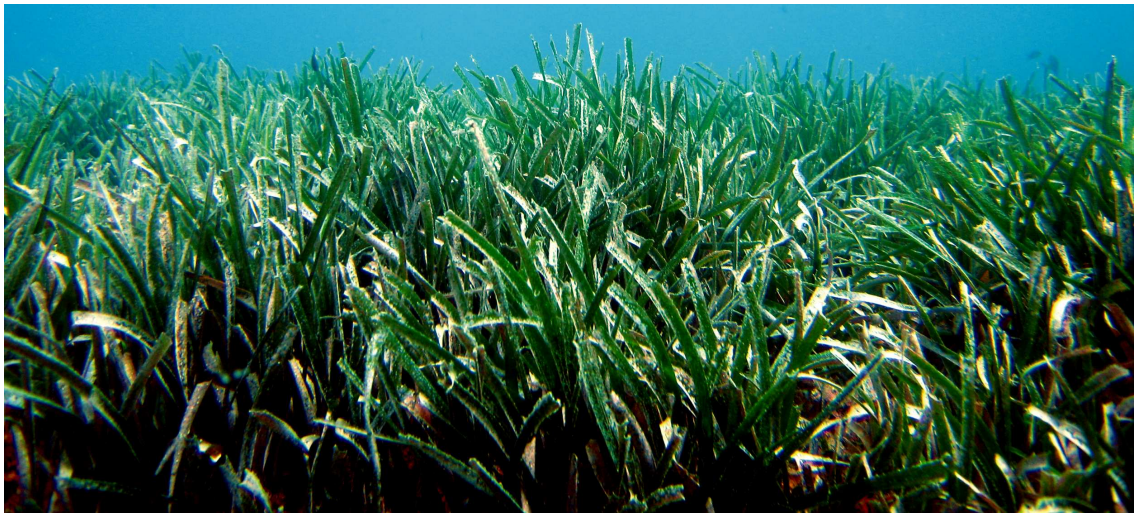


Figure 1.*P. oceanica* meadow to 12 m deep into Mazarrón bay, in Murcia (SE Spain)

In identifying the ecosystem services provided by natural environments, a common practice is to adopt the broad definition of the Millennium Ecosystem Assessment that ‘‘ecosystem services are the benefits that people obtain from ecosystems.’’ Seagrass beds provide a wide range of ecosystem services, including raw materials and food, coastal protection, erosion control, water purification, maintenance of fisheries, carbon sequestration, and tourism yet reliable estimates of the economic values of most of these services are lacking (Barbier et al., 2011).

As Borum et al., (2004) described, *P. oceanica* were highly valued as raw materials and food, for example, the leaves were traditionally used as packing material to transport fragile items (i.e., glassware, pottery) in Mediterranean countries. They were also used to ship fresh fish from the coast to cities. As parasites thrived less in *P. oceanica* leaves than in straw, they were used as cattle bedding in stables and, later, as filling material for mattresses and cushions (Pope Julius III popularized this practice throughout Italy in the 16th century). Respiratory infections seemed to be prevented when sleeping in this type of bedding; other medicinal uses included the alleviation of skin diseases (i.e. acne) and pain in legs caused by varicose veins. When straw was scarce dry *P. oceanica* leaves were used to make adobes, and as roof insulation (e.g., in SE Spain and the Balearic islands).

Likewise, coastal protection and erosion control are often listed as important ecosystem services provided by *P. oceanica*. Gacia and Duarte (2001), examined on an annual time scale, the effect of *P. oceanica* on particle deposition and resuspension. Their results confirmed in the Mediterranean littoral, the presence of *P. oceanica* enhances sediment stability by preventing resuspension.

Seagrasses also generate value as habitat for ecologically and economically important species such as scallops, shrimp, crabs, and juvenile fish (Barbier et al., 2011). *P. oceanica* is an important nursery area for fishes in the Mediterranean Sea. One example is the study developed by Del Pilar Ruso and Bayle-Sempere in 2006 where *P. oceanica* leaf canopy was confirmed as a very important transitional shelter for the early stage larvae of some demersal fish species as Sardinella aurita, Sparidae, Engraulis encrasicolus and Gobiidae.

Seagrasses are involved in carbon sequestration by using carbon dissolved in the seawater (mostly in the form of CO2, but also HCO3-). The detritus burial from vegetated coastal habitats contributes about half of the total carbon burial in the ocean (Duarte et al., 2005). Gacia et al., (2002), quantified the net annual sedimentary flux of carbon and nutrients (N and P) to the sediments under a *P. oceanica* meadow and elucidate the sources and fate of the material deposited. The results presented point to high carbon deposition rates. *P. oceanica* and its associated epiphytes contribute 29% of the sedimentary flux of organic carbon but represent 43% of the organic carbon present

in the sediments, indicating that inputs from below-ground organs and the low decomposition rate of *P. oceanica* relative to other detritus are important. While benthic remineralization rates are threefold higher in the vegetated sediments, the remineralization rate represents a small fraction (10%) of the input, resulting in high carbon and nutrient burial rates in the *P. oceanica* meadows.

Apart of the ecological services produced, it is also important to comment that *P. oceanica* is becoming an important tourist lure to the tourist industry of many Mediterranean countries which supposes direct economic benefits for many local companies. For instance, in Ibiza island, there are a huge offer focused in activities developed on the *P. oceanica* meadow for tourists (e.g diverse companies offer diving with snorkel or taking photos using an underwater camera on the meadow). Also yachts and tourist boats with bottom view to see the *P. oceanica* habitat becomes more popular and scuba diving clubs are also offering submarine itineraries into the *P. oceanica* meadow.

Finally there are some works where medical applications of *P. oceanica* are evaluated. Decoction of the leaves has been quoted to be used as a remedy for diabetes mellitus and hypertension by villagers living by the sea coast of Western Anatolia. A current study driven by Gokce and Haznedaroglu (2008) concluded that oral administration of *P. oceanica* extract (POE) for 15 days, in rats, resulted in a dose-dependent decrease in blood glucose. Likewise, relaxant responses to acetylcholine (ACh) in diabetic thoracic aorta were restored by POE treatment and also attenuated the augmented phenylephrine (PE) and serotonin (5-HT) contractions. At concentration levels of 150 and 250 mg/kg b.wt., POE exerted a protective effect on the significantly decreased levels of antioxidants namely, glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase and nitric oxide (NO).

Likewise phenolics substances excretes by *P.oceanica* and its seeds in stress conditions, is a new promising field into the medical applications. The medical benefit obtained from *P.oceanica* is just another reason to makes the protection and restoration of *P.oceanica* an important challenge for the actual and next generations.

Impacts and conservation of the seagrass P.oceanica

Seagrasses are key components of shallow coastal ecosystems for their contribution to biological productivity and the maintenance of biodiversity, the control of water quality and the protection of the shoreline (Hemminga and Duarte 2000). However, seagrasses are subjected to multiple stressors, including sediment and nutrient runoff, physical disturbance, invasive species, disease, commercial fishing practices, aquaculture, overgrazing, algal blooms, and global warming, causing seagrass declines at scales of square meters to hundreds of square kilometres (Orth 2006).

- *Impacts in P. oceanica*

According to Pergent (2010) in UICN 2012, there have been declines in the population of the seagrass *P. oceanica* due to mechanical damage from trawling and boats, coastal development and eutrophication, with losses particularly observed in the western Mediterranean. The overall decline in the Mediterranean has been measured as approximately 10% over the last 100 years, which does not trigger any of the threatened categories, but further information is needed. Others impacts are becoming important in the last years such coastal salinity changes, introduction of exotic species or global warming. *P. oceanica* is a slow-growing seagrass species with low sexual reproduction what makes that recovery from disturbance to be low.

- *Use of P.oceanica seed as units of transplants*.

Flowering in *P. oceanica* is low, variable and with high rates of fruit abortion and predation, which means that the availability of *P. oceanica* seeds and seedlings is very low and variable, and their use as planting units has been not common. Mature seeds of *P. oceanica* found as drift material in beaches are, however, a valuable source to produce seedlings that could be planted at sea.

For example, *P. oceanica* seedlings from shoreline drift planted in dead matte (the mixture of rhizomes, roots and sediment that this species forms) had a 70%

survivorship after three years while none of those planted in gravel survived (Balestri et al., 1998). Some experiments (Figure 2) driven by our group in the Hornillo Bay (Dominguez et al., 2011) shown the percentage of seedlings surviving after one year in the dead matte was similar to those reported by Balestri et al., (1998) (80 % survival after one year), both for naturally-recruited or planted seedlings, in dead matte at a depth of 10 m in the Ligurian Sea.

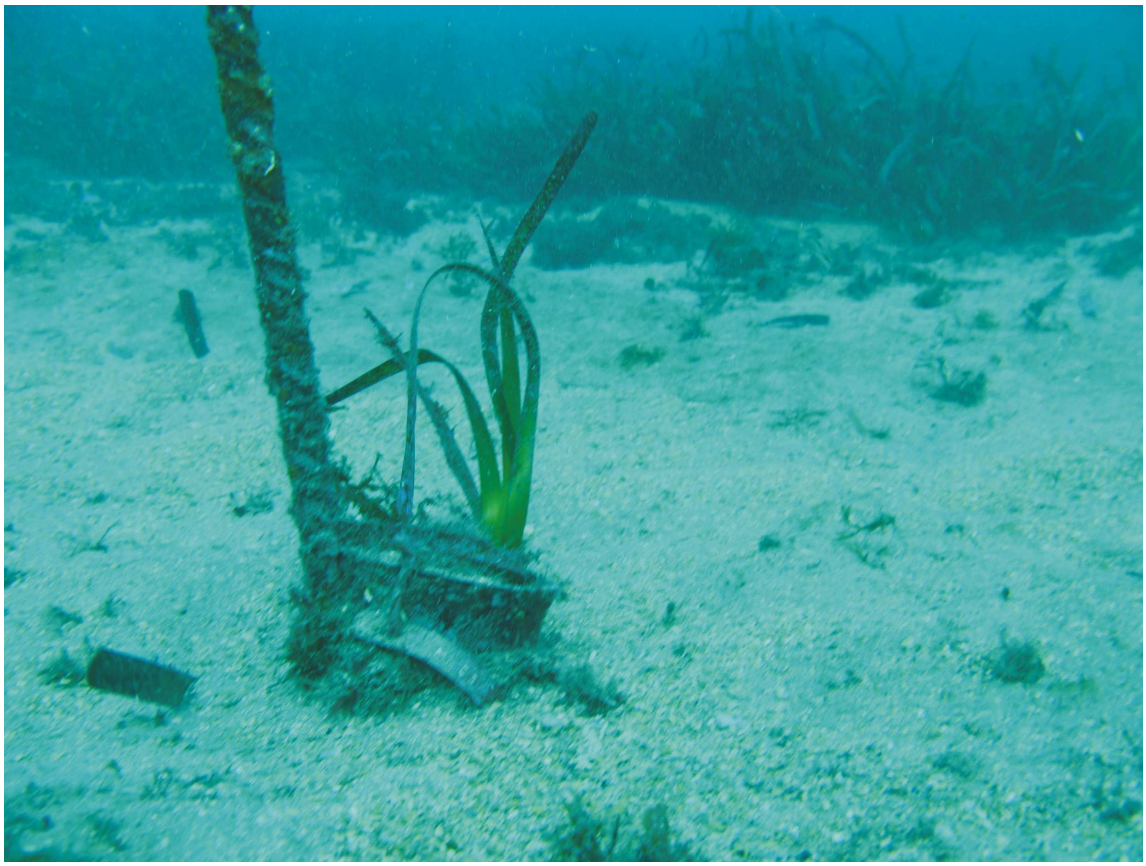


Figure 2 Seedling of *P. oceanica* transplanted in Hornillo bay, Águilas, Murica (SE Spain.). Seedling is attached to a fibrous substrate in a plastic mesh pot.

A similar percentage of naturally-recruited *P. oceanica* seedlings surviving was also found by Piazzi et al., (1999) at the same depth and substratum but it was lower (50 % after one year) at a depth of 2 m (Piazzi et al., 1999). Researching on the conditions and processes that govern the establishment, survival and growth of seagrass planting units, is essential to understand natural colonisation and recovery from disturbance of seagrass meadows and to guide their possible restoration.

Knowledge gaps

There are numerous studies about the understanding of growth dynamics in seagrasses. Lee et al., (2007) synthesizes the current literature on light, temperature and nutrient effects on seagrass physiology and growth. Likewise, Belzunce et al., (2005) described in detail, the mature seed of *P. oceanica* and early plantlet structures by means of anatomical procedures. Despite the extend literature about biology and ecology of seagrasses (Larkum et al., 2006), there is lack of information about some aspects of seed of *P. oceanica* to improve seedling growth as association with other microorganims and early responses of the seed to light.

- *Bacterial association*

Specific association between seed and microorganisms play an important role in plant growth promoting and biological control agents. For example Limura and Hosono in 1998 determined the antifungal activity of a Gram-negative fermentative and oxidative bacteria and Gram-positive bacteria against contaminating fungi on germinating buckwheat seeds. Also Puente et al., (2009) describes a plant–bacterium association between the giant cardon cactus Pachycereus pringlei and endophytic bacteria help seedlings establish and grow on barren rock. There are several examples of association between terrestrial plants and microorganims but this relationships are however unknown in the marine environment.

- *Photosynthesis activity in seeds*

Classically photosynthesis activity in plants is taken place only in leaves, inside the chloroplast at the thylakoid membranes, but seeds of many plant species are green during embryogenesis. Tschiersch et al., (2011) have reported photosynthesis in immature seeds, at some stage during their development in peas and barley caryopsis. All species of genus Posidonia have green seeds and its peculiarity is that these seeds

keep green when they are mature which indicates presence of chlorophyll a and b and possibly photosynthetic activity.

Hypotheses and study objectives

The present Doctoral Thesis studied certain aspects of the non-dormant seed of *P. oceanica* as response to increase the possibilities of survival and growth of the seedling. These strategies are developed in the frame of an oligotrophic environment, the Mediterranean Sea, where nutrients contain is low and organisms develop specified abilities to survive. The study was focused on the microbiologycal and photosynthetical skills of the seed.

In this context, the main hypotheses is based on the idea that the seed of *P.oceanica* is capable of develop some special mechanisms to implement its growth and implement the possibilities of survival not seen in marine plants before. One of these mechanisms is related with the bacterial association with *Marinomonas* to increase the foliar area of the seedling. The second mechanism is about the capacity of developing seed photosynthesis activity to guarantee seedling survival.

According with these two mechanisms, the main goals of this Doctoral Thesis were:

i) To estimate the effect of two bacteria: *Marinomonas posidonica* and *Marinomonas mediterranea* living on the plant in natural conditions respect to the growth of the seedling.

ii) To test photosynthetic activity on the epidermis of the seed (*P. oceanica*) and increasing the knowledge about this mechanism in the first stages of germination, and determining the functionality of the photosynthesis activity in the seedling growth.

iii) To compare photosynthetic activity in other species of the genus Posidonia to determining the role of this feature in the evolution of these seagrasses and the possible ecological implications.

In order to accomplish these tasks, several specific objectives have been developed into four main chapters that correspond to original papers which have been published or sent to scientific journals of international impact:

Chapter 1. Effects of the epibiotic bacteria, *Marinomonas mediterranea* and *Marinomonas posidonica*, on leaf growth and epiphyte communities of the seagrass *Posidonia oceanica.*

The purpose of this chapter was to study the effects of *M. mediterranea* and *M. posidonica* on the leaf growth and the epiphyte community established on *P. oceanica* seedlings. According to this, two assays were run: a growth assay in the laboratory under aseptic conditions and an epiphyte assay in the field under natural conditions.

Chapter 1 was published in 2012:
Celdrán D, Espinosa E, Sánchez-Amat A, Marín A (2012) Effects of the epibiotic bacteria, *Marinomonas mediterranea* and *Marinomonas posidonica*, on leaf growth and epiphyte communities of the seagrass *Posidonia oceanica*. Marine Ecology Progress Series. 456:21-27

Chapter 2. Photosynthetic activity of the non-dormant *Posidonia oceanica* seed

In this chapter, was study the contribution in the leaf area of *P.oceanica* seedlings, by the photosynthesis activity of the seed, during germination, under different dark-light conditions and evaluating the reserves status of the seed to know if photosynthesis activity in non-dormant *P. ceanica* seeds determines its growth.

Chapter 2 was published in 2011: Celdran D, and Marin A (2011) Photosynthetic activity of the non-dormant *Posidonia oceanica* seed. Marine Biology. 158:853–858

Chapter 3. Seed photosynthesis enhances seedling growth in the seagrass *Posidonia oceanica* (L.) Delile

This chapter examined the contribution of seed photosynthesis on leaf growth and mobilisation of the seed reserve and nutrients during the first three months of seedling development of the seagrass *P. oceanica*. To achieve these goals, the changes in leaf growth, concentration of free sugar, starch, and C, N and P in seed of the seedlings in different conditions of light/dark treatments were tested.

Chapter 4. Photosynthesis activity in seed of Australian and Mediterranean Posidonia seagrasses started before continent separation

This chapter testied if the two of the most abundant species of Australian Posidonia (*P. australis* and *P. sinuosa*) display photosynthesis activity as seeds of the Mediterranean specie (*P. oceanica*). Based on the three species of study are not close genetically and geographically separated by thousand of kilometres. The objectives of our study were determined and compare photosynthesis activity and respiration in seeds of *P. oceanica*, *P. australis* and *P. sinuosa* by fluorescence (PAM) and oxygen production during germination.

References

Balestri E, Piazzi L, Cinelli F (1998) Survival and growth of transplanted and natural seedlings of *Posidonia oceanica* (L.) Delile in a damaged coastal area. Journal of Experimental Marine Biology and Ecology. 228:209-225

Barbier EB (2007) Valuing ecosystem services as productive inputs. Economic Policy. 22:177–229

Barbier E, Hacker SD, Koch E, Stier AC, Silliman B (2011) The value of estuarine and coastal ecosystem services. Ecological Monographs. 81 (2)169 – 193

Belzunce M, Navarro RM, Rapaport HF (2005) Seed and early plantlet structure of the Mediterranean seagrass *Posidonia oceanica*. Aquatic Botany. 82:269–283

Borum J, Duarte CM, Krause-Jensen D, Greve TM (2004) European seagrasses: an introduction to monitoring and management. The M&MS project, Copenhagen. ISBN: 87-89143-21-3, pp:95

Boudouresque CF, Bernard G, Bonhomme P, Charbonnel E, Diviacco G, Meinesz A, Pergent G, Pergent-Martini C, Ruitton S, Tunesi L (2006) Preservation et conservation des herbiers a` *Posidonia oceanica*. RaMoGe Publication M onaco. pp:202

Del Pilar Ruso, Y, Bayle-Sempere JT (2006) Diel and vertical movements of preflexion fish larvae assemblages associated with *Posidonia oceanica* beds. Scientia Marina (Barceloma). 70(3):399-406

Duarte CM, Middleburg JJ, Caraco N, (2005) Major role of marine vegetation on the oceanic carbon cycle. Biogeosciences 2:1–8

Francour P, Ganteaume A, Poulain M (1999) Effect of boat anchoring in *Posidonia oceanica* seagrass beds in the Port-Cros national park (north-western

Mediterranean sea). Aquatic Conservation: Marine Freshwater Ecosystem. 9:391-400

Francour P, Magreau JF, Mannoni PA, Cottalorda JM, Gratiot J (2006) Management guide for Marine Protected Areas of the Mediterranean Sea, Permanent Ecological Moorings. Universite´ de Nice-Sophia Antipolis & Parc National de Port-Cros, Nice pp:68

Gacia E, Duate CM (2001) Sediment retention by a Mediterranean *Posidonia oceanica* meadow: The balance between deposition and resuspension. Estuarine Coastal and Shelf Science. 52:505 – 514

Gacia E, Duarte CM, Middelburg JJ (2002) Carbon and nutrient deposition in a Mediterranean seagrass (*Posidonia oceanica*) meadow. Limnology and Oceanography. 47:23-32

Gokce G, Haznedaroglu MZ (2008) Evaluation of antidiabetic, antioxidant and vasoprotective effects of *Posidonia oceanica* extract. Journal of Ethnopharmacology. 115:122-30

Hemminga ME, Duarte CM (2000) Seagrass Ecology. Cambridge University Press, Cambridge. pp:298

Larkum A, Orth JR, Duarte CM (2006) Seagrasses: Biology, Ecology and Conservation. Springer. Dordrecht.

Lee KS, Park RS, Kim KY (2007) Effects of irradiance, temperature, and nutrients on growth dynamics of seagrasses: A review.Journal of Experimental Marine Biology and Ecology. 350:144–175

Limura K, Hosono A (1998) Antifungal Activities of Bacteria Endemic to Buckwheat Seeds. Fagopyrum. 15:42 – 54

MEA [Millennium Ecosystem Assessment] (2005) Ecosystems and human well-being: current state and trends. Coastal systems. Island Press, Washington, D.C., USA.

Orth RJ, Carruthers TJB, Dennison WC, Duarte CM, Fourqurean JW, Heck KLJ, et al. (2006). A Global Crisis for Seagrass Ecosystems. Bioscience. 56(12):987–996

Pergent G, Semroud R, Djellouli A, Langar H, Duarte CM (2010) *Posidonia oceanica*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1.

Piazzi L, Acunto S, Cinelli F (1999) In situ survival and development of *Posidonia oceanica* (L.) Delile seedlings. Aquatic Botany. 63:103-112

Puente ME, Li CY, Bashan Y (2009) Rock-degrading endophytic bacteria in cacti. Environmental and Experimental Botany. 66:389–401

Tschiersch H, Borisjuk L, Rolletschek H (2011) Gradients of seed photosynthesis and its role for oxygen balancing. Biosystem. 103:302-308

Chapter 1: Effects of the epibiotic bacteria, Marinomonas mediterranea and Marinomonas posidonica, on leaf growth and epiphyte communities of the seagrass Posidonia oceanica.

Nihili est qui nihil amat
(Plauto)

Abstract

Beneficial effects of microorganisms on plant productivity are well known in terrestrial ecosystems, but no corresponding effects on marine plants have been described. This work tested the hypothesis that the bacteria *Marinomonas posidonica* and *Marinomonas mediterranea*, which have been described as forming part of the microbiota of *Posidonia oceanica,* influence leaf growth and epiphytic communities of this seagrass. Four treatments were developed: seedlings inoculated with *M. mediterranea* or *M. posidonica* and seedlings to which sterilized seawater (sterile control) or fresh seawater (field control) was added. Two experiments were run: a growth assay in the laboratory in sterile conditions measuring leaf area after three months, and an epiphyte assay in which the seedlings were transferred to the field and at three months a sample of leaves was collected to analyse the community structure of epiphytes. The growth experiment showed that inoculation with *M. posidonica* enhanced leaf growth of *P. oceanica,* compared with the controls to which no bacterial strain was added. In contrast, inoculation with *M. mediterranea* had no stimulatory effect on leaf growth. Likewise inoculation with *M. posidonica* induced changes in epiphyte structure and can be expected to have a regulatory effect on macroepiphytes structure. The effect of *M. posidonica* could have a direct application in seagrass restoration programmes with *P. oceanica.*

Introduction

Specific microbial-plant associations play an important role in promoting plant production in terrestrial ecosystems. For example, Rhizobium spp. and Glomus spp. produce respectively nitrogen-fixing nodules and arbuscular mycorrhizal fungi that enhance plant growth in nutrient-limited soil (Avis et al. 2008). The ecological importance of these associations in terrestrial plants is such that Glomus spp. are obligate symbionts of more than 80% of terrestrial plants (St-Arnaud et al. 1996, Selosse et al. 2006). Other microorganisms, such as Pseudomonas spp., indirectly assist plant productivity through the control of plant pathogens. In particular, Pseudomonas spp. has been widely studied for their ability to reduce the development of various soilborne plant pathogens (Carisse et al. 2003). Numerous modes of action have been reported for Pseudomonas spp., including the production of antimicrobial compounds (Thrane et al. 2000), competition (Ellis et al. 1999) and the induction of plant defence mechanisms (Ongena et al. 2000, 2002). Whether relationships between bacteria and host plant surfaces also play an important role in maintaining productivity of primary producers in marine ecosystems is, however, unknown.

Marine biofouling communities are complex, highly dynamic ecosystems consisting of a wide range of organisms. The development of such communities begins with bacterial attachment followed by colonisation by higher organisms such as invertebrate larvae and algal spores (Richmond & Seed 1991, Rodriguez et al. 1993, Maki 1999, Callow 2000). The initial phases of the colonization of a new surface occur in a period of time lasting from minutes to days. Bacteria becomes established in the first hour, diatoms the first day and larval spores after one week (Wahl 1989).

Bacteria on living plant surfaces appear to form spatially structured, host-specific, and relatively stable communities during the life-span of their host-plants (Bhadury et al. 2004, Pasmore et al. 2003, Egan et al. 2008). Competition between bacteria, the ability to resist grazing, and host-derived factors such as surface-localized secondary metabolites are some of the factors likely to determine the final surface community composition (Givskov 1996, Manefield et al. 2001, Matz et al. 2005, Rao et al. 2005, Egan et al. 2008). In terms of chemical ecology, it seems likely that some

strains of epibiotic bacteria on living surfaces play a 'protective' role, releasing chemicals that prevent biofouling by other organisms (Armstrong et al. 2001, Rao et al. 2005). Studies of different bacterial species have underlined the importance of exopolymeric substances for the activation or inhibition of macrofouling settlement (Young & Mitchell 1973) suggesting that initial bacterial composition would have a cascading effect on the structure of the epiphyte community, although this aspect remains unexplored.

Leaves and rhizomes of *Posidonia oceanica* L (Delile) offer suitable substrata for the settlement and growth of a number of sessile (epiphytic) organisms that form stratified multi-species assemblages (Mazzella et al. 1989). Leaf epiphytes may account for up to 30% of the canopy biomass of *P. oceanica* (Mazzella & Ott 1984), support a substantial community of macro- and micrograzers (Orth & Van Montfrans 1984) and display high species diversity (Boero 1981, Mazzella et al. 1989). Encrusting red and brown algae, filamentous algae, encrusting and erect bryozoans, hydroids and foraminifera are some of the most common morphological groups found in *P. oceanica* blades (Pardi et al. 2006). The abundance and wide species composition of these epiphytes support most of the herbivores that inhabit seagrass patches (Kitting et al. 1984, Nichols et al. 1985, Gleason 1986, Dauby 1989).

This work tests the hypothesis that the bacteria *Marinomonas mediterranea* (Solano et al. 1997) and *Marinomonas posidonica* (Lucas-Elio, Marco-Noales, Espinosa, Ordax, Lopez, Garcias-Bonet, Marba, Duarte and Sanchez-Amat 2010), which form part of the microbiota of *P. oceanica,* would stimulate seagrass growth, inhibit seagrass epiphytes and influence epiphyte community structure on seagrass. The melanogenic marine bacterium *M. mediterranea* displays a rich secondary metabolism. It expresses two different growth-phase-regulated polyphenol oxidases (PPOs), a tyrosinase and a laccase (Lucas-Elio et al. 2002). The tyrosinase is involved in melanin synthesis using tyrosine as substrate (López-Serrano 2004). In addition, *M. mediterranea* synthesizes an antibacterial protein, named marinocine, which shows activity against both gram-positive and gram-negative bacteria. The antimicrobial activity of marinocine is due to the hydrogen peroxide generated by its lysine oxidase activity (Gomez et al. 2006). Contrary to *M. mediterranea*, *M. posidonica* neither synthesizes melanin nor expresses an extracellular lysine oxidase (Espinosa et al. 2010).

M. posidonica, which only have been isolated from the surface of *P. oceanica*, could, however, still play an important roll in influencing plant growth if it facilitates other beneficial bacterial species or secretes nutritional substances of benefit to the plant.

Our aim was to study the effects of *M. mediterranea* and *M. posidonica* on the leaf growth and the epiphyte community established on *P. oceanica* seedlings. Two assays were run: a growth assay in the laboratory under aseptic conditions and an epiphyte assay in the field under natural conditions.

Material and Methods

Bacterial strains

M. mediterranea MMB-1 (ATCC 700492) and *M. posidonica* IVIA-Po-181 (NCIMB 14433) were routinely grown in Marine Broth or Marine Agar 2216.

Growth assay

P. oceanica fruits were collected at Hornillo Bay (Murcia, SE Spain) in April and May 2008. Forty fruits were sterilized in 70% ethanol and bleach (30:70) for 30 minutes and then rinsed three times in sterile distilled water. After sterilization, all the seeds were extracted from the fruits and individually germinated in solid agar substrate in glass bottles with 150 ml. The solid substrate was prepared with sterilized bidistilled water, 8% bacteriological agar and marine salt (PRODAC) (36 g.l-1), following methodology of Balestri et al. (1998).

All cultures were maintained in a Versatile Environmental Test Chamber (Sanyo MLR-351) at 20 ± 1º C with a photoperiod of 16 h light:8 h dark (PAR 90 µmol m-2 s-1). Two weeks after plantation in agar, 200 ml of artificial seawater (prepared with bidistilled water and the above mentioned marine salt (36 g.l-1) was added to every bottle. The germinated seeds (henceforth referred to as seedlings) had a single rhizome with two leaves (mean leaf area: 1.17 cm2) and one root.

Four treatments were developed (n = 8 seedlings per treatment): seedlings inoculated with 108 cells of *M. mediterranea* or *M. posidonica* diluted in 2 ml of sterilized seawater (MM and MP treatments, respectively) and seedlings to which 2 ml of sterilized seawater (sterile control) or fresh seawater (field control) was added (SC and FC treatments, respectively), (Fig. 3). Fresh seawater was collected from Hornillo Bay (Águilas, SE Spain). SC treatment was used to check the plant growth without

bacterial influence while FC treatment was used to compare the influence of the complex interactions of marine bacterial communities.

All the seedlings were incubated in the conditions mentioned above. Plating confirmed that inoculations were successful for each of the bacterial treatments. Tubes in which contamination was observed were eliminated, so that a total number of 32 seedlings were used. Three months after inoculation in the laboratory, the leaf area of every seedling was measured with a ruler.

Epiphyte assay

In July 2008, after three months in the laboratory, seedlings were transplanted to the field, to an unvegetated patch of dead rhizomes at 13 m depth in Hornillo Bay. Prior to transplant, each seedlings was extracted from its glass bottle and transferred to a separate plastic mesh pot (8 cm high x 8 cm diameter) with fiberglass as substrate. The pots were labelled and transported in a plastic container (200 l) with sterile sea water and an air pump to the field area (2h from the laboratory).

The pots with the seedlings were taken to the sea floor, and buried in sediment by scuba divers. Pots of the four treatments were randomly placed every 2 m along three transects. Herbivores were not excluded to avoid caging effects on leaf macroepiphytes. Leaf macroalgal epiphytes were examined after 90 days (September 2008) on the most external leaf of each seedling to ensure that the analysis focused on mature assemblages (Vanderklift & Lavery 2000, Buia et al. 2003, Pardi et al. 2006).

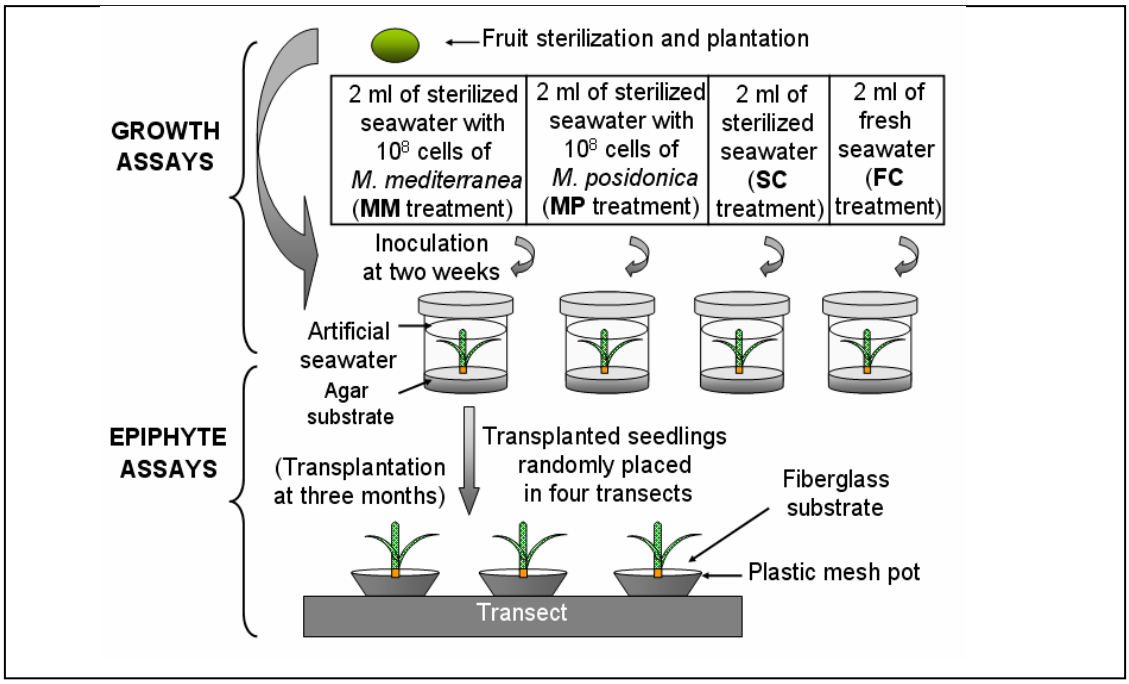


Figure 3. Representation of the growth and epiphyte assays performed

The top 10 cm (from the tip) of seagrass leaves were examined for macroalgal epiphytes under a dissecting microscope fitted with a grid of 100 squares. The macroalgal epiphytes were assigned to morphological groups (filamentous, red crustose, brown crustose, green crustose, red corticated, brown corticated, foliose and articulated calcareous algae), according to Steneck & Dethier (1994). Functional groupings of algae are based on anatomical and morphological characteristics that correspond to ecological conditions. The abundance of each morphological group was obtained by examining 5 visual fields of 1 cm2 on the upper surface of the leaf and recording the presence or absence of that taxon within 9 squares per visual field, as a total of 45 (9 x 5) squares examined. This was considered a sufficient sample size as the cumulative frequency of epiphyte species had reached a plateau at that number of visual fields (species-area curve). The total area examined for epiphytes was 5 cm2, following the methodology of Pardi et al. (2006) and Piazzi et al. (2004). A percentage cover of each morphological group was calculated by determining the proportion of squares out of the total (45) in which it was present.

Data analyses

Differences in leaf area among the four treatments of the laboratory growth assay were compared using a one-way ANOVA. Prior to ANOVA, data were tested using a Kolmogorov–Smirnov test for normality and a Cochran test for homogeneity of variance ($\alpha = 0.05$). After ANOVA, differences between specific treatments were determined with Tukey HSD post-hoc test ($\alpha = 0.05$).

PRIMER (Plymouth Routines in Multivariate Ecological Research, version 6; Clarke & Warwick 2001) was used to examine differences in epiphyte communities among treatments of the field experiment. Hierarchical cluster analysis and multidimensional scaling (MDS) of Bray-Curtis similarities among fourth-root transformed data was used to compare epiphyte community composition between treatments. The similarity analysis (SIMPER, Clarke & Warwick 2001) was used to identify which species made the greatest contributions to those differences. The ratio of the contribution of each species to average dissimilarity/standard deviation (> 1.3) of the Bray-Curtis species dissimilarity between two samples was used to determine those species that contribute much to the dissimilarity between two groups. These species are thus considered good discriminating species (Clarke and Warwick, 2001).

The abundance of epiphyte morphological groups, identified by SIMPER as important contributors to multivariate differences, was compared between treatments, transects and interaction transects-treatments using a two-way ANOVA.

A two-way PERMANOVA was used to determine if significant effects occurred between treatments, transects and if there were any interactive effects between transect and epiphyte community structure (Anderson 2001) with a post hoc pair-wise comparison between treatments (P-values from PERMANOVA analysis were obtained using 9999 permutations). The Bray–Curtis similarity coefficient was employed to construct a similarity matrix from the fourth-root transformed densities of macroepiphytes species recorded for each replicate.

To check that differences in community composition among treatments were not an artefact of the differing leaf areas among these (species-area hypothesis posits an

increase in species richness as the area increases, Cain 1938) a Pearson's correlation between leaf area and the number of functional groups of epiphyte present was run on data from all treatments .

Results

Growth assay

Microbial inoculation had a clear effect on leaf area (Fig. 4), (ANOVA F3, 28 = 6.6, p = 0.002). MP and FC had a larger leaf area than the MM and SC treatments (MP = FC)>(SC = MM), (Tukey-HSD post hoc test, p<0.05). MM showed no significant differences from the SC treatment, and MP and FC were similarly statistically indistinguishable from one another (Tukey-HSD *post hoc* test, p>0.05).

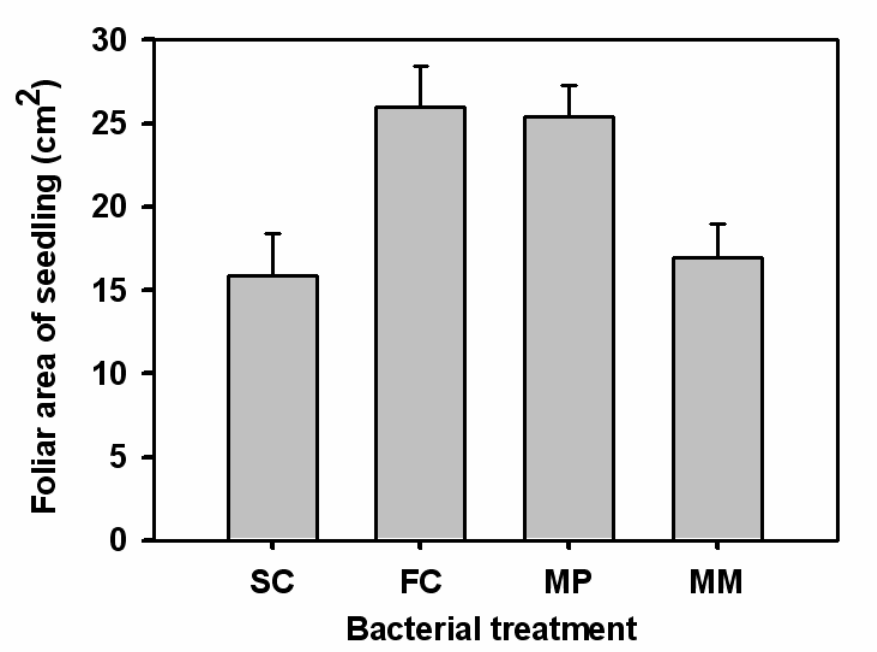


Figure 4. Mean (± SD) leaf area of *P. oceanica* seedlings in the laboratory, three months after inoculation. (MM: *M. mediterranea* diluted in sterilized seawater. MP: *M. posidonica* diluted in sterilized seawater. SC: sterilized seawater control. FC: field seawater control). Letters "a" and "b" refer to two statistically different groups by Tukey HSD post-hoc test ($\alpha = 0.05$).

Epiphyte assay

Prior to the commencement of the field experiment, epiphytes were absent from the SC, MM, MP treatments. Macroalgae were similarly absent from the FC treatment, although some leaves were covered by biofilm.

By the conclusion of the field experiment, the experimental seedlings were colonized by filamentous, red crustose, brown crustose, green crustose, red corticated, brown corticated, foliose and articulated calcareous algae. MDS showed a different epiphyte community structure between the treatments MM and MP (Figure 5) but a high dispersion of replicates within the FC and SC treatments such that these latter two treatments could not be clearly distinguished from one another or the other treatments.

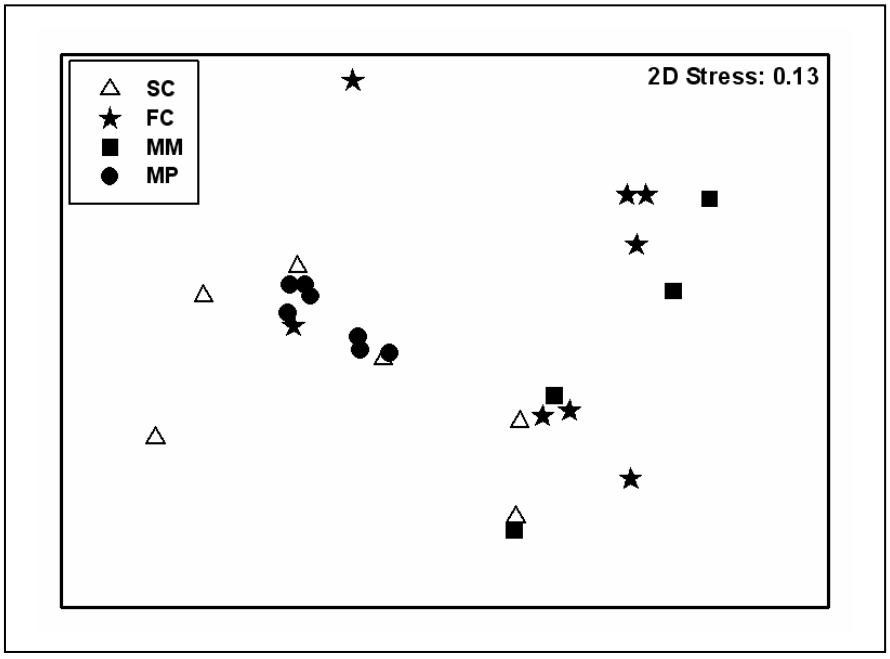


Figure 5. MDS biplot of macroepiphyte similarity in *P. oceanica* seedlings among the four inoculation treatments (MM: *M. mediterranea* diluted in sterilized seawater. MP: *M. posidonica* diluted in sterilized seawater. SC: sterilized seawater control. FC: field seawater control).

The results for a two-way PERMANOVA showed significant differences between treatments (pseudo-$F_{3,15} = 3.17$, $p = 0.01$), but there was no difference between

transects (pseudo-$F_{2,15}$ = 1.16, p = 0.73) or between the interaction of transect location and inoculation treatment (pseudo-$F_{6,15}$ = 0.52, p = 0.73). Pair-wise comparisons among all pairs of inoculation treatments revealed no significant differences except between MP and MM (t = 4.27, p = 0.0003). SIMPER analysis indicated that patterns in the epiphyte macroalgal community were driven mainly by differences in the abundance of red crustose, filamentous and red corticated algae (average dissimilarity/standard deviation > 1.3). However, the, two-way ANOVA analysis did not show significant differences in morphological group of epiphytes between treatment, transect location or the interaction treatments and transect location. The abundance of epiphytes growth forms of the different treatments are shown in Table 1.

Epiphytes growth forms	FC	SC	MM	MP
Filamentous	3.16 ± 1.19	3.27 ± 0.67	3.80 ± 0.98	3.82 ± 0.57
Brown corticated	0	0.42 ± 0.17	0.16 ± 0.13	0
Red corticated	1.2 ± 0.62	0.52 ± 0.35	0.26 ± 0.26	1.45 ± 0.45
Brown crustose	0.03 ± 0.03	0.05 ± 0.05	0.03 ± 0.03	0
Red crustose	4.86 ± 1.23	5.12 ± 0.84	4.50 ± 1.47	7.22 ± 0.20
Green crustose	0	0.07 ± 0.07	0	0
Foloiose	0.1 ± 0.06	0.30 ± 0.19	0	0.27± 0.18
Articulate calcareous	0	0.17 ± 0.175	0	0

Table 1. Abundance of epiphyte growth forms (filamentous, brown corticated, red corticated, brown crustose, red crustose, green crustose, foliose and articulated calcareous) found on seedlings 90 days after of transplantation of the four treatments (mean ± SE).

The Pearson test of linear correlation indicated that there was no relationship between the leaf area and the number of morphological groups of epiphyte present (r^2 = 0.02, p = 0.48).

Discussion

In terrestrial plants, microorganisms which are known to have a beneficial effect on plants are generally classified into two broad groups based on their primary effects: (i) microorganisms with direct growth-promoting effects on plants and (ii) biological control agents that indirectly assist with plant productivity through the control of plant pathogens. The growth assay with seedlings cultured in sterilized media confirmed that inoculation with *M. posidonica* enhanced leaf growth in *P. oceanica* compared to the sterile control with no bacterial strain added. In contrast, inoculation with *M. mediterranea* did not have a stimulatory effect on leaf growth. No significant differences were observed in leaf growth between *M. mediterranea* treatment (MM) and sterile control (SC), suggesting that the stimulatory effect on growth from *M. posidonica* did not occur with *M. mediterranea*. However, similar effects on growth to those observed with *M. posidonica* were observed when non-sterilized seawater was added (FC treatment), suggesting that some microorganisms from the seawater sample were able to colonize seedlings, thereby producing a similar stimulatory effect as the *M. posidonica* treatment (MP).

Several mechanisms could explain the influence of bacterial strains on *P. oceanica* seedlings: a) They play a 'protective' role, releasing chemicals that prevent biofouling by other organisms (Armstrong et al. 2001, Rao et al. 2005), b) They indirectly assist plant productivity through the control of plant pathogens (Carisse et al. 2003), c) They favour growth of organisms that are beneficial to the plant and d) They liberate metabolites or nutrients that induce plant growth. Because the experiments were carried out in sterilized conditions without influence of pathogens or fouling organisms, mechanisms a), b) and c) can be excluded. We could conclude that it is possible that *M. posidonica* secreted metabolites or nutrients that favour seedling growth. More information about the metabolic capacities of *M. posidonica* and *M. mediterranea* is needed to be able to identify the exact mechanisms that influence plant growth.

The epiphyte assay demonstrated that macroalgal epiphyte communities responded to the microbiota present on seagrass. This study showed that patterns in the epiphyte macroalgal community were driven mainly by changes in the abundance of red

crustose, filamentous and red corticated algae, which were more abundant in the *M. posidonica* treatment (MP). The highest abundance of these functional groupings of algae has been found in habitats with high diversity and low disturbance (Steneck & Dethier 1994).

Differences in epiphyte community structure between the *M. posidonica* (MP), the *M. mediterranea* (MM) and the sterile control (SC) treatments could be also explained by the greater leaf area of the MP treatment (species-area hypothesis). However, the results of Pearson test of linear correlation between the leaf area and the number of morphological groups, do not support this hypothesis. In addition, pilot studies indicated that the cumulative frequency of epiphyte species had reached a plateau at the leaf area sub-sampled by this study. This suggests that differences in epiphyte community structure between microbial treatments were due to direct effects of the microbes and not due to the indirect effect of changes in the size of blades, resulting from microbial effects on growth-rate.

Previous studies found high variability in the epiphytes of *P. oceanica* leaves (Piazzi et al. 2004, 2007) and of other species of seagrasses (Vanderklift & Lavery 2000) at a scale of metres. This small-scale variability may be influenced by differences in shoot density and the characteristics of the canopy, which affect light intensity and water movement (Gambi et al. 1989), local hydrodynamic flows that affect the dispersal, settlement and recruitment of propagules (Trautman & Borowitzka 1999, Vanderklift & Lavery 2000), and/or the role of biotic interaction, such as grazing pressure and the influence of established assemblages on potential recruitment (Mazzella & Russo 1989).

In conclusion, the laboratory analysis with sterile seedlings of the seagrass *P. oceanica* showed that inoculation with the bacteria *M. posidonica* enhances leaf growth. In contrast, seedlings inoculated with *M. mediterranea* did not show significant differences from sterile seawater controls. The beneficial effects of microorganisms are well known in terrestrial ecosystems, where they promote plant productivity but no corresponding effects on marine plants have been described.

This study demonstrates that microorganisms can also promote plant productivity in marine ecosystems. Inoculation with *M. posidonica* induced changes in

epiphyte structure and can be expected to have a regulatory effect on macroepiphytic community structure, especially when this bacterium is predominant on the seagrass. This supports the idea that the inoculation of seedlings with bacteria could influence the establishment of the epiphyte community. The effect of *M. posidonica* could have a direct application in seagrass restoration programmes with seedlings with *P. oceanica.*

Acknowledgements

Funds for this study were provided by the research grant 116/SGTB/2007/1.3 of the Ministerio de Medio Ambiente, Rural y Marino of Spain. We wish to thank CULMAREX S.A. group for scuba diving assistance, Francisco Navarrete and Javier Lloret Barba for help during field and laboratory assays. The research was further facilitated by a grant to D. Celdrán from the Ministerio de Ciencia e Innovación Español, Programa Nacional de Formación de Profesorado Universitario, Spain.

References

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecology. 26:32-46

Armstrong, Liming Yan, Kenneth G, Boyd, Phillip C, Wright1, Grant Burgess J (2001) The symbiotic role of marine microbes on living surfaces. Hydrobiologia. 461:37–40

Avis TJ, Gravel V, Antoun H, Tweddell RJ (2008) Multifaceted beneficial effects of rhizosphere microorganisms on plant health and productivity. Soil Biology & Biochemistry. 40:1733–1740

Balestri E, Piazzi L, Cinelli F (1998) In vitro germination and seedling development of *Posidonia oceanica*. Aquat Bot. 60:83-93

Bhadury P, Wright PC (2004) Exploitation of marine algae: biogenic compounds for potential antifouling applications. Planta. 219:561–578

Boero F (1981) Systematics and ecology of the hydroid population of the two *Posidonia oceanica* meadows. PSZNI Mar Ecol.2(3):181-197

Buia MC, Gambi MC, Dappiano M (2003) I sistemi a fanerogame marine. Biologia Marina Mediterranea. 10:145–198

Cain SA (1938) The Species-Area Curve. Am Midl Nat 19:573-581

Callow ME (2000) Algal biofilms. In: Evans LV (eds) Biofilms, Recent Advances in their Study and Control. Overseas Publishing Associates (UK), Amsterdam, p 189-210

Carisse O, Bernier J, Benhamou N (2003) Selection of biological agents from composts for control of damping-off of cucumber caused by Pythium ultimum. Canadian Journal of Plant Pathology. 25:258–267

Clarke KR, Warwick RM (2001) Change in Marine Communities: An Approach to Statistical Analysis and Interpretation, 2nd edition. PRIMER-E, Plymouth, UK

Dauby P (1989) The stable carbon isotope ratios of benthic food webs of the Gulf of Calvi. Corsica Cont Shelf Res. 9:181–195

Egan S, Thomas T, Kjelleberg S (2008) Unlocking the diversity and biotechnological potential of marine surface associated microbial communities. Curr Microbiol. 11(3):219-225

Ellis RJ, Timms-Wilson TM, Beringer JE, Rhodes D, Renwick A, Stevenson L, Bailey MJ (1999) Ecological basis for biocontrol of damping-off disease by Pseudomonas fluorescens 54/96. J Appl Microbiol. 87:454–463

Espinosa E, Marco-Noales E, Gómez D, Lucas-Elío P, Ordax M, Garcías-Bonet N, Duarte CM, Sanchez-Amat A (2010) Taxonomic study of Marinomonas strains isolated from the seagrass *Posidonia oceanica*, with descriptions of Marinomonas balearica sp. nov. and Marinomonas pollencensis sp. nov. Int J Syst Evol Microbiol. 60:93–98

Gambi MC, Giangrande A, Chessa LA, Manconi R, Scardi M (1989) Distribution and ecology of polychaetes in the foliar stratum of a *Posidonia oceanica* bed in the bay of Porto Conte (N.W. Sardinia). In Boudouresque CF, Meisnesz A, Fresi E, Gravez V (eds) International Workshop on *Posidonia oceanica* Beds II. GIS Posidonie, Marseille, p 1452156

Givskov M, de Nys R, Manefield M, Gram L, Maximilien R, Eberl L, Molin S, Steinberg PD, Kjelleberg S (1996) Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. J Bacteriol. 178:6618– 6622

Gleason BF (1986) Utilization of salt marsh plants by postlarval brown shrimp: carbon assimilation rates and food preferences. Mar Ecol Prog Ser. 31:151–158

Gomez D, Lucas-Elio P, Sanchez-Amat A, Solano F (2006) A novel type of lysine oxidase L-lysine-ε-oxidase. Biochim Biophys Act. 1764:1577-1585

Kitting CL, Fry B, Morgan MD (1984) Detection of inconspicuous epiphytic algae supporting food webs in seagrass meadows. Oecologia. 62:145–149

López-Serrano D, Solano F, Sanchez-Amat A (2004) Identification of an operon involved in tyrosinase activity and melanin synthesis in *Marinomonas mediterranea*. Gene. 342:179–187

Lucas-Elio P, Solano F, Sanchez-Amat A (2002) Regulation of polyphenol oxidase activities and melanin synthesis in *Marinomonas mediterranea*: identification of ppoS, a gene encoding a sensor histidine kinase. Microbiology. 148:2457–2466

Lucas-Elío P, Marco-Noales E, Espinosa E, Ordax M, López MM, Garcías-Bonet N, Marbà N, Duarte CM,Sanchez-Amat A (2011) *Marinomonas alcarazii* sp. nov., *M. rhizomae* sp. nov., *M. foliarum* sp. nov., *M. posidonica* sp. nov. and *M. aquiplantarum* sp. nov., isolated form the microbiota of the seagrass *Posidonia oceanica.* Int J Syst Evol Microbiol. 61:2191-2196

Maki JS (1999) The infuence of marine microbes on biofouling. In: Fingerman M, Nagabhushanam R & Thompson MF (eds) Biofilms, Bioadhesion, Corrosion and Biofouling. Vol 3, Science Publishers, New Dehli, p 147- 171

Manefield M, Welch M, Givskov M, Salmond GP, Kjelleberg S (2001) Halogenated furanones from the red alga, Delisea pulchra, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen Erwinia carotovora. FEMS Microbiol Lett. 205:131–138

Matz C, Kjelleberg S (2005) Off the hook—how bacteria survive protozoan grazing. Trends Microbiol. 13:302–307

Mazzella L, Ott JA (1984) Seasonal changes in some features of *Posidonia oceanica* (L.) Delile leaves and epiphytes at different depths. In Boudouresque CF, Juedy de Grissac A, Olivier J (eds) First International Workshop on *Posidonia oceanica* beds, G.I.S. Posidonie Publ Fr. 1:119-127

Mazzella L, Scipione MB, Buia MC (1989) Spatio-temporal distribution of algal and animal communities in a *Posidonia oceanica* meadow. P.S.Z.N: Mar Ecol. 10(2)107-129

Nichols PD, Klump DW, Johns RB (1985) A study of food chains in seagrass communities. III. Stable carbon isotope ratios. Aust J Mar Freshw Res 36:683–690

Ongena M, Daayf F, Jacques P, Thonart P, Benhamou N, Paulitz TC, Be´ langer RR (2000) Systemic induction of phytoalexins in cucumber in response to treatments with fluorescent pseudomonads. Plant Pathology. 49:523–530

Ongena M, Giger A, Jacques P, Dommes J, Thonart P, (2002) Study of bacterial determinants involved in the induction of systemic resistance in bean by Pseudomonas putida BTP1. European Journal of Plant Pathology. 108:187–196

Orth JR, Van Montfrans J (1984) Epiphytes-seagrass relationships with an emphasis on the role of micrograzing: a review. Aquat Bot 18:43-69

Pardi G, Piazzi L, Balata D, Papi I, Cinelli F, Benedetti-Cecchi L (2006) Spatial variability of *Posidonia oceanica* (L.) Delile epiphytes around the mainland and the islands of Sicily (Mediterranean Sea). Mar Ecol. 27:398-399

Pasmore M, Costerton JW (2003) Biofilms, bacterial signaling, and their ties to marine biology. J. Ind. Microbiol. Biotechnol. 30:407–413

Piazzi L, Balata D, Cinelli F, Benedetti-Cecchi L (2004) Pattern of spatial variability in epiphytes of *Posidonia oceanica*. Differences between a disturbed and two references locations. Aquat Bot. 79:345–356

Piazzi L, De Biasi AM, Balata D, Pardi G, Boddi S, Acunto S, Pertusati M, Papi I, Cinelli F, Sartoni GF (2007) Species composition and patterns of spatial variability of morphological forms of macroalgal epiphytic assemblages of the seagrass *Posidonia oceanica*. Vie Milieu 57:1–9

Rao D, Webb JS, Kjelleberg S (2005) Competitive interactions in mixed-species biofilms containing the marine bacterium Pseudoalteromonas tunicata. Appl Environ Microbiol. 71:1729–1736

Richmond MD, Seed R (1991) A review of marine macrofouling communities with special reference to animal fouling. Biofouling. 3:151-168

Rodriguez SR, Ojeda FP, Inestrosa NC (1993) Settlement of benthic marine invertebrates. Mar Ecol Prog Ser. 97:193-207

Selosse MA, Richard F, He X, Simard SW (2006) Mycorrhizal networks : des liaisons dangereuses? Trends Ecol Evol. 21:621-628

Solano F, García E, de Egea EP, Sanchez-Amat A (1997) Isolation and characterization of strain MMB-1 (CECT 4803), a novel melanogenic marine bacterium. Appl Environ Microbiol. 63:3499-3506

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus Glomus intraradices in an in vitro system in the absence of host roots. Mycol Res. 100:328-332

Steneck RL, Dethier MN (1994) A functional group approach to the structure of algal-dominated communities. Oikos. 69:476–498

Thrane C, Nielsen TH, Nielsen MN, Sørensen J, Olsson S, 2000 Viscosinamide producing Pseudomonas fluorescens DR54 exerts a biocontrol effect on Pythium ultimum in sugar beet rhizosphere. FEMS Microbiol Ecol. 33: 139-146

Trautman DA, Borowitzka MA (1999) Distribution of epiphytic organisms on *Posidonia australis* and *P. sinuosa*, two seagrasses with different leaf morphology. Mar Ecol Prog Ser .179:215–229

Vanderklift MA, Lavery PS (2000) Patchiness in assemblages of epiphytic macroalgae on Posidonia coriacea at a hierarchy of spatial scales. Mar Ecol Prog Ser. 192:127–135

Wahl M (1989) Marine epibiosis. I. Fouling and antifouling: some basic aspects. Mar Ecol Prog Ser. 58:175-189

Warwick RM, Clarke KR (1993) Increased variability as a symptom of stress in marine communities. J Exp Mar Biol Ecol. 172:215-226

Young LY, Mitchell R (1973) The role of microorganisms in marine fouling. Int Biodeterior Bull .9:105-109

Chapter 2: Photosynthetic activity of the non-dormant Posidonia oceanica seed

Possunt quia posse videntur
(Virgilio)

Abstract

The photosynthetic adaptive features of non-dormant seeds in *Posidonia oceanica* were studied in order to evaluate the effects of light on germination success. Transmission electron micrographs showed the presence of chloroplasts in the epidermal cells, close to the nucleus at the periphery of the cytoplasm. The well developed thylakoid membranes and the presence of starch granules indicated that the chloroplasts were photosynthetically active. The relationship between photosynthesis versus irradiance in *P. oceanica* seeds incubated at 15 and 21 ºC was analysed. The net photosynthesis in the non-dormant seed of *P. oceanica* was positive and compensated its respiration demand (90 μmol quanta m^{-2} s^{-1}) at both temperatures. Net photosynthesis was negative at the other irradiance values. To test the effects of light on germination success, seeds were placed both in dark and light conditions. Germination success was significantly higher in light rather than in dark condition. The characteristics observed in the photosynthesis in *P. oceanica* seed could be a mechanism to guarantee seedling survival in temperate waters, demonstrating though the specialized nature of this species.

Introduction

Posidonia oceanica (L.) Delile lives in the Mediterranean Sea where it covers 2.5-5 million hectares in the form of extensive submarine meadows (Pergent et al., 1995). It is a slow growing species and constitutes ones of the most productive ecosystems in the word (Pergent et al., 1994). This seagrass has the ability to reproduce sexually, through seeds, or asexualy through the propagation of rhizome fragments (Molinier and Picard 1952). Sexual recruitment seems to be relatively rare and population spread occurs mainly through clonal propagation (Procaccini et al., 2001). However this low recruitment seems to be contradicted bby some events of massive recruitments in particulary favourable local conditions (Balestri et al., 2008). Sexual reproduction, even at a low rate, could play an important role in the colonization of new sites, post-disturbances recovery and the establishment of new genotypes in existing seagrass populations (Alberte et al., 1994; Orth 1999).

P. oceanica is monoecious species with hermaphroditic and male flowers arranged in a peduncle composed of three–four flowered spikes (Balestri et al. 2003). Inflorescences emerge at the start of autumn (September-October) and fruits mature from May to June (Buia and Mazzella 1991). The fruit of *P. oceanica* is ovoid, with a fleshy and spongy pericarp bearing a single seed. Once separated from the plant, the green-coloured fruit floats freely on the sea surface for a few days until dehiscence occurs, and the negative buoyancy of the seed leads it to sink. It is then dragged along the bottom until it becomes fixed (Tomlinson 1982). The fruit dehisces by three longitudinal openings which originate from the base or point of fruit attachment. The non-dormant seed of *P. oceanica* is divided into three areas: apical, central and basal. Within the fruit the seed is positioned with its radical end at the fruit base, and the apical end protected until the seed is completely released (Belzunce et al., 2005).

The seed is characterized by a massive central axis, composed principally of an enlarged hypocotyl, which probably functions as stabilizer during flotation, while providing ample food storage reserves (Tomlinson 1982; Belzunce et al., 2005). Within the apical zone there are leaf primordia, two lateral root primordia and a small part of the hypocotyl. The leaf primordia may reach an advanced stage of development before dehiscence, which is interpreted as a survival mechanism. The central vascular strand

connects the leaf primordia and lateral root primordia with the primary root found in the basal or radical area (Belzunce et al., 2005). *P. oceanica* produce large seeds containing more mineral nutrients (N and P) compared with some closely related species. It seems that during the early seedling developmental phase the seeds act as supplement environmental supplies of N and P to ensure rapid growth of shoot and roots. Seedlings appeared to be primarily limited by P during their second growing season. This lends support the hypothesis that growth limitation due to nutrient deficiency may be a cause of mortality of seedlings established on poor-nutrient patches (Balestri et al., 2009).

The physiological adaptive features of seeds in long-lived seagrasses are not well understood. Because sexual recruitment seems to be relatively rare in *P. oceanica* meadows, the environmental conditions that are conducive to the establishment of *P. oceanica* seedlings need to be elucidated. In this regard, the green colour of *P. oceanica* seed suggests that light may play an important role in seedling recruitment.

The main objectives of this study were to prove that *P. oceanica* seed has photosynthetic activity and test the effects of light on germination success. The photosynthetic activity was examined under physiological (chlorophyll concentration, photosynthetic production versus irradiance and temperature) and ultrastructural analysis. The effects of light on germination success were examined placing seeds in both light and dark conditions.

Material and Methods

Seed sampling

Floating *P. oceanica* fruits were collected along 2000 m of the coastline at Mazarrón Bay (Murcia, SE Spain) in April and May 2008 (Fig. 6 B). Fruits without signs of dehiscence were transported in plastic containers with seawater at a constant temperature to the laboratory, where they were placed in aquaria at 20 ºC and a salinity of 37 psu. Seeds were extracted from selected fruits without sign of dehiscence, herbivorism or mechanical damage.

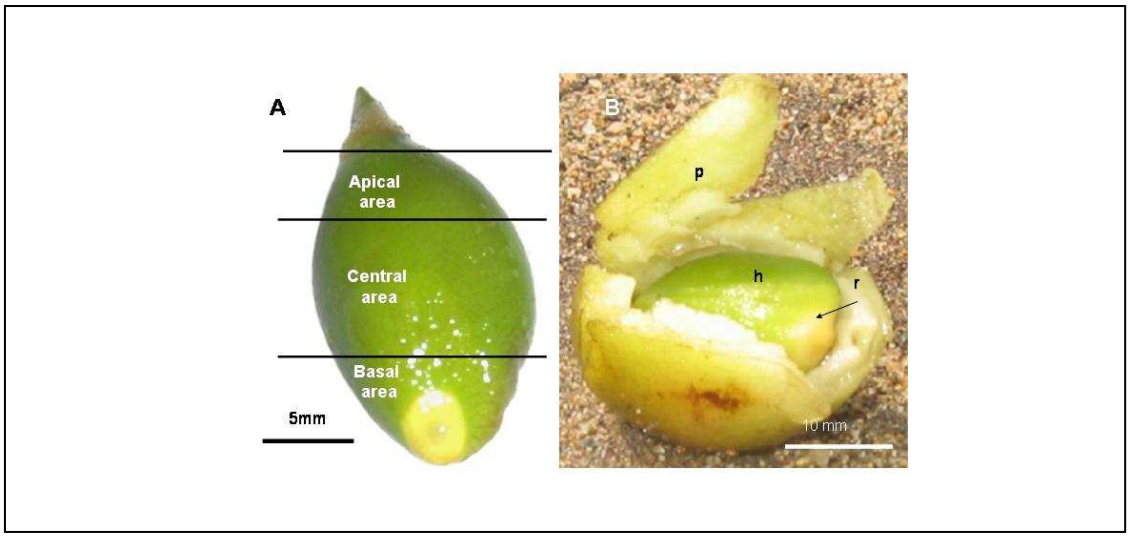


Figure 6. Appearance of *Posidonia oceanica* seeds and fruits collected. (A) different areas of the seed, and (B) open fruit with three longitudinal openings showing the green seed. h: hypocotyls; i: inflorescence; p: pericarp; r: primary root. Scale bars represent 10 mm.

Physiological analysis

Chlorophyll *a* and *b* were extracted from seeds in cold 90% (v/v) acetone and quantified spectrophotometrically (Spectrophotometer 1700 Shimadzu) using the equations of Jeffrey and Humphrey (1975).

Two oxygen production analysis were done, one at 15ºC with seeds collected in April and another at 21ºC with seeds collected in May, (n=10). These temperatures corresponding to the seawater temperature at which the seeds were collected.
The rates of oxygenic photosynthesis and dark respiration (from now on referred to as R) were measured with an oxygen meter (HQ40d, Hach) under rapid mixing conditions placing each seed individually in one 32 ml glass bottle, with sterilized bidistilled water and marine salt (PRODAC) intended for use in aquaria (37 psu). R was measured in a totally dark chamber. Photosynthesis vs. irradiance relationship was generated from measurements made at 35, 70, 90, 154, 300, and 450 μmol quanta m^{-2} s^{-1} (PAR). Each seed was exposed to this range being acclimated 30 minutes among each irradiance.
Incubations were carried out in a Versatile Environmental Test Chamber (Sanyo MLR-351) with irradiance and temperature control. Oxygen values were then normalized to seed biomass, incubation time (2 hours) and the results expressed as mg O_2 g^{-1} dw h^{-1}(this was Net Photosynthesis, from now on referred to as NP). In order to correct possible bacterial respiration a control was incubated at the same conditions of seeds.

Ultrastructural analysis

Small pieces from the apical, central and basal area of the seeds (Fig. 6 A) were fixed in 2.5 % Milloning´s phosphate-buffered glutaraldehyde (pH 7.2-8.2) for 1 h. The pieces were washed in 2.5 % NaHCO3 in distilled water (60 min at 25 ºC) postfixed in 2% OsO4 in 1.25% NaHCO3 for 1 h, dehydrated through an ethanol series, and then embedded in Epon, thinly sectioned and stained with uranyl acetate and lead citrate.

Germination success

To test the effects of light on germination success, seeds were placed both in dark and light conditions. Seeds without signal of germination such as incipient leaves were individually planted in plastic pots with fiberglass as substrate in aquaria with artificial seawater prepared with sterilized bidistilled water and marine salt (PRODAC).

Seeds from light treatment (n= 40) were maintained with a photoperiod of 16 h light: 14 h dark (PAR 90 µmol m-2 s-1) and constant environmental conditions (21ºC and 37 psu). Seeds from dark treatment (n= 40) had the same conditions except that the aquaria was covered with aluminium foil. The numbers of deaths in light and dark conditions were registered during three months.

Data analyses

Analyses of variance (one-way ANOVA) were used to examine differences in NP in the range of irradiances at 15 and 21ºC. Prior to one-way ANOVA, data were tested using Kolmogorov–Smirnov test for normality and Cochran test for homogeneity of variance (α=0.05). If data was not parametric then a Kruskal-Wallis was applied. After one-way ANOVA and Kruskal-Wallis a Tukey HSD post-hoc test (α = 0.05) and a Dunns post-hoc test (α = 0.05) were run respectively.

In the germination success assays, the frequencies of mortality between light and dark conditions were analyzed with a Chi-square test.

Results

Physiological analysis

The chlorophyll analysis demonstrated the presence of photosynthetic active pigments in *P. oceanica* seed. The seed chlorophyll content was 0.26 ± 0.06 mg Chl a·gdw-1 and 0.16 ± 0.04 mg Chl b·gdw-1. The chlorophyll a/b ratio was 1.62.

The NP versus irradiance relationships of seeds incubated at 15 and 21 ºC were compared; R was also included in the figure 7. The results of the ANOVA revealed that there were significant differences in NP for the range of irradiances at 15ºC (ANOVA, $p<0.0001$). The Tukey HSD post-hoc test indicated that the differences were due to 35 and 90 µmol quanta m-2 s-1 ($p<0.05$) being these irradiances the lowest and highest values to the photosynthesis production respectively (Tukey HSD post-hoc test: 35<70=154=300=450<90). Also, there were significant differences between the range of irradiances at 21ºC (Kruskal-Wallis test, $p<0.0001$). These differences were due to the higher (35 and 450 µmol quanta m-2 s-1) and lower irradiance values (90 and 154 µmol quanta m^{-2} s^{-1})(Dunns post-hoc test: , which results: (35=450<70=300<90=154).

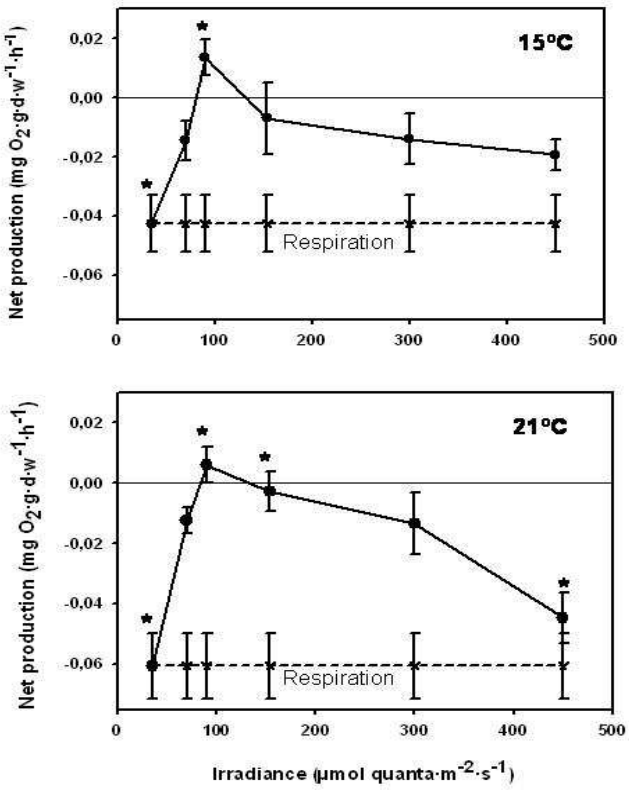


Figure 7. Net Photosynthesis (solid line) and Respiration (dash line) versus irradiance in *Posidonia oceanica* seed at 15 ºC and 21 ºC, (n= 10). Asterisks indicate significant differences (Tukey HSD or Dunns post-hoc tests ;α = 0.05).

The higher NP was observed at 90 μmol quanta m^{-2} s^{-1} at both incubation temperatures (0.013±0.06 mg O_2·g dw^{-1}·h^{-1} at 15ºC, and 0.006±0,005 mg O_2·g dw^{-1}·h^{-1} to 21ºC). At this irradiance value, NP compensated R. NP was negative to the others irradiances values at 15 and 21ºC. The R value was highest at 21ºC (-0.060±0.010 mg O_2·g dw^{-1}·h^{-1}) than at 15ºC (-0.042±0.009 mg O_2·g dw^{-1}·h^{-1}).

Germination success

The germination assays showed that 18 out of 40 seeds on dark died after three months, while only 6 out of 40 seeds on light died (Fig. 8). The Chi-square value was highly significant (p = 0.0034), thus germination success was significantly higher in light rather than in dark condition.

Ultrastructural analysis

Cross sections of the external part of the hypocotyl showed an epidermis composed of cells with chloroplasts together with the main mass of the embryonic body with high starch content. The epidermis consisted of relatively small, regular cells tending toward rectangular shape with a central vacuole (Fig. 9 B). Subtending cells below the epidermis were large with most of the cytoplasm occupied by a light electrodense vacuole.

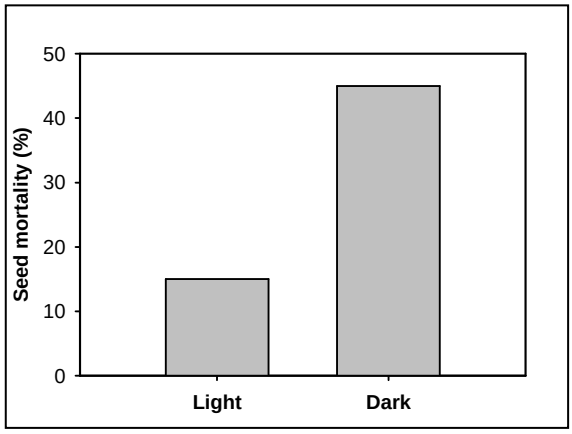
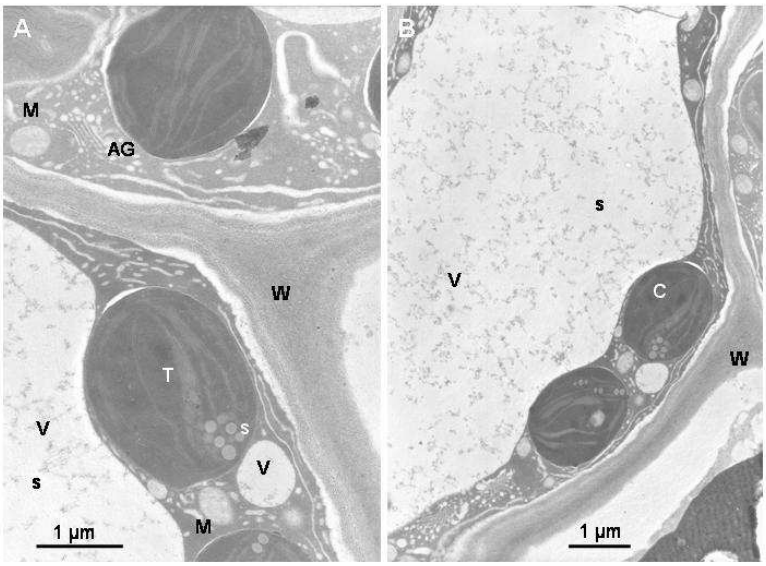


Figure 8. Germination success of *Posidonia oceanica* seeds on light and dark conditions

Transmission electron micrographs showed the presence of chloroplasts in the epidermal cells. The cross sections of the apical area of the seed showed groups of epidermal cells forming 3 or 4 layers of cells with chloroplasts. However, in sections of the central and basal areas of the seed, the epidermal cells containing chloroplast only

formed a monolayer. Chloroplasts were found close to the nucleus at the periphery of the cytoplasm. The well developed thylakoid membranes and the presence of starch



granules suggested that the chloroplasts were photosynthetically active (Fig. 9 A).

Figure 9. Micrographs of the epidermis cells from *Posidonia oceanica* seeds (central area). (A) Detail of cells with chloroplasts showing starch granules and well developed thylakoids (scale bar= 1µm); (B) Epidermis cells containing chloroplasts and a central vacuole (scale bar= 1 µm). G: Golgi apparatus; M: mitochondria; S: starch granules; T: thylakoids; V: vacuole; W: cellular wall.

Discussion

This study demonstrated photosynthesis activity in the non-dormant seed of *P. oceanica*. There was a relatively high abundance of chlorophyll b containing peripheral antennae (the Lhca's and Lhcb's) in the thylakoid membranes (the chlorophyll a/b ratio was approximately 1.62). Plants grown in shadowing habitat have a bigger antenna size and reduced chlorophyll a/b ratio, while plants grown in sunny habitats have smaller antennae and increased chlorophyll a/b ratio (Gopal et al. 2005). In "shadow" plants, chloroplasts normally contain very large grana stacks. This is not the case of the non-dormant seed of *P. oceanica*. Only according to chlorophyll a/b ratio, can the non-dormant seed of *P. oceanica* be considered as a shadow plant.

The non-dormant seeds of *P. oceanica* have photosynthetic characteristics that showed the specialized nature of this species. The NP was significantly higher at 90 µmol quanta m-2 s-1 (15 ºC) and 90-154 µmol quanta m-2 s-1 (21 ºC), suggesting lower dependence of the seed to the starch reserves for these irradiance values. This indicates that seedling recruitment in natural populations could have a higher probability of success when seeds drop in places within these irradiance values. Further experiments should investigate the importance of other factors such as quality and intensity light in the seed germination success. This light range did not match the photosynthesis versus irradiance curve generated by adult leaves. For small leaf sections of *P. oceanica* (Alcoverro et al. 1998) observed light saturation at 257 µmol quanta m−2 s−1, and light compensation at 37 µmol quanta m−2 s−1. Because seeds drop to the sea bottom following the opening of ripe fruit, the slightly lower light compensation and saturation values for *P. oceanica* seed compared with adult leaves could indicate an adaptation to a slightly lower environmental irradiance during germination.

Our results indicate that favourable seed photosynthesis could occur in microhabitats from 5 to 25 m depth where *P. oceanica* meadows spread in the Mediterranean. Piazzi et al. (1999) indicated that the survival and growth of *P. oceanica* seedling strongly depended on depth due to environmental factors such as light and water movement. Shoot survival was greater at 10 m than at 2 m (69.6% at 10 m and 40.5% at 2 m) on dead matte. *P. oceanica* seems to grow best at 17 to 25 ºC (Drew 1978). We observed a higher photosynthesis rate at 15ºC, rather than 21 ºC, which

suggests that seeds released in early spring (April) could be more successful in terms of photosynthesis than seeds liberated in late spring (May) with higher temperatures.

Photosynthesis in non-dormant *P. oceanica* seeds could be a mechanism to guarantee seedling survival in temperate waters with an unfavourable seasonal period. This is why the period of maximum leaf biomass is weakly related to annual production in temperate seagrasses (Duarte 1989).

Growth and production patterns of adult *P. oceanica* are seen as adaptations to competition for space and nutrients with epiphytes and phytoplankton, which lack the stored carbon necessary to use the abundant nutrients during winter at low temperature and irradiance levels (Ott 1980). In autumn, the high photosynthetic activity of a relatively low leaf biomass, supported by the mobilisation of starch in the rhizomes, enables *P. oceanica* to double its biomass during winter. Higher increase of biomass in spring is due to photosynthetic activity only; and in summer, relatively low photosynthesis in a considerable amount of leaf biomass produces a carbon surplus, which is stored in the rhizome as starch (Pirc 1986). Relationship between the seasonal cycle of adult *P. oceanica* leaves and photosynthesis in seeds has not yet been elucidated. It is clear that seedlings must adapt to this seasonal cycle described in *P. oceanica* beds, since seed reserves may be critical for seedling growth processes during the first year. In a study carried out with *P. oceanica* seedlings (Piazzi et al. 1999), indicated that seedlings showed continuous development of the rhizome and a cycle similar to adult plants.

Seagrass seedlings depend primarily on stored carbohydrate reserves, complemented by autotrophic production until the photosynthetic apparatus of the seedling is capable of completely supporting the plant's carbon demands (Kaldy and Dunton 1999). Seedlings of the seagrass Thalassia testudinum Banks ex Konig become photosynthetically self-sufficient between 2 and 6 months after germination (Kaldy and Dunton 1999). Abundant seed storage appears to be a characteristic of Posidonia sp. (Belzunce et al. 2005; Balestri et al. 2009), allowing seedling establishment in an unpredictable marine environment. The main carbohydrate stored in mature seagrass seeds (*P. australis, P. sinuosa, P. coriacea, Thalassia hemprichii*) is starch (Kuo and McComb 1989; Kuo et al. 1990), which is presumably hydrolyzed to sugar and used to nourish early seedling growth (Hocking et al. 1981). In laboratory cultures, nutrient

enrichment of *P. oceanica* seeds did not promote germination, and actually it has inhibited growth (Caye and Meinesz 1989; Balestri et al. 1998).

In conclusion, this study has demonstrated the photosynthetic activity of the non-dormant *P. oceanica* seed. The data confirmed that the R values of the non-dormant *P. oceanica* seed were considerable and could represent a high cost of starch. According to this, the importance of the photosynthetic activity is not to reach high NP, but to compensate the R and to prevent exhaustion of the starch reserves. The most suitable irradiance levels and temperatures should be taken into account for an optimal germination of this species. Finally, the germination success test demonstrated that adequate light conditions in the non-dormant *P. oceanica* seed reduced its mortality. Early photosynthetic activity represents a new strategy to improve the possibilities of survival not seen in marine plants before.

Acknowledgements

This work was funded by the Ministerio de Medio Ambiente y Medio Rural y Marino from Spain (project code 116/SGTB/2007/1.3). The authors kindly thank Javier Lloret for the constructive criticism on this paper. This work was supported by a grant (D. Celdrán) from Ministerio de Ciencia e Innovación (Programa Nacional de Formación de Profesorado Universitario).

References

Alberte RS, Suba GK, Procaccini G, Zimmerman RC, Fain SR (1994) Assessment of genetic diversity of seagrass population using DNA fingerprinting: implications for population stability and management. Proceedings of the National Academy of Sciences of the United States of America. 91:1049–1053

Alcoverro T, Manzanera M, Romero J (1998) Seasonal and age-dependent variability of *Posidonia oceanica* (L.) Delile photosynthetic parameters. J Exp Mar Biol Ecol. 230 (1):1-13

Balestri E, Cinelli F, Lardicci C (2003) Spatial variation in *Posidonia oceanica* structural, morphological and dynamic features in a northwestern Mediterranean coastal area: a multi-scale analysis. Mar Ecol Prog Ser. 250:51–60

Balestri E, Piazzi L, Cinelli F (1998). In vitro germination and seedling development of *Posidonia oceanica*. Aquat Bot. 60:83-93

Balestri E, Lardicci C (2008). First evidence of a massive recruitment event in *Posidonia oceanica*: Spatial variation in first-year seedling abundance on a heterogeneous substrate. Est Coast Shelf Sci. 76: 634-641

Balestri E, Gobert S, Lepoint G, Lardicci C (2009). Seed nutrient content and nutritional status of *Posidonia oceanica* seedlings in the northwestern Mediterranean Sea. Mar Ecol Prog Ser. 388: 99-109

Belzunce M, Navarro RM, Rapaport HF (2005) Seed and early plantlet structure of the Mediterranean seagrass *Posidonia oceanica*. Aquat Bot. 82:269-283

Buia MC, Mazzella L (1991) Cymodocea nodosa (Ucria) Ascher. and Zostera noltii Hornem. Aquat Bot. 40:345–362

Caye G, Meinesz A (1989) Cultures en milieu artificial de *Posidonia oceanica* a partir de graines. In: Boudouresque CF, Meinesz A, Fresi E, Gravez V (eds). Int. Workshop on *Posidonia oceanica* Beds, GIS Posidonie Publ. Vol. 2, pp 293-299

Drew A (1978) Factors affecting photosynthesis and its seasonal variation in the seagrasses Cymodocea nodosa (Ucria) Aschers, and *Posidonia oceanica* (L.) Delile in the Mediterranean. J Exp Mar Biol Ecol 31:173-194

Duarte CM (1989) Temporal biomass variability and production/biomass relationships of seagrass communities. Mar Ecol Prog Ser. 51:269-276

Gopal K, Pattanayak, Ajaya K, Biswal, Vanga S, Reddy, Baishnab C, Tripathy 2005. Light-dependent regulation of chlorophyll b biosynthesis in chlorophyllide a oxigenase overexpressing tobacco plants. Biochem Biophys Res Commun. 320: 466-471

Hocking PJ, Cambridge ML, McComb AJ (1981) Nutrient accumulation in the fruits of two species of seagrasses *Posidonia australis* and *Posidonia sinuosa*. Ann Bot. 45:149–161

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls A, B, C1 and C2 in higher-plants, algae and natural phytoplankton. Biochemie Physiologie Der Pflanzen. 167:191-194

Kaldy JE, Dunton KH (1999) Ontogenic photosynthetic changes, dispersal and survival of Thalassia testudinum (turtle grass) seedling in a sub-tropical lagoon. J Exp Mar Biol Ecol. 240:193–212

Kuo J, Kirkman H (1990) Anatomy of viviparous seagrasses seedlings of Amphibolis and Thalassodendron and their nutrient supply. Bot Mar. 33:117–126

Kuo J, McComb AJ (1989) Seagrass Taxonomy, Structure and Development. Elsevier, Amsterdam.

Molinier R, Picard J (1952) Recherches sur les herbiers de phanerogames marines du littoral mediterrane´en franc¸ais. Ann Inst Oceánog. 27:157–234

Orth RJ, Harwell MC, Fishman JR (1999) A rapid and simple method for thransplanting eelgrass using single, unanchored shoots. Aquat Bot. 64:77-85

Ott JA (1980) Growth and production in *Posidonia oceanica* (L.) Delile. Marine Ecology. 1:47-64

Pergent G, Romero J, Pergent-Martini C, Mateo MA, Boudouresque CF (1994) Primary production, stocks and fluxes in the Mediterranean seagrass *Posidonia oceanica*. Mar Ecol Prog Ser. 106:139–146

Pergent-Martini C, Pergent G (1995) Impact of a sewage treatment plant on the *Posidonia oceanica* meadow: assessment criteria. In: Proceedings of the second International conference on the Mediterranean coastal environment. MEDCOAST'95, pp 1389–1399

Piazzi L, Acunto S, Cinelli F (1999) In situ survival and development of *Posidonia oceanica* (L.) Delile seedlings. Aquat Bot. 63:103-112

Pirc H (1986) Seasonal aspect of photosynthesis in *Posidonia oceanica*: influence of depth, temperature and light intensity. Aquat Bot. 26:203-212

Procaccini G, Orsini L, Ruggiero MV, Scardi M (2001) Spatial pattern of genetic diversity in *Posidonia oceanica*, an endemic Mediterranean seagrass. Molec Ecol. 10:1413–1421

Tomlinson PB (1982) Anatomy of the Monocotyledons VII. Helobiae (Alismatidae). Claredon Press, Oxford.

Chapter 3: Photosynthesis made by Posidonia oceanica seed epidermis determines seedling growth

Non scholae sed vitae discimus
(Seneca)

Abstract

The seed of *P. oceanica* shows photosynthetic activity during the development of seedlings. which suggests that photosynthesis made by the seed body can contribute to the seedling growth. This work examined the contribution of seed photosynthesis on leaf growth and mobilisation of the seed reserve and nutrients during the first three months of seedling development of the seagrass *P. oceanica*. To this was examined the changes in leaf growth, concentration of free sugar, starch, C, N, and P in seed of the seedlings in different conditions of light/dark treatments. The results proved that photosynthetic activity in seed made implement significantly the foliar area. Starch and free sugar contain and nutrients mobilization was not modified by exposition to light or dark which suggest that photosynthetic production contributed by the seed is invested totally in growth of the seedling not in an increment of reserves contain. Results showed that photosynthesis contribution by the seed of *P.oceanica* was functional and in a similar measure as photosynthesis made by only leaves.

Introduction

Seagrasses have key ecological roles in coastal ecosystems and can form extensive meadows supporting high biodiversity (Short et al.. 2007). Seagrasses have developed unique ecological. physiological. and morphological adaptations to a completely submersed existence. including internal gas transport. epidermal chloroplasts. submarine pollination. and marine dispersal (den Hartog 1970. Les et al. 1997). The relatively limited phylogenetic diversity of seagrasses results in a limited range of life history strategies. Seagrasses are clonal plants that have adapted to the marine environment and complete their entire life-cycle in a saline medium. including flowering. pollen transport and seed germination (Phillips and Meñez. 1988). The bulk of seagrass bed expansion occurs through clonal growth (Lewis and Phillips. 1980; Phillips et al.. 1981) however. seeds are important to the maintenance of genetic variation within the population (Alberte et al.. 1994; Williams and Orth. 1998) and as agents of long distance dispersal.

Posidonia oceanica (L.) Delile is a seagrass endemic to the Mediterranean Sea which plays a major role in providing habitat for commercially. recreationally and ecologically important fish and shellfish species as well as it is also important in reducing wave and current energy and trapping and binding of fine grained sediments (Pérès and Picard. 1964; Green and Short. 2003). *P. oceania* is a slow-growing K strategist that invests in multi-year vegetative growth. which results in extensive meadows that dominate shallow. subtidal. Mediterranean landscapes.

Likewise this marine phanerogam is long-lived and forms a deep “matte” of root and rhizomematerial which may be several meters deep and thousands of years old. *P. oceanica* has the ability to reproduce sexually. through seeds. or asexually through propagation of rhizome fragments (Molinier and Picard 1952). Flowering and fruit production is highly variable in space and in time. with intensive synchronized flowering episodes occurring at 8 to 10 yr intervals (Balestri and Cinelli 2003. Diaz-Almela et al.. 2006). The mature positively buoyant fruit consists of a single green seed that displays no seed dormancy. The buoyant fruits are transported by currents away from parental shoots. enabling dehisced seeds to colonize sites outside the range of

vegetative growth (Buia & Mazzella 1991). While the fruits of *P. oceanica* float. the seeds sink immediately upon release from the fruit. settling with the flat side down. germinating within a few days (Caye and Meinesz. 1984). The green seed remain attached to the young plant for 1–2 years after germination. The seeds supplies C. N and P to the developing seedling until 1 yr old months after germination (Balestri et al.. 2009).

It seems that during the early seedling developmental phase the seeds act to supplement environmental supplies of N and P to ensure rapid growth of a photosynthetic and roots capable of supporting the plant. Additionally. seed have photosynthetic activity during the development of seedlings (Celdrán and Marin. 2011). which suggests that it could contribute photosynthetically to the seedling. These authors also indicated that seed survival was higher in light that dark conditions which suggests that seedling could depend of seed photosynthesis. Photosynthesis in non-dormant *P. ceanica* seeds could be an additional mechanism to supply C during the 1st year of seedling that enhances plant growth. Nothing is known about the role of seed photosynthesis associated with seedling development and reserve mobilisation. yet this process is critical to understanding the factors influencing seedling survival. Although seedling recruitment may be of great importance in the process of recolonisation after disturbance and colonization of areas outside the range of vegetative growth (Balestri and Lardicci. 2008). little is still known about the environmental conditions that are conducive to the establishment.

The aim of this work was to examine the contribution of seed photosynthesis on leaf growth and mobilisation of the seed reserve and nutrients during the first three months of seedling development of the seagrass *P. oceanica*. To achieve these goals. we examined the changes in leaf growth. concentration of free sugar. starch. C. N and P in seed of the seedlings in different conditions of light/dark treatments.

Material and methods

Seed collection

Fruits of *P. oceanica* without sign of dehiscence. herbivorism or mechanical damage were collected of the coastline of Murcia. south-east of Spain during May and June 2010. They were carrying by a plastic container at constant temperature to the laboratory. There were placed in aquaria of 200L with the same conditions as were found in the field. 35 psu and 19ºC. After extract seeds from fruits. were individually placed on a plastic mesh pot (8 cm high x 8 cm diameter) with fiberglass as substrate. This substrate favoured high oxygen diffusion to the roots when plastic film was used as covering for light treatments. Because the seed of *P. oceancia* has no dormancy. germination occurred after maturation into the seed. Mature seed extracted from fruits were in the early stages of germination when were planted in pots

Foliar growth

The effect of illumination on seedling growth was investigated exposing the seeds to four light treatments (n=20) during 3 months with the following treatments:

-Full light illumination (FL). Seeds covered by a transparent plastic film with a small hole in the apical side of the seed where leaves went through towards outside. In this treatment seeds and leaves were under light conditions.

- Partial light illumination (PL). Seeds covered by a black plastic film with a small hole in the apical side of the seed where leaves went through towards outside. In this treatment seeds were maintained in dark but leaves were under light conditions.

- Full dark (FD). Seeds covered by a black plastic film with a small hole in the apical side of the seed where leaves went through towards outside. In this treatment both seeds and leaves were under dark conditions.

-Plastic control (PC): Seed not covered by any plastic film. Seed and leaves were illuminated. The four treatments are represented in the figure 10.

Treatments with light conditions (FL. HL and PC) were placed in aquaria of 200L filled with artificial seawater (35 psu) at the same temperature that Mediterranean seawater during seed recollection (19ºC). Water was not enriched with any nutrient. The aquarium was illuminated with halogen lamps to a photoperiod of 16:8 h of light-dark respectively and delivering PAR light levels around 300 µmol photons m^{-2} s^{-1}at the seed level. Water level and salinity within aquaria were checked every second or third day. Full dark treatment (FD) was placed in another aquarium with the same conditions. covered completely by aluminium paper to avoid light entrance. All aquaria were connected by a pump with a filter system that recirculated the water in order to get a homogenous water conditions in all treatments.

Seedling leaf growth was assessed using a plastic rule at the end of the experiment (12 weeks). Also photosynthesis status of seeds was measured at twelfth week through a chlorophyll fluorometer. pulse amplitude modulation PAM. (Heinz Walz GmbH). The maximum efficiency of photosystem II (Y) and the electronic transport rate (ETR) to 160 PAR was recorded.

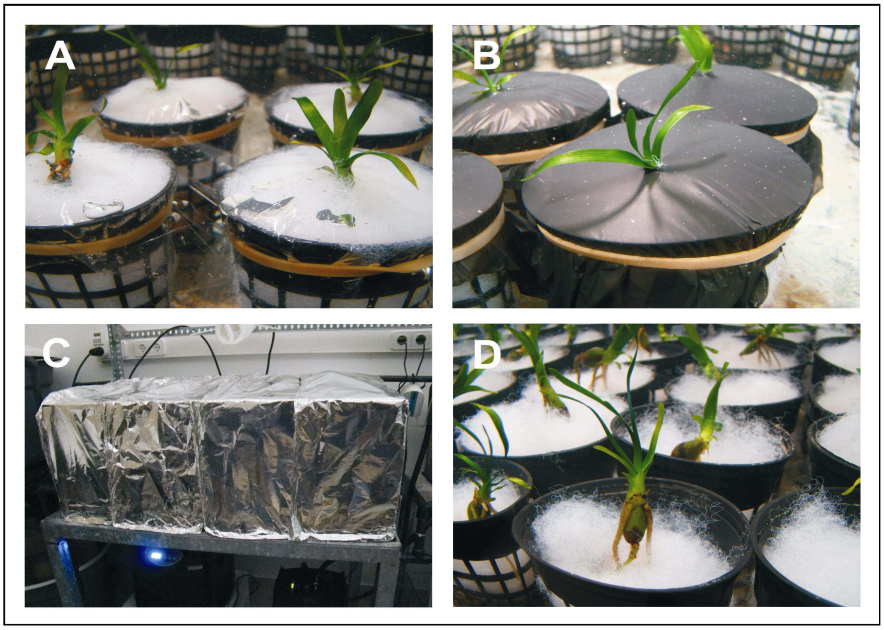


Figure 10. Four treatments in controlled conditions. (A) Image of the full light illumination treatment (FL). (B) Image of the partial light illumination treatment (PL). (C) Image of the full dark treatment (FD). And (D). image of the plastic control treatment (PC).

Mobilisation of seed reserves and nutrients

Seedling growth could be influenced by mobilization of seed reserves in the different light treatments. Free sugar and starch were assessed at the end of the experiment. Free sugars were extracted from the seeds of three month seedlings old. in hot ethanol (80ºC) (Zimmerman et al 1989). The extracts were evaporated by heating block. redissolved in distilled water and analyzed spectrophotometrically using resorcinol 1% assay standardized to sucrose (Huber & Israel 1982). Starch was extracted from ethanol-insoluble residues in 1N KOH. and analyzed spectrophotometrically using anthrone assay standardized to sucrose (Yemn & Willis 1954).

For C. N. P analysis. seeds were dried at 70 ºC and then ground into a homogeneous powder (MM 301 microgrinding device. Retch). Elemental analyses of C and N were performed on dry samples (2 to 6 mg) using a C:N:S analyser (NA 1200. Carlo Erba). Elemental analysis of P was performed using an inductively coupled plasma-mass spectrometer (Elan DCR II) on a 10 mg distilled water (DW) sample after digestion with HNO3 and H2O2 in an Ethos D microwave digester.

Data analysis

Differences in leaf area among the four treatments. reserves and nutrients concentrations in seeds were examined using a one-way ANOVA. Data were tested for homogeneity of variances using Levene's test (α=0.05) and normality using the Shapiro–Wilk test (α=0.05). After ANOVA. differences between specific treatments were determined with a Tukey HSD post-hoc test (α = 0.05). Another one-way ANOVA was made of the values of (Y) and (ETR) between the four treatments.

Results

Foliar growth

There were significant differences in leaf area between light treatments (one-way ANOVA; $p < 0.05$) after 12 weeks since seed germination. Post-hoc analysis confirmed that seedlings with seeds exposed to light (FL and PC) showed higher leaf surface than seedlings with seeds in darkness (PL and FD). (Figure 11). Seedlings that seeds were maintained in dark but leaves were under light conditions did not present significant differences with seedling in full dark (Tukey-HSD *post hoc* test. $p<0.05$). Likewise. the results of the two one-way ANOVA of the values of PAM (Y and ETR) did not showed significant differences. which confirmed that seeds of all treatments were photosynthetically active but differences in leaf growth were not caused by differences in photosynthetic status of seed at the beginning of the experiment.

Dependent variable	MS	F	p	Tukey's test
Foliar area	109.423	8.7412	<0.001	FL=PC>PL=FD
(Y)	0.0001	0.23	0.877	ns
(ETR)	2.07	0.1265	0.943772	ns
Free sugars	1.0188	1.3022	0.28	ns
Starch	0.0480	2.1548	0.11	ns
C	6.12	1.424	0.27	ns
N	0.3157	1.3546	0.29	ns
P	0.007488	0.2413	0.86	ns
C:N	7.934	0.7635	0.53	ns
N:P	3.1075	0.4151	0.74	ns

Table 2. Results of one-way ANOVAs testing for the effects of seedling illumination treatments on: Foliar area. (Y). (ETR). C. N and P concentrations. and atomic C/N and N/P ratios for seed. Results of Tukey's tests are also reported where values differ significantly. (ns: not significant) n = 20.

Mobilisation of Seed reserves and nutrients

The carbohydrates reserves (free sugar and starch) in seeds did not show significant differences between treatments (one-way ANOVA. $p>0.05$). Similar results were found for percentage of elemental C. N and P. There was no significant differences between treatment in elemental C. N and P contents (one-way ANOVA. $p>0.05$). (Table 2). Analysis of fluorescence showed no significant differences between treatments light/dark. And all treatments were photosynthetically active: (Y) = 0.64 ± 0.06 and (ETR) = 17.80 ± 4.56. (mean ± SE).

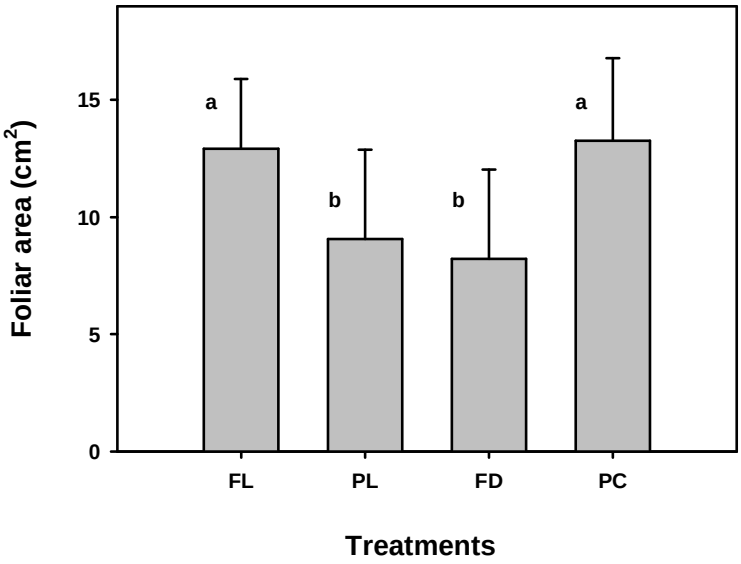


Figure 11. Foliar area (cm^2) of the four treatments of light-dark: FL. PL. FD and PC. Letters 'a' and 'b' refer to 2 statistically different groups (Tukey's HSD post hoc test. $p < 0.05$).

Discussion

Results demonstrated significant differences in the response of the seedling growth which seeds were exposed to light. Seedlings with seeds illuminated (FL and PC) presented a significantly higher leaf area than treatments where seeds were not illuminated (PL and FD) which evidenced a clear influence of seed photosynthesis in seedling growth. In this way the results indicated a similar growth of seedlings completely in light (seed and leaves) than seedlings where only leaves were in light. This suggests that production obtained from the seed photosynthesis was as important as the production from the leaves photosynthesis at least during the initial development period (3 months). In addition. there was a progressive leaf growth in all seedlings independently of light treatment. which suggest that seed illumination was no a trigger mechanist for plant growth. This could be a security mechanism of survival when seedlings are buried for wave action. Posidonia seedling has a relatively long shade tolerance. being able to survive during three months after germination. albeit at significantly reduced foliar growth which leave to the seedlings in unfavourable conditions for survive during the limiting light winter period. Burial of seagrass seeds (shading conditions) has been linked to seedling mortality. Rollon et al.. (2003) found that experimental burial of *Thalassia hemprichii* seeds by 5 cm of sand caused rapid and total seed mortality after only 6 days.

There were no differences in carbohydrates content and nutrients of the seed. which indicates that the higher leaf growth observed in illuminated seed was due to photosynthetic activity of seeds and not to higher starch. free sugar and nutrients mobilization. Imbalances in the carbon budget of seagrass plants from shade-induced reductions in photosynthetic rates are frequently linked to reduced growth. utilisation of carbohydrate reserves and. eventually. changes in the biomass distribution of the plants (Alcoverro et al.. 1999). In this study reduced leaf growth occurred in full dark treatment (FD) and also in plants that partially the seeds were in dark (PL). but plants at both sites remobilised the same carbon from seed carbohydrate reserves than lighted seed-leaves seedlings (FL and PC). Sugars contained in the rhizome predominantly supplemented growth during shading. a commonly observed response in seagrasses (Burke et al.. 1996; Lee & Dunton. 1997; Longstaff et al.. 1999). However. in seeds of seedlings of the four treatments there were no differences in carbohydrates reserves

between treatments. which suggest that carbohydrates reserves and nutrient mobilization was an independent process from photosynthesis.

Tschiersch et al.. 2011 confirmed photosynthesis in immature seeds of the barley caryopsis and pea. but this activity declines during maturation. and by the time of grain maturity. photosynthetic ETR was completely abolished. Also affirmed there was no evidence for photosynthetic activity of the embryo at this early stage of germination. However the seed of *P. oceania* showed photosynthesis activity during a relative long time after germination. which suggest the functionality of the seed photosynthesis to the seedling growth.

In conclusion, our results proved that photosynthetic activity in seed made implement seedling growth in the form of higher foliar area. Starch and free sugar contain and nutrients mobilization was not modified by exposition to light or dark which suggest that photosynthetic production contributed by the seed is invested totally in growth of the seedling not in an increment of reserves contain. Finally, results demonstrate. photosynthesis contribution by the seed of *P. oceanica* was functional and in a similar measure as photosynthesis made by only leaves. More studies are needed to implement the knowledge about the reach of this strategy and the ecological implications.

Acknowledgements

We wish to thank David López Vivancos. Elena Lozano Guardiola and Laura Otero Rodriguez for their help during field and laboratory assays. The research was further facilitated by a grant to DC from the Ministerio de Ciencia e Innovación Español. Programa Nacional de Formación de Profesorado Universitario. Spain.

References

Alberte RS. Suba GK. Procaccini G. Zimmerman RC. Fain SR (1994) Assessment of genetic diversity of seagrass population using DNA fingerprinting: implications for population stability and management. Proceedings of the National Academy of Sciences of the United States of America 91:1049–1053

Alcoverro T. Zimmerman RC. Kohrs DG. Alberte RS (1999) Resource allocation and sucrose mobilization in light-limited eelgrass Zostera marina. Marine Ecology Progress Series 187:121-131

Balestri E. Cinelli F. Lardicci C (2003) Spatial variation in *Posidonia oceanica* structural. morphological and dynamic features in a northwestern Mediterranean coastal area: a multi-scale analysis. Mar Ecol Prog Ser 250:51–60

Balestri EC. Lardicci (2008) First evidence of a massive recruitment event in *Posidonia oceanica*: Spatial variation in first-year seedling abundance on a heterogeneous substrate. Estuarine.Coastal and Shelf Science 76:634-641

Balestri E. Gobert S. Lepoint G. Lardicci C (2009) Seed nutrient content and nutritional status of *Posidonia oceanica* seedlings in the northwestern Mediterranean Sea. Mar Ecol Prog Ser 388:99– 109

Buia MC. Mazzella L (1991) Cymodocea nodosa (Ucria) Ascher. and Zostera noltii Hornem. Aquat Bot 40:345–362

Burke MK. Dennison WC. Moore KA (1996) Non-structural carbohydrate reserves of eelgrass Zostera marina. Marine Ecology Progress Series. 137:195-201

Caye G. Meinesz A (1984) Observations sur la floraison et la fructifiration de Posidonla oceanica dans la Bale de Ville- franche ct en Corse du Sud. In. Boudouresque CF. Jeudy de Grissac A. Olivier J (ed) International Workshop on Posidonia oceanica Beds. Vol 1 GIS Posidonie. Marseille. p 193-201

Celdran D and Marin A (2011) Photosynthetic activity of the non-dormant *Posidonia oceanica* seed. Marine Biology 158:853–858

Den Hartog C (197 0) The sea-grasses of the world. Amsterdam: North-Holland Publishing Co.

Diaz-Almela E. Marbà N. Álvarez E. Balestri E. Ruiz-Fernãndez JM. Duarte CM (2006) Pattern in seagrass (*Posidonia oceanica*) flowering in the Western Mediterranean. Mar Biol 148:723-742

Green EP and Short FT (2003) World Atlas of Seagrasses. University of California Press. Berkeley.

Huber SC. Israel DW (1982) Biochemical Basis for Partitioning of Photosynthetically Fixed Carbon between Starch and Sucrose in Soybean (Glycine max Merr.) Leaves. Plant Physiol 64: 749-753

Lee K and Dunton KH (1997) Effects of in-situ light reduction on the maintenance. growth. and partitioning of carbon resources in Thalassia testudinum Banks ex Konig. Journal of Experimental Marine Biology and Ecology 210:53-73

Les DH. Cleland MA. Waycott M (1997) Phylogenetic studies in the Alismatidae. II: Evolution of the marine angiosperms (seagrasses) and hydrophily. Systematic Botany 22: 443–463

Lewis RR and. Phillips RC (1980) Seagrass mapping project. Hillsborough County. Florida. Rept. to the Tampa Port Authority. P 15

Longstaff BJ and Dennison WC (1999) Seagrass survival during pulsed turbidity events: the effects of light deprivation on the seagrasses Halodule pinifolia and Halophila ovalis. Aquatic Botany 65:101-121

Molinier R. Picard J (1952) Recherches sur les herbiers de phane´rogames marines du littoral mediterrane´en franc¸ais. Ann Inst Ocea´nogr 27:157–234

Pérès JM. Picar J (1964) Nouveau manuel de bionomie benthonique de la Méditerranée. Recueil des Travaux de la Station Marine d´Endoume 31 :1–137

Phillips RC and Meñez EG (1988) Seagrasses: Washington. D.C.. Smithsonian Institution Press. Smithsonian Contributions to the Marine Science series 34:104

Phillips RC. McMillan C. Bridges KW (1981) Phenology and reproductive physiology of Thalassia testudinum from the western tropical Atlantic. Aquat Bot 11:263–277

Rollon RN. Vermaat JE. Nacorda HME (2003) Sexual reproduction in SE Asian seagrasses: the absence of a seedbank in Thalassia hemprichii. Aquat Bot 75:181–185

Short RJ. Carruthers T. Dennison W. Waycott M (2007) Global seagrass distribution and diversity: A bioregional model. Journal of Experimental Marine Biology and Ecology 350: 3–20

Tschiersch H. Borisjuk L. Rolletschek H (2011) Gradients of seed photosynthesis and its role for oxygen balancing. Biosystem. 103:302-308

Williams SL and Orth RJ (1998) Genetic diversity and structure of natural and transplanted eelgrass populations in the Chesapeake and Chincoteague Bays. Estuaries 21:118–128

Yemen EW and Willis AJ (1954) The estimation of carbohydrates in plant extracts by anthrone. J Bio Chem 57:508.

Zimmerman RC. Smith RD. Alberte RS (1989) Thermal acclimation and whole-plant carbon balance in Zostera manna L. (eelgrass). J Exp Mar Biol Ecol 130:93-109

Chapter 4: Photosynthesis activity in seeds of Australian and Mediterranean Posidonia seagrasses started before continent separation

Nosce te ipsum
(Delfos)

Abstract

The existence of species of the genera Posidonia in two distant part of the world has make us question if two of the most abundant Australian species (*Posidonia australis* and *Posidonia sinuosa*) share the recent photosynthetic activity ability discovered in the seed of *Posidonia oceanica*. the Mediterranean specie. We have used analysis of fluorometry (PAM) and oxygen production to compare photosynthetic; the maximal efficiency of photosystem II (Y). the electron transport rate (ETR) which was represented *vs* Irradiance. the photochemical quenching (PQ). the non photochemical quenching (NPQ) and oxygen production parameters: respiration (R). gross production (GP). and net production (NP). After statistic tests. the results revealed photosynthesis activity in the three species which suggests that photosynthetic capacity was a skill acquired during before Late Eocene from a common ancestor. Likewise. within every specie. (NP) compensated (R) rates which suggest to be a compensatory mechanism to relive the lack of O_2 into the seed. Finally. the relative high oxygen production and higher ETR values in the seed of *P. oceanica*. respect the two Australian species. reveal a strategy develop by this specie to survive in oligotrophic environments as the Mediterranean Sea.

Introduction

Seagrasses. marine flowering plants. are widely distributed along temperate and tropical coastlines of the world. Seagrasses have key ecological roles in coastal ecosystems and can form extensive meadows supporting high biodiversity. The global species diversity of seagrasses is low (<60 species). but species can have ranges that extend for thousands of kilometers of coastline (Short et al.. 2007). Seagrass distribution is a product of combined plant sexual reproduction and clonal growth. influenced by dispersal and environmental limitations (Spalding et al.. 2003).

The seagrass Posidonia dominates the western and southern coasts of Australia and the Mediterranean Sea but is found nowhere else in the world. The Western Australia contains some of the largest seagrass meadows in the world. and is the most diverse in the number of Posidonia species (eight species) (Cambridge and Kuo. 1979; Kuo and Cambridge. 1984). In the Mediterranean Sea. there is only one species. *Posidonia oceanica*. but this seagrass ranges approximately 2% of the seafloor and extend for 17.000 km of coastline. This bipolar distribution in Posidonia is also a relic of an once continuous distribution (Kuo and Hartog 2000).

The nine current species of Posidonia are probably descended from the Tethyan fossil species (e.g. *Posidonia cretacea*. *P.perforate*. and *P. parisiensis*)(Stockmans 1932) but the divergence in their DNA sequences (Waycott and Les 2000) indicates that the separation of the Mediterranean and Australian species took place at a relatively early time in the history of the seagrasses. probably during the Late Eocene (37.2 million to 33.9 million years ago) (Hemminga and Duarte 2000). Some seagrasses are short-lived. with fast growth and a high production of seeds. but other species as Posidonia populations are highly clonal. largely relying on asexual reproduction for population maintenance (Rasheed 1999; Waycott et al.. 2006).

A reproductive strategy involving clonal growth and production of long-lived. locally dispersed seeds may provide an evolutionary advantage to plants growing in environments subject to temporally unpredictable major disturbances (Rasheed 2004). Posidonia species releases floating fruit that contain a single negatively buoyant seed that lacks dormancy. After dehiscence. the green seed. due to negative buoyancy. sinks

to the bottom where rapidly develops primary roots and leaf system. The green seed remain attached to the young plant for 1–2 years after germination.

In the Mediterranean species. *P. oceanica.* sexual recruitment seems to be relatively rare. and population spread occurs mainly through clonal propagation (Procaccini et al.. 2001). In contrast. *P. australis* flowers frequently produce large numbers of seeds (Cambridge and Hocking 1997; Marba and Walker 1999). The combined ability of *P. australis* and *P. sinuosa* to disperse over longer distances using floating fruit. as well as its extensive clonal growth and persistence. appears to have contributed to the dominance of this genus in the Western Australia (Ruiz-Montoya et al .. 2012).

The green seeds of the Mediterranean *P. oceanica* have an extraordinary feature; seeds have photosynthetic activity during the development of seedlings (Celdrán et al.. 2011).This photosynthetic activity of the seed of *P. oceanica* was corroborated with IMAGIM-PAM analysis (previous measurement). Pictures offered a gradient distribution of seed photosynthesis on the skin of the seed and on leaves (Figure 12 B) where the IMAGINM-PAM program displayed. in the central part of the seed. a maximal efficiency of photosystem II (Y)= 0.667 represented by a strong blue colour whereas the leaves showed a (Y) slightly lower = 0.64 with a green-blue colour.

This peculiarity has been not described in other seagrasses. however seeds of Australian Posidonia species are also green coloured. According to this. our main hypothesis was testing if the two of the most abundant species of Australian Posidonia (*P.australis* and *P. sinuosa*) display photosynthesis activity as seeds of the Mediterranean specie (*P. oceanica*). Based on the three species of study are not close genetically (Larkum 2006). and geographically separated by thousand of kilometres. the second hypothesis. is if the three species display similar values of photosynthesis parameters. To this. the objectives of our study were determined and compare photosynthesis activity and respiration in seeds of *P. oceanica*. *P. australis* and *P. sinuosa* by fluorescence (PAM) and oxygen production during germination.

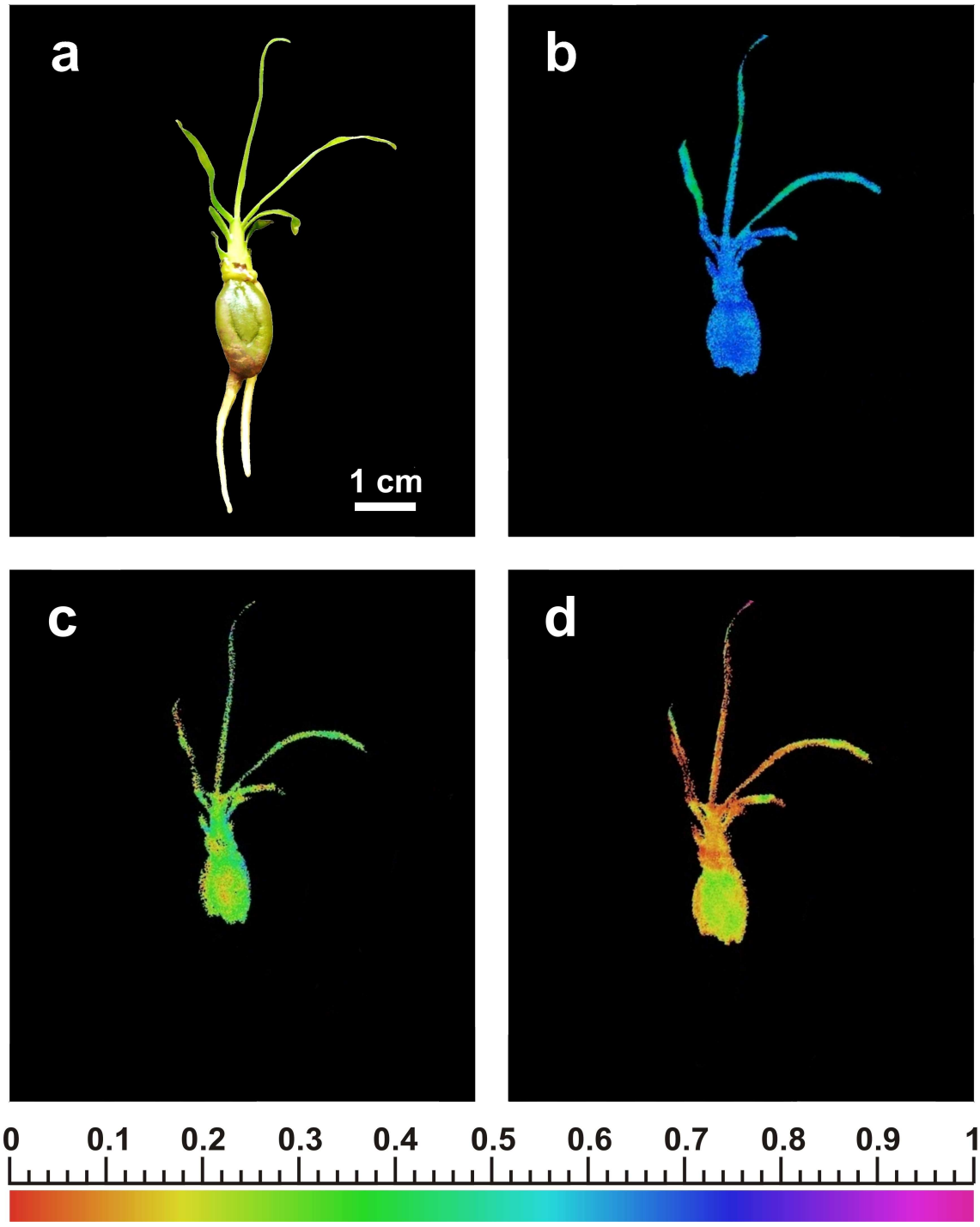


Figure 12. Gradient distribution of seed photosynthesis in *P. oceanica* by PAM fluorescence. The scale shows the relationship between the colour and the fluorescence parameter. (A) Image of a representative *P. oceanica* seed. (B) Image of maximal efficiency of photosystem II. (C) Image of photochemical quenching. (D) Image of non photochemical quenching. All measured at 310 µmol quanta $m^{-2}s^{-1}$.

Materials and methods

Site collection seed

Australian fruits of *P. australis* and *P. sinuosa* were collected in beaches of Woodman Point Regional Park Perth of Perth. (SW Australia) on November 2011. while Mediterranean fruits of *P. oceanica* were collected in beaches of Mazarron. in Murcia (SE Spain) on May 2012. The collection in both places. were made by hand from raw material deposited in shore. Seeds were extracted from mature fruits that showed no sign of dehiscence. herbivory or mechanical damage. Mediterranean and Australian seeds were germinated in aquaria illuminated with 20W halogen lamps during a month. All cultures were maintained at 19 ± 1 ºC. 35 psu. in a photoperiod of 12 h light: 12h dark (PAR 300 µmol m^{-2} s^{-1}).

Fluorescence analysis

To fluorescence and gross production analysis was used 15 seeds of every species of Posidonia after one month from germination. This time assures to use seeds completely mature. viable and where photosynthetic activity is totally developed. Also. analysis on seed of one month old. let us reject a possibly photosynthesis activity from non developed tissues as Tschiersch et al.. (2011) reported in immature seeds. at some stage during their development in peas and barley caryopsis.

Photosynthesis activity was assessed using an underwater pulse amplitude fluorometer (Diving-PAM). Seeds were acclimated to dark during 30 minutes before measurements. then. were fixed to the PAM clip. Light curves were run to analyze the maximal efficiency of photosystem II (Y). the electron transport rate (ETR) which was represented *vs* Irradiance. the photochemical quenching (PQ) and the non photochemical quenching (NPQ). Light curves were made through eight saturated pulse where basal PAR was increased to: 53. 162. 310. 517. 767. 1053 and 1551µmol m^{-2} s^{-1} with duration of 15 s among every pulse. ETR values were calculated as Φ_{PSII} x PAR x 0.5 x ETR factor. Φ_{PSII} is the effective quantum yield in steady-state illumination (not

acclimated to dark). The value of 0.5 concern to that half of the photons absorbed are absorbed by photosystem II. The ETR factor corresponds to the fraction of incident light absorbed by a leaf and is a specific parameter of every species. To calculate (ETR) the ETR factors used were; 0.64 and 0.59 to *P. australis* and *P. sinuosa* respectively (Horn 2006) and 0.88 to *P. oceanica* (Enriquez et al.. 1992). (PQ) was calculated as (Fm´- F)/(Fm´-Fo) and (NPQ) as (Fm-Fm´)/(Fm´) to 310 PAR. There were one value of (PQ) and (NPQ) to every PAR radiation. but were selected 310 PAR because was the PAR which the maximum (ETR) is obtained.

Photosynthetic production of seeds

Seeds were placed individually in 13 ml glass bottle. with sterilized bidistilled water and marine salt (PRODAC) intended for use in aquaria (36 psu). Previously. leaves were cut to avoid interaction with the seed production. The Net Production (NP) and respiration (R) were measured in light and dark conditions respectively with an oxygen meter (HQ40d. Hach). Gross production (GP) was calculated as (NP + R). Incubations were carried out during 1 hour in a Versatile Environmental Test Chamber (Sanyo MLR-351) with controlated irradiance (approximately 300 μmol m^{-2} s^{-1}) and temperature (19ºC) which corresponding approximately to the seawater temperature at which the seeds were collected in Australia and Spain. Incubations for seed respiration (R) were realized at the same temperature but in dark conditions. Oxygen values were then normalized to seed biomass. and the results expressed as mg O_2 gdw^{-1} h^{-1}. With the purpose of correct possible bacterial respiration. a control was incubated at the same conditions of seeds.

Finally. a new set of 10 seed of every species was used to measure and compare dry weight. Seeds were dried in oven during two days to 60ºC.

Data analysis

Analysis of variance one-way ANOVA were applied to fluorescence data: (ETR). (Y). (PQ). (NPQ). to oxygen production and respiration data: (R. PN and PB)

and weight of seed to assess significant differences in seeds of the three species of Posidonia. Prior to ANOVA a Shapiro-Wilk´s W test were run to analyze normality of data and Conchran test for homogeneity of variance (α=0.05). If data was not parametric then a Kruskal-Wallis was applied. After one-way ANOVA and Kruskal-Wallis a Tukey HSD post-hoc test (α = 0.05) and a Dunns post-hoc test (α = 0.05) were run respectively.

Results

Fluorescence analysis

The three species of Posidonia (*P. australis*. *P. sinuosa* and *P. oceanica*) showed photosynthetic activity and similar (Y). (PQ) and (NPQ) values with no significant differences ($p>0.05$). There were significant differences in all (ETR) values from the eight PAR radiations ($p<0.05$). The (ETR) vs Irradiance curves shown *P. oceanica* seed had higher values of (ETR) than to *P. australis* and *P. sinuosa*. The shapes in three cases were similar of the three species. with a general increasing of (ETR) until 310 PAR (Figure 13). up to this saturating point. *P. australis* and *P. sinuosa* showed accentuated decreasing. while *P. oceanica* had a gentler decreasing. The Tukey HSD post-hoc or Dunns test indicated that the differences in (ETR) were due to *P. oceanica* to all values of PAR used ($p<0.05$). There were no significance differences in (Y). (QP) and (NPQ) (Table 3). Dry weight of seeds of *P. australis*. *P. sinuosa* and *P. oceanica* was: 0.10±0.02. 0.08±0.01 and 0.34±0.09 (mean ± SE) respectively. There were significant differences in weight ($p<0.05$) and Duns post-hoc indicated they were due to *P. oceanica.*

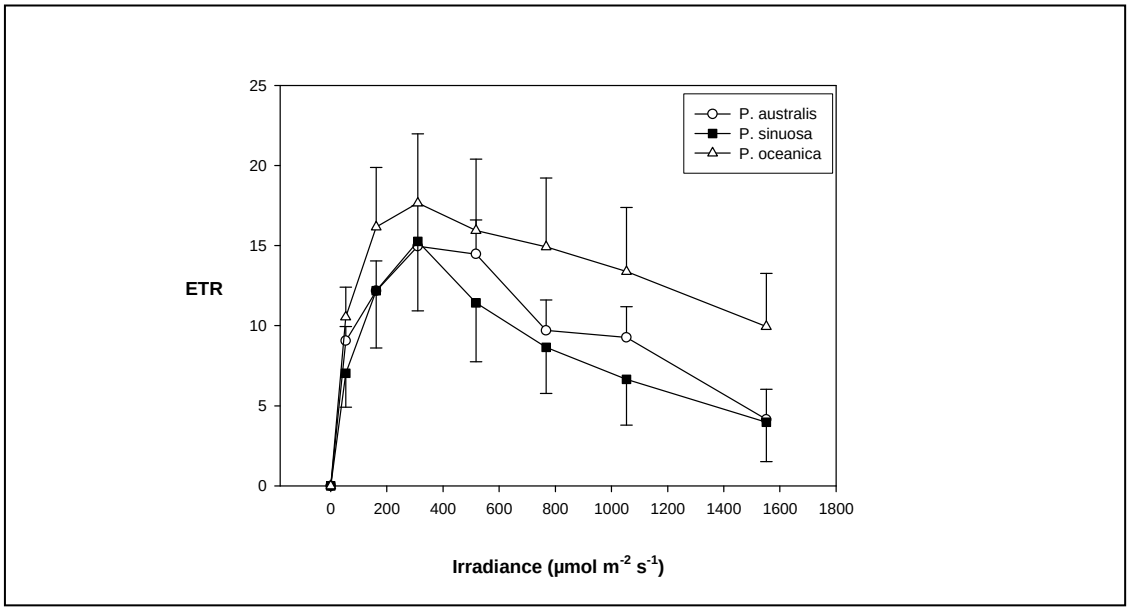


Figure 13. Electronic transport rate (ETR) of seeds of *P. australis*. *P. sinuosa* and *P. oceanica vs* irradiation (310 µmol m^{-2} s^{-1}) after one moth from germination.

	P. sinuosa	***P. australis***	***P. oceanica***
Y	0.635 ± 0.068	0.647 ± 0.063	0.673 ± 0.041
QP	0.250 ± 0.086	0.168 ± 0.115	0.155 ± 0.071
NPQ	0.200 ± 0.144	0.172 ± 0.040	0.139 ± 0.070

Table 3. Maximum quantum yield (Y). Photochemical Quenching (QP) and the Non-Photochemical Quenching (NPQ) values to *P. sinuosa. P. asutralis* and *P. oceanica*. (mean ± SE).

Photosynthetic production of seeds

P. oceanica showed higher rates of oxygen production and respiration than *P. australis* and *P. sinuosa*. however. *P. sinuosa* generated a positive (NP) as average. (Figure 14). There were significant differences in oxygen production and dark respiration between the three species; R ($p<0.001$). (NP) ($p=0.001$) and (GP) ($p=0.037$). Post-hoc analysis showed that significance differences in (R) were due to *P. oceanica.* in (NP) due to *P. sinuosa* and in lesser extend to *P. oceanica* and in (GP) to *P. sinuosa* ($p<0.05$).

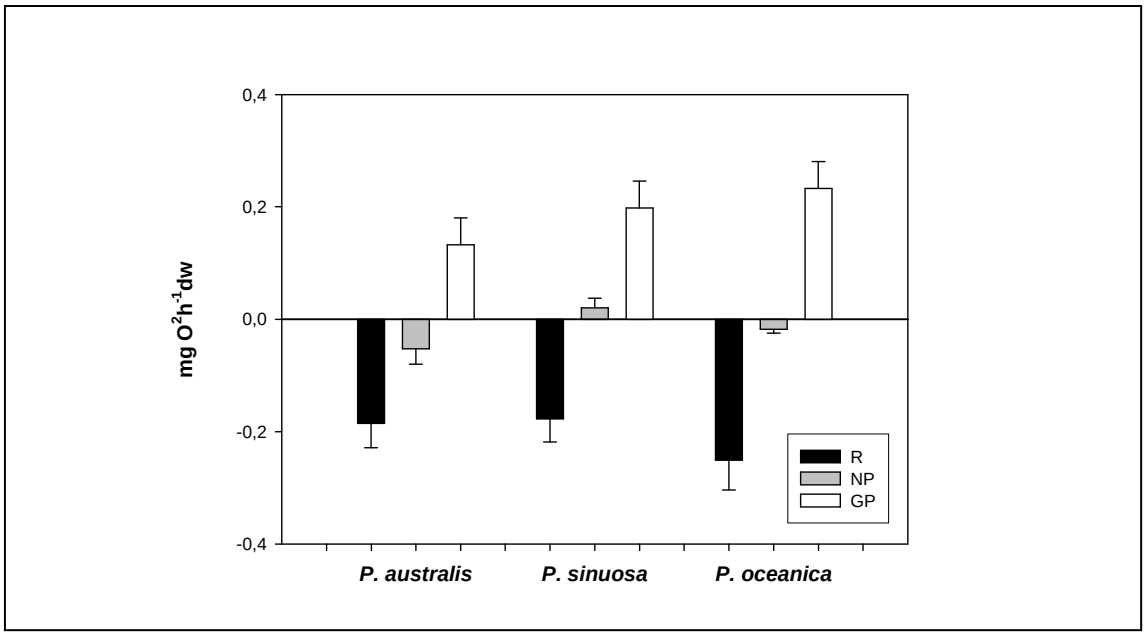


Figure 14. Respiration, Net Production and Gross Production of seed of *P. australis*, *P. sinuosa* and *P. oceanica* at 300 µmol m^{-2} s^{-1} and 19ºC. Oxygen expressed as mg O_2 gdw

Discussion

Fluorescence and oxygen production analysis confirm photosynthetic activity of the seeds of *P. australis. P. sinuosa* and *P. oceanica* after one month from germination. The higher (ETR) obtained from seeds of *P.oceanica* respect to the two Australians species. could be based on a higher amount of light absorbed by the photosynthetic antennae chlorophyll (Beer et al 1998) which was supported also by a higher value of (Y). Representation of (ETR) values vs Irradiation curves showed that *P. oceanica* was more photosynthetic active in all PAR values than Australian species. The three species exhibited similar (PQ). which suggest a similar energy invested in the photochemistry and similar (NPQ) which reflects heat-dissipation of excitation energy system and is related with the xanthophylls cycle (photoprotection of the photosynthetic apparatus).

Results in oxygen production evidences within every of the three species. (R) was practically compensated by (NP). In one hand. the higher cost of oxygen in the metabolism of reserves to *P. oceanica*. could be explained according to Balestri et al 2009. who compared the mean total nitrogen (N) and phosphorus (P) content of *P. oceanica* with Australian Posidonia species obtaining higher nutrient and store reserve in *P. oceanica*. Respect to the significant lower (GP) showed by seeds of *P. sinuosa*. this specie also showed significant lower weight than *P. oceanica* and slightly less than *P.australis* and thus less reserves. As a result. to *P. sinuosa.* can compensates easily (R) showing a positive (NP). In the other hand the compensatory mechanism displayed by the three species. is supported by the conclusions of the first study made on non-dormant seed of *P. oceanica* about photosynthetic activity (Celdran and Marin 2011).

Posidonia forms large meadows in oligotrophic areas subjected to seasonal variation of nutrients and irradiation. In some Australian *Posidonia* species (*P. australis*. *P. sinuosa* and *P. coriacea* Kuo and Cambridge). the seeds supplies C. N and P to the developing seedling until 4–6 months after germination (Kuo and Kirkman 1996; Walker et al.. 2004). while *P. oceanica* still provides this reserves to 1 yr old seedlings (Balestri et al .. 2009). It seems that during the early seedling developmental phase the seeds act to supplement environmental supplies of N and P to ensure rapid growth of shoot and roots. The ability of *P. oceanica* to produce nutrient-rich seeds may be a strategy to allow prolonged seedling development in environments where nutrients

are in concentrations low enough to limit the establishment of other seagrass species. In the Mediterranean Sea. there are relatively high concentrations of N and P in late autumn-winter and low concentrations in spring-summer. while irradiation is low and high respectively (Ballesteros 1989. Alcoverro et al.. 1995. 1997. Vidondo and Duarte 1995). *P. oceanica* produce large seeds containing more mineral nutrients (N and P) compared with Australian related species. The seeds of *P. oceanica* contained. 6.78 mg of N and 0.63 mg of P per seed. whereas this amount reached only 2.80 mg of N and 0.47 mg of P per seed in *P. australis* seeds (Hocking et al.. 1980; Balestri et al.. 2009). The Posdidonia ancestor probably. as nowadays Australian and Mediterranean Posidonia. was characterised as slow-growing with closed nutrient-cycling systems and high reserves content in seeds. In addition. separation of the continents promoted isolation of Mediterranean Sea in an enclosed basin connected to the Atlantic Ocean by the narrow sill of the Strait of Gibraltar.

The Mediterranean Sea has long been known as an impoverished area with nutrient levels low compared with other parts of the world's oceans. Seagrass ecosystems are often nitrogen- orphosphorus-limited. depending upon sediment composition (Kenworthy and Fonseca 1992; Touchette and Burkholder 2000; Alcoverro et al.. 2001). Nutrient limitations have been confirmed in nutrient addition studies in both the field and mesocosm systems (Kenworthy and Fonseca 1992; Peralta et al.. 2003). The seed of almost all our major crops as well as those of their wild relatives have photosynthetic pigments at some stage during their development (Tschiersch et al.. 2011). For example. the green oilseed *Brassica napus* seeds are adapted to utilize low light in photosynthesis during developing that receive inside of pods.

The photosynthesis during embryogenesis of seeds could be due to that seeds may experience hypoxia. as observed in developing legumes (Rolletschek et al.. 2002; Vigeolas et al.. 2003). In *Hordeum vulgare*. oxygen deficiency is more pronounced in the central than in the peripheral regions of the endosperm (Borisjuk and Rolletschek. 2009). When the internal O_2 level is low enough to limit respiration (i.e.. hypoxia). raising the supply of O_2 through seed photosynthesis could in principle alleviate this stress. by promoting the supply of respiratory energy (Tschiersch et al.. 2011). Seed photosynthesis in the Posidonia species studied seems to be a compensatory mechanism for alleviate the high respiration demand. The biggest size of the seed of *P. oceanica*

probably increased respiration demand respect to the two Australian species. and was forced to optimize seed photosynthesis (e.g. higher seed surface and higher ETR).
The photosynthetic activity of Posidonia on both geographical areas suggests that photosynthetic capacity was a skill acquired during before Late Eocene (37.2 million to 33.9 million years ago) from a common ancestor. The compensatory mechanism could appear when ancestral Posidonia colonized with great success tempered nutrient-poor habitats from Tethys Sea. Fossil evidence indicates that several of the now existing seagrass genera. such as Posidonia. Cymodocea. Thalassodendron and Thalassia had already evolved in the Eocene and possibly were widely distributed (Larkum and den Hartog. 1989).

There is a limited supply of nutrients to the surface waters of the Mediterranean Sea. both from its lower layers and from external sources (the Atlantic inflow. river discharges. atmospheric input) but the principal reason for this poverty is related to the Mediterranean's hydrology and circulation as a concentration basin (Souvermezoglou. 1988). Only one species. *P. oceanica*. could adapt to this impoverished area increasing the nutritional reservoir of seed. Australian Posidonia species evolved in a continent with a broad latitudinal gradient temperature allowed species to move gradually with the latitudinal advance and retreat of cooler conditions. which have favoured higher species diversity. The adaptation to the higher diversity of environmental conditions from Australian waters could to explain the variability in the size. nutrient content and photosynthetic activity of *P. australis* and *P. sinuosa*.

In resume. the evidence of photosynthetic activity in mature and germinated seeds of *P. australis*. *P. sinuosa* and *P. oceanica* point the photosynthetic activity as feature inherited from a common ancestor. Likewise. the relative high oxygen production and higher ETR values in the seed of *P. oceanica*. respect the two Australian species. reveal a strategy develop by this specie to survive in a oligotrophic environment such the Mediterranean Sea. Finally. and connecting with the idea of a relative high contain of nutrients in seed of the genera Posidonia is that photosynthesis activity could be a mechanisms to avoid hypoxia processes into the seed. This explains the similar values of NP and R as a compensatory mechanism to relive the lack of O_2 into the seed during seedling development.

Acknowledgements

We thank Jose Antonio Hernández Cortés. vicepresident of CEBAS-CSIC for help with the IMAGIM-PAM. laboratory assays. This research was further facilitated by a grant to D.C. from the Ministerio de Ciencia e Innovación Español. Programa Nacional de Formación de Profesorado Universitario. Spain.

Literatured Cited

Alcoverro T. Duarte CM. Romero J (1995) Annual growth dynamics of *Posidonia oceanica*: contribution of largescale versus local factors to seasonality. Mar Ecol Prog Ser. 120:203–210

Alcoverro T. Romero J. Duarte CM. López NI (1997) Spatial and temporal variations in nutrient limitation of seagrass *Posidonia oceanica* growth in NW Mediterranean Mar Ecol Prog Ser. 146:155-161

Alcoverro T. Cerbian E. Ballesteros E (2001) The photosynthetic capacity of the seagrass *Posidonia oceanica*: influence of nitrogen and light. J Exp Mar Biol Ecol. 261:107–120

Balestri E. Gobert S. Lepoint G. Lardicci C (2009) Seed nutrient content and nutritional status of *Posidonia oceanica* seedlings in the northwestern Mediterranean Sea. Mar Ecol Prog Ser. 388:99– 109

Beer S. Ilan M (1998) In situ measurements of photosynthetic irradiance responses of two Red Sea sponges growing under dim light conditions. Mar Biol. 131:613–617

Belzunce M. Navarro RM. Rapoport HF (2008) *Posidonia oceanica* seeds from drift origin: viability. germination and early plantlet development. Bot Mar. 51:1–9

Borisjuk L. Rolletschek H (2009) The oxygen status in the developing seeds. New Phytol. 182:17-30

Cambridge ML. Hocking PJ (1997) Annual primary production and nutrient dinamics of the seagrasses *Posidonia sinuosa* and *Posidonia australis* in south-west Australia. Aquat Bot. 59:277–295

Cambridge ML. Kuo J (1979) Two new species of seagrasses from Australia. *Posidonia sinuosa* and P. angustifolia (Posidoniaceae). Aquat Bot. 6:307–328

Celdran D. Marin A 2011 Photosynthetic activity of the non-dormant *Posidonia oceanica* seed. Mar Biol. 158:853–858

Enriquez S. Agusti S. Duarte CM. (1992) Light absorption by seagrass (Posidonia oceanica (L.) Delile) leaves. Mar Ecol Prog Ser. 86:201-204

Hemminga MA. Duarte CM (2000) Seagrass Ecology. Cambridge University Press. UK.

Hocking PJ. Cambridge ML. McComb AJ (1980) Nutrient accumulation in the fruits of two species of seagrass. *Posidonia australis* and *Posidonia sinuosa*. Ann Bot. 45:149-61

Horn Lotte (2006) The measurement of seagrass photosynthesis using pulse amplitude modulated (PAM) fluorometry and its practical applications. specifically in regard to transplantation. PhD thesis. Murdoch University.

Kenworthy WJ. Fonseca (1992) MS The use of fertiliser to enhance growth of transplanted seagrasses Zostera marina L. and Halodule wrightii Aschers. J Exp Mar Biol Ecol. 163:141–161

Krom MD. Brenner S. Kress N. Gordon LI (1991) Phosphorus Limitation of Primary Productivity in the E.Mediterranean Sea. Limn Ocean. 36:424-432.

Kuo J. Kirkman H (1996) Seedling development of selected Posidonia species from southwest Australia. In: Kuo J. Phillips RC. Walker D. Kirkman H (eds) Seagrass biology: proceedings of an international workshop. Rotnest Island. Western Australia. p 57-64

Kuo J. Hartog D (2000) Seagrasses: A profile of an ecological group. Biol Mar Med. 7 (2):3-17

Kuo J. Cambridge ML (1984) A taxonomic study of the Posidonia ostenfeldii complex (Posidoniaceaea with description of four new Australian seagrasses. Aquat Bot. 20:267–295

Larkum AWD. Den Hartog C (1989) Evolution and biogeography of seagrasses. In: Larkum. AWD. RJ Orth. CM Duarte (2006). Seagrasses: biology. ecology and conservation. Springer. Dordrecht. pp 691

Marba N. Walker DI (1999) Growth. flowering. and population dynamics of temperate Western Australian seagrasses. Mar Ecol Prog Ser. 184:105–118

Peralta G. Bouma TJ. Soelen JV. Perez-Llorens JL. Hernandez I (2003) On the use of sediment fertilization for seagrass restoration: a mesocosm study on Zostera marina L. Aquat Bot 75:95–110

Procaccini G. Orsini L. Ruggiero MV. Scardi M (2001) Spatial pattern of genetic diversity in *Posidonia oceanica*. an endemic Mediterranean seagrass. Mol Ecol. 10:1413–1421

Rasheed MA (1999). Recovery of experimentally created gaps within a tropical Zostera capricorni (Aschers.) seagrass meadow. Queensland Australia. J Exp Biol Ecol. 235:183-200

Rasheed MA (2004). Recovery and succession in a multi-species tropical seagrass meadow following experimental disturbance: the role of sexual and asexual reproduction. Journal of Experimental Marine Biology and Ecology 310: 13-45

Rolletschek H. Borisjuk L. Koschorreck M. Wobus U. Weber H (2002) Legume embryos develop in a hypoxic environment. J Exp Bot 53: 1099–1107

Ruiz-Montoya L. Lowe RJ. Van Niel KP. Kendrick GA (2012) Title The role of hydrodynamics on seed dispersal in seagrasses J Limn Ocean.57:257-1265

Short FT. Carruthers TJB. Dennison WC. Waycott M (2007) Global seagrass distribution and diversity: A bioregional model. J Exp Mar Biol Ecol 350:3–20

Spalding M. Taylor M. Ravilious C. Short FT. Green E (2003) in World Atlas of Seagrasses. eds Green EP. Short FT (Univ of California Press. Berkley. CA). pp 5–26

Stockmans F (1932) Posidonia perforate Saporta et Marion des mares de Gelinden (Paleocene). Bull Mus Hist nat Belgyque. 8 (27):1-9

Touchette BW. Burkholder JM (2000) Review of nitrogen and phosphorus metabolism in seagrasses. J Exp Mar Biol Ecol. 250:133–167

Tschiersch H. Borisjuk L. Rolletschek H (2011) Gradients of seed photosynthesis and its role for oxygen balancing. Biosystem. 103:302-308

Vidondo B. Duarte CM (1995) Seasonal growth of Codium bursa. a slow growing Mediterranean macroalga: in situ experimental evidence of nutrient lim~tation. Mar Ecol Prog Ser. 123:185-191

Vigeolas H. van Dongen JT. Waldeck P. Hühn D. Geigenberger P (2003) Lipid storage metabolism is limited by the prevailing low oxygen concentrations within developing seeds of oilseed rape. Plant Physiol 133: 2048–2060

Walker DI. Campey ML. Kendrick GA (2004) Nutrient dynamics in two seagrass species. Posidonia coriacea and Zostera tasmanica. on Success Bank. Western Australia. Estuar Coast Shelf Sci. 60:251-260

Waycott M. Les DH (2000) Current perspective on marine angiosperm evolution. Biol Mar Med. 7:160–163

Waycott M. Procaccini G. Les DH. Reusch TBH (2006) Seagrass evolution. ecology. and conservation: a genetic perspective. In: Larkum. A.W.D.. Orth. R.J.. Duarte.

C. (Eds.). Seagrasses: Biology. Ecology. and Conservation. Springer. The Netherlands. pp. 25–50

General Discussion

Amor Omnia Vincit
(Virgilio)

Discussion

Flowering in the seagrass *P. oceanica* is relatively rare, and population spread occurs mainly through clonal propagation (Procaccini et al. 2001). Also, the low recruitment of seed of *P. oceanica* is usually attributed to seed loss in sites unsuitable for establishment (Buia and Mazzella, 1991), fruit abortion and predation on inflorescences (Buia and Mazzella, 1991; Piazzi et al., 2000). In addition to this, maturation of fruits takes approximately only four months (Buia and Mazzella 1991). Moreover, the success collecting fruits is highly chance due to the unpredictability of the fruit deposit in beaches by waves and the highly dispersion of fruits attached to the plant. Collectors has to sampled long coasts transects or diving many hours with low possibilities of achieve. These peculiarities make the progress of the study of the seed of *P. oceanica* difficult due to the scarcity and unpredictability of this experimental material.

There is only a few studies of the seed of *P. oceanica* and has been focussed in describing the mature seed and early plantlet structures, germination in vitro of the seed, and distribution of seedlings over spatial scales (Balestri et al., 1998, 2008, 2009 Belzunce et al., 2005). It seems surprising nobody suspected that the green colour of the seed of *P. oceanica* could indicate photosynthesis activity on it. Likewise there is a knowledge gap respect to possibly relationships among the seed and the marine microbiota. Our results respect to the seed of *P. oceanica* made us find several features of the seed and some of them suppose a new paradigm to the classical biology.

The culture of *M. posidonica* on seed of *P. oceanica* revealed this Marinomona enhances seedling growth increasing the foliar area (Celdran et al., 2012). The stimulatory effect was possibly carried out trough liberation of metabolites or nutrients that induce plant growth. Stimulatory effects of microorganims on plants have been widely studied (St-Arnaud et al., 1996, Selosse et al., 2006 Carisse et al., 2003) however there is no information about this stimulatory effects on seagrasses. This is the first time it is proved microorganisms can also promote plant productivity in marine ecosystems. Inoculation with *M. posidonica* also changed patterns in the epiphyte macroalgal biota (Figure 15), which suggest microoganisms associated to *P. oceanica* not only regulate

seedling growth but have a significant effect on the epiphyte community. *Marinomonas mediterranea* did not stimulate leaf growth or induced changes in epiphyte structure

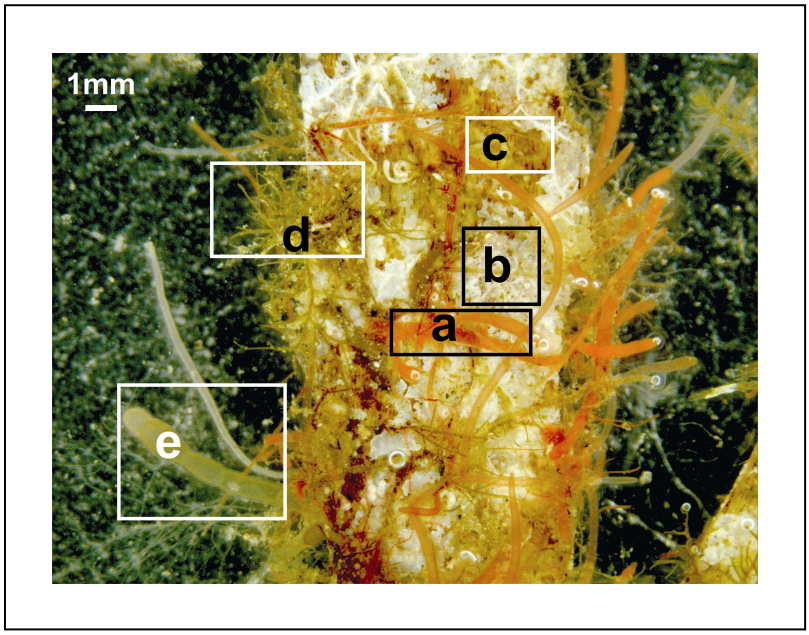


Figure 15. Six month old leaf of *P. oceanica* seedling covered by algal epiphytes. a: Red corticated. b: red crustose. c: brown crustose. d:filamentous. e: brown corticated

The increasing of the foliar area and determining of a certain epiphyte community in its surface by microrganisms is one of the singular skills unknown so far of the seed of *P. oceanica*. The green colour of seeds was the sign of possible photosynthesis activity as a strategy not seen before in any seed of seagrass.

The description of photosynthesis in mature seed of *P. oceanica* (Celdran and Marin 2011) make the classical photosynthesis theories have to been reviewed. The information that can be obtained from analysis of photosynthesis is very valuable. For instance, in the experiment "photosynthesis activity in non-dormant seed of *P.oceanica*" the chlorophyll "a/b" ratio of the seed was calculated and pointed to classify the seed as a shadow plant while leaves of adult *P. oceanica* plant have higher chlorophyll "a/b" ratio (Rotini 2010) than seed. Moreover, the deep where the seed show positive net production was calculated deep and goes from 5 to 25m deep while *P. oceanica* can live almost in the surface at less than 1m deep. These two data plus the saturation point of

the seed is lower than the adult plant suggest the seed is adapted to slightly lower environmental irradiance during germination. Likewise high photosynthesis rate at 15ºC, rather than 21ºC indicated a higher photosynthetic success to seeds that released in early spring than seeds liberated in late spring. Seeds liberated in early spring are conditioned by a lower irradiation and photoperiod than seeds released in late spring, possibly to supply this deficiency, seeds that mature in first months of spring present higher net photosynthesis.

Photosynthesis tested in mature seeds of *P. oceanica* after three months from germination evidenced that the seed continues having photosynthetic activity a relative long time after germination and contribute to the seedling growth and increased its germination achievement, however more studies are need to evaluate the time that the seed still have photosynthesis activity. Likewise the photosynthetic contribution of the seed was similar than photosynthetic contribution of leaves of seedlings which highlights the importance of the photosynthesis activity of the seed. These results give us an idea of such functional is the photosynthesis of the seed to the seedling. However there was a progressive leaf growth in all seedlings independently of light treatment, which suggest that seed illumination was no a trigger mechanist for plant growth. This could be a security mechanism of survival when seedlings are buried for wave action.

The second and fourth chapter corroborate that net production was proximal to "zero", due to the equilibrium between respiration and photosynthesis. In the second experiment the photosynthetic measurements were made on seed just extracted from fruits and in the fourth experiment were used seed of three months old. Both results were obtained from a concise time in the daily metabolism of the seed. In the third chapter leaf growth represented the balance of three months of seed metabolism. In this balance, was probed that photosynthetic contribution of the seed was invested totally in growth of the seedling not in an increment of reserves contain. These arguments unite a same affirmation; the contribution of the seed photosynthesis is invested to compensate respiration product of the seedling growth.

This compensatory mechanism could be related to the hypoxia that seeds experiments during its embryogenesis, as observed in developing legumes (Rolletschek et al., 2002; Vigeolas et al., 2003). According to this, photosynthesis activity could be a

mechanism to avoid hypoxia processes into the seed, which explain the similar values of NP and R as a compensatory mechanism to relive the lack of O_2 into the seed during seedling development.

Photosynthesis activity in seed of *P. oceanica* as compensatory mechanism of the respiration produced by the seedling growth determined in the second third and fourth experiments is also supported by the results of the analysis of carbohydrate reserve and nutrient contain of the second experiment. Starch, free sugar and nutrients mobilization seems to be an independent process from photosynthesis.

Photosynthesis activity was also probed in seed of *P. ustralis* and *P. sinuosa*. Results of fluorescence and oxygen production analysis corroborated that photosynthesis activity in seed of the Australian species of Posidonia were also compensatory mechanism to counteract respiration rates. The presence of photosynthetic activity in Mediterranean and Australian Posidonia species (Figure 16) suggests that photosynthetic capacity was a skill acquired during the Late Eocene (37.2 million to 33.9 million years ago) from a common ancestor. The three species displayed a similar photosynthetic parameters; the maximal efficiency of photosystem II (Y), photochemical quenching, (PQ) and non photochemical quenching,(NPQ) values, which suggest a similar photosynthetic efficiency, energy invested in the photochemistry and similar reflects heat-dissipation of excitation energy system (related with the xanthophylls cycle, photoprotection of the photosynthetic apparatus) respectively.

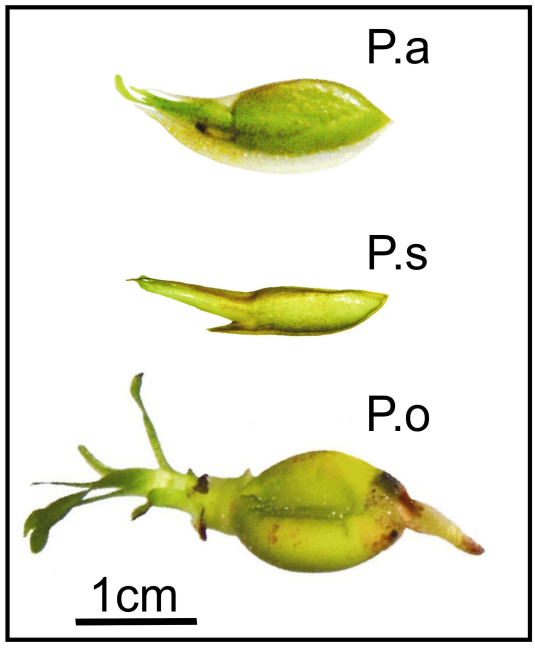


Figure 16: Seed of *Posidonia australis* (P.a), *Posidonia sinuosa* (P.s) and *Posidonia oceanica* (P.o), one week after extraction from fruits.

However, Mediterranean and Australian species of Posidonia are not close genetically (Larkum 2006) and some differences were observed in the photosynthetic tests. The higher (ETR) values of *P. oceanica* showed a higher photosynthetic activity in all PAR values than Australian species. *P. oceanica* is also the specie with more nutrient contain and higher weight of the three species. These differences could respond to the conditions that *P. oceanica* faced in a very oligotrophic environment, the Mediterranean Sea.

This Sea has a limited supply of nutrients to the surface waters, both from its lower layers and from external sources (the Atlantic inflow, river discharges, atmospheric input) but the principal reason for this poverty is related to the Mediterranean's hydrology and circulation as a concentration basin (Souvermezoglou, 1988). Australian Posidonia species evolved in a continent with a broad latitudinal gradient temperature allowed species to move gradually with the latitudinal advance and retreat of cooler conditions, which have favoured higher species diversity. The adaptation to the higher diversity of environmental conditions from Australian waters could to explain the variability in the size, nutrient content and photosynthetic activity of the Australian Posidonia species.

References

Balestri E, Piazzi L, Cinelli F (1998) In vitro germination and seedling development of *Posidonia oceanica*. Aquat Bot 60:83–93

Balestri E, Lardicci C (2008) First evidence of a massive recruitment event in *Posidonia oceanica*: spatial variation in first-year seedling abundance on a heterogeneous substrate. Estuar Coast Shelf Sci 76:634–641

Balestri E, Gobert S, Lepoint G, Lardicci C (2009) Seed nutrient content and nutritional status of *Posidonia oceanica* seedlings in the northwestern Mediterranean Sea. Mar Ecol Prog Ser 388:99–109

Belzunce M, Navarro RM, Rapaport HF (2005) Seed and early plantlet structure of the Mediterranean seagrass *Posidonia oceanica*. Aquat Bot 82:269–283

Buia MC, Mazzella L (1991) Reproductive phenology of the Mediterranean seagrasses *Posidonia oceanica* (L.) Delile, Cymodocea nodosa (Ucria) Aschers., and Zostera noltii Hornem. Aquat Bot 40:343–362

Carisse O, Bernier J, Benhamou N (2003) Selection of biological agents from composts for control of damping-off of cucumber caused by Pythium ultimum. Can J Plant Pathol 25: 258−267

Celdran D and Marin A (2011) Photosynthetic activity of the non-dormant *Posidonia oceanica* seed. Marine Biology 158:853–858

Celdran D, Espinosa E, Sanchez-Amat A, Atucha A. Effects of epibiotic bacteria on leaf growth and epiphytes of seagrass. *Posidonia oceanica*. Mar Ecol Prog Ser 2012; 456:21-27;

Larkum, AWD, RJ Orth, CM Duarte (2006). Seagrasses: biology, ecology and conservation. Springer. Dordrecht. pp 691

Piazzi, L., Balestri, E., Cinelli, F., 2000. Grazing on inflorescences of the seagrass *Posidonia oceanica*. Bot. Mar.43, 581–584.

Procaccini G, Orsini L, Ruggiero MV, Scardi M (2001) Spatial pattern of genetic diversity in *Posidonia oceanica*, an endemic Mediterranean seagrass. Mol Ecol. 10:1413–1421

Rolletschek H, Borisjuk L, Koschorreck M, Wobus U, Weber H (2002) Legume embryos develop in a hypoxic environment. J Exp Bot 53: 1099–1107

Rotini A (2010) Biochemical and molecular tools to monitor *Posidonia oceanica* (L.) Delile meadows. PhD Thesis.

Selosse MA, Richard F, He X, Simard SW (2006) Mycorrhizal networks: des liaisons dangereuses? Trends Ecol Evol 21: 621−628

Souvermezoglou E. 1988. Comparaison de la distribution et du bilan d'échanges de sels Nutritifs en Méditerranée et en mer Rouge. Oceanologica Acta, Océanographie pélagique mediterranéenne, ed. H.J.Minas et P. Nival, No SP:103-109.

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus Glomus intraradices in an in vitro system in the absence of host roots. Mycol Res 100: 328−332

Vigeolas H, van Dongen JT, Waldeck P, Hühn D, Geigenberger P (2003) Lipid storage metabolism is limited by the prevailing low oxygen concentrations within developing seeds of oilseed rape. Plant Physiol 133: 2048–2060

Conclussions

Ab imo pectore
(J. Cesar)

Conclussions

1. Inoculation with the bacteria *Marinomonas posidonica* on *P.oceanica* seedling enhances leaf growth, which confirms microorganisms can also promote plant productivity in marine ecosystems. Likewise, macroalgal epiphyte communities grown on seedlings, responded to the inoculation with *Marinomonas posidonica.* Main changes in the abundance of the epiphyte community were due to red crustose, filamentous and red corticated algae, which were more abundant in seedlings treat with *Marinomonas posidonica*. However *Marinomonas mediterranea* does not stimulate leaf growth or induced changes in epiphyte structure.

2. The chlorophyll analysis demonstrates the presence of photosynthetic active pigments (0.26 ± 0.06 mg Chl a·gdw-1 and 0.16 ± 0.04 mg Chl b·gdw-1) in *P. oceanica* seed. The higher NP obtained from the seed of *P. oceanica* is at 90 µmol quanta m-2 s-1 at both incubation temperatures (0.013 ± 0.06 mg O2·g dw-1·h-1 at 15ºC, and 0.006 ± 0,005 mg O2·g dw-1·h-1 to 21ºC). At this irradiance value, NP compensates R. Finally, germination success is significant higher in seed illuminated than in seed not illuminated.

3. Photosynthesis in the seed of *P. oceanica* is "functional", which enhances the seedling growth. The seed, that remain attached to seedling, shows photosynthesis activity during at least three months after germination. Seedlings with seeds exposed to light showed higher leaf surface than seedlings with seeds in darkness. Photosynthetic contribution of the seed of *P. oceanica* is similar than seedling leaves.

4. The carbohydrates reserves (free sugar and starch) and nutrients (C, N and P) in seeds not show significant differences between seed of seedling illuminated respect to seedling not illuminated. Mobilization of reserves and nutrients in the seed is independent to photosynthesis. Likewise, there is a progressive leaf growth in all seedlings independently of light, which confirm that seed illumination is not a trigger mechanist for plant growth.

5. Seed photosynthetic activity was found in Australian seeds of *Posidonia australis* and *Posidonia sinuosa*. The presence of photosynthetic activity in Mediterranean and Australian Posidonia species indicates that photosynthetic capacity was a skill acquired during the Late Eocene (37.2 million to 33.9 million years ago) from a common ancestor.

6. Mediterranean and Australian species show similar photosynthetic efficiency, energy invested in the photochemistry and similar reflects heat-dissipation of excitation energy system (related with the xanthophylls cycle, photo-protection of the photosynthetic apparatus) respectively. However *P. oceanica* shows higher (ETR) values in all PAR values than Australian species.