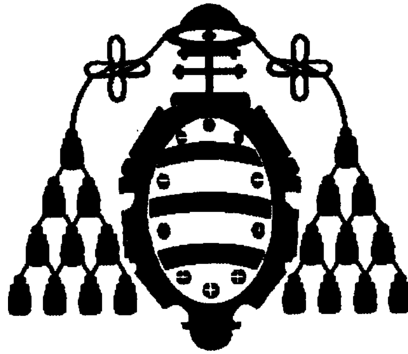

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Departamento de Biología de Organismos y Sistemas

Programa de Doctorado: "Biología aplicada a la sostenibilidad de recursos naturales (Mención de Calidad)"

"Bases fisiológicas y moleculares de la reproducción sexual y axesual en el gametofito de helechos"

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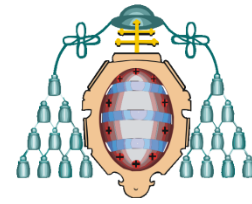

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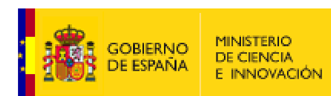

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Lista de Abreviaturas.

2-DE, Two dimensional electrophoresis

ABA, Abscisic acid

AC, Active charcoal

AF, acidic ethyl acetate fraction

IAA, Indol-3-acetic acid

BA, 6-Benzylaminopurine

cZ , cis-zeatin

cZR, cis-zeatin riboside

CCB, Coomasie Brilliant Blue

CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate

ddH2O, distilled and deionized water

DHZ, dihydrozeatin

DHZR , dihydrozeatin riboside

DTT, dithiothreitol

DW, Dry weight

EtOAc, ethyl acetate

HG, Homogenized mature gametophytes

HPLC, High performance liquid chromatography

GAs, Gibberellins

GA_3, Gibberellic acid

GA_{4+7}, Mixture of GA4 and GA7 gibberellins

GC–MS, Gas chromatography–mass spectrometry

IAA, Indole-3-acetic acid

IEF, Isoelectric focusing

IPG, Inmobilized pH gradient

iP, Isopentenyladenine

iP9G, Isopentenyl adenine-9-glucoside

iPR, Isopentenyladenosine

KNN, K nearest neighbours

LC-MS/MS, Liquid Cromatography coupled to Mass Spectrometry-Mass Spectrometry

MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization – Time of Flight

Mr, Molecular relative weight

MS, Murashige and Skoog mineral medium (1962)

MeOH, methanol

NAA, Naphtaleneacetic acid

NF, Neutral ethyl acetate fraction

***m/z*,** ratio of mass to charge

PAGE, Polyacrilamide gel electrophoresis

PC, Principal Component

PCA, Principal Component Analysis

p*I*, Isoelectric Point

PMF, Peptide Mass Fingerprinting

PMSF, Phenylmethylsulphonylfluoride

PTM, Post translational modification

PVP, Polyvinylpirrolidone

RP-HPLC, reverse-phase HPLC

SDS, Sodium Dodecyl Sulfate

SG, Spore-derived gametophytes

tZ, trans-zeatin

tZR, trans-zeatin riboside

WMS, MS medium without the cellular contents released during homogenization

BASES FISIOLÓGICAS Y MOLECULARES DE LA REPRODUCCIÓN SEXUAL Y ASEXUAL EN EL GAMETOFITO DE HELECHOS

1. INTRODUCCIÓN

La reproducción es una de las características esenciales de la vida, común a todos los seres vivos, en todas sus formas. Mishler (1988) define la reproducción como la producción de una planta nueva, fisiológicamente independiente, y este concepto ha sido completado posteriormente por Mogie (1992), como un evento que inicia una recapitulación de la ontogenia. Hay numerosos mecanismos reproductivos que van desde la simple división de las células hasta complicados sistemas que incorporan varios estados dentro de un ciclo reproductivo. La reproducción es tanto sexual, con gametos haploides que se fecundan e intercambian la información genética durante la recombinación, o asexual, básicamente divisiones celulares mitóticas que transmiten material genético idéntico desde el parental a la progenie. A través de la reproducción sexual los seres vivos replican su material genético de una manera eficaz para así poder reparar posibles daños en él, mejorando con ello las posibilidades de supervivencia de una especie. Sin embargo, el sexo no es necesario para la reproducción (Ghiselin 1988; Mogie 1992). El coste energético, genético y ecológico de la reproducción sexual es alto (Crow 1994) y la multiplicación sin sexo ha sido una forma efectiva para mantener las poblaciones aún con riesgo de pérdida de variación genética (Klekowski 1997; McLellan et al. 1997).

1.1. Determinación sexual en plantas

La determinación sexual es un proceso que conduce a la distinción y separación de estructuras responsables de la producción de gametos masculinos y femeninos, cuyo objetivo último es favorecer el intercambio genético entre individuos apareantes aumentando con ello la heterosis y disminuyendo la endogamia (Chuck 2010).

El fenotipo sexual en las plantas va desde los individuos hermafroditas, en los cuales los órganos sexuales masculinos y femeninos están en la misma flor, hasta las especies dioicas en las cuales las flores masculinas y femeninas se hallan en individuos separados. Si bien las plantas son generalmente hermafroditas, disponen de distintos mecanismos encaminados a favorecer el cruzamiento entre diferentes individuos tales como la autoincompatibilidad genética o la “heterostyly”, plantas que presentan estilos de diferente longitud (Chuck 2010).

La determinación sexual en las plantas es un proceso importante en su ciclo vital, que además de contribuir a la supervivencia de la especie puede tener importantes repercusiones económicas en especies de interés agronómico (Tanurdzic y Banks 2004).

1.2. Mecanismos de la determinación sexual en plantas

En plantas se han descrito diferentes formas de controlar la determinación sexual, en determinados sistemas experimentales. Así, por ejemplo, se han descrito los loci responsables de la generación de tipos sexuales masculinos, femeninos y hermafroditas en el helecho *Ceratopteris richardii* (Tanurdzic y Banks 2004). En algunos helechos, el sexo del gametofito está mediado por la acción epigenética de unos compuestos denominados anteridiógenos, por su acción inductora de órganos sexuales masculinos o anteridios (Tanurdzic y Banks 2004). Entre las plantas con flor, la determinación sexual ha sido bien estudiada en la especie *Silene latifolia*, en la cual el fenotipo sexual está ligado a los cromosomas sexuales XY como ocurre en animales (Charlesworth 2008) y en otras especies tales como *Zea mays*, o las cucurbitaceas *Cucumis sativa* y *Cucumis melo*, el sexo está íntimamente asociado a los reguladores del desarrollo. Por último, cabe comentar la incidencia de mecanismos epigenéticos, tales como la metilación del DNA, en la determinación sexual y en el desarrollo floral (Vyskot et al. 1993; Janoušek, Siroky y Vyskot 1996; Vyskot 1999; Spielman 2001).

1.3. Bases fisiológicas y moleculares del desarrollo sexual en plantas.

Estudios llevados a cabo en especies de angiospermas de una gran importancia económica como el maíz o las cucurbitaceas pepino y melón, han arrojado mucha luz sobre las bases fisiológicas y moleculares del dimorfismo sexual resultante de un programa de determinación sexual. En dichas especies, la determinación sexual ocurre por la detención selectiva del desarrollo de los estambre o de los carpelos. Desde un punto de vista fisiológico, los reguladores del desarrollo han sido propuestos como reguladores en la determinación sexual.

La implicación de las hormonas o reguladores del desarrollo vegetal en la determinación sexual fue puesta de manifiesto de dos maneras: a) mediante la administración de estos compuestos a las plantas, y b) a través del análisis de los niveles en que aquellos se hallan presentes en las plantas (Duran y Duran 1984; Chailkyan y Khryanin 1987; Khryanin 2002). A pesar de que en muchos experimentos se intenta modificar la expresión sexual en plantas mediante su aplicación, pocas veces encontramos publicaciones sobre niveles endógenos de reguladores en esas plantas.

1.3.1. *Determinación sexual en Cucurbitaceae*

Algunas especies de la familia de las Cucurbitaceas han sido modelos de estudio del dimorfismo sexual durante décadas. Estas plantas son principalemtne monoicas pero puden ser dioicas o hermafroditas, dependiendo del genotipo. En *Cucumis sativus* L., el sexo está genéticamente determinado por tres loci, F, A y M y ello puede ocasionar la formación de un espectro más amplio de fenotipos sexuales desde la monoecia hasta la andromonoecia o ginomonoecia. Además, también se ha visto que la aplicación de GAs promueve la masculinización mientras que el etileno favorecería la feminización (Perl-Teves 1999). Tratamientos con etileno de líneas monoicas favorecen el aumento de flores femeninas (Iwahori et al. 1970; MacMurray y Miller 1968; Shanon y de la

Guardia 1969). Sin embargo, estos datos no simplemente indican que cada hormona controla cada sexo o que el balance de las dos hormonas controla la determinación en esta especie. Tratamientos de líneas di-mórficas sexualmente por ambas hormonas o mediante el empleo de inhibidores apuntan hacia el etileno como principal determinante de la expresión sexual (Yin y Quinn 1995). Enzimas implicados en la síntesis de etileno (CS-ACS1 o 1-aminocyclopropane-1-carboxylic acid sintasa) o en la percepción de la señal estarían detrás de los loci descritos inicialmente y de la expresión sexual en dicha especie (Kamachi et al. 1997; Yamasaki et al. 2001; Milbus y Tatlioglu 2004).

En *Cucumis melon* L. también se ha descrito un sistema uni-hormonal basado en el etileno como factor clave en la determinación sexual en esta otra cucurbitacea. En melón, este proceso estaría gobernado por dos alelos: a (andromonoecious) and g (gynoecious), siendo el genotipo A-G- monoecious, mientras que las plantas aagg serían hermafroditas. Además, las plantas aaG- serían andromonoecious y aquellas AAgg serían gynoecious (Kenigsbuch y Cohen 1989). El modelo para la determinación sexual basado en la expresión de los genes G y A se representa en la Fig. 1, así como la conexión con el etileno. De acuerdo con él, G tendría una función dual reprimiendo el desarrollo de los carpelos y promoviendo el de los estambres a través de la represión de A en flores monoicas. En flores gynomonoecious, G no se expresa, permitiendo el desarrollo de carpelos y la expresión de A, que bloquea el desarrollo de los estambres. En hermafroditas, la ausencia de G permite el desarrollo de carpelos y la expresión de a, que sería una variante atenuada de A que tendría un aminoácido sustituido en el sitio activo (Boualem et al. 2009), y por ello tendría reducida su función y no reprimiría el desarrollo de los estambres. Resulta interesante que genes que controlan la biosíntesis de una hormona difusible como el etileno sean capaces de reprimir el desarrollo floral de una manera tan precisa.

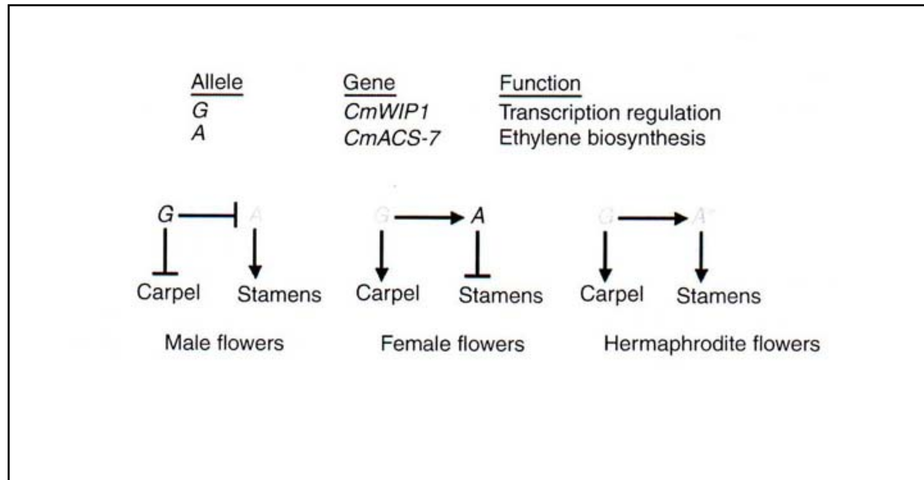


Figura 1.1. Determinación sexual en *Cucumis melo*. Modelo para la determinación sexual en melón, basado en la expresión de los genes G y A (Martin et al. 2009), donde G es represor de carpelos y A de estámenes. Las letras en gris representan inactivación. El alelo A* tiene actividad reducida debido a la sustitución de un aminoácido y por ello no reprime estámenes (Boulaem et al. 2008).

1.3.2. *Determinación sexual en Zea mays L.*

El efecto de los reguladores del desarrollo en la determinación sexual ha sido bastante estudiado en maíz. En esta planta monoica, las flores unisexuales masculinas y femeninas ocupan posiciones diferentes en el tallo, estando las flores estaminadas en inflorencencias terminales (tassel) y las pistiladas en inflorescencias laterales (ear). La determinación del sexo en maíz se logra mediante el aborto selectivo de los primordios pistilares en las flores estaminadas en desarrollo y los primordios estaminales en las pistiladas. Dicho aborto selectivo operaría correctamente gracias a la acción conjunta de dos rutas genéticas que actuarían de forma independientemente. La existencia de mutantes en maíz para el metabolismo de las giberelinas o en la percepción tales como dwarf (d) y anter-ear,(an), indicaron que las GAs promovían el desarrollo pistilar y la supresión del desarrollo estaminal.en las inflorescencias femeninas "ear" (Phinney 1981; Phinney y Spray 1982; Dellaporta y Urrea-

Calderón (1994). Por otra parte, Dellaporta y Calderón-Urrea (1994) propusieron mediante otra serie de mutantes ligados a determinación sexual tales como silkless (sk1) y tasselseeds (ts) que Sk1 y Ts2, actuarían de modo antagonista, reprimiendo mutuamente la expresión uno del otro, de manera que en Ts2 se expresa en las inflorescencias masculinas, reprimiendo la acción de Sk1, abortando los pistilos y madurando los estambres. En las inflorescencias femeninas operarían a la inversa. Recientemente, (Acosta et al. 2009), ha sido relacionado el mutante ts1 con los niveles de ácido jasmónico, implicando también a esta hormona en la determinación sexual en maíz, al tiempo que también se pregunta por un posible papel de las citoquininas (Gan y Amasino 1995; Young et al. 2004). Tal vez sea posible conectar todas las rutas hormonales implicadas en la determinación del sexo en pepino, melón y maíz con las GAs. De hecho, múltiples rutas hormonales parecen converger en la ruta de señalización DELLA (Achard et al. 2006), incluyendo al ácido jasmónico (Navarro et al. 2008)

1. 4. A la búsqueda de nuevos sistemas experimentales

La determinación sexual en plantas es el resultado de la expresión del genoma del gametofito haploide. En la mayoría de las plantas estudiadas, plantas de semilla, la fase gametofítica haploide se halla muy reducida y completamente dependiente de la fase esporofítica. El análisis de la determinación del sexo en plantas de semilla es por lo tanto muy complicado debido a la interferencia del esporofito en la expresión del genoma del gametofito. Goldberg et al. (1993) sugirieron que hay unos 25000 genes expresados en la antera de tabaco en el momento en que el núcleo de la microspora empieza a dividirse, de los cuales unos 10.000 son específicos de la antera. Por lo tanto, es muy difícil dilucidar qué genes son genes gametofíticos cuyos productos están implicados en la determinación del género y cuáles son esporofíticos cuyos productos afectan a los genes gametofíticos o a sus productos.

Durante la última centuria, numerosos organismos verdes desde algas hasta angiospermas han sido propuestos como sistemas experimentales para llevar a cabo estudios en campos tales como la fisiología, bioquímica, genética o biología del desarrollo (Pryer et al. 2002). Entre las características que hacen candidatos adecuados para estos propósitos están tiempo de generación corto, tamaño pequeño, abundante descendencia, capacidad de cruzamiento y relativa facilidad de manipulación en condiciones de laboratorio, invernadero o campo. La fase haploide del helecho ha mostrado algunas importantes ventajas para su uso en estudios biológicos tales como fácil manipulación *in vitro*, buena disponibilidad de material, fácil de observar al microscopio, poca complejidad de desarrollo comparado con otros eucariotas fotosintéticos, haciendo todo ello del gametofito un material adecuado para estudios sobre el desarrollo sexual como el propuesto aquí. Durante las últimas décadas, hemos acumulado en nuestro laboratorio un cierto grado de conocimiento sobre cómo proceder en el cultivo *in vitro* de estas plantas para diferentes propósitos (Fernández y Revilla 2003).

Aparte de las características anteriormente descritas, se tienen o se deben tener en cuenta los siguientes criterios a la hora de elegir una especie como modelo de estudio: la posición filogenética y el tamaño del genoma. De los principales linajes vegetales, las angiospermas son las mejor caracterizadas hasta la fecha. Sin embargo, si los esfuerzos siguen concentrándose únicamente en estas plantas, escasamente se producirán avances en el conocimiento comparativo de la estructura y función del genoma vegetal y de su evolución. De hecho, se recomienda añadir a los estudios vigentes, al menos, dos representante de las principales líneas vegetales. Ello incluiría: 1) coniferas, cycales, Ginkgo y gnetofitos; 2) helechos y colas de caballo; 3) lycofitos; 4) musgos, hepáticas y líquenes; y 5) algas verdes (Pryer et al. 2002).

Como regla general, un genoma pequeño ha sido considerado una buena guía a la hora de decantarse por una especie. El ADN C-value es un índice que indica la cantidad de ADN presente en el genoma haploide, (expresado en pg) y

se usa como indicador de la idoneidad de la especie para llevar a cabo estudios moleculares. En el caso de *Arabidopsis* es uno de los más bajos, C=0.18, entre las angiospermas.

El helecho *Ceratopteris richardii* representa a día de hoy un sistema experimental válido para llevar a cabo estudios sobre el desarrollo vegetal (Hickok et al. 1995). En esta especie, la determinación sexual está epigenéticamente controlada por la feromona anteridiógeno, la cual es secretada por el gametofito hermafrodita y dirige el desarrollo sexual masculino de un gametofito joven aún no determinado sexualmente. La separación de machos y hembras en esta especie ha contribuido grandemente a analizar los genes que controlan la masculinidad y femeneidad bajo la acción del anteridógeno (Banks 1999).

1.5. Reproducción sexual en Pteridophyta

En las plantas sin semillas como helechos, licopodios y colas de caballo predomina la homosporia, con la formación de un único tipo de espora, que formará un gametofito haploide. Cuando la formación de nuevos individuos ocurre por mecanismos sexuales, es necesario la formación de los órganos sexuales masculinos o anteridios y femeninos o arquegonios, en el gametofito. En helechos se describen dos sistemas de apareamiento: intergametofítico e intragametofítico (Klekowski 1969). El apareamiento intergametofítico es la fusión de gametos procedentes de dos gametofitos distintos, los cuales pueden haberse originado a partir del mismo esporofito o bien de esporofitos diferentes. El apareamiento intragametofítico es la fusión de gametos producidos por el mismo gametofito, y conduce, obligatoriamente, a la formación de un individuo homozigoto.

Si bien hay una tendencia inicial en la mayoría de los helechos homosporeos a experimentar apareamiento intragametofítico, hay

adaptaciones que incrementan o disminuyen dicha probabilidad. Según Klekowski (1969) estas adaptaciones pueden ser morfológicas, poblacionales y genéticas. Entre las primeras estaría la secuencia en la que se forman los gametangios. En este sentido, la formación inicial de anteridios seguida de una fase hermafrodítica prolongada sería una adaptación para el apareamiento intragametofítico y, posiblemente, la más frecuente en estas plantas. Sin embargo, las posibilidades de apareamiento intergametofítico aumentan con una secuencia ontogénica donde se formarían inicialmente arquegonios, seguido de una fase hermafrodítica, como es el caso de muchas especies estudiadas de la familia Blechnaceae (Klekowski 1969), entre otras.

En todos los helechos homospóreos, la labilidad en la expresión sexual se considera la regla, (Korpelainen 1998). Un ejemplo típico de cómo el sexo es determinado por el ambiente lo encontramos en la acción ejercida por los anteridiógenos, los cuales promueven el apareamiento intergametofítico y aseguran el mantenimiento de la variabilidad genética. La determinación sexual en estas plantas consiste en cómo el sexo del gametofito es determinado independientemente del genotipo

1.5.1. *Bases fisiológicas de la reproducción sexual en Pteridophyta*

Entre los factores más importantes que controlan la diferenciación sexual en el gametofito del helecho se encuentran los anteridiógenos, también llamados feromonas. Se trata de sustancias endógenas secretadas al medio de cultivo por gametofitos que han alcanzado un cierto grado de desarrollo, tanto hermafroditas como femeninos y que inducen la formación de los órganos sexuales masculinos en gametofitos con un menor grado de desarrollo (Yamane 1998). Estos compuestos anteridiógenos son los responsables de que se produzcan muchos gametofitos masculinos alrededor de un pequeño número de gametofitos femeninos, minimizando el apareamiento intragametofítico y favoreciendo el intergametofítico (Takeno y Furuya 1987).

Estas feromonas han sido identificadas o sugeridas en muchos helechos, incluyendo las familias *Aspidiaceae, Blechnaceae, Parkeriaceae, Pteridaceae,* and *Schizaeaceae* (o Anemiaceae and Lygodiaceae) (Miller 1968; Näf et al. 1975; Tanurdzic y Banks 2004). Los anteridiógenos se agrupan en tres categorías, según su grado de especificidad (Kazmierczak 2006). (I) Anteridiógenos altamente específicos, que sólo se activan en gametofitos de su propia especie, ej. *Ceratopteris richardii*, *C. angustifolium*, *C. thalictroides, Lygodium japonicum*, and *Onoclea sensibilis*, (II) Anteridiógenos con doble especificidad, que son activos en su propia especie y en otra especie, escrita entre paréntesis. Ej. *Dryopteris filix-mas (Pteridium aquilinum*), *Blechnum gibbun (Onoclea sensibilis*), y *Anemia phyllitidis (L. japonicum*), (III) Anteridiógenos de *P.aquilinum* (A_{Pt}) and Pteris vittata (A_{Ps}), que son poliespecíficos ya que inducen anteridiogénesis en la menos 38 especies de helechos (Chiou y Farrar 1997), ej. *C. angustifolium, C. phyllitidis, Lepisorus thunbergianus, Microgramma heterophylla, Phymatopsorus scolopendria, Polypodium pellucidum, O. sensibilis* y *Phlebodium aureum.*

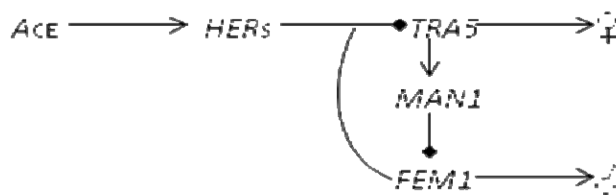
Los resultados obtenidos del empleo de biotests bien mediante el uso de inhibidores de la síntesis de giberelinas como AMO-1618 (Naf et al. 1975; Rhagavan 1989; Yamane 1998; Tanurdzic y Banks 2004), de TLC (cromatografía en capa fina), HPLC (cromatografía líquida de alta resolución) y de la espectrometría de masas (Nakanishi et al. 1971; Yamane 1998), revelan que todos los anteridiógenos caracterizados hasta la fecha, -fundamentalmente en helechos de la familia Schizaeaceae-, tienen una estructura química relacionada con el grupo de las giberelinas. Por otra parte, en los gametofitos de *A. phyllitidis,* la anteridiogénesis puede ser inducida por las principales giberelinas descritas en plantas superiores de acuerdo al siguiente orden de actividad: GA7=GA4>GA1>GA3>GA9 (Schraudolf 1964). En gametofitos de esta misma especie, se ha publicado recientemente que las giberelinas y/o los anteridiógenos y el etileno podrían cooperar en la inducción de la anteridiogénesis (Kažmierczak 2010).

El ácido anterídico (A_{An}) producido por los gametofitos de *A. phyllitidis* fue el primer compuesto química y biosintéticamente caracterizado y sintetizado (Nakanishi et al. 1971; Corey y Myers 1985; Corey et al. 1986; Yamauchi et al. 1997). El hecho de que la GA_7 se pudiera transformar en ácido anterídico dejaba entrever que estas sustancias poseían la configuración química de las giberelinas (Furber y Mander 1987). El ácido anterídico también estaba presente como el principal anteridiógeno en otros miembros del género *Anemia* como *A. hirsuta* (Zano et al. 1972), *A. rotundifolia* y *A. flexuosa* (Yamane et al. 1987). Otros compuestos como 3α-hydroxi-9,15-cycle-GA_9 (GA_{107}) y 3-epi-GA_{63} identificados como anteridiógenos menores en *A. phyllitidis* (Yamauchi et al. 1991; 1995). Los gametofitos de *A. mexicana* producen GA_{104} como principal anteridiógeno mientras que los de las especies *L. japonicum*, *L. circinnatum* y *L. flexuosum* también contienen metil éster de GA_9 y de metil éster de GA_{73} (Yamauchi et al. 1997). La caracterización parcial del anteridiógeno de *Ceratopteris* indica que se trata de una pequeña molécula hidrofóbica con una masa molecular de 300D (Koitabashi 1996), similar a la de otras GAs.

1.5.2. *Bases moleculares del desarrollo sexual en Pteridophyta*

A pesar de los esfuerzos realizados en diferentes especies, se puede decir que hay relativamente poca información acerca de las bases moleculares de la determinación sexual. Hasta la fecha, se han seguido dos estrategias para intentar aislar los genes responsables de dicho proceso en plantas: una sería el empleo de homólogos de genes que se sabe están implicados en desarrollo floral en plantas modelo como *Arabidopsis* o *Anthirrhinum*, y, en segundo lugar, usando estrategias de clonación implicando enriquecimiento de secuencias cromosómicas sexuales o enriquecimiento de transcritos ligados al sexo (Ainsworth, 2000). En realidad, estamos empezando a comprender cómo estos genes actúan y cómo tiene lugar el desarrollo de los órganos sexuales en diferentes grupos de plantas. Los estudios comparativos proporcionarán en un futuro nuevos conocimientos en los mecanismos que actúan en el desarrollo de los órganos sexuales.

Durante las últimas décadas, se ha llevado a cabo un excelente trabajo de investigación en el gametofito de *Ceratopteris richardii*, para intentar identificar los genes implicados en la determinación sexual (Banks 1999). Mediante dicho estudio, se obtuvieron una serie de gametofitos mutantes que presentaban alteraciones sexuales, los cuales permitieron establecer un modelo simple sobre la ruta de la determinación sexual asociada a la señal del anteridiógeno, que actúan en esta especie. De acuerdo con este modelo, hay dos clases principales de genes implicados, uno que promueve la masculinidad (el gen FEMINIZATION O FEM 1) y otro que promovería la feminidad (los genes TRANSFORMER O TRA). Que se expresen unos u otros dependerá de la señal del anteridiógeno, la cual activa otro grupo de genes, los genes HER (Banks 1999). La activación por anteridiógeno provoca que los genes HER junto con FEM 1, repriman la masculinidad. En ausencia de anteridiógeno, los genes TRA no están reprimidos, FEM1 se reprime y el gametofito desarrolla líneas femeninas (Fig. 1.2).



GENOTIPO	ACTIVIDAD DE GENES DE DETERMINACIÓN SEXUAL	FENOTIPO
wt -ACE	HERS **TRA 5** **MAN1** FEM1	FEMALE
wt +ACE	**HERS** TRA 5 MAN1 **FEM1**	MALE
tra5 -ACE	HERS TRA 5 MAN1 **FEM1**	MALE
fem1 -ACE	HERS **tra 5** **MAN1** fem1	FEMALE
tra5 fem1 -ACE	HERS tra 5 MAN1 fem1	ASEXUAL

Figura 1.2. Modelo de determinación sexual en Ceratopteris (Banks, 1999). La presencia o ausencia de anteridiógeno (ACE) en el medio de cultivo, así como el genotipo del gametofito influyen sobre la actividad de los genes responsables de la determinación sexual en esta especie.

1.6. Reproducción asexual en Pteridophyta

Aparte del ciclo vital sexual típico en plantas vasculares donde se produce la alternancia de generaciones, cada una desarrollando un cuerpo multicelular, que son el gametofito y el esporofito, se pueden encontrar variaciones y algunas especies completan el ciclo vital asexualmente. Las variaciones mejor descritas incluyen la apomixis de las gramíneas y la apogamia obligada en helechos (Cordle et al. 2010).

La formación de esporofitos a partir de células somáticas, es decir, sin la intervención de los órganos sexuales se denomina apogamia. Con el término apogamia obligada se hace referencia al ciclo en el cual un esporofito es habitualmente producido de esta manera. Aproximadamente, el 10% de helechos y un porcentaje impreciso de plantas inferiores tienen ciclos de este tipo. Sin fertilización, debido a la ausencia de órganos sexuales, el esporofito se producirá a partir de células vegetativas del gametofito mediante el proceso apogámico (Smith 1979; White 1979). El esporofito apogámico posee el mismo número de cromosomas que el gametofito y ello es posible porque durante la esporogénesis se produce un mecanismo compensatorio (Klekowski 1979; Walker 1979). En el proceso apogámico no se requiere la presencia de agua para favorecer la fertilización y se considera una adaptación a ambientes secos (White 1979). De hecho, muchos helechos del género Cheilantes usan esta estrategia para sobrevivir en dichos habitats (Mickel 1979). La apogamia puede ser inducida impidiendo la fertilización en helechos que normalmente se reproducen sexualmente (Lang 1898) o bien cultivando los gametofitos en medio enriquecido en azucar (Whittier y Steeves 1962). En el helecho *Ceratopteris richardii* es posible inducir la apogamia en dichas condiciones y en un breve periodo de tiempo (Cordle et al. 2007). En medio inductivo ha sido publicado que el tiempo de inicio del proceso apogámico coincide con la formación del primer arquegonio, estableciendo una posible relación entre la formación del aquegonio y el inicio de dicho proceso (Cordle et al. 2010).

Mientras que la mayoría de plantas con flor se reproducen sexualmente para formar las semillas, unas 400 especies pertenecientes a 40 familias de angiospermas han desarrollado rutas que derivan en la formación de semillas sin fertilización, denominándose apomixis a dicho fenómeno (Nogler 1982). Los helechos, aún siendo plantas sin semilla, son embriofitas, con un gametofito accesible experimentalmente, que lo hace adecuado para comparar su ciclo con la apomixis en angiospermas, donde ha sido bastante estudiado (Ozias-Akins 2006; Tucker y Kultunov 2009). El origen del embrión asexual pueden ser: a) células diploides de tejidos adyacentes al embrión sexual (embriones adventicios), b) célula madre de la megaspora que falla al iniciar o completar la meiosis antes de la mitosis, originando un saco embrionario que contiene una célula tipo huevo que experimenta un proceso embrionario sin fertilización (diplosporia), y c) células somáticas próximas a la célula madre de la megaspora acaban formando embriones asexuales (aposporia). En todos estos procesos se formará un embrión asexual diploide genéticamente idéntico al progenitor dado que no hay ni fertilización ni recombinación meiótica.

La apogamia en helechos ha sido comparada con la apomixis diplosporea; la diferencia está entre la formación directa de un embrión asexual esporofítico en angiospermas y la formación de un gametofito independiente previo a la formación del esporofito apogámico, en helechos. En el musgo *Physcomitrella patens,* cuando el gen del musgo ortólogo del de *Arabidopsis* CURLYLEAFF(CLF)/MEA/SWINGER (SWG) está ausente, el musgo se vuelve apogámico (Okano et al. 2009).

1.7. Nuevos enfoques para el estudio de las bases moleculares de la determinación sexual y asexual en plantas

Durante los últimos años, ha tenido lugar una explosión de nuevas técnicas al servicio de la Biología celular y de la “Biómica. La Biómica incluiría la Genómica,

Transcriptómica, Proteómica, Metabolómica, así como el estudio de otros compuestos celulares (Chen 2008).

La Proteómica va ganando adeptos lentamente entre los investigadores de la biología vegetal (Rose et al. 2004; Carpentier et al. 2008; Jarrín-Novo et al. 2009). Por una parte, la proteómica da cuenta de la expresión de las moléculas que más directamente influyen en la bioquímica celular. Además, otra cuestión importante es la capacidad de aislar fracciones proteicas subcelulares y adquirir conocimiento sobre localización o función incluso de complejos proteicos aislados cuyos constituyentes peptídicos no pueden predecirse a partir de secuencias de DNA o de la abundancia del ARNm.

El término proteoma, quimera de proteína y genoma, se usó por vez primera en 1997 por Wilkins et al. El proteoma ha sido definido como el conjunto de proteínas, incluyendo las modificaciones post-transcripcionales encontradas en una célula, tejido u órgano. Dicho término se usa habitualmente para describir la unión entre la espectrometría de masas de alta resolución con algoritmos para identificar una proteína en base a la masa del péptido o ésta más el resultado de la información de la fragmentación (MS/MS) (Ahn et al. 2007).

Las estrategias a seguir en los análisis de proteómica son las siguientes. Después del aislamiento de las proteínas, se pueden analizar usando tanto la técnica basada en gel o bien libre de gel. En el primer caso, las proteínas se separan mediante electroforesis uni o bi dimensional, y, a continuación, los spots son aislados del gel y digeridos con tripsina. Los péptidos resultantes son analizados por espectrometría de masas y los datos obtenidos usados en la identificación de proteínas. En la segunda estrategia, las proteínas aisladas se digieren en una solución que contiene tripsina. En esta metodología, los péptidos resultantes son separados mediante cromatografía líquida y analizados

por espectrometría de masas. En cualquier caso, los datos obtenidos se usan para buscar en las bases de datos usando uno o más algoritmos bioinformáticos para identificar las proteínas (Fig. 1.3.).

La metodología basada en la digestión libre de gel está en alza. En la llamada tecnología de identificación de proteínas multidimensional (MudPIT), la proteína total se digiere en una solución y posteriormente se separa mediante 2D-LC. El sistema de cromatografía líquida puede acoplar con la fuente de iones del espectrómetro de masas, disminuyendo las pérdidas e incrementando la sensibilidad (Yates et al. 2009). Hasta la fecha, sólo un limitado número de trabajos sobre reproducción vegetal han sido publicados (Agrawal et al. 2008; Feng et al. 2009) pero se espera que esta tendencia cambie en el futuro.

Hasta la fecha, la mayoría de los estudios de proteómica se dividen en dos categorías básicas. En primer lugar, la **proteómica descriptiva** o perfiles de proteína de materiales biológicos con el objetivo de separar, secuenciar y catalogar el máximo número de proteínas. Una estrategia complementaria para reducir la complejidad de un extracto en concreto sería la obtención de proteomas subcelulares. Una segunda categoría se podría denominar **proteómica comparativa** o Expresión diferencial proteica, donde el objetivo es caracterizar diferencias entre diferentes poblaciones proteicas. En esta categoría, se pueden incluir proteínas de tejidos en diferente estado de desarrollo o bien estadios en la respuesta a estímulos externos. Otras áreas tales como **modificaciones posttranscripcionales, interactómica y proteinómica** se hallan en desarrollo.

En cualquier caso, los análisis proteómicos necesitan ser validados y comparados con aquellos obtenidos usando la transcriptómica, la biología celular y las aproximaciones clásicas fisiológicas y bioquímicas, especialmente cuando se trata de análisis a partir de plantas no modelo o con genomas

pobremente caracterizados. Como resultado de la combinación de las nuevas omics y de las técnicas bioquímicas y de biología celular básicas, cada vez se tiene más conocimiento acerca del proteoma en plantas.

La proteómica vegetal está comenzando a tener algunas contribuciones prácticas en campos como la biomedicina y la agronomía. Se espera que los estudios de proteómica en helechos ayuden a comprender los mecanismos implicados en reproducción vegetal y puedan contribuir a aumentar nuestro conocimiento tanto en cuestiones de carácter básico como aplicado.

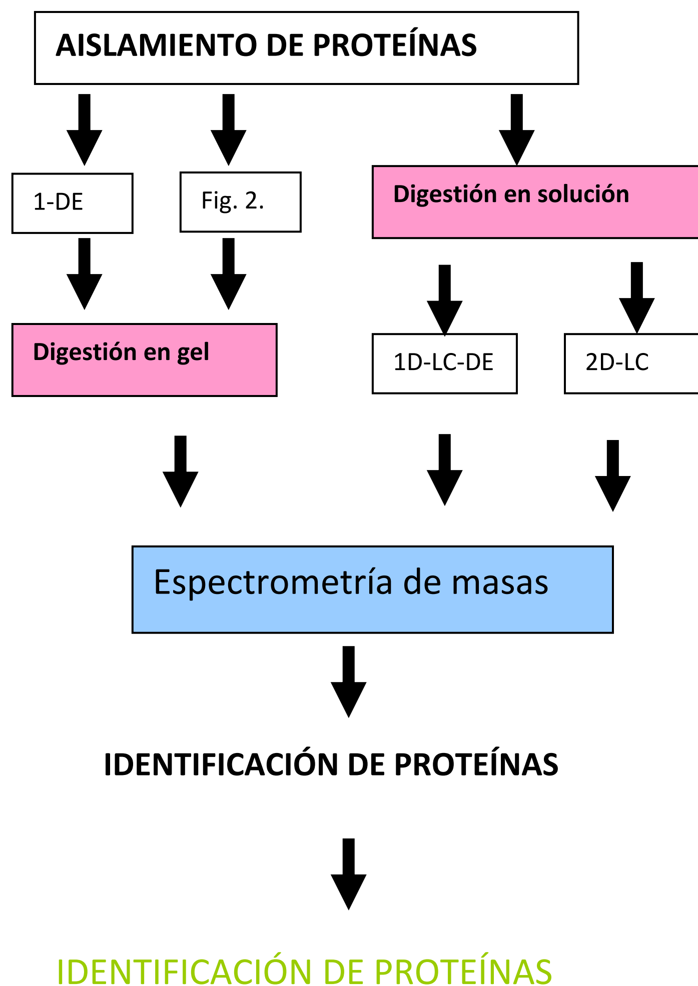


Fig. 1.3. Estrategias en los análisis de proteínas.

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OBJETIVOS

Objetivo general

En este trabajo se plantea estudiar la reproducción sexual y asexual en las especies *Blechnum spicant* y *Dryopteris affinis ssp. affinis*, para analizar el papel de los reguladores del desarrollo, así como la utilidad de nuevas herramientas como la proteómica para avanzar en el conocimiento de las bases moleculares.

Objetivos parciales

Para conseguir el objetivo general definido, se proponen a continuación una serie de objetivos parciales

1. Relacionar tanto la aplicación exógena como los niveles endógenos de reguladores del desarrollo, con el sexo del gametofito en *B. spicant* L.

2. Verificar la presencia de un sistema anteridiógeno en *B. spicant* L.

3. Aislar y purificar el/los compuesto/s anteridiógenos.

4. Relacionar tanto la aplicación exógena como los niveles endógenos de reguladores del desarrollo con la apogamia en *D. affinis ssp. affinis*.

5. Poner a punto las técnicas de la proteómica en el gametofito del helecho para encontrar genes implicados en reproducción.

CHAPTER 1- GROWTH AND GENDER IN THE GAMETOPHYTE OF BLECHNUM SPICANT L.

Abstract

The growth and gender in the gametophyte of Blechnum spicant L. were strongly affected by its origin, spore or mature homogenized gametophytes, and also by the addition of plant growth regulators to the culture medium. Spore-derived gametophytes cultured in Murashige and Skoog liquid medium were females and heart-shaped, whereas those derived from mature homogenized gametophytes were shorter and male or asexual (1:3) because of the release of antheridiogen to the culture medium. In the latter, maleness was especially increased by the addition of BA. This cytokinin is a strong inductor of maleness in homogenized cultures; however, even though BA influenced sexual organ formation in spore-derived gametophytes, it does not change the female sexual pattern that these gametophytes have. Separate male and female populations of gametophytes were obtained in this work.

Keywords:. Antheridiogen, Blechnum spicant L., Fern, Gametophyte, Tissue culture

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INTRODUCTION

The main economic value of ferns is as popular or-namental foliage plants. Blechnum spicant L. is an important plant because of its ornamental appeal. Traditionally, ferns are propagated by two methods: the sexual and the vegetative. The sexual method of propagation involves raising plants from spores. The use of spores to initiate cultures avoids contamination concomitant with the culture of explants taken from sporophytes grown in the field (Deberg 1994). However, propagation through spores requires knowledge about the biology of the gametophyte, which is formed after spore germination and represents the generation of the fern life cycle where sporophyte formation takes place either by sexual or asexual mechanisms (Fernández and Revilla 2003). Fernández et al. (1997) observed that when B. spicant gametophytes are cultured on solid medium, the archegoniate sexual condition persisted together with the absence of sporophytes. The failure in the development of antheridia and, consequently, sporophyte formation in the gametophytes of B. spicant cultured on MS solid medium, in addition to the diffusible nature associated with antheridiogen in other species, encouraged us to test new culture systems.

This work deals with the study of the effect of the origin of a gametophyte (spore or mature homoge-nized gametophytes) and the effect of growth regulators (auxins, cytokinins, and gibberellins) on growth and gender, in gametophytes of *B. spicant* cultured in a medium other than a solid medium. Gametophytes of *B. spicant* were cultured to induce abundant antheridia, which could contribute in increasing our knowledge on some basic aspects concerning its biology. The knowledge gained could allow us to understand better the antheridiogen activity and sexual determination in this species, which may have repercussions on sporophyte formation, which is interesting from a practical point of view.

MATERIALS AND METHODS

Culture of spores and mature homogenized gametophytes in MS liquid medium

Spores (20 mg) of *B. spicant* L. obtained from sporophytes growing in the forest of Turo´n (Asturias, Spain) were soaked in water for 2 h and then washed with a solution of NaClO (5 g/l) containing Tween 20 (0.1%, v/v) for 10 min. Then, they were rinsed thrice with sterile distilled water. Spores were centrifuged at 2,000 rpm for 3 min between rinses. Homogenized cultures from mature gametophytic tissue were grown according to Fernández et al. (1993). For this pur-pose, 0.3 g of mature female gametophytes, which have been maintained in culture for 2 years, were fragmented mechanically using an ultraturrax for 15 s under aseptic conditions. The sterilized spores and homogenized tissue were separately cultured in 250-ml Erlenmeyer flasks containing 50 ml of Murashige and Skoog (1962) liquid culture medium (MS) with concentrations of growth regulators as shown in Table 1. Media were supplemented with 2% sucrose (w/v) and 0.7% Difco Bacto-agar, pH adjusted to 5.7 with NaOH (1 or 0.1 N) unless otherwise noted. Cultures were maintained at 25 ºC under cool-white fluorescent light (40 µmol/m^2/s) with a 16-h photoperiod.

In addition, spores were cultured in 250-ml Erlenmeyer flasks containing 50 ml of MS medium supplemented with an aqueous extract (ca. 5 ml) of gametophytes after homogenization (0.3 g). The cultures were then placed on a gyratory shaker (75 rpm).

Treatment number	PGRs concentration (µM)
1	MS
2	MS + GA_{4+7} 1 mg/l
3	MS + GA_3 2.8 µM
4	MS + IAA 5.7 µM
5	MS + BA 4.4 µM
6	MS + GA_{4+7} 0.1 mg/l
7	MS + GA_3 0.28 µM
8	MS + IAA 0.57 µM
9	MS + BA 0.44 µM

Table 1. Treatments assayed for growing spore-derived gametophytes and homogenized gametophytes of Blechnum spicant L.

Microscopic observations

Morphological studies were conducted under an optical microscope (Nikon Eclipse E600), fresh gametophyte samples were taken and classified according to the morphology of the gametophyte: filamentous (one-dimensional growth), spatula (initial bi-dimensional growth), spatula–heart (apical notch in progress), and heart (apical notch and lobes well defined); growth in length and width; and gender including type (male, female, hermaphrodite, and asexual), frequency, and number of sexual organs (archegonia and antheridia).

Data collection and statistical analyses

One hundred gametophytes were observed in each treatment and the experiments were repeated twice. Deviation from normality and homogeneity of variance were tested with Shapiro–Wilk and Barlett–Box tests, respectively. For parametric data, an analysis of variance (ANOVA) with post-hoc Scheffe´ compari-sons was used. Nonparametric data (morphotype and gender) were analyzed by means of the Chi-square test of frequency of gametophytes showing a particular morphological or sexual condition. The level of significance was set at $\alpha = 0.05$ for all tests (Zar 1998)

RESULTS

Figure 1 shows the data of gametophyte morphology (filamentous, spatula, spatula–heart, and heart), growth in length and width, and gender of B. spicant gametophytes after 2 months of culture. The devel-opmental stage of spore-derived gametophytes (Fig. 1a) was mainly spatula–heart shaped (80%) when no growth regulators were used. The addition of growth regulators to the MS medium influenced the morphology of the gametophytes. The tested gibberellins showed contrasting effects: 1 mg/l of GA_{4+7} promoted meristem progress, enhancing the heart-shaped gametophytes ($\chi^2_{(100)} = 19.71$, $p < 0.001$),

whereas GA_3 delayed gametophyte development and the gametophytes remained as spatulas ($\chi^2_{(100)}$ = 54.95, p<0.001). BA (4.44 µM) inhibited gametophyte development and 60% of the gametophytes remained filamentous.

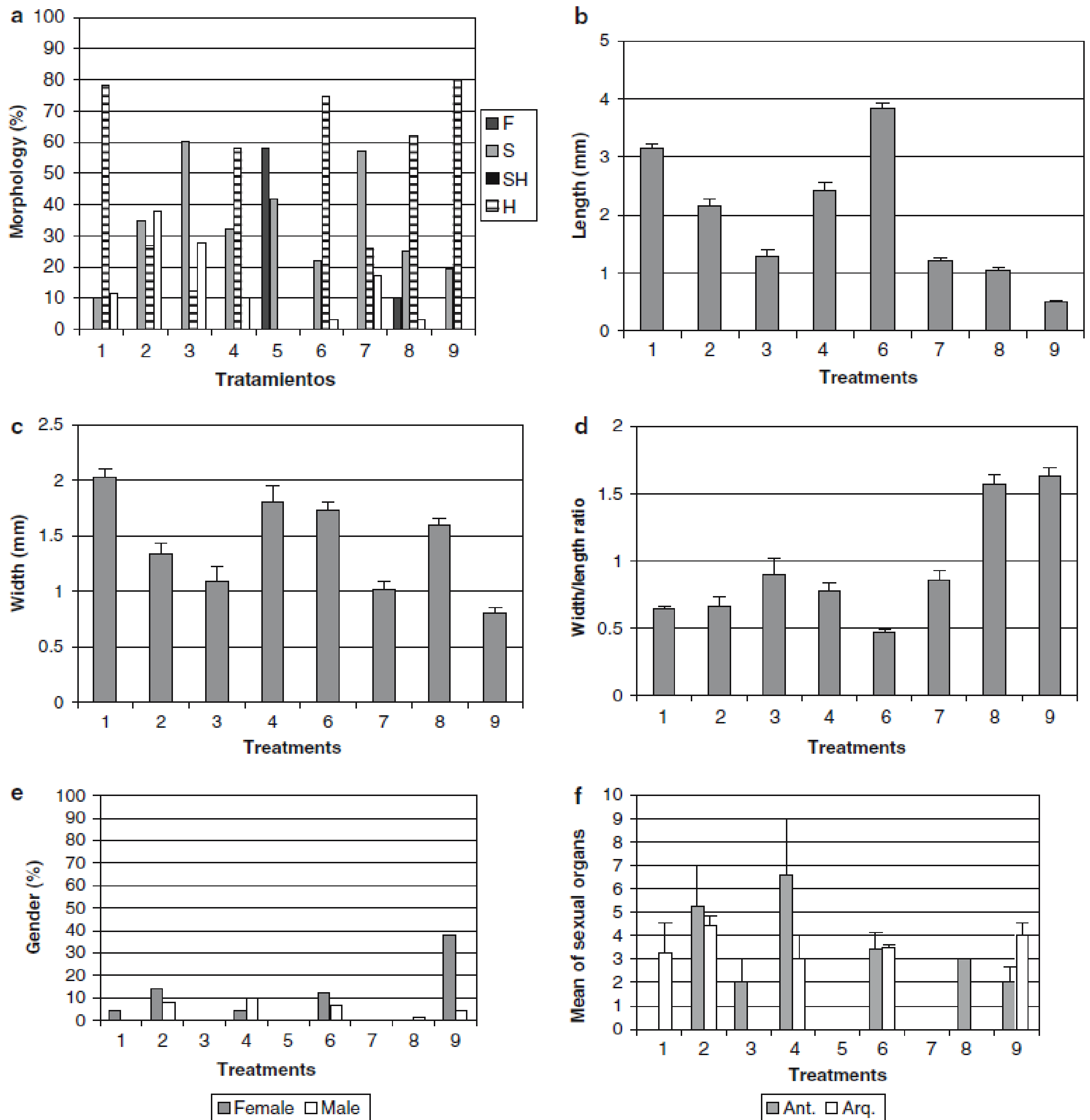

Fig. 1 Growth and gender in spore-derived gametophytes of *Blechnum spicant* L. cultured in MS medium supplemented with GA_{4+7},GA_3, IAA, or BA.and H = heart-shaped gametophytes. Data about length, width and sexual organs (Ant = antheridia and Arch = archegonia) were estimated in the spatula–heart-shaped gametophytes. Data after 60 days are shown as mean+SE. F = filamentous, S = spatula, SH = spatula–heart, and H= heart-shaped gametophytes.

The growth parameters of gametophytes (length and width) are shown in Fig. 1b, c. As the spatula– heart-shaped gametophytes were numerous in almost all the treatments assayed (except for those cultured with BA [4.44 µM]; treatment 5), the length and width were measured in all treatments except in treatment 5 (the data of treatment 5 is not included in Fig. 1b–d). Gametophyte elongation was promoted by GA_{4+7} (0.1 mg/l) (one-way ANOVA test, $F_{7,338} = 324$, $p<0.001$) and reduced by GA_3, IAA ($p< 0.001$), and BA ($p < 0.001$). Gametophyte width decreased with GA_3 (test, $F_{7,338}= 25.91$, $p< 0.001$), GA_{4+7} (1 mg/l) ($p< 0.01$), and BA ($p < 0.001$). Finally, the width/length ratio (Fig. 1d) increased with IAA (0.57 µM) ($F_{7,338}= 63.41$, $p< 0.0001$) and BA (0.44 µM) ($p< 0.0001$), the width being 1.5 times higher than the length.

Exogenous growth regulators influenced the development of sexual organs. The percentage of female gametophytes increased with the use of BA (0.44 µM) ($X^2_{(100)} = 37.58$, $p < 0.001$) and GA_{4+7} (1 mg/l) ($X^2_{(100)} = 4.92$, $p < 0.05$). The percentage of male gametophytes increased with the use of GA_{4+7} mg/l) ($X_{(100)} = 6.19$, $p < 0.05$) and IAA (5.7 lM) ($X^2_{(100)} = 6.19$, $p < 0.05$). The mean values of sexual organs did not show significant differences between treatments (Fig. 1f).

Culture of homogenized gametophytes in MS liquid medium

Gametophyte regeneration from homogenized cultures occurs in a few days (Fernández et al. 1993). Figure 2 shows the data of gametophyte morphology (filamentous, spatula, spatula–heart, and heart), growth in length and width, and gender of B. spicant gametophytes after 2 months of culture. A common feature observed in all the treatments assayed was a delay in the development of gametophytes with regard to spore cultures, as indicated by the predominant spatula shape of the gametophytes (Fig. 2a). Exogenous growth regulators influenced the gametophytic growth. That is, GA_{4+7} (1 mg/l) or GA3 (2.8 µM) inhibited gametophyte development as the filamentous gametophytes represented one-half of the morpho-types present in the culture ($\chi^2_{(100)} = 47.85$,

$p < 0.001$, and $\chi^2_{(100)} = 42.85$, $p < 0.001$, respectively). Also, the addition of BA (0.44 µM) signifi-cantly enhanced the spatula-shaped gametophytes ($\chi^2_{(100)} = 11.75$, $p < 0.001$). An opposite effect on growth was induced by the IAA (5.7 lM), which promoted gametophyte development by reducing the number of filament-shaped gametophytes ($\chi^2_{(100)} = 10.53$, $p<0.01$) and increasing the presence of both spatula–heart and heart-shaped gametophytes at the lowest concentration ($\chi^2_{(100)}= 4.20$, $p<0.05$, and $\chi^2_{(100)} = 19.78$, $p < 0.001$, respectively).

The length and width of the gametophytes were measured only in the more frequent morphologies in these cultures: the spatula-shaped gametophytes. With regard to length, significant differences among treatments were observed (one-way ANOVA, $F_{8,456} = 28.72$, $p < 0.01$): increased by GA_3 (28 µM) ($p<0.001$) and decreased by the two concentrations of BA assayed ($p < 0.01$ for the highest and $p<0.001$ for the lowest) (Fig. 2b). Width expansion (Fig. 2c) was also affected by the exogenous growth regulators ($F_{8,456} = 10.65$): the width was increased by the lowest concentration of IAA ($p < 0.001$) and by both concentrations of BA ($p<0.01$ and $p<0.05$ for the highest and lowest concentrations, respectively). Finally, significant differences were observed in the width/length ratio ($F_{8,456} = 31.55$, $p < 0.01$), which increased in the presence of IAA and BA, being especially high with cytokinins ($p<0.001$) (Fig. 2d). The growth of gametophytes cultured in the presence of BA was similar in both width and length.

Antheridia were observed in all the treatments assayed, but archegonia were never observed (Fig. 2e). In the control, antheridia were present on spatula-and spatula–heart-shaped gametophytes. GA_{4+7}, at the highest dose, increased the percentage of male spat-ula-shaped gametophytes. The percentage of maleness in the culture with GA3 did not differ from the control, but the formation of antheridia was delayed with regard to the rest of the treatments (data not shown). BA induced maleness at the two tested

concentrations ($\chi^2_{(100)} = 71.22$, $p < 0.001$, and $\chi^2_{(100)}=32.34$, $p<0.001$, respectively) (Fig. 2f, g).

Finally, spore-derived gametophytes, cultured in a medium supplemented with an aqueous extract obtained during the homogenization process of mature gametophytes, formed antheridia actively, and of these, 95% were males.

Discussion

This work reports on the effect of cultural conditions such as the origin of gametophyte and the effect of the addition of growth regulators to the cultire medium on growth and gender in *B.spicant* L. gametophytes.

The differences in growth and sexual development observed between spore-derived gametophytes and those regenerated from mature gametophytes, when cultured without growth regulators, could be associated to the culture medium itself. In the homogenized cultures, both the delayed growth and the male sexual pattern observed in the gametophytes could be because of the protocol used to prepare this type of culture. The use of the type of culture prepared involves changes in the composition of the medium by the excretion of undefined compounds from the broken mature gametophytic tissue, including pheromone antheridiogens, which induce maleness in the youngest gametophytes (Döpp 1950; Näf 1956; Cousens 1979). Indeed, the induction of maleness in spore-derived gametophytes cultured in medium supplemented with an aqueous extract obtained during the homogenization process of mature gametophytes conclusively proves the epigenetic action on sexual development by some compounds present in the mature gametophyte of

B. spicant. In addition, it also indicates that spore-derived gametophytes cultured in liquid medium are sensitive to antheridiogens and respond to the induction factor when it is present in the culture medium.

In general, gametophytic growth was hardly enhanced by exogenous growth regulators when cultured spore-derived gametophytes was used, and it was more susceptible to increase in the gametophytes derived from mature homogenized gametophytes. With regard to gender, quantitative variations in maleness occurred in gametophytes from mature homogenized gametophytes, but qualitative changes (male by female or vice versa) in both types of gametophytes were minimal. In fact, the persistent maleness shown by gametophytes regenerated from mature homogenized gametophytes and the continued femaleness condition observed in the spore-derived gametophytes was not changed much by the inclusion of the exogenous growth regulators.

It can be inferred that GA_{4+7} favors growth in length in both types of gametophytes whereas GA_3 facilitated growth only in homogenized cultures, which may be because of the stimulation of gametophytic cell expansion induced by gibberellins (Kázmierczak 1998). GA_{4+7} affects sexual organ formation but does not determine the gender. However, GA_3 does not affect sexual development in this species, our results being in disagreement with those reported by Schraudolf (1962) and recently by Kázmierczak (1998, 2003), who found antheridia induction in the fern Ceratopteris by this compound. The most remarkable result obtained in the current work was the effect of the addition of the cytokinin BA to the culture medium. BA is a strong growth inhibitor in B. spicant gametophytes, irrespective of their origin being through spores or through homogenized mature gametophytes. Gametophytes cultured with BA were widened and lacked meristem. This cytokinin is a strong inductor of maleness in homogenized cultures and even though BA influenced sexual organ formation in spore-derived gametophytes, it does not change the female sexual pattern that these gametophytes have. Finally, IAA promoted growth and our

results are in agreement with those published by Miller (1968).

Gibberellic acid and cytokinins have been reported as important phytohormones responsible for expressing male or female sex in flowering plant species, and has been reported to be mediated by their influence on the plant growth rate (Khryanin 2002). In this regard, an enhanced maleness caused by gibberellin was always related to shoot elongation, whereas an enhanced femaleness brought about by cytokinin was related to growth inhibition (Khryanin 2002). From the results of the current work, it can be assumed that the effect of gibberellin GA_{4+7} and mainly cytokinin BA on gender is more likely to be linked to the presence of antheridiogen in the culture medium than the gametophytic growth rate. That is, BA induced maleness in gametophytes derived from mature homogenized gametophytes, which were cultured in a medium with antheridiogen, whereas it induced femaleness in cultures of spore-derived gametophytes, where antheridiogen is apparently absent, regardless of the gametophytic growth rate in both cases. It has been extensively reported that the same hormone may have a diametrically opposite effect on sex, depending on the species studied (Duran and Duran 1984, 1990). The current work contributes to the fact that the same hormone could have different effects in a process because of synergistic or antagonistic relationships with other compounds. Furthermore, it could also depend on the physiological status of the cultured explant.

In summary, this work shows the lability of both growth and sexual development observed in ancient vascular plants such as ferns, and provide us with an opportunity to apply the obtained protocols to grow populations of B. spicant gametophytes entirely composed of female or male individuals. These protocols represent simple experimental systems that help in studies on plant sex expression, and conclusively increase our knowledge on how the pheromone antheridiogens are involved in sex expression.

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CHAPTER 2- GIBBERELLINS AND ANTHERIDIOGEN ON SEX IN BLECNUM SPICANT L.

The role of gibberellins (GAs) in determining sex in the gametophyte of the fern *Blechnum spicant* L. was studied through (a) the effect of exogenous GA_{4+7} and GA_3 (b) quantitation of the endogenous levels of GA_1, GA_3, GA_4, GA_7, GA_9, and GA_{20} in male and female gametophytes, and (c) the effect of flurprimidol, a GAs biosynthesis inhibitor of the steps of oxidation of ent-kaureno to ent-kaurenoic acid. Our results show that GA_{4+7} had a slight effect of inducing either male or female sexual organs, antheridia and archegonia, respectively. The endogenous GAs content was not significantly different between sexes, but the GA_4, GA_7, and GA_{20} levels were raised above those of the other GAs in both sexes. Neither antheridiogen biosynthesis nor antheridia formation was inhibited by flurprimidol. Finally, antheridiogen activity was studied concerning: a) when do gametophtes begin to synthesise these compounds, b) when do gametophytes become sensitivity to its action, and c) to isolate and purify them.

Key words Antheridiogen, Blechnum spicant, Fern, Gibberellins, Sexual development.

***V. Menéndez, M.A. Revilla, P. Bernard, V. Gotor and H. Fernández. Plant Cell Reports (2006) 25: 1104-1110.**

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INTRODUCTION

The control of sex expression in plants is far less understood than that in animals. Traditionally, studies on sexual development have been conducted on species with unisexual flowers, monoecious species and dioecious ones, although dioecious plants are particularly well suited for a study of the development of sexual organs because all the processes involved in an induced program of genetic expression cannot interfere with the development of the other sex (Chailakhyan and Khryanin 1986).

The determinants of sexual phenotype in plants are diverse, ranging from sex chromosomes in *Marchantia polymorpha* and *Silene latifolia* to hormones in *Zea mays* and *Cucumis sativa* and pheromones in some homosporous ferns (Tanurdzic and Banks 2004). Where the determinants are pheromones, the sex in several species is determined epigenetically by the antheridiogen pheromones. Since their discovery by Döpp (1950) in the fern *Pteridium aquilinum*, antheridiogens have been identified and characterized for many species of leptosporangiate ferns (Näf 1979; Yamane 1998).

An antheridiogen system affecting the formation of the male sexual organs has been described in the gametophyte of the fern *Blechnum spicant* L. (Näf 1956; Voeller 1964; Cousens 1979; Fernández et al. 1997; 1999), and although the structure of the *Blechnum* antheridiogen is unknown, all other fern antheridiogens characterized to date are mostly novel gibberellins (Yamane 1998). Studies on monoecious and dioecious plants of exogenous applications and endogenous measurements of hormones provide us with numerous examples that GAs seem to be particularly important for male fertility (Chailakhyan and Khyryanin 1986; Huang et al. 2003).

The masculinizing role attributed to GAs and the fact that antheridiogens are chemically related to these hormones, encouraged us to study a possible involvement of GAs in the formation of both gametangia (antheridia and archegonia) and antheridiogen in *B. spicant*. We used recent findings made in our laboratory such as that of gametophytes entirely compounded by only one sexual category, male or female (H. Fernández, unpublished observations), when cultured, and we also used homogenate cultures obtained using mature gametophytes, which accumulate antheridiogen and promote antheridiogenesis. The experiments were conducted at three levels: (a) determining the effect of exogenous GAs on spore-derived gametophytes cultured in vitro, (b) quantitation of the endogenous contents of the active GAs and the precursor in male and female populations, and (c) determining the effect of flurprimidol, an inhibitor of the oxidation of ent-kaureno to ent-kaurenoic acid in antheridiogenesis in homogenate cultures. Finally, antheridiogen activity was studied in order to test: a) when do gametophtes begin to synthesise these compounds, b) when do gametophytes become sensitivity to its action, and c) to isolate and purify these compounds.

MATERIALS AND METHODS

Culture of spores and HG

Spores (5 mg) of *B. spicant L.* obtained from sporophytes growing in the forest of Turón (Asturias, Spain) were soaked in water for 2 h and then washed for 10 min with a solution of NaClO (0.5%) containing Tween 20 (0.1%). Then, they were rinsed three times with sterile distilled water. Spores were centrifuged at 2000 rpm for 3 min between rinses and cultured in 100-mlflasks containing 20ml of Murashige and Skoog medium (MS) supplemented with 2% sucrose (w/v) and 0.7% Difco-Bacto agar, pH adjusted to 5.7 with 1 or 0.1 N NaOH, and unless otherwise noted, cultures were maintained at 25 ºC under cool-white fluorescent light (40 µmolm^{-2}s^{-1}) with a 16-h photoperiod. Once germinated, gametophytes were transferred periodically to fresh medium. Homogenate

cultures from mature gametophytic tissue were performed following the procedure of Fernández et al. (1993). Mature female gametophytes (0.3g) that had been maintained in culture for 2 years were mechanically fragmented using an Ultraturrax for 15 s under aseptic conditions. The homogenized tissue was separately cultured in 250-ml Erlenmeyer flasks containing 50 ml of MS liquid medium supplemented with 2% sucrose (w/v), pH adjusted to 5.7. All liquid cultures were placed on a gyratory shaker (75 rpm).

Culture of gametophytes with GA_{4+7} or GA_3

Sterilizedsporeswerecultured in 250-ml Erlenmeyerflasks containing 50 ml of MS supplemented with 2% sucrose (w/v) and 0.1 and 1 mg/l GA_{4+7} or 0.28 and 2.8 µM GA_3, pH adjusted to 5.7.

Quantitation of gibberellins

The endogenous GA_1, GA_3, GA_4, GA_7, GA_9, and GA_{20} content was analyzed in female and male populations of *B. spicant* gametophytes. Female cultures were compounded by 2-year-old gametophytes, approximately 1 cm in length, with numerous archegonia on the cushion that had been maintained on MS solid medium. Male gametophytes were derived from HG grown in MS liquid medium supplemented with BA (0.44 µM).

Samples for quantitation of GAs (200 mg DW) were frozen in liquid nitrogen, lyophilized, and stored at −20 ºC until analysis. Plant material was homogenized in 15 ml of cold 80% methanol containing 30.000 dpm of tritiated GAs (GA_1, GA_4, GA_8, and GA_{20}) and 100 ng of deuterated GA_1, GA_3, GA_4, GA_7, GA_9, and GA_{20} and extracted overnight in darkness at 4 ºC. After filtration, the residue was re-extracted using 5 ml 80% methanol for 2 h and was refiltered. The extract was purified through a C18 Seppack cartridge, and the eluate was absorbed onto 0.5 g of Celite, purified using a column of silicic acid (SiO_2), and

fractionated using reverse-phase high performance liquid chromatography (HPLC) on a Kromasil 100 C18 (5 µm, 250 mm ×4.6 mm, Sharlab S.L). A mobile phase of methanol with a linear gradient of 10–73% inanaqueous solution of 1% acetic acid was used for 60 min with a flow rate of 1.5 ml min^{-1}. Forty-five fractions were collected each minute, and the fractions containing radioactive GAs were collected, pooled, evaporated to dryness, and methylated using ethereal diazomethane and trimethylsilylated using 50 µl *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% (v/v) trimethylchlorosilane for 30 min at 65 ºC. Samples were dried and dissolved in chloroform and injected into a 5% PhMeSiloxano (60 m ×250 um ×0.25 nominal) capillary column installed in a Hewlett Packard 6890 GC coupled to a Hewlett Packard 5973 mass detector. The injection and interface temperatures were 270 and 280 ºC, respectively. The column temperature was maintained at 60 ºC for 1 min, increased at 20 ºC min^{-1} to 200 ºC, and then increased at 4 ºC min^{-1} to 250 ºC. The endogenous GA levels were determined by measuring the abundance of ion pairs: 506/508 and 491/493 *m*/*z* for GA_1, 504/506 and 489/491 *m*/*z* for GA_3, 418/420 and 284/286 *m*/*z* for GA_4, 416/418 and 384/386 *m*/*z* for GA_7, 298/300 and 270/272 *m*/*z* for GA_9, and 418/420 and 375/377 for GA_{20}. The ratio of the integrated peak areas for the endogenous and deuterated ions was used to calculate the amount of endogenous hormones present.

Culture of HG with flurprimidol

DMSO was used as a solvent (final concentration 0.001% for 3.2 and 32 µM treatments and control). Flurprimidol was filter sterilized (0.2 µm) into autoclaved and cooled MS medium supplemented with 2% sucrose (w/v) in 250-ml Erlenmeyer flasks. Flurprimidol assays were performed with HG, according to the procedure of Fernández et al. (1993).

Culture of HG in WMS with AC or ABA

HG were washed freely three times with sterile water in a test tube over 2 h to remove the cellular compounds excreted during homogenization and then cultured in 50 ml of MS liquid medium with 2% sucrose (w/v) and supplemented with AC (0.1 or 1 g/l) or ABA (0.037, 0.37, and 3.7 µM) in 250-ml Erlenmeyer flasks placed on a gyratory shaker (75 rpm).

Antheridiogen production in gametophytes of different time in culture

Gametophytes of *B. spicant* cultured for 75 days, 6 months and two years in MS medium with 2% sucrose and 0.7% agar, were liophylized and stored at -20º C until analysed for testing antheridiogen production. For this purpose, 1 g of gametophytic tissue was triturated in 2 ml of water, filtered and finally the crude extract obtained was frozen until use.

To test the capacity of gametophytes, which have been maintained in culture for different periods of time, to synthesize antheridiogen, 15 days-old spatulated-shaped gametophytes (that had been growth in liquidMS medium) were transferred to 3 cm diam. Petri dishes containing MS medium with 2% sucrose and 0.7% agar and supplemented with the crude extracts (10% v/v) obtained previously and esterilized through filters. .Data of both growth and sexual organ formation in the gametophytes were scored after two months of culture.

Antheridiogen sensitivity

Gametophytes of *B.spicant* cultured for 15, 30, 45 and 60 days in MS medium with 2% sucrose and 0.7% agar were transferred to fresh MS medium supplemented with crude extract obtained from gametophytes which have been maintained for two years in culture. Data of both growth and sexual organ formation in the gametophytes were scored after 15 days of culture.

Partition of crude extract obtained from gametophytes

Female two-year-old gametophytes were taken for analyses and they were harvested and frozen in liquid nitrogen, lyophilized, and stored at -20 ºC until use. For antheridiogen extraction, 11 g dry weight was homogenized in 800 ml of cold 80% MeOH, and after filtration, the residue was re-extracted with 200 ml of 80% MeOH for 2 h and re-filtered, then, MeOH was evaporated under vacuum. The residue was dissolved in 200 mL of phosphate buffer (0.1 M; pH 7.8), pH was adjusted to 7 and extracted 3 times with 100 mL of EtOAc to give a neutral EtOAc fraction (NF). The aqueous phase (Aq) was adjusted to pH3 and extracted 3 times with 100 mL of EtOAc to give an acidic EtOAc phase (AF). Both NF, AF and the remaining Aq fraction were evaporated under vacuum until dryness.

Sep-Pak Cartridge Treatment

NF fraction was dissolved in 20 mL of MeOH:H_2O (40:60, v/v) and passed through a Sep-Pak C18 cartridge (Waters). The cartridge was washed with 5 mL of MeOH:H_2O (40:60, v/v) and eluted with 10 mL of MeOH. AF was dissolved in 20 mL of MeOH:H_2O (50:50, v/v) and passed through a Sep-Pak C18 cartridge (Waters). The cartridge was washed with 5 mL of MeOH:H_2O (50:50, v/v) and eluted with 10 mL MeOH:H_2O (80:20, v/v). Both eluates were named as AF1 and NF1. Then, a second elution was done with MeOH 100% and the eluates were named as AF2 and NF2. All eluates were aliquoted in 2 mL and evaporated to dryness under vacuum.

The First RP-HPLC

The Sep-Pak eluate was dissolved in 1 mL MeOH:H_2O (40:60, v/v) and filtered before injection. Then, it was fractionated using reverse-phase high performance liquid chromatography (HPLC) on a Kromasil 100 C18 (5 µm, 250 mmx4.6 mm, Sharlab S.L.). A mobile phase of MeOH in aqueous solution with

the following gradient was used: t=0 min (50% MeOH), t=30 min (100% MeOH), t=40 min (100% MeOH), t=50 min (0% MeOH). The flow rate was 0.5 mL/min. Following injection, fractions were collected every 5 min., and then tested for antheridiogen activity. The fractionation step was done nine times to acumulate antheridiogen enough to detect biological activity in the bioassay.

Antheridium-formation bioassay

One month-old gametophytes of *B. spicant* L. derived from spore were cultured on 10 mL solid MS medium containing a test sample in 3 cm diameter Petri dishes. Gametophytes were cultured for 30 days and then checked under a microscope to score antheridia formation.

Morphological studies

Observations of gametophytes were conducted using an optical microscope (Nikon Eclipse E600) and microphotographic equipment (DS Camera Control Unit DS-L1), taking samples of fresh gametophytes and scoring them for morphology [filamentous (one-dimensional growth), spatula-shaped (initial bidimensional growth), spatula-heart-shaped (meristem in progress), and heart-shaped (meristem and lobes well defined)], increase in length and width; and sex (male, female, and hermaphrodite).

Data collection and statistical analysis

A total of 100 gametophytes were observed for each treatment and experiments were repeated two times. Deviation from normality and homogeneity of variance were tested through the Shapiro–Wilk and Barlett–Box tests, respectively. For parametric data, an analysis of variance (ANOVA) was used, and post hoc comparisons of means were performed using the Scheffe's test. The frequency of morphology and gender of gametophytes were analyzed

using the chi-square test. The level of significance was set at α=0.05 for all tests.

RESULTS

Effect of GA_{4+7} and GA_3 on gender

The effect of the gibberellins GA_{4+7} and GA_3 added to the culture medium is shown in Fig. 1. GA_{4+7} at a dose of 1 mg/l favored a significant increase in the percentage of female gametophytes (χ^2=4.92, P<0.05) and at 0.1 mg/l a significant increase of the percentage of male gametophytes (χ^2=6.19, P<0.05). GA_3 had no effect on the development of sexual organs.

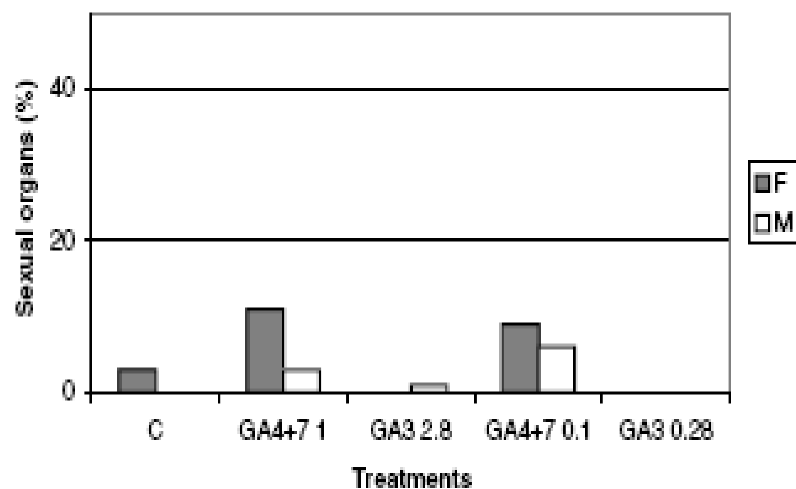

Fig.1. Effect of GA_3 (0.28 and 2.8 μM) and GA_{4+7} (0.1 and 1 mg/l) on sexual development in gametophytes of *Blechnum spicant* L. cultured in MS liquid medium with 2% sucrose

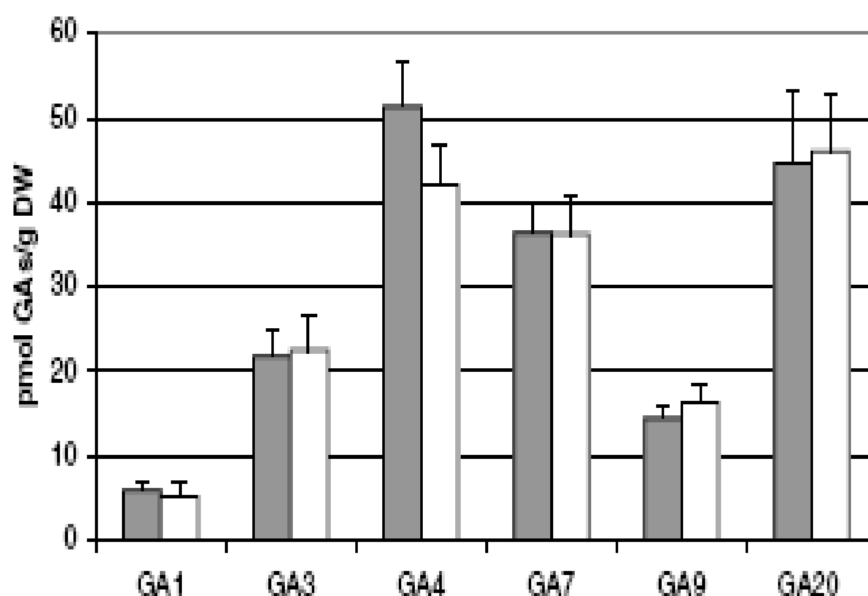

Fig. 2. Endogenous gibberellin content in *Blechnum spicant* L. gametophytes. Data are mean+SE.

Quantitation of GA_1, GA_3, GA_4, GA_7, GA_9, and GA_{20}

The endogenous contents of these gibberellins in male and female gametophytes are shown in Fig. 2. No differences were found between female and male populations of gametophytes. The lowest levels of GAs detected corresponded to GA_1, and the highest to GA_4, GA_7, and GA_{20}.

Effect of flurprimidol on HG

Flurprimidol affected both the growth and sex of *B. spicant* gametophytes (Fig. 3). This compound promoted an increase in gametophyte length ($F_{1,65}$ =44.95, P<0.0001) and width ($F_{1,65}$=144.58, P<0.0001) (Fig. 3a and b) and promoted the formation of antheridia (Fig. 3c) ($U_{6120,8415}$ =2465, P<0.0001). The gametophytes formed numerous lobes, and the apical notch was not defined (Fig. 3d).

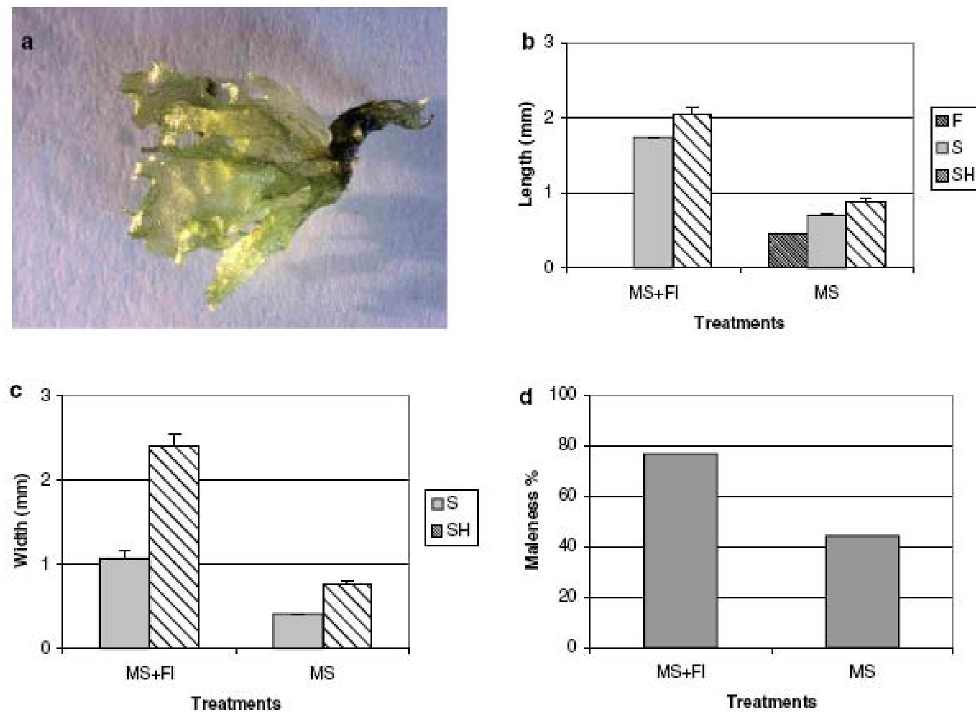

Fig. 3. Effect of Flurprimidol (F) on growth and gender of *Blechnum spicant* L. gametophytescultured in MS liquid medium with 2% sucrose (w/v) or MS liquid medium with 2% sucrose and and 3.2 µM flurprimidol (MS ±Fl). Data are mean ±SE. Data obtained after 2 months of culture. F, filamentous; S, spatula-shaped; SH, spatula-heart-shaped

Effect of WMS medium, AC and ABA on growth and sex in HG

The growth and gender of gametophytes derived from HG were influenced by washing of the culture medium (Fig. 4). When cultured in WMS medium, without the cellular compounds released during homogenization, these gametophytes completed their development and were mostly heart-shaped (Fig. 4a) (χ^2=85.71, P<0.0001), longer, wider (one-way ANOVA test $F_{1,134}$ =16.12, P<0.0001 and $F_{1,134}$ =62.72, P<0.0001, respectively) (Fig. 4b), and mostly males (χ^2 =69.79, P<0.0001) (Fig. 4c). In contrast, when they grew in a MS medium with cellular debris, the gametophytes were mostly spatula-shaped (χ^2=54.95,

P<0.0001). No archegonia were observed during the experiment in both culture conditions.

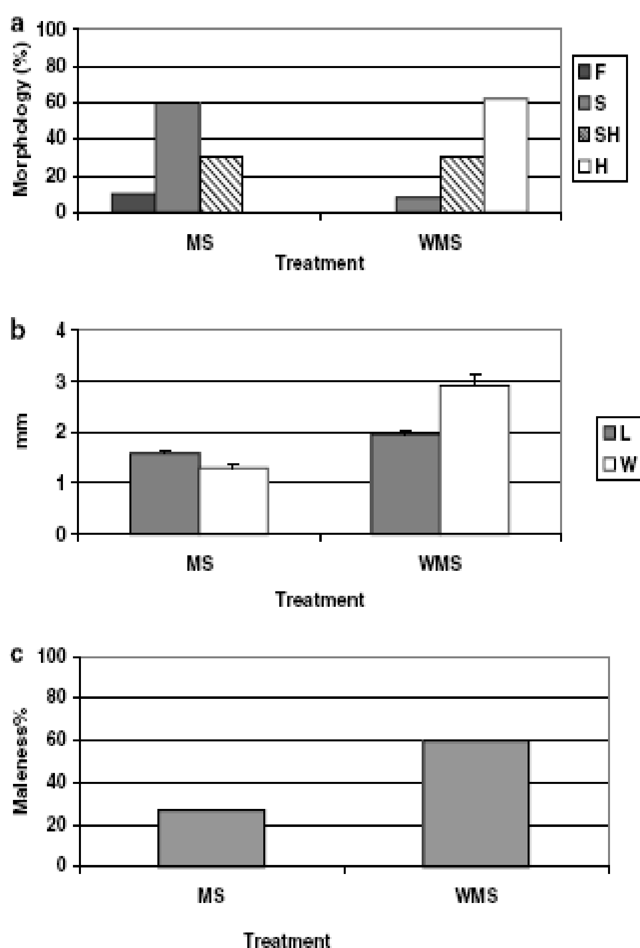

Fig. 4. Effect of washing for 2 h in homogenized mature gametophytes of *Blechnum spicant* L., before culture in MS liquid medium with 2% sucrose (w/v) on gametophyte shape (a), increase in length (*L*) and width (*W*) (b), and sexual development (c). Different letters mean significant differences at α=0.05. Data obtained after 2 months for MS medium and 1 month for WMS medium. F, filamentous; S, spatula-shaped; SH, spatula-heart-shaped; H, heart-shaped.

On the other hand, addition of AC or ABA influenced gametophyte development (Fig. 5). The formation of antheridia was significantly diminished by addition of active charcoal (χ^2=11.75, *P*<0.001), almost zero with the highest concentration tested. ABA favored the formation of antheridia at the highest concentration (χ^2=140.15, *P*<0.005) (Fig. 5b).

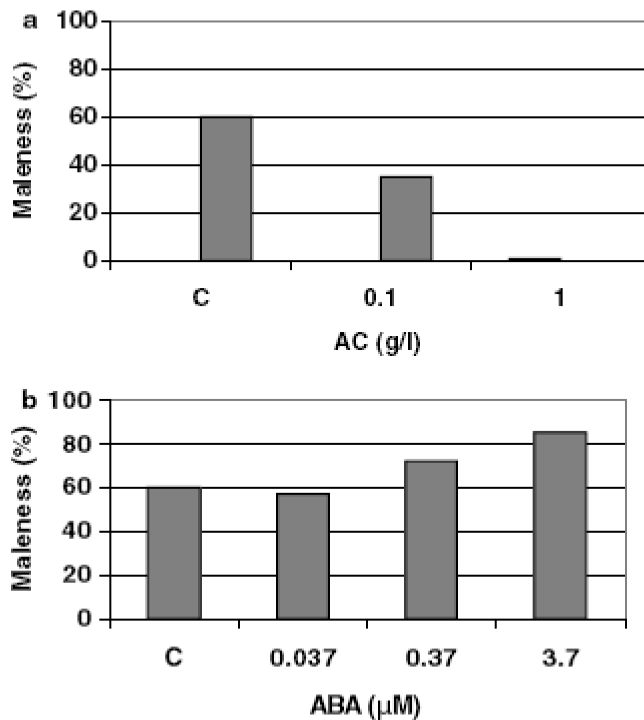

Fig. 5. Effect on sexual development of a) active charcoal (AC) at o.1 and 1 mg/l and b) exogenous supply fo ABA (0.035, 0,35, and 3.5 μM) in homogenized mature gametophytes of Blechnum spicant L. Data obtained after 2 months of culture.

Antheridiogen production in gametophytes of different time in culture

Data of both gametophyte development and sexual organ formation in gametophytes cultured on a medium supplemented with a crude extract obtained from gametophytes which have been maintained in culture for 75 days, 6 months and two years are shown in Fig. 6. No significant differences were found with regard the proportion of spatulated and heartead gametophytes (Fig. 6a). The addition of a crude extract from the studied gametophytes promoted antheridia formation (Fig. 6b).

Antheridiogen sensitivity

The results about the sensitivity to antheridiogen shown by gametophytes of 15, 30, 45 and 60 day-old (from the date of spore culture) are given in Fig. 7. Gametophytes 15 day-old are able to respond to the antheridiogen added to the

culture medium and exhibit high mortality. From this developmental stage, gametophytes showed the highest sensitivity to antheridiogen.

Purification of antheridiogen activity

Antheridiogen activity was checked along the different purification steps done to a crude extract (Fig. 8). In the control, the most part of gametophytes are asexual being male gametophytes almost absent. Antheridiogen activity significantly increased in the neutral and acid fraction either before and after passing through Sep-Pak cartridge.

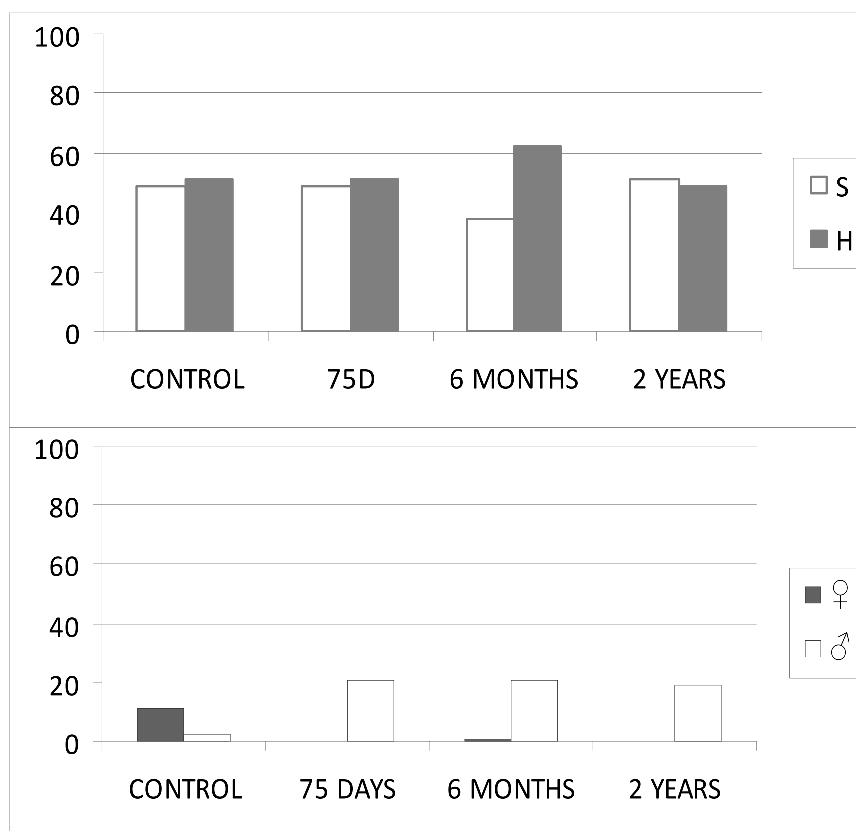

Fig. 6. Gametophyte development (a) and sexual organ formation (b) in gametophytes of *Blechnum spicant* L cultured on MS medium supplemented with a crude extract from gametophytes maintained in culture for different periods of time from spore inoculum.

	15 days	30 days	45 days	60 days	control
Antheridia development	+	++	++	++	-
Gametophyte mortality	+++	-	-	-	-
Gametophytic basal-proliferation	-	-	+	+++	+

Fig. 7. Sensitivity to antheridiogen in gametophytes of *B. spicant* L. of 15, 30, 45 and 60 days from spore inoculum, cultured on solid MS medium with 2% sucrose supplemented with crude extract obtained from gametophytes which have been maintained for two years in culture. Data obtained after 15 days of culture.

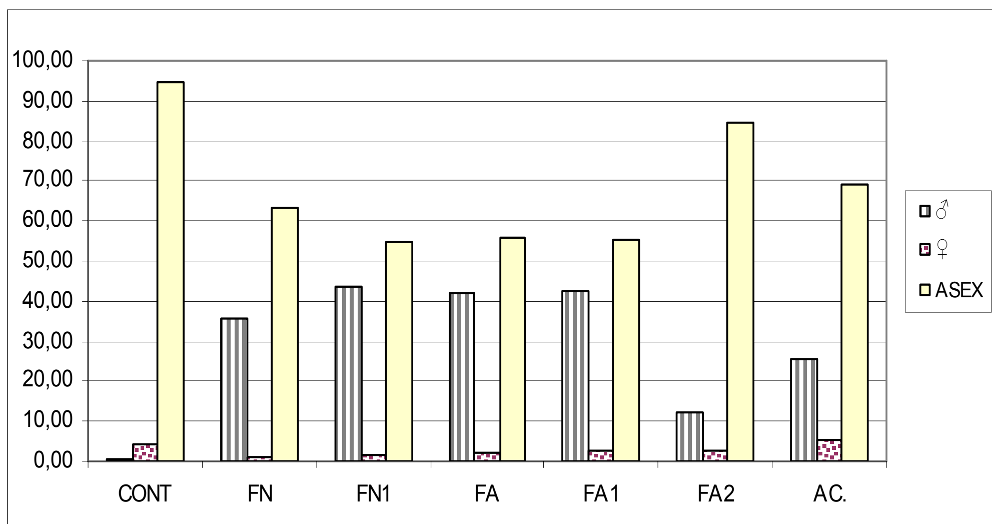

Fig. 8. Partition of a crude extract obtained from gametophytes of Blechnum *spicant* L. maintained for two years in culture from spore inoculum, with ethyl acetate . FN, netral fraction; FA, acidic fraction; AC, aqueous fraction; FA1, acid faction after Sep-pack purification; FA2, acidic fraction after washing Sep-Pack with 100% MeOH;FN1, neutral fraction after Sep-Pack purification.

Fractionation of FN1 fraction through HPLC revealed that antherdiogen activity is mostly retained in F2 (Fig. 9).

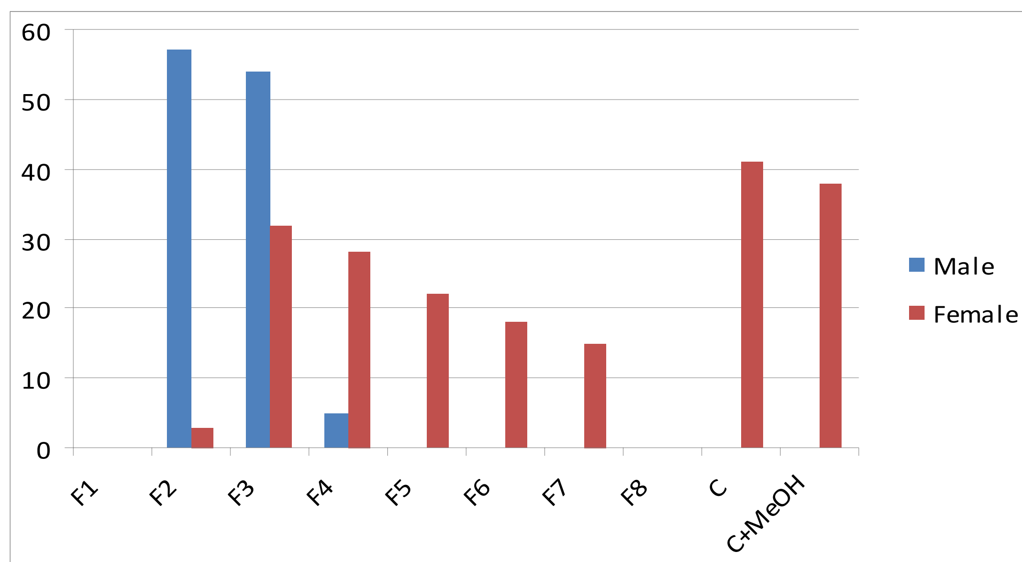

Fig. 9. Fractionation through HPLC of the neutral fraction (FN) obtained by partition with ethyl acetate of a crude extract derived from gametophytes of *Blechnum spicant* L. maintained for two years in culture.

Discussion

In this work, the effect of exogenous GAs has been studied and their endogenous contents have been analyzed for the first time in the fern *B. spicant*. Unfortunately, the large numbers of experiments that have been conducted in an attempt to modify sexual expression in dioecious and monoecious plants have not always been followed by analyses of the endogenous levels of phytohormones (Chailakhyan and Khryanin 1986; Duran and Duran 1990). In particular, analyses of the endogenous levels of phytohormones in the gametophyte of ferns are scarce.

The experiment performed to study the effect of exogenous gibberellins

shows that GA_{4+7} slightly induced either antheridia or archegonia. In previous reports (Schraudolf 1966; Takeno and Furuya 1987; Sugai et al. 1987), authentic GA_4 and GA_7 have been found to lead to higher antheridium activity in prothallia of schizaeaceous ferns such as *Anemia and Lygodium* than did GA_9, GA_3, and GA_1, but GA_4 and GA_7 inhibited the development of archegonia. GA3 has been shown to induce the male sex and inhibit the female sex in angiosperms (Metzger 1995; Lebel-Hardenack and Grant 1997), algae (Kazmierczak et al. 1999), and ferns (Schraudolf 1962, 1966; Näf et al. 1975; Kazmierczak 1998, 2003), but exogenous application of this compound to the culture medium did not promote the development of sexual organs in *B. spicant.* The effect of exogenous GA_{4+7} observed in this species correlated with higher levels of GA_4 and GA_7 found in male and female gametophytes. In Pinaceae, the exogenous effect of GA_{4+7} inducing flowering was in agreement with high levels of GA_4 detected in male cone buds (Fernández et al. 2003). King et al. (2001) observed in *Lolium temulentum* high levels of GA_1 and GA_4 along the floral development. The low levels of GA_1 and GA_9 detected indicate that these GAs are not directly involved in sexual organ development in *B. spicant.* However, Evans and Poething (1995) established the influence of GA_1, GA_3, and GA_5 floral development by means of analyses done in maize mutants and Moritz et al. (1989) reported that shoots from physiological mature, flowering trees of *Picea sitchensis* contained mainly GA1 and GA_3 while young plants contained predominantly GA_4 and GA_9. On the other hand, Hisamatsu et al. (2000) have verified that GA9 is not an active gibberellin per se neither in vegetative growth nor in flower promotion in *Matthiola incana* when applying this gibberellin exogenously. Finally, to give a broad explanation about the role of phyto-hormones on a biological process such as sex determination or flowering we must take into account that sometimes the same hormone often exhibits opposite effects, depending on the systematic group of the experimental plants (Duran and Duran 1990).

Overall, it can be suggested that the lack of differences in the endogenous content of GAs observed between sexes in *B. spicant* means that

these hormones could contribute to the development of both types of sexual organs, but not to the development of a particular organ in *B. spicant*.

Another way of analyzing the role of GAs in sexual development is through homogenate cultures using mature gametophytes. These gametophytes produce antheridiogens, which induce the formation of antheridia in regenerated gametophytes. The increased number of antheridia observed in the gametophytes treated with the lowest concentration of the inhibitor flurprimidol does not support a direct effect of gibberellins on antheridiogenesis. The induction of antheridia by flurprimidol observed in gametophytes means that antheridiogen biosynthesis and antheridia formation are not inhibited when the oxidation steps in the biosynthetic pathway of GAs are blocked. Warne and Hickok (1989) find that the compounds AMO 1618 and ancymidol, which inhibit the initial cyclization of geranylgeranyldiphosphate and the oxidation steps from ent-kaureno to ent-kaurenoic acid, inhibit the formation of antheridia in *Ceratopteris richardii*, but not completely. These authors also observe that addition of GAs to the culture medium or ent-kaurenoic acid has no effect on the formation of antheridia, suggesting that biosynthesis of *Ceratopteris* antheridiogen occurs via a different pathway that may include steps in common with GAs biosynthesis. Concerning growth, the effect of flurprimidol stimulating width of gametophyte, in particular, agrees with that reported by Kende and Zeevaart (1997) who said that GAs inhibit lateral expansion.

Some novel features of the antheridiogen operating in *B. spicant* were discovered from the last experiment. Firstly, the finding ofinhibitionofmalenessinwashedhomogenate cultures supplemented with active charcoal proved the production and release of antheridiogen to the culture medium by the regenerated gametophytes, but the increase in the formation of antheridia observed in these gametophytes in the early developmental stages means that they produce antheridiogen before attaining maturity. In contrast,

spore-derived gametophytes with a similar age or maturity level neverformantheridiabutformarchegonia (Fernández et al. 1997). Our results are not in agreement with those of Banks et al. (1993), who report that antheridiogen in *Ceratopteris* is not released by the hermaphrodite until after it loses the competence to respond to its male-inducing effects, which corresponds to the initiation of the meristem. The presence of male well-developed cordate gametophytes forming numerous antheridia in MSW medium indicates that maleness is not necessarily associated with growth reduction, our results being in agreement with those of Näf (1956), who established that the biologically active factor was a natural metabolite specifically involved with the induction of an organ (antheridia), and the more recent results of Fernández et al. (1997). Furthermore, the accelerated growth noted in the gametophytes regenerated from mature gametophytes in WMS medium indicates the possibility that inhibitory compounds could be released from the broken tissue, which could be removed after washing. We suspected that this could be due to the release of ABA to the culture medium from the broken tissue during the homogenization process. ABA has been considered an antagonist of antheridiogen (Hickok 1983, 1985; Warne and Hickok 1991; Banks 1999). Our results demonstrate that the exogenous supply of ABA does not block the formation of antheridia in the gametophyte of *B. spicant*. Therefore, compounds other than abscisic acid and antheridiogens may be responsible for the inhibited growth observed in the homogenate cultures.

The mode of action of the antheridiogen activity detected in *B.spicant* as well as the effectiveness of the protocol assayed to isolate and purify these compounds is reported. Analysing the time required for gametophytes of *B. spicant* to produce antheridiogens, it was observed that crude extracts from gametophytes maintained a minimum of 75 days in culture from spore inoculum, induces antheridia formation in young sensitive gametophytes. No shorter period of time in culture were assayed so that we can not precise the minimum time for gametophytes to become producers of antheridiogens.

Certainly, the time necessary to start producing antheridiogens depends on the own developmetal rytm of each species (Näf 1958, 1960). The capacity to produce antheridiogens has been associated to the heart developmental stage of gametophytes, which is reached 8 days after inoculation of spores in *P. aquilinum*, or 4-6 weeks in *Anemia phyllitidis* or *Lygodium japonicum* (Näf 1958, 1960

A second feature of the mode of action of antheridiogens is the time when gametophytes become sensitive to them. In this work, gametophytes cultured for 15, 30, 45 and 60 days were checked and all of them showed antheridia. As the period of time in culture increase, and due to the high basal gametophyte proliferation, there is an assortment of gametophytes in different developmental stages, being probably the youngers that respond to the antheridiogen extract added to the medium. As the prothallium matures it becomes insensity to the antheridiogen (Döpp 1959). The loss of sensitivity is reported to make coincidence with the development of the apical notch or meristem (Banks et al. 1993). Concerning antheridiogens isolation, the activity after reverse-phase HPLC fractionation of FN1 was retained in the F2 fraction, which correspond to 60 % MeOH. Unfortunately, identification of these compounds was not possible.

In summary, it seems that GAs do not have a significant role in determining sex or in the biosynthesis of the antheridiogens in *B. spicant*; only GA_4 and GA_7 seem to have a small, but unclear role in male and female organ formation. On the other hand, gametophytes derived from homogenate cultures synthesize antheridiogen before they attained maturity, being males, and display at this point a physiological behavior different from that of spore-derived gametophytes of a similar age, which are females. Studies to obtain molecular markers associated with antheridiogen activity and consequently sex determination in this species are in progress.

Acknowledgments

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CHAPTER 3- THE EFFECT OF CYTOKININS ON GROWTH AND SEXUAL ORGAN DEVELOPMENT IN THE GAMETOPHYTE OF BLECHNUM SPICANT L.

In this study, we report the role of exogenous and endogenous cytokinins on growth and sexual organ development in the fern *Blechnum spicant* L. Spore derived gametophytes (SG) were cultured in full-strength Murashige and Skoog (1962) liquid medium supplemented with (a) 4.44 µM N^6-benzyladenine (BAP), (b) a crude extract from mature female gametophytes, and (c) 4.44 µM BAP in combination with the crude extract from mature gametophytes, respectively. Both BAP and the crude extract delayed the gametophyte development, and this effect was increased when they were added together. With respect to sexual organ development, BAP inhibited the sexual organ formation, while the crude extract favored antheridia formation; however, when added together, the percentage of antheridia decreased. The endogenous level of the cytokinins cis-zeatin (cZ), cis-zeatin-riboside (cZR), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR), isopentenyl adenine (iP), isopentenyl adenosine (iPR), isopentenyl-9-glucoside (iP9G), trans-zeatin (tZ), and trans-zeatin riboside (tZR) were analyzed in female and male gametophytes of B. spicant L. The endogenous levels of cytokinins tZ, cZ, DHZ, cZR, iP, and iPR were higher in female gametophytes than in male gametophytes, with the endogenous iP and iPR content being increased more than 300 and 400 times, respectively.

Keywords: Antheridiogen, Blechnum spicant, Cytokinin, Fern, Gametophyte, Sex.

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INTRODUCTION

The control of sex expression in plants has been mainly studied on species with unisexual flowers, either monoecious or dioecious, although dioecious plants are particularly appropriate because all the processes involved in an induced program of genetic expression cannot interfere with the development of the other sex. In line with it, the gametophyte of the ferns has been proposed as an interesting model for studying several processes related to the plant growth and development, particularly the mechanisms underlying sex determination (Banks 1994). The differentiation of plant sexual organs can be determined by chromosomes as it has been reported in *Marchantia polymorpha* and *Silene latifolia*, by the endogenous plant growth regulators (PGR) balance in *Zea mays* and *Cucumis sativa*, or by the epigenetic action of pheromones in some homosporous ferns (Tanurdzic and Banks 2004). In the last case, it was Do¨pp (1950) who observed that the culture medium of maturing prothallia of the bracken fern *Pteridium aquilinum* hastened the onset of antheridium formation in juvenile prothallia of *Dryopteris filix-mas* as well as *P. aquilinum*. Do¨pp concluded that the maturing prothallia produced a hormonal substance regu-lating antheridial formation, which was active in many species extending to over at least five families, and termed it as antheridiogens (Näf 1966). All the antheridiogens characterized so far are gibberellins-related compounds (Yamane 1998)

Blechnum spicant L. belongs to one of the most ancient groups of ferns. Sexual development in the gametophyte of this species is carried out through epigenetic action of the unknown antheridium-inducing substances. When cultured *in vitro*, initially the gametophyte develops female sexual organs or archegonia, and produces and excretes antheridiogens into the medium, which induce the formation of male sexual organs or antheridia in the youngest gametophytes, which are filamentous or spatulate-shaped (Klekowski 1969; Cousens 1979; Fernández et al. 1997).

The possible effects of plant growth regulators (auxins, cytokinins, and gibberellins) on the sexual organ development mediated by antheridiogen in *B. spicant* L. have been investigated in two types of gametophytes: sporederived gametophytes (SG) and gametophytes regenerated from homogenized mature gametophytes (HG) (Menéndez et al. 2006a). It had been observed that in the absence of exogenous growth regulators, SG became predominantly females, while HG were mainly asexuals or males. The maleness observed in HG might be associated with the release of antheridiogen from the mature gametophytes into the culture medium during homogenization (Meneéndez et al. 2006b). Of all the phytohormones tested (Menéndez et al. 2006a), only the cytokinin BAP strengthened the antheridiogen extract activity in the HG. However, SG cultured with BAP and without antheridiogen extract remained as females.

To examine whether the stimulatory effect of an antheridiogen-rich medium on male gametophyte production is amplified by BAP application, the SG were cultured in the presence of BAP, antheridiogens, and their combination, respectively. Apart from this preliminary study related to the effect of exogenous cytokinins on the sexual organ development, the endogenous levels of cytokinins in gametophytic cultures that were mostly male or female, were analyzed.

MATERIALS AND METHODS

Spores (5 mg) of *B. spicant* L. obtained from sporophytes growing in the forest of Turón (Asturias, Spain) were soaked in water for 2 h, and then sterilized for 10 min with a solution of NaClO (0.5%) containing Tween-20 (0.1%). Subsequently, they were rinsed thrice with sterile distilled water and centrifuged at 2,000 rpm for 3 min between the rinses, and cultured in 500 ml Erlenmeyer flasks containing 100 ml full-strength Murashige and Skoog (MS) (1962) liquid medium

supplemented with (a) 4.44 µM BAP, (b) a crude extract from 0.5 fresh weight mature female gametophytes, and (c) 4.44 µM BAP plus the crude extract, respectively. The pH was adjusted to 5.7 and, unless otherwise noted, the cultures were maintained at 25 ºC under cool-white fluorescent light (40 $\mu mol/m^{-2}s^{-1}$) with a 16 h photoperiod. The cultures were grown on a gyratory shaker (75 rpm).

The crude extract added to the culture medium was prepared as follows: 0.5 g of fresh gametophytes was homogenized with an Ultraturrax in 5 ml sterile distilled water for 10 s. Then, 1 h after the extraction, the liquid without the gametophytic tissue was recovered with a sterile Pasteur pipette and incorporated into a fresh MS medium.

The endogenous cytokinin content was analyzed in two types of cultures of B. spicant L. gametophytes, labeled A and B. Culture A was compounded by 2-year-old gametophytes, approximately 1 cm in length and mostly females, which were maintained on solid MS medium; culture B was compounded by 40-day-old gametophytes obtained from homogenized cultures of mature female gametophytes, and were mostly males (Table 1). Homogenized cultures from the mature gametophytic tissue were prepared according to the protocol by Fernández et al. (1993). For this purpose, mature female gametophytes (0.3 g) that had been maintained in culture for 2 years were mechanically fragmented using an Ultraturrax for 5 s under aseptic conditions. The homogenized tissue was washed thrice with sterile distilled water. Then, the gametophytic tissue was separately cultured in 250 ml Erlenmeyer flasks containing 50 ml of liquid MS medium supplemented with 2% sucrose (w/v).

Morphological studies were carried out under an optical microscope (Nikon Eclipse E600). Fresh gametophyte samples were taken and classified according to morphology of the gametophyte: filamentous (one-dimensional growth), spatulate (initial bidimensional growth), spatulate-heart (apical notch in progress), and heart (well-defined apical notch and lobes), and sexual

development (male, female, hermaphrodite, and asexual).

Sexual condition (%)	Types of cultures	
	A	B
Male	28	65
Female	60	10
Hermaphrodite	10	2
Asexual	2	23

Table 1 Percentage of sexual phenotypes in the two types of cultures of gametophytes of Blechnum spicant L. A. Gametophytes cultured on solid MS medium. B. Gametophytes regenerated from mature homogenized

Samples of the gametophytes (200 mg dry weight) were extracted overnight in 1.8 ml of Bieleski solvent (chloroform/methanol/formic acid/water—25:60:5:10, Bieleski 1964) at 4 ºC. Then, 10 pmol of deuterated cytokinins ([^{2}H5]tZ, [^{2}H5]tZR, [^{2}H5]DHZ, [^{2}H5]9RDHZ, [^{2}H6]iP, [^{2}H6]iPR; Old-ChemIm, Olomouc, Czech Republic) were added. The samples were centrifuged (20,000g, 20 min, 4 ºC), re-extracted for 1 h in 1 ml 80% MeOH, and re-centrifuged. Both the supernatant fractions were pooled and passed through a C18 SPE (500 mg) to remove the pigments. Organic solvents were evaporated under reduced pressure at 40 ºC, the pH of the water phase was adjusted to 7, and distilled water was added to a final volume of 20 ml. The sample was applied onto a preequilibrated DEAE-Sephadex A-25 (Amersham Pharmacia Uppsala, Sweden) (2 ml NH4HCO3 form) with a preequilibrated C18 SPE cartridge (BondElut.Varian, Hamburg, Germany) coupled underneath. The cartridges were rinsed with an additional 20 ml of distilled water. Then, the C18 cartridge was removed and eluted with 10 ml of 80% methanol to recover the free bases, ribosides, and 9-glucosides. These cytokinins were further purified using an immunoaffinity column according to the manufacturer's instructions (OldChemIm, Olomouc, Czech Republic). The cytokinins purified by this way were dissolved in 20% methanol and analyzed by micro liquid chromatography coupled to electrospray tandem mass spectrometry (LC-(ES+)-MS/MS). The LC

system used was a Kontron 325 pump, and the samples (30 µl) were injected using a Kontron 465 injector. The LC system was coupled to a Quattro II triple quadruple mass spectrometer (VG Micromass, Manchester, UK) equipped with an electrospray interface [(+)ES LC-MS/MS) and a Z-spray source (source temperature: 80 ºC, capillary voltage: 3.5 kV, cone voltage: 20 V]. The column was a Phenomenex Synergy Max-RP 80 A (10 cm 9 1mm, 4 µm). Cytokinins were eluted using a gradient from 20% MeOH in 0.01 M ammonium acetate to 90% MeOH for 2 min, held in those conditions for 3.5 min, and returned to the initial conditions in isocratic mode for 5 min (flow rate: 60 µl/min). Quantification was done using Multiple Reaction Monitoring of $[M + H]^+$ ion (dwell time: 0.1 s) and the appropriate product ion. All the data were processed by Masslynx software (VG Micromass, Manchester, UK).

One hundred gametophytes were collected for each treatment and the experiments were repeated twice. Statistical analyses of the data were carried out using SigmaStat v3.1 software. Nonparametric data (morphotype and sexual condition) were analyzed using the χ^2 test of frequency of gametophytes showing a particular morphological or sexual phenotype. The level of significance was set at a = 0.05 for all the tests.

Results

Spores of *B. spicant* germinated in all the media assayed in 1 week, and the gametophyte development occurred (Fig. 1a). Significant differences with respect to the gametophyte development were observed among the treatments χ^2 116:527, P \ 0.01). In the control med ium, initially the gametophytes were filamentous, then spatulate, and finally, grew up until they achieved a typical heart shape. On the other hand, those cultured with BAP or the crude extract

did not reach the typical heart shape and were mostly spatulated χ^2 54:192, P \ 0.001; χ^2 64:826, P \0.01), and no significant differences were observed between these two treatments (χ^2 0:294, = 0.588). The addition of both BAP and crude extract together into the culture medium increased the presence of the spatulate morphology in the gametophytes with respect to the control (χ^2 113:916, P \ 0.001). Significant dif ferences were also observed when comparing the percentage of spatulate gametophytes present in the MS medium with both the crude extract and BAP together with respect to the addition of BAP (χ^2 9:841, P = 0.002) or crude extract (χ^2 6:362, P = 0.012) separately.

In terms of sexual organ development, significant differences were observed among the treatments (Fig. 1b) ((χ^2 243:152, P \ 0.001). The SG cultured in liquid MS medium were mostly female; the addition of BAP diminished the archegonia formation significantly with respect to the control (χ^2 83:946, P \ 0.001), while the addition of the extract promoted maleness, either alone (v¼ 64:826, P \ 0.001) or with BAP ((χ^2 14:275, P \ 0.001), even though the percentage of antheridia significantly decreased when added together ((χ^2 81:106, P \ 0.001).

The endogenous levels of several cytokinins were analyzed by HPLC-MS/MS in cultures of gametophytes that were predominantly males or females. The coexistence of more than one sexual condition—male, female, hermaphrodite, and asexual—in the gametophytes cultured in either in vivo or in vitro conditions, makes it difficult to carry out this type of analyses in ferns. Nevertheless, we can obtain gametophytic cultures in which one type of sexual phenotype is predominant, by adequately handling the culture. The results of endogenous cytokinin concentration (pml/g dry weight) are shown in Fig. 2. Significant differences were found in the endogenous levels of trans-zeatin (tZ), cis-zeatin (cZ), dihydrozeatin (DHZ), cis-zeatin-riboside (cZR), isopentenyl

adenine (iP), and isopentenyl adenosine (iPR) in female and male gametophytes. The endogenous levels of these cytokinins were higher in female gametophytes than in males. The endogenous content of iP and iPR detected in the female gametophytes strongly increased—the level of iP and iPR was around 300 and 400 times higher, respectively, in female than in male gametophytes of *B. spicant*.

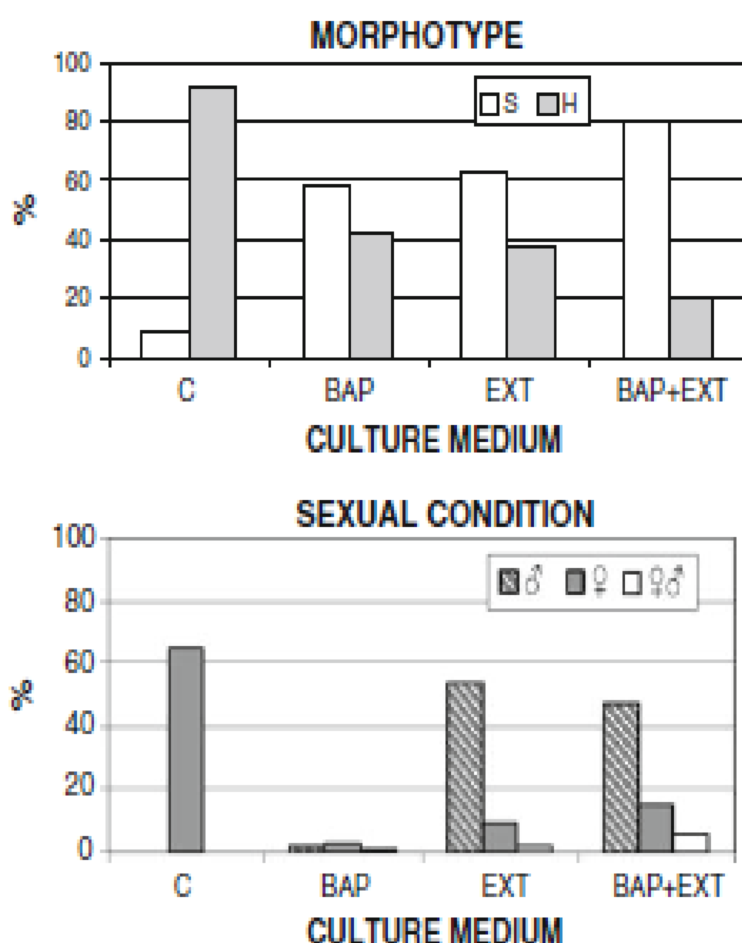

Fig. 1 Data of growth and gender in spore-derived gametophytes of Blechnum spicant L. cultured in liquid MS medium for 75 days. S = spatula, H = heart-shaped gametophytes, C =control medium, BAP = medium supplemented with 4.4 µM N^6-benzylaminopurine, EXT = medium with an extract from mature female gametophytes, antheridiogen-rich; BAP+EXT = medium with 4.4 µM BAP plus extract from mature female gametophytes

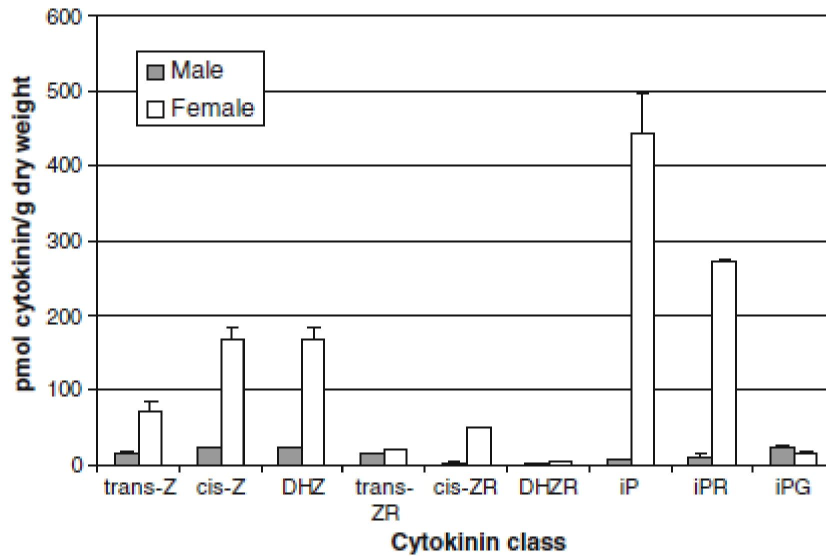

Fig. 2. Endogenous cytokinin 600 content in female and male gametophytes of B. spicant L. Female gametophytes were cultured in solid MS medium and male gametophytes were obtained from mature homogenized gametophytes cultured in liquid MS medium.

DISCUSSION

In this study, a possible role of cytokinins on both the growth and sexual organ development mediated by an antheridium-inducing hormonal substance, known as antheridiogen, in the gametophyte of the fern *B. spicant* is reported.

In an earlier work done in our laboratory, the effect of the exogenous plant growth regulators: indol-3-acetic acid (IAA), BAP, and the gibberellins, GA_3 and GA_{4+7}, on the growth and sexual organ development in the SG and HG of B. spicant was reported (Menéndez et al. 2006a). The results obtained revealed that HG cultured in a medium without exogenous plant growth regulators showed an inhibitory effect on their development, as well as a stimulatory effect

on the antheridia formation (presumably owing to the fact of being cultured in an antheridiogen-rich medium), which was enhanced by the addition of the cytokinin, BAP, to the culture medium. When comparing these results with those obtained with SG presented in this work, particularly, those referring to the BAP+crude extract treatment, the differences between them were obvious. In HG, the addition of BAP to the antheridiogenrich medium in which they were cultured (Menéndez et al. 2006b) enhanced the antheridia formation. On the contrary, in SG, the addition of BAP to the medium with or without crude extract (which also includes antheridiogens), did not enhance the antheridia formation, and moreover, the sexual organ development diminished or strongly diminished, respectively. Furthermore, converse effects were detected for the cytokinin, BAP, on the antheridiogen activity in each type of the gametophytes.

It has been extensively reported that the same plant growth regulator may have a diametrically opposite effect on sex, depending on the species studied (Duran and Duran 1990). Our study went further and demonstrated how the same plant growth regulator could have different effects on the sexual development in the gametophytes of different origins within the same species. The cytokinin, BAP, enhanced the positive effect of the antheridiogens on antheridia formation in HG and inhibited the sexual organ formation (mainly archegonia) in SG, or diminished the effect of antheridiogens on antheridia formation when these compounds (BAP and antheridiogens) present in the crude extract were added together to the culture medium. These findings also contribute to study on the lability of sex expression reported in the more ancient plant forms, such as mosses and ferns, when compared with many angiosperms (Korpelainen 1998).

The fact that exogenous cytokinins, such as BAP, affect the sexual organ development mediated by antheriogens in the gametophyte of B. spicant, inspired us to analyze the endogenous levels of several cytokinins in the

gametophytes of different sexes. The endogenous level of the cytokinins cZ, cZR, DHZ, dihydrozeatin riboside (DHZR), iP, iPR, isopentenyl-9-glucoside (iP9G), tZ, and transzeatin riboside (tZR) were analyzed, and the results revealed that the endogenous levels of these cytokinins were higher in female gametophytes than in the male gametophytes, with the endogenous iP and iPR content being increased more than 300 and 400 times, respectively.

The organogenesis of sexual organs can be considered as resulting from the expression of differentiation programs in which the plant growth regulators are considered to be involved. To determine the importance of plant growth regulators in sex expression, two different approaches have been employed: the exogenous administration of growth regulators to the developing plants and the quantitative analyses of endogenous phytohormones (Duran and Duran 1984; Chailkyan Kh and Khryanin 1987). In the light of these data, gibberellins and cytokinins have been reported as the major plant growth regulators responsible for expressing the male or female sex in the flowering plant species. In particular, the predominance of cytokinins favored female-sex expression and that of gibberellins contributed to male-sex expression (Khryanin 2002).

No differences in the endogenous content of the gibberellins GA_1, GA_3, GA_4, GA_7, GA_9, and GA_{20} were found between the sexes in the gametophytes of B. spicant (Menéndez et al. 2006b); however, differences in the endogenous content of the cytokinins mentioned earlier were observed. Not much is known about the influence of particular cytokinin metabolites on sex expression. In the dioecious species, Leuceadendron rubrum, a correlation between the increased content of the cytokinin, iP, and the development of female plants was reported (Dekock et al. 1994). In the most extensively studied system, Mercurialis, where a number of genes control the sex expression, specific cytokinins have been linked with sex differentiation in comparisons done between the wild-type male and female plants. The results showed that the nucleotide iP, although shared

by both the sexes, was accumulated in females. Besides, the zeatin-free base was in higher quantity in females, whereas the males lacked it (Duran and Duran 1990). Furthermore, a direct link between the feminizing alleles and the female-specific cytokinin metabolite, tZ, was also suggested. In males, the zeatin pathway stops at its immediate precursor, tZR, whereas the tZ mononucleotide gets accumulated (Yang et al. 1998). In addition, Yang et al. (1998) reported on a female-specific DNA and the existence of a female-specific enzyme capable of converting tZR into the specific feminizing cytokinin, tZ. More recently, studies on over-expressed zeatin O-glucosylation genes demonstrated the formation of tassel seed in the homozygous transformants in maize, revealing a link between cytokinins and sex-specific floral development in monocots (Pineda Rodo´ et al. 2008).

In summary, our data revealed that the effect of an exogenous plant growth regulator, such as BAP, may change depending on the origin of the plant material, in our case, the spores and homogenized mature gametophytes. It is possible to find either a synergistic or an antagonistic action among different growth substances as presented in this study for antheridiogen and cytokinins. Further research is required to understand the antagonistic effect of BAP on the antheridiogen activity in each type of gametophytes. However, analysis of the endogenous hormonal level would be a desirable starting point. On the other hand, the analyses of endogenous content of these compounds in different sexes revealed, for the first time, that the male and female individuals have significant differences in their endogenous cytokinin levels in this species, with female gametophytes being several time richer in tZ-and cZ-free bases, especially in iP and its riboside. The analyses of molecular markers expressed by the action of antheriodiogens and cytokinins are in progress in our experimental systems of *B. spicant* gametophytes.

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CHAPTER 4- EXOGENOUS AND ENDOGENOUS GROWTH REGULATORS ON APOGAMY IN DRYOPTERIS AFFINIS (LOWE) FRASER-JENKINS SSP. AFFINIS.

This work showed for the first time the relationship between the effect of exogenous auxins and gibberellins on apogamy in *Dryopteris affinis* (Lowe) Fraser-Jenkins ssp. *affinis* and its endogenous contents during early apogamic events. The addition of NAA (0.53 and 5.37 µM) or GA_3 2.8 µM to an MS solid medium significantly increased apogamous sporophyte formation. BA induced brown callus that regenerated sporophytes in a hormone free medium. The endogenous contents of GA_1, GA_3, GA_4, GA_7, GA_9 and IAA were determined by GC–MS in gametophytes cultured on MS solid medium, before and during early stages of apogamous embryo development. The accumulation of both GA_9 and IAA before embryo development was evident as high levels of GA_4 in the earliest analysed stage of embryo development and high levels of GA_3 in elongating shoots were found. The role of gibberellins on apogamy was also supported by data showing a decrease in the percentage of gametophytes developing embryos because of the addition of flurprimidol to the culture medium.

Keywords: Apogamy, *Dryopteris affinis sp. affinis,* Fern, Gametophyte

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Introduction

When the sporophyte originates from somatic gametophyte cells, i.e. without the intervention of sexual organs, it is termed apogamy. "Obligate apogamy" describes a cycle in which a sporophyte is regularly produced from gametophyte without sexual fusion. Approximately 10% of ferns and an unknown proportion of other Pteridophyta have life cycles of this kind (Sheffield and Bell 1987).

According to Salvo (1990), apogamous embryos are formed by proliferation of one or more vegetative cells near the apical notch and they parallel the normal embryo development. This lacks sufficient evidence based on existing histological continuity between gametophytic and sporophytic tissues in the apogamous ferns. However, in most of the reports on either obligate or induced apogamy, the authors always refer to the origin of apogamous sporophytes by means of expressions such as"bud development", "sporophytic outgrowths", etc. without explicit reference to an embryogenic process.

Since reported in tobacco pith callus by Skoog and Miller (1957), it has been well known that the promotion of organogenesis or embryogenesis in plant tissues is generally dependent on the addition of exogenous hormones to the culture medium. The induction or promotion of apogamy has been investigated by Whittier and colleagues emphasising the effect of factors such as carbohydrates (Whittier and Steeves 1960, 1962; Whittier 1964, 1965), osmotic conditions (Whittier 1975), and growth regulators such as ethylene (Elmore and Whittier 1975a,b), auxins, and cytokinins (Whittier 1966). However, to our knowledge, no study has tried to delve into the physiological, biochemical, and molecular events occurring during the formation of an apogamous embryo in the gametophyte.

Dryopteris affinis (Lowe) Fraser-Jenkins ssp. *affinis* is a diploid fern with

an apomictic life cycle and it probably originates from the crossing of *D. walichiana* and *D. oreades*. It is broadly distributed in the Mediterranean, Macaronesia, and western Eurosiberia regions (Salvo 1990). When cultured *in vitro*, apogamy in this species is evident as gametophyte develops a brown meristematic area near the apical indentation that evolves into a new sporophyte. Sexual reproduction is not possible due to the lack of archegonia (Fernández et al. 1996a).

The objectives of this investigation are (a) to determine the effect of exogenous growth regulators on apogamy in a species which is obligate apogamous and (b) to analyse possible variations in the endogenous hormone levels during embryo development. The wide range of GAs analysed included gibberellins that are ending products with biological activity according to the main biosynthetic pathway reported. Finally, gametophytes were cultured in a medium with flurprimidol, an inhibitor of GAs biosynthesis to let clear the possible role of GAs on apogamy.

MATERIALS AND METHODS

Culture of spores

Spores (20 mg) of *D. affinis* (Lowe) Fraser-Jenkins ssp. *affi-nis* were obtained from sporophytes growing in the forest of Tur´on (Asturias, Spain), soaked in water for 2 h, and then washed with a solution of NaClO (5 g/l) containing Tween 20 (0.1%) (v/v) for 10 min. Afterwards, they were rinsed three times with sterile distilled water. Spores were centrifuged at 2,000 rpm for 3 min between rinses. The sterilised spores were cultured in 100-ml flasks containing 20 ml of Murashige and Skoog (1962) culture (MS) medium supplemented with 2% sucrose (w/v) and solidified with 0.7% Difco Bacto-agar (w/v).

Culture of homogenised gametophytes on solid media

Homogenisation of the gametophytes was done from 4-month-old spore-derived gametophytes (0.3 g), which were fragmented mechanically using a Waring blender for 15 s under aseptic conditions (Fernández et al. 1993) and then cultured in 100-ml flasks containing 20 ml of the following media: full-strength MS medium with 2% or 4% sucrose (w/v), full-strength MS medium with 2% sucrose (w/v), and one of the following growth regulators: NAA (0.53, 5.37, 26.5 µM), GA_3 (0.28, 2.8, 14 µM) or BA (0.44, 4.44, 22 µM). The culture media were solidified with 0.7% Difco Bacto-agar. Unless otherwise stated, pH was adjusted at 5.7, and cultures were kept at 25 ºC under a 16-h photoperiod provided by white fluorescent light of 40 µmol m^{-2} s^{-1}. Five replicates were used for treatment and the experiment was repeated twice.

Culture of homogenised gametophytes in liquid medium with BA

Homogenised gametophytes were cultured in 250-ml Erlenmeyer flasks containing 50-ml MS medium supplemented with 2% sucrose (w/v) and BA of 4.44 µM. Cultures were placed on an orbital shaker at 75 rpm. After 2 months, gametophytes were transferred to a hormone-free MS medium solidified with 0.7% Difco Bacto-agar.

Quantitation of endogenous levels of GA_1, GA_3, GA_4, GA_7, GA_9 and IAA

Gametophytes derived from spore were cultured as described previously. They were also periodically checked under an optical microscope (Nikon Eclipse E600) and a microphotographic equipment (DS Camera Unit DS-L1). Samples corresponding to the following developmental stages were taken for analyses: 1: 50-day-old gametophytes, with no signs of initiation of apogamous embryos; 2: 65-day-old gametophytes, drawing a tan area composed of dividing small cells near to the apical notch; 3: 72-day-old gametophytes with an emerging apical bud, resembling a button; and 4: 80-day-old gametophytes with an apogamous sporophyte growing up.

Each sample (200 mg DW) was frozen in liquid nitrogen, lyophilised, and finally stored at −20 ºC until analysed. Plant materials were homogenised in 15 ml of cold 80% methanol containing 30,000 dpm of tritiated GA_1 and GA_4 and 100 ng of deuterated GA_1, GA_3, GA_4, GA_7, GA_9 and IAA, and extracted overnight in darkness at 4 ºC. After filtration, the residue was re-extracted with 5 ml of 80% methanol for 2 h and refiltered. The extract was purified through a C18 Sep-pack cartridge and the eluate was absorbed onto 0.5 g of Celite 545 (ProLab), purified by step-elution silicic acid (SiO_2) partition chromatography running into a buffer ethyl acetate–hexane (95:5), and then fractionated by reverse-phase HPLC in a column Kromasil 100 C18 (Scharlau Science GmbH, 250 mm×4.6 mm, 5 µm). The mobile phase was a linear gradient from 10 to 73% methanol in an aqueous solution with 1% acetic acid (v/v) during 60 min at 1 ml min^{-1} flow rate. Measurement of radioactivity was done using a scintillation counter (Packard 2500) and a scintillation liquid (Gold XR, Perkin-Elmer). The fractions containing radioactive GAs were collected, pooled, evaporated to dryness, methylated using ethereal diazomethane and trimethylsilylated using 50 µl *N*,*O*-bis(trimethylsilyl) trifluoroacetamide with 1% (v/v) trimethylchlorosilane for 30 min at 65 ºC. Samples were dried and dissolved in chloroform before injecting onto a capillary column HP-5MS (Agilent Technology), 60 m ×250 µm ×0.25 nominal installed in a GC Hewlett-Packard 6890 coupled to a mass detector Hewlett-Packard 5973. Injection and interface temperatures were 270 and 280 ºC, respectively. The column temperature was maintained at 60 ºC for 1 min, then increased by 20 ºCmin^{-1} to 200 ºC,followed by 4 ºCmin^{-1} to 250 ºC.Levelsofendogenous GAs were determined by measuring the abundance of ion pairs: both *m*/*z* 508/506 and 493/491 for GA_1, both *m*/*z* 506/504 and 491/489 for GA_3, both *m*/*z* 420/418 and 286/284 for GA_4, both *m*/*z* 418/416 and 386/384 for GA_7, and both *m*/*z* 300/298 and 272/270 for GA_9, and finally *m*/*z* 266/261 and 207/202 for IAA. The ratio of integrated peak areas between endogenous [^{1}H]GAs and deuterated [^{2}H]GAs was used to calculate the amount of endogenous GAs.

Culture of gametophytes with flurprimidol

Flurprimidol (Duchefa Biochemie B.V.) was prepared using DMSO as a solvent (final concentration 0.001% for treatment and control). An aliquot was filter sterilised (0.2 µm pore size) to give a final concentration of 3.2 µM and poured into cooled MS medium supplemented with 2% sucrose (w/v) and 0.7% Difco Bacto-agar, contained in 100-ml flasks. Flurprimidol assays were performed with 2-month-old spore-derived gametophytes.

Histological procedure

Gametophytes developing apogamous embryos were fixed in 3% glutaraldehyde in buffer phosphate 0.2 M pH 5.8, dehydrated in a series of tertiary butyl alcohol solutions, embedded in Paraplast (Fisher Scientific Co.), sectioned at 8 µm and stained with blue toluidine O (Johansen 1940).

Data collection and statistical analyses

One hundred gametophytes were observed for each treatment and the experiments were repeated twice. Deviation from normality and homogeneity of variance were tested with Shapiro–Wilk and Barlett–Box tests, respectively. For parametric data, an analysis of variance (ANOVA) with *post-hoc* Scheffés comparisons was used. The frequency of gametophytes bearing apogamous embryos were analysed by means of the chi-square test. The level of significance was set at α=0.05 for all tests.

RESULTS

Effect of sucrose concentration and growth regulators on apogamy

In the fern *D. affinis* ssp. *affinis*, sporophytes arose from an apogamous embryo (Fig. 1a). Gametophytes of this species can form antheridia (Fig. 1b) but not archegonia.

Apogamous sporophyte development on MS solid medium was affected by exogenous growth regulators but not by culture conditions such as sucrose concentration (Table 1). The addition of the growth regulators NAA (0.53 and 5.37 µM) and GA_3 (2.8 µM) favoured sporophyte development (Fig. 1c).

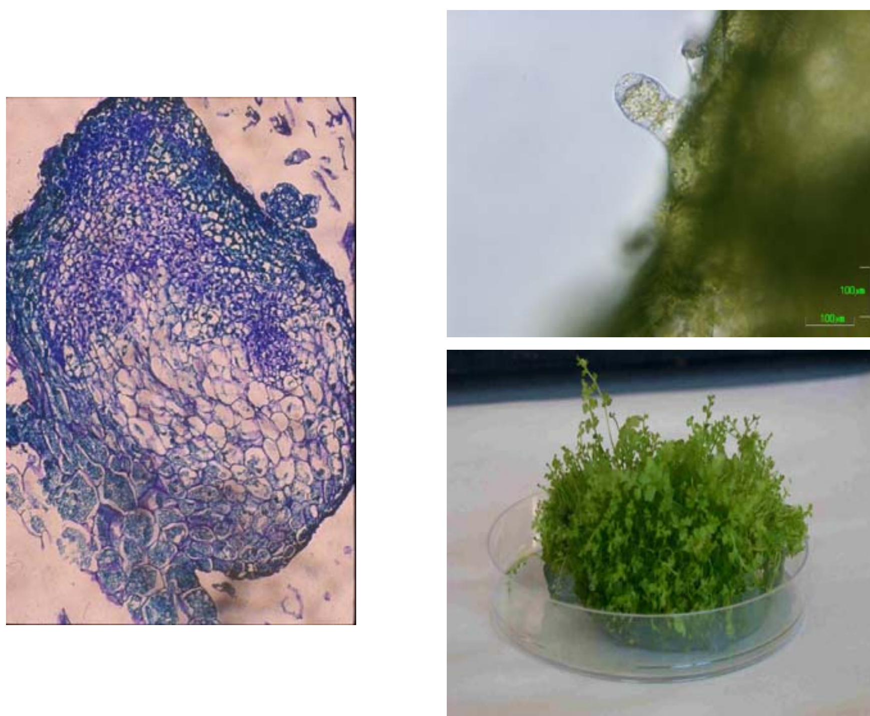


Fig. 1. Apogamy in homogenised gametophytes of *Dryopteris affinis* ssp. *affinis.* (a) Longitudinal section of embryo: g=gametophyte, rm=root meristem, cm=apical meristem, (x60); (b) Gametophyte forming antheridia; (c) Apogamous sporophytes in gametophytes cultured on MS solid medium with NAA of 5.37 µM for 3 months.

The addition of BA to MS solid medium induced callus which became necrotic if cultured for an extended period of time. Sporophytes were not observed unless they were transferred to a fresh hormone-free medium prior turning brown. On the other hand, NAA and GA_3, at the highest concentration, delayed gametophyte development and, in the case of GA_3, induced aggregates of incipient filamentous-shape gametophytes which did not progress.

Culture media	Mean of sporophyte number/flask	Callus
MS+S 2%	1.33 ± 0.57	*
MS+S 4%	0.2 ± 0.1	
MS+NAA 0.53 µM	>50	*
MS+NAA 5.3 µM	>50	*
MS+NAA 26.5 µM	0.56±0.38	
MS+BA 0.44 µM	–	*
MS+BA 4.44 µM	–	***
MS+BA 22 µM	–	***
MS+GA_3 0.28 µM	–	
MS+GA_3 2.8 µM	>50	*
MS+GA_3 14 µM	–	***

Asterisk indicates callus formation (*, low; ***, high). Data after 3 months are presented as mean ± SE

Table 1. Effect of culture conditions on apogamy in homogenised gametophytes of *Dryopteris affinis* ssp. *affinis* cultured on full-strength MS medium with 2% or 4% sucrose (w/v), full-strength MS medium with 2% sucrose (w/v), and one of the following growth regulators: NAA (0.53, 5.37, 26.5 µM), GA_3 (0.28, 2.8, 14 µM) or BA (0.44, 4.44, 22 µM).

Homogenised gametophytes cultured in liquid medium with BA. After 2 months, homogenised gametophytes cultured in MS liquid medium with or without growth regulators grew as spherical cell aggregates, which were entirely formed by filamentous-shape gametophytes. Green-pale callus actively regenerating sporophytes is observed in these cultures (Fig. 2). An extended contact of gametophytes with BA induced the browning of callus.

Fig. 2. Sporophyte regeneration from callus tissue induced in homogenised gametophytes of *Dryopteris affinis* ssp. *affinis* cultured in MS liquid medium with BA 4.44 µM for 2 months and then subcultured on a hormone-free medium.

Quantitation of endogenous content of GA_1, GA_3, GA_4, GA_7, GA_9 and IAA

Apogamousgametophytedevelopmentalstagesconsidered for analyses are shown (Fig. 3a). The data of endogenous levels of plant growth regulators are also given (Fig. 3b). High levels of both GA_9 and IAA were found in gametophytes without signs of cellular organisation near the apical notch (one-way ANOVA test, $F_{2,6}$=6.48, p<0.05 and $F_{3,9}$=13.6, p<0.01, respectively). By the time a brown circle of meristematic cells is organised near the apical notch, the endogenous levels of GA_4 significantly increased ($F_{3,8}$=4, p=0.05); the apparent increase of

GA_7 was not significant. Finally, the endogenous content of both GA_1 and GA_3 increased in elongating shoots, with levels of GA3 being particularly high ($F_{3,4}$=20.48, p<0.01).

Effect of flurprimidol

The addition of flurprimidol, an inhibitor of the three oxidation steps from ent-kaurene to ent-kaurenoic acid (of the GA biosynthesis pathway) to the culture medium prevented apogamy (χ^2=33.7, p=0.0001). Differences among gametophyte shapes (spatulate, spatulate-heart, and heart shapes) were non-significant (Table 2).

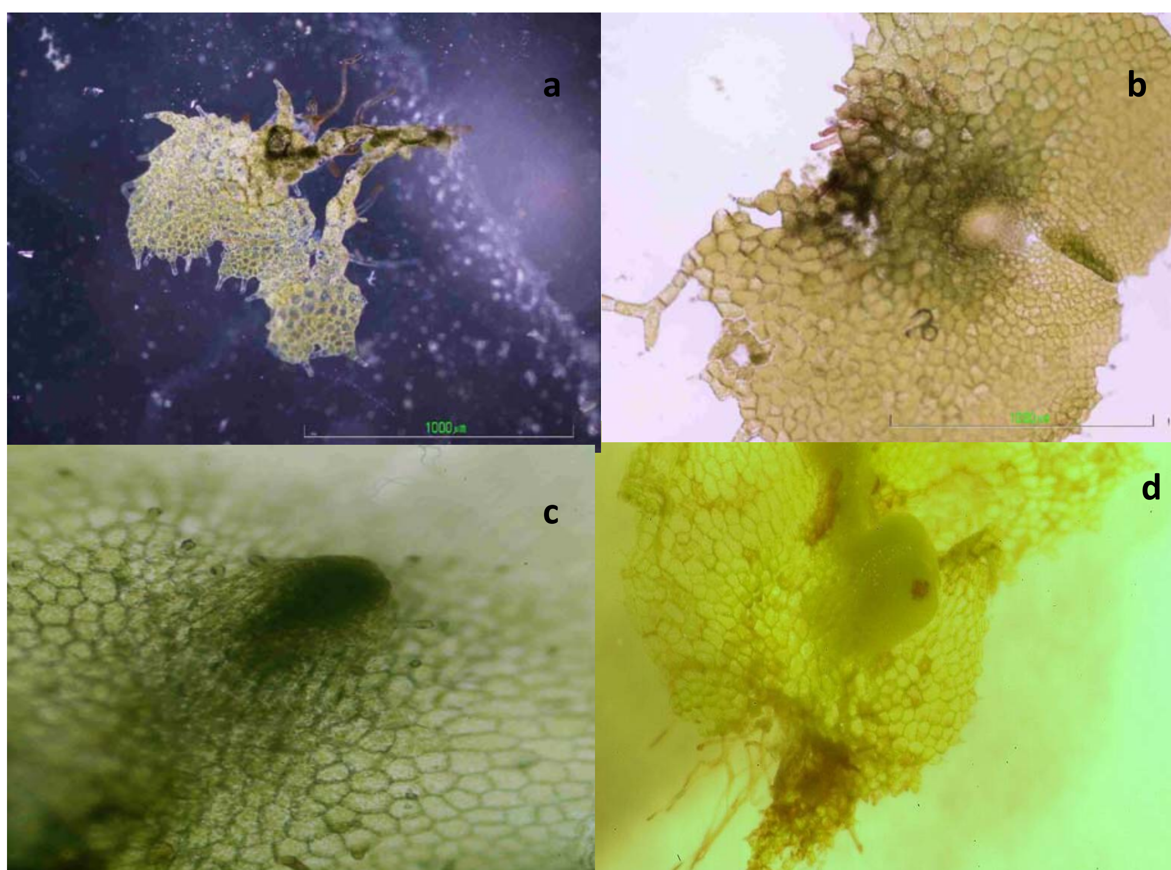
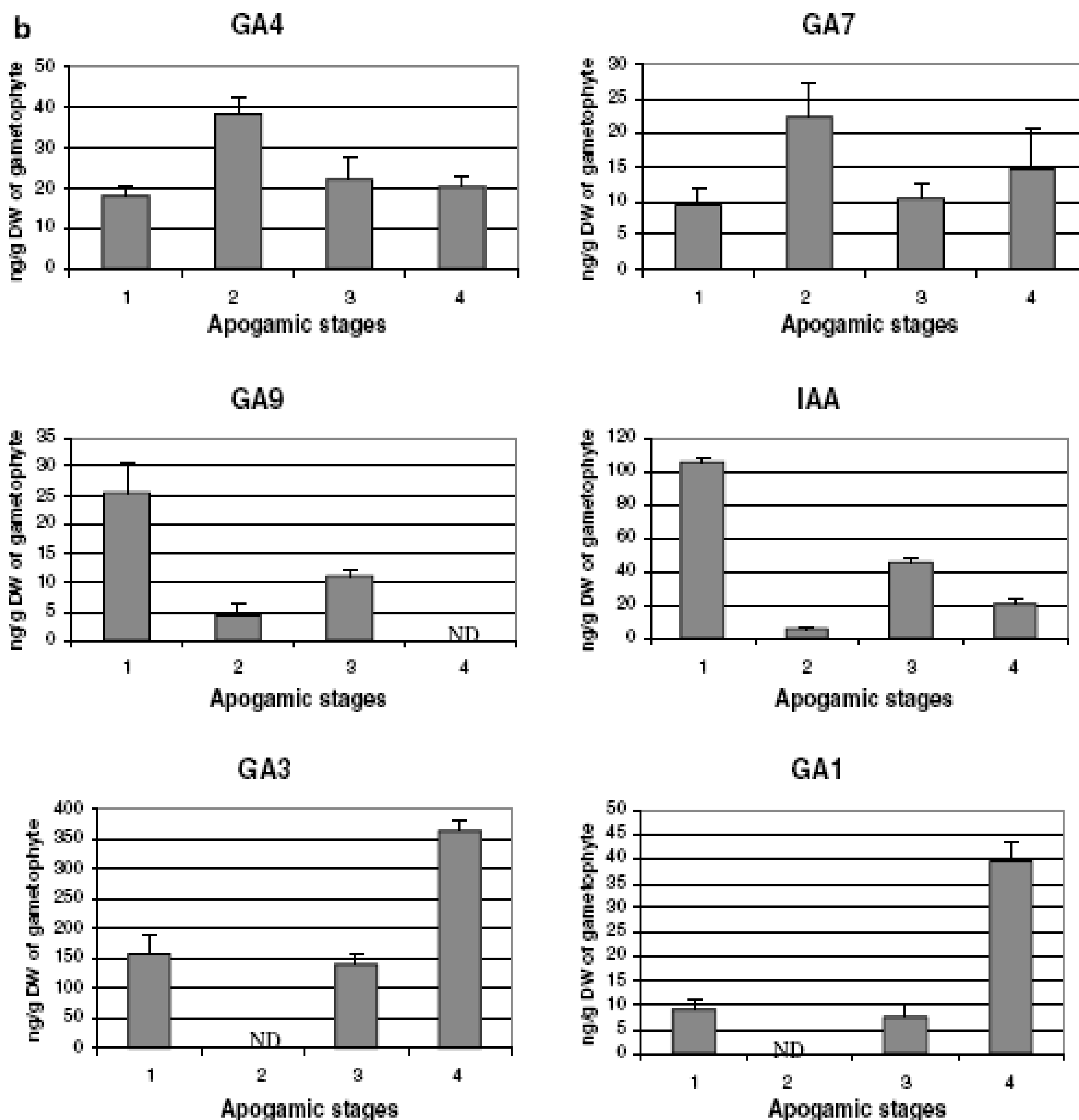


Fig. 3. Endogenous growth regulators on apogamy in *Dryopteris affinis* sp. *affinis*. (a) Developmental stages of gametophyte considered for analyses: **1**= 50-day-old gametophytes, with no signs of initiation of apogamous embryos; **2**= 65-day-old gametophytes, forming a tan area composed of small cells near the apical notch, **3**= 72-day-old gametophytes with an emerging apical bud, resembling a button, and **4**= 80-day-old gametophytes with an apogamous shoot growing up. (b) Endogenous content of IAA and GAs. Data are presented as mean±SE. ND=not determined.



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Treatments	Morphology (%)			Apogamous gametophytes (%)
	Spatulate	Spatulate-heart	Heart	
MS	6	4	90	68
MS+F	8	7	85	27

Data after 2 months

Table 2. Effect of flurprimidol on both gametophyte morphology and apogamy, in 2-moth-old sporophytes of *Dryopteris affinis ssp. affinis* cultured on MS solid medium and MS solid medium supplemented with flurprimidol 3.2 µM (MS+F).

DISCUSSION

The present experimental approach relates the effect of exogenous growth regulators on apogamy with additional analyses of endogenous hormones along this process, in *D. affinis* ssp. *affinis*. Considerable work has been done using tissue culture techniques for plant regeneration and large-scale propagation of various fern species through the addition of auxins and cytokinins to the culture medium, preferably those linked to bud induction from sporophytic tissues (Padhya and Mehta 1982; Fernández et al. 1996b, 1997; Higuchi et al. 1986; Higuchi and Amaki 1989). *In vitro* culture techniques have also been used to carry out studies concerning growth and development of gametophytes and sporophytes (Hickok et al. 1987). However, only scattered reports are available on apogamy until now.

In *D. affinis* ssp. *affinis*, sporophyte is formed without intervention of sexual mechanisms because the gametophyte is able to form antheridia but not archegonia. It is widely recognised that antheridia may be present in abundance

on apogamous prothalli but archegonia may or may not be present, and if so, is usually a non-functional structure (Laird and Sheffield 1986).

The current work shows a promoter effect on apogamy by the addition of NAA or GA_3 to the culture medium. Kwa et al. (1995) reported on the promotion of apogamy in *Platycerium coronarium* (Koening) Desv. gametophytes by IAA and Whittier (1966) also published that NAA and gibberellic acid induced apogamous shoots in *Pteridium* gametophytes. We observed that BA induced callus from the gametophyte either applied in solid or liquid medium. Initially, this callus is green-pale coloured, and it becomes brown faster in solid than in liquid medium. Green-pale callus regenerates sporophyte when transferred to a hormone-free medium and then brown callus can regenerate gametophytes. Our results are in agreement with those previously reported by Kwa et al. (1997). An extended period of culture with BA induced necrosis of gametophytes that we had to manipulate this hormone carefully in cultured *D. affinis* ssp. *affinis* gametophytes. The role of cytokinins inducing apogamous sporophytes may or may not be linked to an intermediate callus phase. Kuriyama et al. (1990) obtained good results by using BA not less than 0.5 and 1 µM, with gametophytes of *Equisetum arvensis*; higher concentrations induced callus. Later, Kuriyama and Maeda (1999) induced globular cell masses from spores that developed into sporophytic plants of the same species with BA 1 µM.

Concerning the effect of sucrose concentration on apogamy, there are several reports stating that high concentrations of sugar is required for apogamy (Whittier and Steeves 1960, 1962; Whittier 1964, 1965). However, it was not the case of this species with the tested concentrations.

In the second part of this work, we tried to find out whether observations done about the effect of exogenous growth regulators implied the existence of possible variations in the endogenous content of auxins and gibberellins during apogamous embryo development, in *D. affinis* ssp. *affinis*.

Before any sign of differentiation was visible, the accumulation of both IAA and GA_9 was detected in the gametophyte. It is well known that auxins, either in the presence or absence of cytokinins, induce somatic embryogenesis (Ammirato 1977; Dodeman et al. 1997; Raghavan 1986). In addition, these hormones are generally considered necessary for the acquisition of embryogenic competence (Ammirato 1983; Charrière et al. 1999). On the contrary, we do not have complete understanding of the involvement of gibberellins in the regulation of somatic embryo induction and development. Exogenous and endogenous GAs may have a positive or negative role, depending on the species or genus (Jiménez et al. 2001). We found increased levels of GA_9 before visible embryo development and that of GA_4 at the time the differentiation process occurring near the apical notch was visible. Our results agree with those published by Noma et al. (1982) who found high levels of GA_{4+7} and low levels of GA1-like substances in developing somatic embryos of carrot. Thus, GA_9 could be converted to GA4 in lower plants parallel to occur in the major GA biosynthesis pathway in higher plants where the conversion from GA_9 to GA_4 is well known (Hedden and Kamiya 1997). The highest levels of gibberellins found in our work refer to those of GA_3*.* As it is well known, GAs stimulate stem elongation by stimulating cell division elongation (Crozier 1983). The role of GAs on apogamy in *D. affinis* ssp. *affinis* is also supported by the effect of the inhibitor flurprimidol.

In conclusion, the present investigation reports for the first time how auxins and gibberellins play a stimulatory role during the induction and differentiation of apogamous embryo development in *D. affinis* ssp. *affinis*.

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CHAPTER 5. PROTEOMIC PROFILE IN MALE SEXUAL DEVELOPMENT MEDIATED BY ANTHERIDIOGEN IN BLECHNUM SPICANT L.

In this paper we present the first report of a proteomic comparation between male and female gametophytes of a fern Blechnum spicant. Female gametophytes were obtained in MS medium directly from spores and the males ones were obtained growing the gametophytes in the same medium supplemented with an extract of mature gametophytes showing anteridiogenic effects (NF).Gametophytes were collected separately, frozen in liquid nitrogen and.lyophilized. Proteins were extracted using Phenolic method. After 2-DE ,581 spots were detected in Coomasie stained gels within the 3-11 pH range and 10-100 kDa Mr ranges. 2-D gel image analysis revealed quantitative and qualitative differences in spot intensity between controls and NF plants. During the growth with the NF an increased number of spots compared with the controls.was observed Only 91 spots were assumed to be differentially accumulated between treatments. We found proteins related to central metabolism (13 proteins), mainly involved in the photosynthesis (8 proteins), one-carbon metabolism (2 proteins), glycolysis (1 proteins), Cysteine and ATP biosynthesis (1 protein each). Defense and response to stress (7 proteins), protein biosynthesis (6 proteins) and transport and cell division (1 protein each).

Keywords: Blechnum spicant, antheridiogen, sexual differentiation

INTRODUCTION

Sex expression in plants is a fundamental process and it is particularly important for economic reasons, especially in the case of commercially important crops (Tanurdzic and Banks 2004). In plants, sexual development is linked to the gametophytic phase, which is extremely reduced in flowering plants. Analysis of gender determination in seed plants is therefore greatly complicated by sporophytic effects on the expression of the gametophyte genome. During the last century, several green organisms from algae to angiosperms, have been proposed as experimental models to carry out studies in fields such as physiology, biochemistry, genetics or developmental biology (Pryer et al. 2002). The haploid phase of ferns has shown some important advantages for use in biological studies such as to be easy of handling in "tissue culture", ready availability of material, easy of microscopic observations, very simple growth and differentiation compared with other photosynthetic eukaryotes, making gametophyte suitable for leading studies in areas such as developmental control of sexual reproduction as it has been proposed here. During last decades, a certain degree of knowledge has been accumulated about how to proced to culture *in vitro* these plants for different purposes (Fernández and Revilla 2003).

Blechnum spicant L. belongs to one of the most ancient groups of ferns. Sexual development in the gametophyte of this species is carried out through the epigenetic action of the unknown antheridium-inducing substances. When cultured *in vitro*, initially the gametophyte develops female sexual organs or archegonia, and produces and excretes antheridiogens into the medium, which induce the formation of male sexual organs or antheridia in the youngest gametophytes, which are filamentous or spatulate-shaped (Klekowski 1969; Cousens 1979; Fernández et al. 1997, 1999; Menéndez et al. 2006).

Proteomic analyses have been conducted in flowering plants in relation to reproduction (Miernyk et al. 2010). Concretely, several reports have been published on male gametophyte development (Kalinowski et al. 2002; Kerim et al. 2003, Miki-Hirosige et al. 2004; Holmes-Davis et al. 2005; Noir et al. 2005; Imin et al. 2006, Dai et al. 2006, 2007a,b; Sheoran et al. 2006, 2007). The aim of this work is determining the proteome profile along maleness becomes setting up in the gametophyte of *B.spicant* L.

MATERIAL AND METHODS

Plant Material

Spores (15 mg) of *B. spicant L.* obtained from sporophytes growing in the forest of Turón (Asturias, Spain) were soaked in water for 2 h and then washed for 10 min with a solution of NaClO (0.5%) containing Tween 20 (0.1%). Then, they were rinsed three times with sterile distilled water. Spores were centrifuged at 2000 rpm for 3 min between rinses, and then cultured in 500-ml Erlenmeyer flasks containing 100 ml of Murashige and Skoog medium (MS) supplemented with 2% sucrose (w/v), pH 5.7 (adjusted with NaOH). All liquid cultures were placed on a gyratory shaker (75 rpm) and maintained at 25ºC under cool-white fluorescent light (40 µmol m^{-2} s^{-1}) with a 16-h photoperiod. After 30 days, the developing gametophytes were transferred into plastic Petri dishes (3,5 cm diameter) containing 10 mL of MS medium supplemented with 2% sucrose (w/v) and 0.7% Difco-Bacto agar (Fem medium) or MS medium supplemented with 2% sucrose (w/v), 0.7% Difco-Bacto agar and the neutral fraction with antheridiogen activity obtained from mature gametophytes (Ant medium). Life cycle of *B. spicant* and materials used in this work are described in Figure 1.

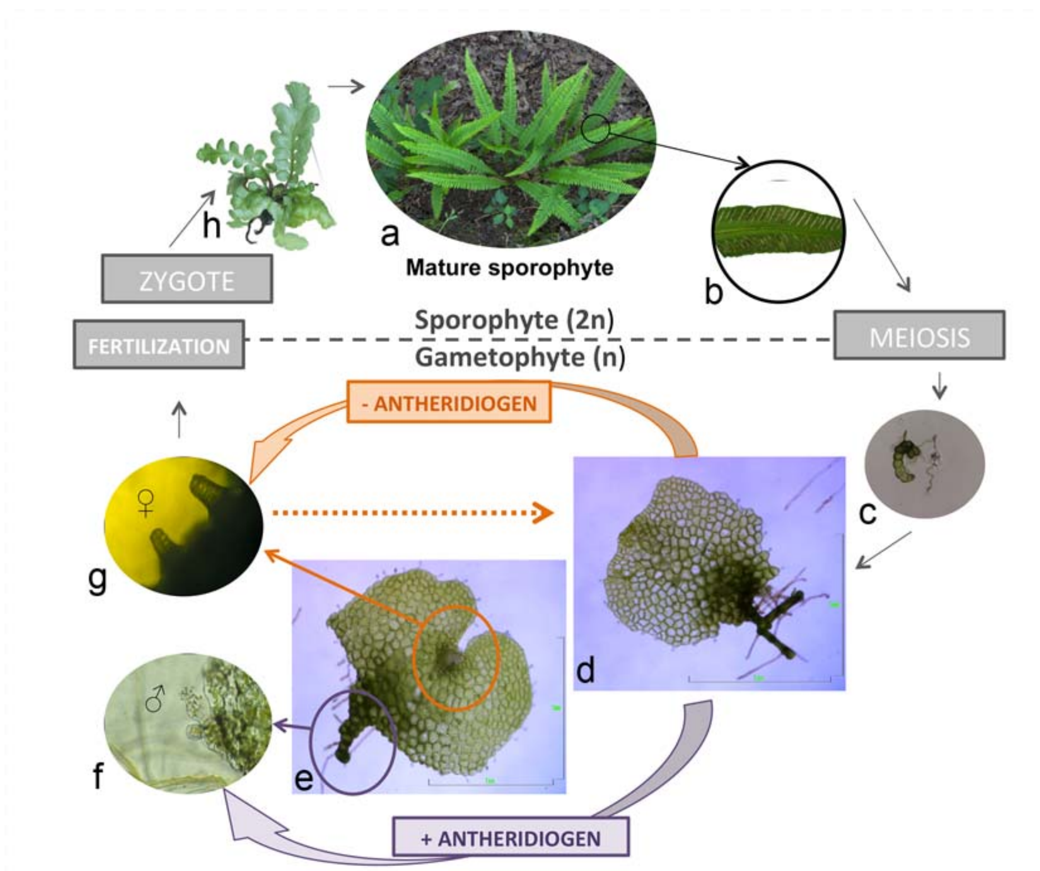

Figure 1: *Blechnum spicant* life cycle. Mature sporophytes (a) have reproductive leaves with sori (b). Each sorus is a cluster of sporangia in which spores are produced via meiosis. The fern spores develop into a small gametophyte (c) that sustains itself by photosynthesis. Young gametophytes are spatulated-shaped (d) and quickly maturate into a heart shape (d). The gametophytes with fastest growth differentiate as a female (arquegonia, e), and, along with this maturation they produce and excrete the antheridiogen to the media. The presence of this pheromone will induce the male differentiation of the surrounding immature gametophytes (antheridia, e). Anteridium produces fern sperm (f) that uses flagella to swim through moisture from antheridia to fertilize the eggs in the arquegonia (g). After fertilization a zygote develops into a new young sporophyte (h).

The neutral fraction (NF) showing antheridiogen activity was extracted from mature female gametophytes that had been maintained in culture for 2 years (Menéndez et al.2004). These gametophytes were ground to a fine powder by using a mortar and liquid nitrogen, after lyophilisation samples were stored at -20ºC until extraction.

The neutral fraction was extracted from 11 g (dry weight) of female gametophytes, by adding 800 mL of cold 80% methanol and incubating overnight in darkness at 4ºC. The sample was then filtered and re-extracted for two hours in 500 ml of cold 80% methanol. The organic solvent of the filtrate was evaporated under reduced pressure at 37ºC. The aqueous layer were dissolved in 200 mL of phosphate buffer (0.1 M, pH 7.8), adjusted to pH 7 with KCl 12 N and extracted three times with 200 mL of Ethyl acetate (EtOAc) to give a neutral EtOAc fraction (NF). The remaining aqueous layer (200 mL) was adjusted to pH 3 with HCl 12N and extracted three times with EtOAc, to give an acidic EtOAc fraction (AF) and a aquous remainder (Aq). FN, AF and Aq fractions were evaporated to dryness in vacuo.

Antheridiogen activity assay

NF was dissolved in 4 ml of 40% methanol, AF was dissolved in 4 mL of 50% methanol and Aq was dissolved in 20 mL of sterile milliQ water. 100 µL of FN, 100 µL of FA or 3 mL of Aq were filter sterilized and added to 100 mL of still warm autoclaved MS medium supplemented with 2% sucrose (w/v) and 0.7% Difco-Bacto agar and poured into sterile 3.5 cm diameter Petri dishes (10 mL of media each). One-month-old gametophytes were aseptically inoculated in the surface of these dishes. After 20 days of culture at 25ºC under cool-white fluorescent light (40 µmol m-2 s-1) with a 16-h photoperiod, the gametophytes were scored for morphology and sex development traits under an optical microscope (Nikon Eclipse E600).

Protein extraction

Gametophytes of 50 days old, growth for 20 days in Ant or Fem medium before sampling, were collected separately, frozen in liquid nitrogen and then lyophilized. 50 mg (dry weight) of each sample were ground into a fine powder with liquid nitrogen by using a potter-elvehjem tissue homogenizer. The powder was suspended in 1 mL of cold phenol extraction buffer (0.7 M Sucrose, 0.5 M

Tris-HCl pH 7.5 buffer, 50 mM EDTA, 0.1 M KCl, 4 mM DTT, 1 mM PMSF; PVPP 10 mg/mL) and mixed vigorously for 5 min at 4ºC. Then 600 µL of Tris-saturated phenol (pH 7.9) were added, and the mixture was shaked for 1 hour at 4ºC. After centrifugation at 21000 g for 5 min at 4ºC, the phenolic phases were collected into new tubes. An equal volume of washing buffer (extraction buffer without PVPP) was then added to phenolic phase. Tubes were vortexed for 30 s and then centrifuged in the same conditions. Phenolic phase is recovered and washing step is repeated once. One mL of ice-cold 0.1 M ammonium acetate in methanol was added to the washed phenolic phase and the proteins were allowed to precipitate overnight at -20ºC. After centrifugation at 21000g, 5 min at 4ºC the supernatant was removed and the pellet was rinsed three times in 1 ml ice-cold methanol/0.07% DTT and then three times in 1 ml acetone/0.07% DTT. Between the rinsing steps, the samples were vortexed and/or sonicated (if necessary) until the pellet was completely disaggregated. The final pellet was air-dried and solubilised in 200 µl of rehydration buffer (7M Urea, 2M Thiourea, 2% CHAPS, 1.2% deStreak solution (GE Healthcare) and 0.5% µL IPG-buffer 3-11(,Biorad)). Insoluble material was removed by centrifugation and the protein concentration was determined using Bradford Assay (Biorad) with ovoalbumin as a standard. Samples were stored at -80ºC until isoelectrofocussing (IEF).

2-DE

Immobilized pH gradients (IPG) strips (Immobiline DryStrip pH 3-11 NL, 24 cm; GE Healthcare BioSciences AB) were passively rehydrated for 12 hours at 20ºC with 600 µg of protein in 450 µL of IEF rehydration buffer. The strips were loaded onto a Ettan IPGphor 3 Isoelectric Focusing System (Amersham Bioesciences) and proteins were electrofocused at 20ºC with current limit 50 µA/strip following manufacturer´s intructions. Strips were immediately equilibrated according Carpentier *et al.,* (2005). The separation in the second dimension was performed on the Ettan DALTsix System (Amersham Bioesciences) with lab cast 1.5 mm SDS polyacrylamide gels (13%) setting the

temperature at 30ºC: 10 mA/gel and 80V during 1 hour; 12 mA/gel and 150V during 17 hours.

Staining and Image Analysis

Gels were stained three times with colloidal CBB G-250 (Bio-Rad) for 20 h following the method described by Mathesius *et al.,* (2001). Images were immediately adquired whit a GS-800 callibrated densitometer (Biorad) to avoid fading. Digitalized gel images were analyzed with PDQuest 8 basic (Bio-Rad) using eight folds over background as a minimum criterium for presence/absence for the guided protein spot detection method.

A spot by spot visual validation of automated analysis was done to increase the reliability of the matching (Chich *et al*., 2007). Spots intensities were obtained for later quantitative analysis. pI calibration in IEF gels was determined using IEF standards (Bio-Rad). M_r values were calculated by mobility comparisons with protein standard markers (SDS molecular weight standars, Broad range, Bio-Rad) run in a separate lane in the gel.

Statistical analyses

Statistical analysis was performed following the recommendations proposed by Valledor and Jorrín (2011). In brief, spot volumes were pre-processed before statistical analyses. Missing spots volumes were estimated from the dataset employing a sequential K-Nearest Neighbour algorithm 1.0.1. This procedure was only performed if the spot appeared in at least 3 out the 5 replicates of each kind of gametophyte culture. After missing values imputation, total spot intensity per gel was used to normalize spots intensities (% of individual spot intensity/Σ% spot intensity of each gel) to compensate the variations between gel replicates and then log transformed.

Spot values passed the Levene's homoscedasticity and Kolmogorov-Smirnov normality tests. Differentially abundant spots were defined after applying a one way ANOVA with block effects, employing FDR to control the error rates. The joint spot analysis was performed following three different multivariate approaches. Principal Component Analysis (PCA) (centered normalized spot values, un-rotated solution) and heat map clustering (employing Euclidean distance and Ward's aggregation method). Five biological and one technical replicates were done for each treatment All of the statistical analyses were performed In R environment v 2.12 (R development Core Team, 2011) employing its core functions and the packages SeqKNN and gplots.

MS-Analysis and Protein identification

Spots proteins were identified as described in Quiala et al. (under review) In brief spots were manually excised from the gel and transferred to multiwell 96 plates. The spots were digested following the protocol described by Shevchenko *et al.* [18], with minor variations, then dried and later purified employing C-18 microcolumns (ZipTip, Millipore).

Peptides were deposited onto MALDI plate and analyzed in a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems) following a top 3 method. A combined PMF search (MS plus MS/MS) was performed using GPS Explorer™ software v 3.5 (Applied Biosystems) over no redundant NCBI databases using the MASCOT search engine (Matrix Science, London; http:// www.matrixscience.com. The confidence in the PMF matches was based on the MOWSE score and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum and protein scores with a p-value lower than 0.05.

RESULTS

2-DE Protein profiles

Developing gametophytes were grown in non limiting MS media with or without NF with antheridiogen activity to stimulate the sexual differentiation. Protein yield obtained after tissue dehydration and phenol extraction was evaluated. Protein yields were the same between treatments, in the range of 10.41 ± 2.6 µg per mg of dried tissue. The same extraction protocol was tested over fresh tissues but extractions were less efficient and the resulting 2-DE profiles dirtier (data not shown).

After 2-D electrophoresis and coloidal coomassie staining, 581 spots were resolved in the 3-11 pH range and 10-100 kDa Mr. 2-D gel image analysis revealed quantitative and qualitative differences in spot intensity were observed between controls and NF plants. During the growth with the NF we observed an increased number of spots compared with the controls (574 and 558 respectively). Interestingly, 23 new spots were found in NF plants, which only miss 7 spots from the controls. The mean coefficient of variation of analyzed spots was estimated into 28.3%,

Quantitatively, the same behavior was observed in both treatments, most spots intensities tend to adjust in the same way during cultivation. After applying a one-way ANOVA only 91 spots were assumed to be differentially accumulated between treatments (5% FDR; q-value < 0.05; 4.56 false positives expected).

To obtain further information, different and additional approaches were performed. First of all, a data reduction to the whole dataset by means of PCA analysis was applied. Out of the potential 581 principal components (PC)

extracted, the first 8 PCs accounted for 96.5% of the biological variability (Supplementary table 2). The use of these components in a 2-D representation (plotting PC1 and PC3) allowed the effective separation of samples (Figure 3a). PC1 accounts for 48.98% of the variance, while PC3 9.2%. PC2 explains 15.17% of the variance, but was not considered for analysis since it explains the variance related to the different batches of electrophoresis. The correlation of each particular spot to PC 1 and 3 was determined from the loading matrix generated during the PCA. The spots showing the highest correlations with each PC 1 and 3 were determined, establishing the threshold in 0.11 after studying the loadings distribution (Figure 2). Out of these spots 13 correlated to PC1 and 5 to PC3 were subjected to MS analysis (discussed later).

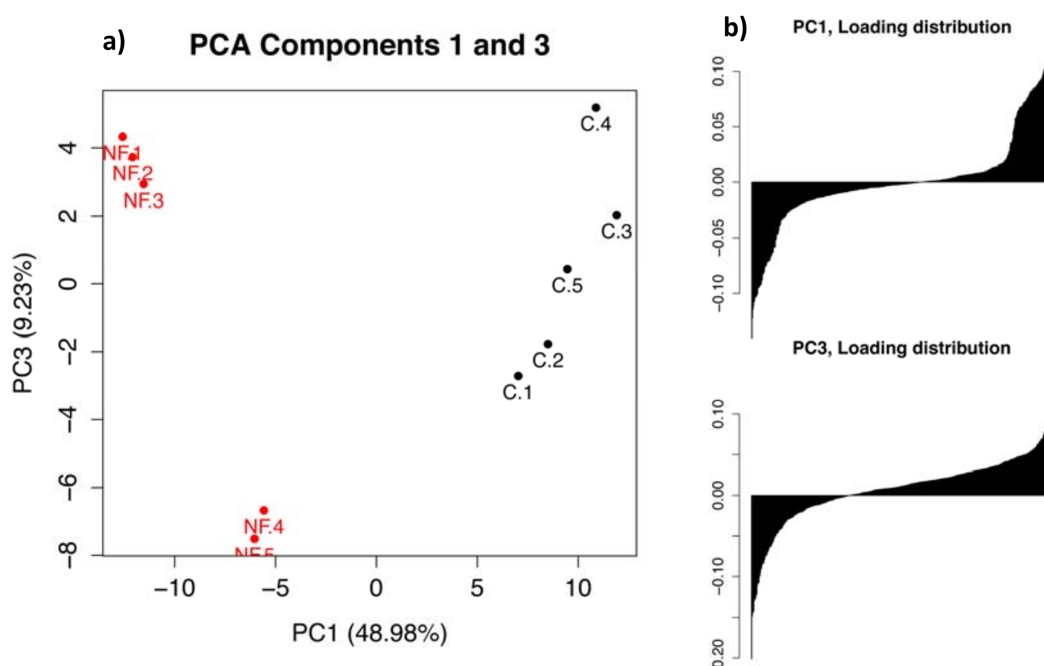

Figure 2: 2-D representation of the main principal components obtained by PCA over all spots dataset (a) and distribution of the loadings of the showed principal components (b)

In order to find further support to understand the behavior of the different groups of proteins identified, spots were clustered employing Euclidean distance and Ward's aggregation method (Figure 3). The samples corresponding to each treatment group together showing a close intra-group distance. With regard to the analyzed spots they grouped into four main groups. First group is mostly represented by variable spots present in both treatments and to non differential spots (each in different branches), second and third group by characteristic spots for each treatment and, the fourth group, includes most of the non differential spots. As is it represented inter-group distances are significant, while intra-group distances are small.

MS analysis and protein identification

60 variable spots, chosen from the most abundant, were excised and analyzed by MALDI-TOF-TOF MS after trypsin digestion. Results obtained were compared to the Viridiplantae and UniProt databases allowing the identification of 28 unique proteins. Resulting proteins are summarized in Table 1. A good correlation between theoretical and experimental pI was obtained whereas some differences in Mr were observed. For some spots, a higher Mr than reported in database was observed maybe due to the absence of mature forms and the presence of sequences corresponding only to a fragment of the protein.

Within the proteins identified, we found proteins related to central metabolism (13 proteins), mainly involved in the photosynthesis (8 proteins), one-carbon metabolism (2 proteins), glycolysis (1 proteins), Cysteine and ATP biosynthesis (1 protein each). Defense and response to stress (7 proteins), protein biosynthesis (6 proteins) and transport and cell division (1 protein each).

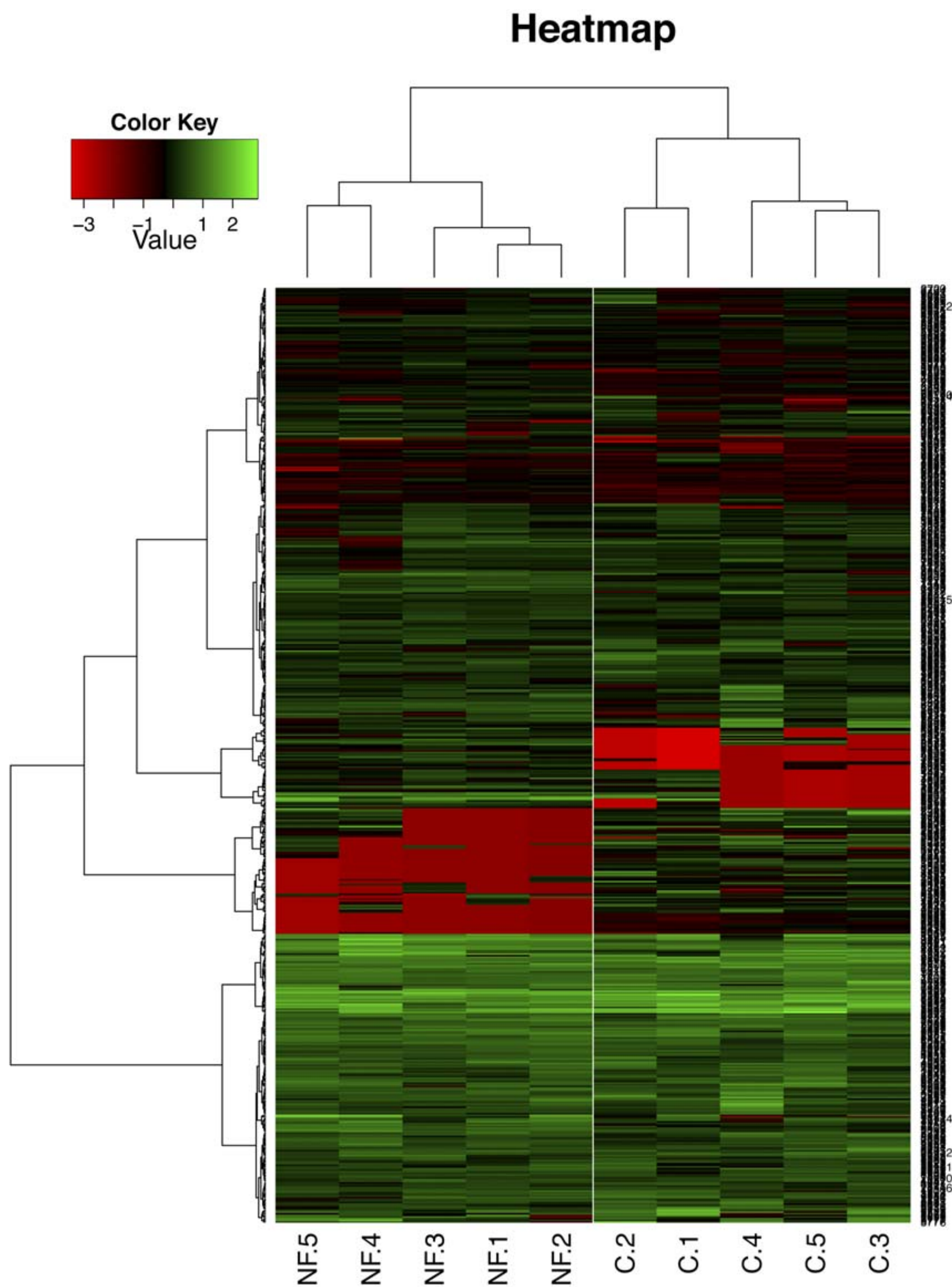

Figure 3: Hierarchical clustering employing Euclidean distance and Ward's aggregation method of all of the analyzed protein spots. Four main groups can be distinguished, the first one corresponding to low abundance proteins expressed in both kind of samples, the second and third classifies spots characteristic of NF or control samples, respectively. The last group classifies abundant proteins common to both treatments.

SSP	Protein name	Reference species	Accession	Mascot Score	Control Mean	Control SD	Ant. NF Mean	Ant. NF SD	ANOVA p-value	trend	
[illegible]	**2-Cys-peroxiredoxin**	***Riccia fluitans***	**gi|7[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	^	**Response to oxidative stress**
2214	2-Cys-peroxiredoxin	*Riccia fluitans*	gi|7339568	180	2,243	0,148	2,652	0,179	0,006	^	Response to oxidative stress
[illegible]	**Thioredoxin peroxidase**	***Nicotiana tabacum***	**gi|[illegible]**	**[illegible]**	**0**	**0**	**[illegible]**	**[illegible]**	**0**	^	**Response to oxidative stress**
3328	Cysteine synthase A	*Vitis vinifera*	gi|225439147	96	0,948	1,299	2,554	0,182	0,025	^	Metabolism
[illegible]	**Uncharacterized protein (putative LHC protein)**	***Picea sitchensis***	**gi|[illegible]**	**170**	**0**	**0**	**[illegible]**	**[illegible]**	**0**	^	**Photosynthesis**
4611	Ribulose-1,5-bisphosphate carboxylase/oxygenase Large subunit	*Polystichum subacutidens*	gi|98978841	493	0,706	0,967	2,271	0,701	0,027	^	Photosynthesis
[illegible]	**Glutathione peroxidase**	***Medicago truncatula***	**gi|[illegible]**	**[illegible]**	**0**	**0**	**[illegible]**	**[illegible]**	**0**	^	**Response to oxidative stress**
6316	Hypothetical protein (putative Glutathione S-Transferase superfamily)	*Vitis vinifera*	gi|225440534	117	1,685	0,387	2,508	0,2	0,003	^	Response to oxidative stress
[illegible]	**Polyphenol oxidase**	***Triticum aestivum***	**gi|[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	^	**Catechol oxidase**
6555	Ribulose-1,5-bisphosphate carboxylase/oxygenase Large subunit	*Sinopteris albofusca*	gi|885869	506	0,929	1,284	2,516	0,628	0,052	^	Photosynthesis
[illegible]	**Ribulose-1,5-bisphosphate carboxylase/oxygenase Large subunit**	***[illegible]***	**gi|[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	^	**Photosynthesis**
7206	Glutathione peroxidase	*Hordeum vulgare*	gi|6179602	73	0	0	2,239	0,462	0	^	Response to oxidative stress
[illegible]	**Putative hypothetical protein**	***Picea sitchensis***	**gi|[illegible]**	**110**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	^	
8268	Peptidyl-prolyl cis-trans isomerase	*Populus trichocarpa x P. deltoides*	gi|118489052	120	0,549	1,228	2,59	0,502	0,012	^	Protein biosynthesis
[illegible]	**Predicted protein (probable prohibitin)**	***Physcomitrella patens***	**gi|[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**0**	^	**Response to stress**
8323	Fructose bisphosphate aldolase	*Picea sitchensis*	gi|116780982	189	1,474	0,453	2,042	0,275	0,021	^	Glycolysis

Table 1: Simplified list of identified proteins which are differentially expressed between control and neutral fraction with antheridiogen. Proteins were classified according to Uniprot gene ontology categories.

Table 1: Simplified list of identified proteins which are differentially expressed between control and neutral fraction with antheridiogen. Proteins were classified according to Uniprot gene ontology categories.

					Control		Ant. NF		ANOVA		
SSP	Protein name	Reference species	Accession	Mascot Score	Mean	SD	Mean	SD	p-value	trend	
1370	**Predicted protein (putative member of Light Harvesting complex)**	***Physcomitrella patens***	**[illegible]**	**77**	**[illegible]**	**[illegible]**	**0**	**0**	**0**	**V**	**Photosynthesis**
2520	ATP synthase beta chain	*Microgramma percussa*	gi|42558432	417	2,329	0,208	0	0	0	V	ATP Synthesis
[illegible]	**[illegible] CARBOXYPEPTIDASE-LIKE [illegible] PRECURSOR**	***[illegible]***	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**0**	**0**	**0**	**V**	**Protein biosynthesis**
3665	Putative protein (homolog to translation initiation factor)	*Picea sitchensis*	gi|116786878	103	1,558	0,144	0	0	0	V	Protein biosynthesis
[illegible]	**Predicted protein (AAA+-type ATPase containing the peptidase M41 domain)**	***Physcomitrella patens***	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**0,01**	**V**	**Protein biosynthesis**
3703	Predicted protein (homolog to Ftsh metalloprotease)	*Physcomitrella patens*	gi|168016254	96	2,032	0,223	0	0	0	V	Cell division
[illegible]	**Putative Light Harvesting Complex [illegible]**	***[illegible]***	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**0**	**0**	**0**	**V**	**Photosynthesis**
4448	Rubisco activase	*Datisca glomerata*	gi|3687676	349	2,624	0,183	0,917	1,256	0,026	V	Photosynthesis
[illegible]	**S-Adenosylmethionine synthetase**	***[illegible]***	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**1,117**	**[illegible]**	**V**	**One-carbon metabolism**
5526	Elongation factor TuB (EF-TuB), chloroplastic	*Nicotiana sylvestris*	gi|218312	114	2,667	0,52	2	0,303	0,06	V	Protein biosynthesis
[illegible]	**S-adenosylmethionine synthetase 1 (SAMS1)**	***Arabidopsis thaliana***	**[illegible]**	**275**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**V**	**One-carbon metabolism**
5563	S-adenosylmethionine synthetase 1	*Zea mays*	gi|195621568	154	2,471	0,336	1,741	0,352	0,016	V	One-carbon metabolism
5719	**Transketolase 1**	***[illegible]***	**[illegible]**	**80**	**[illegible]**	**0,317**	**[illegible]**	**[illegible]**	**0,003**	**V**	**Photosynthesis**
5768	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Prosaptia palauensis*	gi|42540709	320	1,901	0,263	0,56	0,889	0,017	V	Photosynthesis
[illegible]	**[illegible] ABC transporter**	***Oryza sativa***	**[illegible]**	**[illegible]**	**[illegible]**	**0,407**	**0**	**0**	**0**	**V**	**Transport**
6456	NB-ARC domain containing protein, expressed	*Oryza sativa*	gi|77552477	115	1,549	0,18	0	0	0	V	Apoptosis/Defense
[illegible]	**Ribulose-1,5-bisphosphate carboxylase/oxygenase**	***[illegible]***	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**0**	**0**	**0**	**V**	**Photosynthesis**
7236	Predicted protein (homolog to oxigen-evolving protein family)	*Physcomitrella patens*	gi|168022403	92	1,766	0,34	0,573	0,951	0,033	V	Photosynthesis
[illegible]	**Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit**	***[illegible]***	**[illegible]**	**[illegible]**	**[illegible]**	**[illegible]**	**0**	**0**	**0**	**V**	**Photosynthesis**

DISCUSSION

Blechnum spicant L. belongs to one of the most ancient groups of ferns in which sexual development in the gametophyte is determined by the action of still unknown antheridium-inducing substances (Cousens 1979; Fernández et al. 1997). It is clear that this induction should be reflected in a complete reprogramming of the cell fate and function, since there are not only functional but structural changes during this process. Considering the effect shown by the NF of antheridiogen over the immature gametophytes towards the induction of antheridia, in this work we studied the changes in the proteome of *B. spicant* gametophytes during this process.

The most important observation was the dramatic changes found in some spots related to metabolism and stress response, despite the similar protein content and number of spots observed during the treatment. It has to be considered that in 2D-gels, only a fraction of the total proteome can be observed, mainly proteins which are the most abundant such as the housekeeping proteins (Buxbaum, 2010). From these results we conclude that antheridiogen induction is a very stressing process for the gametophytes.

PCA analysis allowed a clear classification of samples. The plot of PC1 (49%) and PC3 (9.2%) shows a great distance between control and NF induced samples. This supports above indicated hypothesis in terms of the non- similarity found between gametophyte behavior during the induction of antheridiogen. This idea was confirmed after the application of a heat map clustering techniques that clearly distinguish between those characteristic proteins of antheridiogen-induced and control gametophytes. On the other hand it can be observed that most of the proteins remain unchanged, showing that only specific cellular activities are changed as a consequence of antheridiogen induction. Most of the spots with high loading over PC1 and PC2 have been also

considered differential after ANOVA analysis. This adequate statististical power has been achieved due to the employ a relative high number of biological replicates (compared to other plant proteomic studies; Jorrín et al. 2009), manual supervision of all of the stages in image analysis, and data pre-processing strategy that had led to a low CV% and a good ANOVA power. The employ of this statistical workflow has been previously recommended for analyzing 2-DE proteomic data (Valledor and Jorrín 2011; Chich et al. 2007).

It is relevant that we have identified most of the enzymes involved in cell defense and stress responses are over-accumulated in induced gametophytes. It is obvious that the neutral fraction, obtained by ethyl acetate fractionation, contains compound with antheridiogen activity (masculinity inductors) but also it may contain other factors, with inhibitory function. However, these response have been reported to occur during “natural” organogenic responses. It has been previously reported in *Arabidopsis* that organogenic response is associated to the pathogen independent activation of stress and defense pathways (Quirino et al. 1999), activating also polyphenol oxidases (like catechol oxidase). Most of these activities have been related to a signaling process mediated by cytokinins and ethylene. In this sense it has been proved that *Arabidopsis* ecotypes with different sensitivity to ethylene have different capacity to shoot regeneration, being the more sensitive the most responsive (Chatfield et al., 2008).

It is conceivable to pose the importance of the changes in the SAM synthetase in this process, acting as a marker of a complete organogenic induction, since the presence of this protein species is significantly lower in NF induced gametophytes. Ethylene is produced from methionine *via* S-adenosylmethionine (SAM) and ACC (1-aminocyclopropane-1-carboxylic acid; Wang, Li and Ecker 2002) by nearly all plant organisms: angiosperms, ferns (*P. aquilinum*, *Matteuccia strutiopteri* and *Polystrichum munitum*; Tittle 1987),

mosses (*Funaria hygrometrica*; Rohwer and Bopp 1985) and lichens and also by some microorganisms (Bleecker and Kende 2000).

Ethylene and gibberellin cooperation may depend on the regulation of their concentrations which is mediated by a direct ethylene precursor, ACC (Kaneta, Kakimoto and Shiboaka 1997; van der Knaap et al. 2000; Rijnders et. al. 1997). ACC (or ethylene) may participate in antheridiogenesis and in development of *A. phyllitidis* gametophytes as the second messenger because without gibberellic acid no antheridia are formed (Kaźmierczak 2006; Kaźmierczak and Kaźmierczak 2009). GA_3 activates antheridiogenesis along two parallel pathways without or with cooperation with ethylene. In the former, the cell cycle is directly induced by GA_3 activating specific CDK (cyclin dependent kinase) and cyclins (Kaźmierczak 2006), and in the latter

The importance of the antheridiogen induced signalling cascade is indubitable, but how does this pathway acts for inducing organogenesis? It is clear that the formation of a new organ implies apoptosis and cell reprogramming, for gaining new shape and function. In this sense we have found that MRP-Like ABC transport disappear in the NF-induced gametophytes. These transporters are very selective, and the basis of sensing internal and external stimuli (Knöller and Murphy, 2011). These transporters have been proved to mediate in the phytohormone-responsive phosphoproteins in *Arabidopsis* (Chen et al. 2010), mediating in signalling pathways. Maybe this transporter disappears after first sensing of antheridiogen for protecting the gametophyte of over induction. We cannot forget the changes in the protein biosynthesis pathways, that seems to be reduced into the NF induced gametophytes, may reflect the end of organogenic pathway, the loose of morphogenic competence and the maturation of tissues, similar effects related to maturation have been previously described in other plants (Valledor et al 2010). This hypothesis is supported by the fact that the spots corresponding to

two proteins related to apoptosis and cell division cannot be detected in NF gametophytes, maybe indicating that the organogenic process is finished since no new cell formation or apoptosis is required.

Despite we don't have a definitive answer, the induction of masculine gametophytes by antheridiogens lead to a global increase in stress and defense related pathways, which affecting also flavonoid signaling and cell division while reducing protein biosynthesis pathways. On the other hand the photosynthesis and other energy related pathways are affected during this induction. Since photosynthesis is very sensitive to cell homeostasis this changes may be another indicator of how stressed are the gametophytes during induction process. This is, to our knowledge, the first contribution in the proteomic study of fern gametophyte.

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DISCUSIÓN GENERAL

En esta memoria de Tesis doctoral se aborda el estudio de los mecanismos reproductivos que actúan en dos especies de helechos, en las que la formación del esporofito ocurre de dos maneras muy distintas. En la especie *B.spicant* encontramos un modelo de reproducción sexual, con la formación de órganos sexuales femeninos y masculinos, productores de gametos, cuya fusión conlleva a la formación del esporofito. Como ocurre en algunas especies de helechos, en el gametofito de *B.spicant* opera un sistema anteridiógeno que favorece el intercambio genético. Por su parte, en la especie *D.affinis ssp. affinis* la formación del esporofito tiene lugar mediante un proceso apogámico, sin la intervención de gametos, dada la incapacidad del gametofito para formar órganos sexuales femeninos.

En el laboratorio de Fisiología Vegetal se ha trabajado con helechos desde hace mucho tiempo, casi siempre en proyectos con una vertiente de propagación de especies de interés ornamental (Fernandez y Revilla 2003), sin dejar no obstante de apreciar algunas características interesantes de cara a utilizar estas plantas como sistemas experimentales para tratar temas o procesos relacionados con desarrollo vegetal. En este sentido, el gametofito del helecho, por su simplicidad morfológica, su facilidad de cultivo, la alta disponibilidad de material, y su independencia del esporofito, le hace acreedor de una cierta idoneidad para ser tenido en cuenta como material experimental con el que llevar a cabo estudios de diversa índole (ver introducción).

En el primer capítulo de la memoria, se decide comparar el desarrollo vegetativo y sexual del gametofito de *B. spicant*, de dos orígenes distintos: espora y triturado de gametofito maduro, que muestra una enorme capacidad de regeneración en cultivo *in vitro*. Asimismo, se estudia el efecto de los

reguladores del desarrollo en el crecimiento y maduración sexual del gametofito. El patrón de desarrollo vegetativo y sexual se vió, inicialmente, que era dependiente de las condiciones de cultivo. Así, mientras que el gametofito obtenido de espora evoluciona hacia la feminidad, el gametofito regenerado tiene una fuerte tendencia a la masculinidad, debido, aparentemente, a la liberación al medio de cultivo de sustancias durante el proceso mecánico de trituración del tejido, que inciden tanto en el desarrollo vegetativo como sexual. La adición al medio de cultivo de los reguladores del desarrollo ensayados no modificaba cualitativamente la condición sexual del gametofito si bien se encontró en la citoquinina un fuerte potenciador de la actividad anteridiógena en gametofitos regenerados. La aportación al objetivo general del trabajo de tesis de este capítulo radica en la posibilidad de disponer de cultivos de gametofito de diferente condición sexual: masculina y femenina, con los que poder llevar a cabo los experimentos de los siguientes capítulos.

En los capítulo segundo y tercero, se analiza el papel los reguladores del desarrollo: citoquinas y giberelinas, tradicionalmente implicados en desarrollo sexual, en la reproducción sexual del gametofito de *B. spicant*. La organogénesis de los gametangios se puede considerar como el resultado de la expresión de programas de diferenciación en los cuales los reguladores del desarrollo pueden estar implicados. Para determinar la importancia de dichos compuestos, dos aproximaciones se han venido haciendo: la administración exógena y los análisis de los niveles endógenos (Duran and Duran 1984; Chailkyan Kh and Khryanin 1987). A la luz de estos datos, las giberelinas y las citoquininas se han mostrado como candidatos de la expresión del sexo. En concreto, la predominancia de citoquininas favorecería la expresión femenina mientras que las giberelinas favorecerían la masculina (Khryanin 2002).

El capítulo segundo, dedicado a las giberelinas, se aborda mediante dos estrategias habituales: a) el análisis de los niveles endógenos de aquellas

giberelinas que son productos finales de las rutas de biosíntesis, y b) el empleo de inhibidores de dicha biosíntesis, como el flurprimidol, que bloquea las etapas oxidativas del ent-kaureno a ácido ent-kaurenoico. El efecto inductor de órganos sexuales masculinos y femeninos observado con GA_{4+7} en esta especie correlaciona con la detección de niveles más altos de GA_4 y GA_7 en gametofitos machos y hembras. Encontramos referencias en la bibliografía que indican variaciones en los niveles de determinadas giberelinas durante el desarrollo floral (Moritz et al. 1989; Evans y Poething, 1995; King et al. 2001; Villacorta et al. 2008). En concreto, en En Pinaceae, también hemos visto en nuestro laboratorio que el efecto inductor de la floración al aplicar exógenamente GA_{4+7} también se corresponde con los niveles de GA_4 medidos en yemas masculinas (Fernández et al. 2003).

En relación al presunto carácter inhibitorio atribuido a los compuestos anteridiógenos, se realizan una serie de tratamientos con la intención de aportar algo a esta cuestión, como son el lavado del extracto, o la adición de carbono activo o de ácido abscísico al medio de cultivo. El lavado del extracto permite un mayor desarrollo vegetativo del gametofito, que se acompaña de la formación de anteridios, poniendo en entredicho el carácter inhibitorio de los supuestos anteridiógenos. Por su parte, la adición de carbono activo bloquea la formación de anteridios, reforzando la idea de la producción y liberación al medio de cultivo de sustancias con actividad anteridiógena en el gametofito de *B. spicant*. El hecho de que el lavado del extracto favoreciera el desarrollo vegetativo del gametofito hizo pensar en otros posibles compuestos inhibitorios del desarrollo, tales como el ácido abscísico, considerado antagonista de la acción de los anteridiógenos no hallando en este experimento ningún efecto inhibitorio de dicha actividad por parte de dicho compuesto.

En el tercer capítulo, se analiza el papel de las citoquininas en el desarrollo sexual del gametófito de *B. spicant* mediante dos actuaciones. Se

había visto, en el primer capítulo, que la citoquinina BA tenía un efecto amplificador de la actividad anteridiógena en gametofitos regenerados. En este trabajo, se cultivaron esporas con BA y extracto, añadidos por separado y juntos. En gametofitos derivados de espora, la presencia de BA en el medio no favorece la formación de órganos sexuales en ningún caso, ni en presencia ni en ausencia de extracto. No resulta extraño que un mismo regulador pueda tener efectos opuestos sobre el sexo dependiendo de la especie (Duran and Duran 1990) pero en este caso, el efecto de la hormona no es el mismo en gametofitos procedentes de espora que en gametofitos regenerados.

El hecho de que la citoquinina exógena afectara a la formación de órganos sexuales invitaba a analizar los niveles endógenos de estos compuestos en gametofitos de diferente sexo, encontrando variaciones en los niveles de algunos de ellos. Así, se observó que los niveles de iP e iPR incrementaban hasta 300 y 400 veces, respectivamente en gametofitos femeninos de *B. spicant*. En la bibliografía se han descrito correlaciones entre el contenido de citoquininas y el desarrollo de plantas femeninas en otras espeecies (Dekock et al. 1994; Duran y Duran 1990; Yang et al. 1998; Pineda Rodó et al. 2008).

Tras una serie de experimentos que apuntan a la presencia de compuestos anteridiógenos en el gametofito de esta especie, como se ha visto, se investiga en la actividad fisiológica de dichos compuestos, en concreto, lo referente al momento del desarrollo del gametofito en que empieza a producirlos y a volverse insensible, y, finalmente se aplican protocolos para purificar y aislar estos compuestos de cara a una futura identificación de la naturaleza química, tradicionalmente asociada con la familia química de las giberelinas. Los gametofitos que llevan 60 días en cultivo seguían mostrando sensibilidad a dichos compuestos y extractos obtenidos de cultivos de 75 días de gametofitos eran capaces de estimular la formación de anteridios. Respecto a la purificación de estos compuestos, la partición del extracto crudo de

gametofitos maduros con acetato de etilo arrastra actividad anteridiógena en las fracciones neutras y ácida, y el fraccionamiento de la fracción neutra mediante cromatografía líquida de alta resolución recuperaba la actividad en una fracción correspondiente al 60% de metanol.

En el capítulo cuarto, se presenta el trabajo realizado acerca del proceso apogámico responsable de la formación de esporofitos en el gametofito de *D.affinis ssp. affinis*. En esta especie no es posible la reproducción sexual debido a la ausencia de órganos sexuales femeninos, si bien se han visto anteridios (Fernández et al. 1996). La formación del esporofito ocurre a partir de determinadas células somáticas, ubicadas donde tiene lugar habitualmente la formación de órganos sexuales femeninos o arquegonios, en especies con reproducción sexual.

La adición de los reguladores NAA, GA_3 o BA la medio de cultivo favorece en ocasiones la respuesta apogámica, coincidiendo con otros resultados publicados con anterioridad (Whittier 1966; Kuriyama et al. 1990; Kwa et al. 1995, 1997; Kuriyama and Maeda 1999). En el caso de la aplicación de la citoquinina tenía lugar la formación de callo, que tras la retirada de regulador del medio de cultivo formaba esporofitos con una enorme capacidad morfogénica, no observada nunca en ninguna otra especie de helecho cultivada en el laboratorio.

La segunda parte de este capítulo describe las variaciones detectadas en los niveles endógenos de auxinas y giberelinas, al cabo de 50, 65, 72 y 80 días de iniciado el cultivo de esporas. La caracterización de cada muestra resultó realmente difícil al tener que identificar gametofito a gametofito al microscopio y asegurar que se trataba de una muestra homogénea en cuanto al estadio del proceso. Antes de cualquier signo visible de diferenciación se detectaban acumulaciones de AIA y GA_9. La apogamia descrita en este helecho se asemeja a

un proceso de embriogénesis somatica, en la que las auxinas, con o sin citoquininas, han sido consideradas necesarias para inducir la respuesta (Ammirato 1983; Raghavan 1986; Ammirato 1977; Dodeman et al. 1997; Charrière et al. 1999). Sin embargo, no se tiene la misma evidencia del papel de las giberelinas en la regulación de la embriogénesis somática, que puede ser positiva o negativa según la especie (Jiménez et al. 2001). En nuestro caso, encontramos niveles elevados de GA_9 antes de ser aparente el desarrollo del embrión, y de GA_4 al tiempo en que se hace visible un centro de diferenciación formado por células de coloración oscura y contorno isodiamétrico, en la hendidura apical. Noma et al. (1982) describe variaciones en los niveles de GAs durante la embriogénesis somática en zanahoria. En nuestro trabajo, los niveles más elevados de GAs correspondían a GA_3, asociada con elongación celular (Crozier 1983) y se refuerza el papel de las giberelinas en este proceso con la aplicación del inhibidor flurprimidol. En este capítulo se aportan evidencias por vez primera de la influencia de auxinas y giberelinas en el proceso apogámico en esta especie.

El último capítulo de la tesis representa el primer intento de aplicar las técnicas de la proteómica a la búsqueda de cambios en el perfil proteico durante el desarrollo sexual masculino y femenino en el gemetofito del helecho, en este caso *B. spicant*. Pare ello, se obtuvieron cultivos enteramente formados por individuos masculinos y femeninos. Un punto crítico en este estudio lo representaba la puesta a punto de un protocolo de extracción de las proteinas que ofreciese resultados cualitativos y cuantitativos satisfactorios. Los protocolos habitualmente empleados en otras especies vegetales no permitían extraer la cantidad necesaria de proteina para realizar los análisis. El pequeño tamaño del gametofito y su alto contenido en agua fue un factor limitante a la hora de diseñar un protocolo de extracción. La comparación de los perfiles proteicos de gametofitos masculinos y femeninos reveló la abundancia de proteinas implicadas en procesos de estrés y de defensa en los gametofitos masculinos, que habían sido cultivados en un medio inductor de anteridios que

contenía extracto de gametofitos maduros. En estas condiciones de cultivo, el gametofito originado de espora, que estaba inicialmente programado para seguir un patrón de diferenciación sexual femenino, recibe del medio señales químicas que le obligan a variar dicho patrón. Este cambio de patrón organogénico podría justificar la expresión de proteinas relacionadas con el estrés. Por otra parte, el extracto añadido al medio de cultivo que contiene antieridiógenos no es un extracto puro, por lo tanto junto con los anteridiógenos no se descarta la presencia de sustancias de diversa índole (inhibidores de desarrollo, etc.) que una vez liberados al medio de cultivo pueden desencadenar en el gametofito la activación de mecanismos relacionados con estrés y defensa. Otro de los resultados encontrados fue el descenso de la actividad de SAM sintetasa en los gametofitos cultivados en el medio inductor de anteridios. Esta enzima participa en la síntesis de etileno y ha sido asociada su actividad con la expresión femenina en el modelo de estudio *Cucumis*. En helechos, por el contrario, Kamierckaz (2010), trabajando con la esepcie *Anemia*, ha estalecido la cooperación entre GA_3 y el etileno, en calidad este último de segundo mensajero, en la anteridiogénesis. En el caso de *B. spicant*, el gametofito no responde a la aplicación de GA_3 como en *Anemia*, ni tampoco exite una inducción de la síntesis de etileno. Por lo tanto, el papel de esta hormona en la diferenciación sexual en *B. spicant* sería más próximo a lo que sucede en el modelo de Cucumis.

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CONCLUSIONES GENERALES

El objetivo general de la tesis fue el estudio del papel de los reguladores del desarrollo en el gametofito de dos especies de helechos con reproducción sexual y asexual, y abordar un estudio molecular a través de la proteómica para determinar posibles genes asociados a estos procesos. Las conclusiones son las siguientes:

1. El crecimiento y desarrollo sexual del gametofito del helecho es sensible a las condiciones de cultivo y a través del cultivo *in vitro* del gametofito es posible obtener cultivos de gametofitos masculinos o femeninos, válidos para profundizar en la expresión sexual.
2. El efecto de la citoquina BA en lel desarrollo sexual en B. spicant depende del origen del gametofito

3. Las GAs no tienen un papel significativo en la determinación del sexo ni en la biosíntesis de anteridiógenos en *B. spicant,* al contrario de lo publicado en otras especies de helechos.

4. La feminidad en el gametofito de *Blechnum spicant* L. está asociada con aumentos de los niveles de las bases libres tZ, cZ, iP y su riboside.

6 Las auxinas y las giberelinas influyen en el inducción y diferenciación del embrión apogámico en *D. affinis* ssp. *affinis.*

7. En este trabajo se consigue purificar y aislar los compuestos con actividad anteridiógena, quedando pendiente su identificación

8. Mediante la aplicación de las técnicas de la proteómica se constatan variaciones en los niveles de expresión de SAM y de proteínas relacionadas con estrés, en el desarrollo sexual del gametófito de B. spicant.