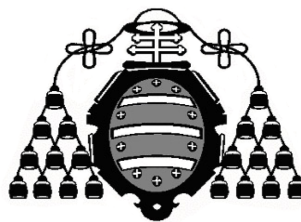

Universidad de Oviedo

Departamento de Biología de Organismos y Sistemas

TESIS DOCTORAL

BASES FISIOLÓGICAS Y MOLECULARES EN LA MICROPROPAGACIÓN DE FAMILIAS DE *Pinus pinea* L.

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Bases fisiológicas y moleculares en la micropropagación de familias de Pinus Pinea L.

Candela Cuesta Moliner

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FINANCIACIÓN Y ORGANISMOS IMPLICADOS

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ABREVIATURAS

½LP: Medio LePoivre con Macroelementos Diluidos a la Mitad

½LPC: Medio LePoivre con Macroelementos Diluidos a la Mitad y Carbón Activo

AFLP: Amplificación de Fragmentos de Longitud Variable ("Amplified Fragment Length Polymorphism")

AHK: Receptor Histidin-Quinasa de *Arabidopsis thaliana* ("*Arabidopsis thaliana* Histidine Kinase")

AHP: Proteína de Transmisión de Fosfatos de *Arabidopsis thaliana* ("*Arabidopsis thaliana* Histidine Phosphotransfer Protein")

AMOVA: Análisis de la Varianza Molecular ("Analysis of Molecular Variance")

ANOVA: Análisis de la Varianza ("Analysis of Variance")

AMP/ADP/ATP: Adenosina 5'-Mono/Di/Trifosfato ("Adenosine 5'-Mono/Di/Triphosphate")

ARR: Regulador de Respuesta de *Arabidopsis thaliana* (""*Arabidopsis thaliana* Response Regulator")

BA: Benciladenina ("Benzyladenine")

BA3G: 3-Glucósido de Benciladenina ("Benzyladenine 3-Glucoside")

BA7G: 7-Glucósido de Benciladenina ("Benzyladenine 7-Glucoside")

BA9G: 9-Glucósido de Benciladenina ("Benzyladenine 9-Glucoside")

BA9GR: Ribósido del 9-Glucósido de Benciladenina ("Benzyladenine 9-Glucoside Riboside")

BAR: Ribósido de Benciladenina ("Benzyladenine Riboside")

BARMP: Ribótido de Benciladenina ("Benzyladenosine 5'-Monophosphate")

BFC: Capacidad de Formación de Yemas ("Bud Formation Capacity")

CCR: Cotiledones con Respuesta Caulogénica ("Cotyledons with Caulogenic Response")

CK: Citoquinina ("Cytokinin")

CTAB: Bromuro de Cetiltrimetilamonio ("Cetyl Trimethylammonium Bromide")

***c*Z**: *cis*-Zeatina ("*cis*-Zeatin")

***c*ZR**: Ribósido de *cis*-Zeatina ("*cis*-Zeatin Riboside")

***c*ZRMP**: Ribótido de *cis*-Zeatina ("*cis*-Zeatin Riboside 5'-Monophosphate")

DHZ: Dihidrozeatina ("Dihydrozeatin")

DHZ9G: 9-Glucósido de Dihidrozeatina ("Dihydrozeatin 9-Glucoside")

DHZOG: *O*-Glucósido de Dihidrozeatina ("Dihydrozeatin *O*-Glucoside")

DHZR: Ribósido de Dihidrozeatina ("Dihydrozeatin Riboside")

DHZRMP: Ribótido de Dihidrozeatina ("Dihydrozeatin Riboside 5'-Monophosphate")

DMAPP: Dimetilalil Difosfato ("Dimethylallyl Diphosphate")

DW: Peso Seco ("Dried Weight")

FW: Peso Fresco ("Fresh Weight")

HPLC: Cromatografía Líquida de Alta Resolución ("High Performance Liquid Chromatography")

IAA: Ácido 3-Indolacético ("3-Indolylacetic Acid")

iP: Isopenteniladenina ("Isopentenyladenine")

iP7G: 7-Glucósido de Isopenteniladenina ("Isopentenyladenine 7-Glucoside")

iP9G: 9-Glucósido de Isopenteniladenina ("Isopentenyladenine 9-Glucoside")

iPR: Ribósido de Isopenteniladenina ("Isopentenyladenosine")

iPRMP: Ribótido de Isopenteniladenina ("Isopentenyladenosine 5'-Monophosphate")

IPT: Isopenteniltransferasa ("Isopentenyltransferase")

ISSR: Microsatélites Anclados ("Inter Simple Sequence Repeat")

MEP: Metileritritol ("Methylerythritol")

MRM: Monitorización Multireacción ("Multireaction Monitoring")

MS: Espectrómetro de Masas ("Mass Spectrometer")

***m*T**: *meta*-Topolina (*"meta*-Topolin")

***m*T7G**: 7-Glucósido de *meta*-Topolina (*"meta*-Topolin 7-Glucoside")

***m*T9G**: 9-Glucósido de *meta*-Topolina (*"meta*-Topolin 9-Glucoside")

***m*TR**: Ribósido de *meta*-Topolina (*"meta*-Topolin Riboside")

MVA: Mevalonato ("Mevalonate")

NAA: Ácido Naftalenacético ("Naphtalen Acetic Acid")

OP: Polinización Abierta ("Open-Pollinated")

***o*T**: *orto*-Topolina (*"ortho*-Topolin")

***o*T7G**: 7-Glucósido de *orto*-Topolina (*"ortho*-Topolin 7-Glucoside")

***o*T9G**: 9-Glucósido de *orto*-Topolina (*"ortho*-Topolin 9-Glucoside")

***o*TR**: Ribósido de *orto*-Topolina (*"ortho*-Topolin Riboside")

PCR: Reacción en Cadena de la Polimerasa ("Polymerase Chain Reaction")

ppb: Porcentaje de Bandas Polimórficas ("Percentage of Polymorphic Bands")

ppc: Porcentaje de Combinaciones Polimórficas ("Percentage of Polymorphic Combinations")

***p*T**: *para*-Topolina (*"para*-Topolin")

***p*TR**: Ribósido de *para*-Topolina (*"para*-Topolin Riboside")

RAPD: ADN Polimórfico Amplificado al Azar ("Random Amplified Polymorphic DNA")

RFLP: Polimorfismo de Longitud de Fragmentos de Restricción ("Restriction Fragment Length Polymorphism")

RI: Índice de Enraizamiento ("Rooting Index")

RL: Restricción/Ligación ("Restriction/Ligation")

SAMPL: Amplificación Selectiva de Microsatélites de *Loci* Polimórficos ("Selective Amplified Microsatellite Polymorphism *Loci*")

SE: Error Estándar ("Standard Error")

SEC: Capacidad de Elongación de Tallos ("Shoot Elongation Capacity")

SSR: Microsatélites ("Simple Sequence Repeat")

***t*Z**: *trans*-Zeatina ("*trans*-Zeatin")

***t*Z7G**: 7-Glucósido de *trans*-Zeatina ("*trans*-Zeatin 7-Glucoside")

***t*Z9G**: 9-Glucósido de *trans*-Zeatina ("*trans*-Zeatin 9-Glucoside")

***t*ZOG**: *O*-Glucósido de *trans*-Zeatina ("*trans*-Zeatin *O*-Glucoside")

***t*ZR**: Ribósido de *trans*-Zeatina ("*trans*-Zeatin Riboside")

***t*ZRMP**: Ribótido de *trans*-Zeatina ("*trans*-Zeatin Riboside 5'-Monophosphate")

***t*ZROG**: Ribósido *O*-Glucósido *trans*-Zeatina ("*trans*-Zeatin Riboside *O*-Glucoside")

UPGMA: Método de Agrupamiento Por Pares No Ponderados con Media Aritmética ("Unweighted Pair-Group Meted with Arithmetic mean")

UPLC: Cromatografía Líquida de Ultra Resolución ("Ultra Performance Liquid Chromatography")

Z: Zeatina ("Zeatin")

ZR: Ribósido de Zeatina ("Zeatin Riboside")

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INTRODUCCIÓN

Imagen modificada de "Lambert, Aylmer Bourke (1803-1807). *A description of the genus* Pinus. *Vol. 1*. Londres (Reino Unido)".

El pino piñonero (*Pinus pinea* L.)

La especie

El pino piñonero (*Pinus pinea* L.) es un árbol de distribución mediterránea con una característica silueta en forma de parasol (Figura 1). Esta conífera, perteneciente a la familia de las pináceas, se caracteriza por ser xerófila, heliófila y relativamente termófila; vive en suelos franco-arenosos pobres, bien drenados y con fuerte sequía estival (Gordo *et al.* 1999).

Figura 1. Silueta característica de *Pinus pinea*.

La distribución natural de *P. pinea* ha estado condicionada por la gestión y manejo del hombre desde época pre-romana, lo que dificulta la definición de sus límites geográficos (Martínez y Montero 2004). En la actualidad se encuentra distribuido por toda la cuenca mediterránea, desde Portugal al Líbano, con poblaciones importantes en Francia, Italia y el Norte de África (Figura 2).

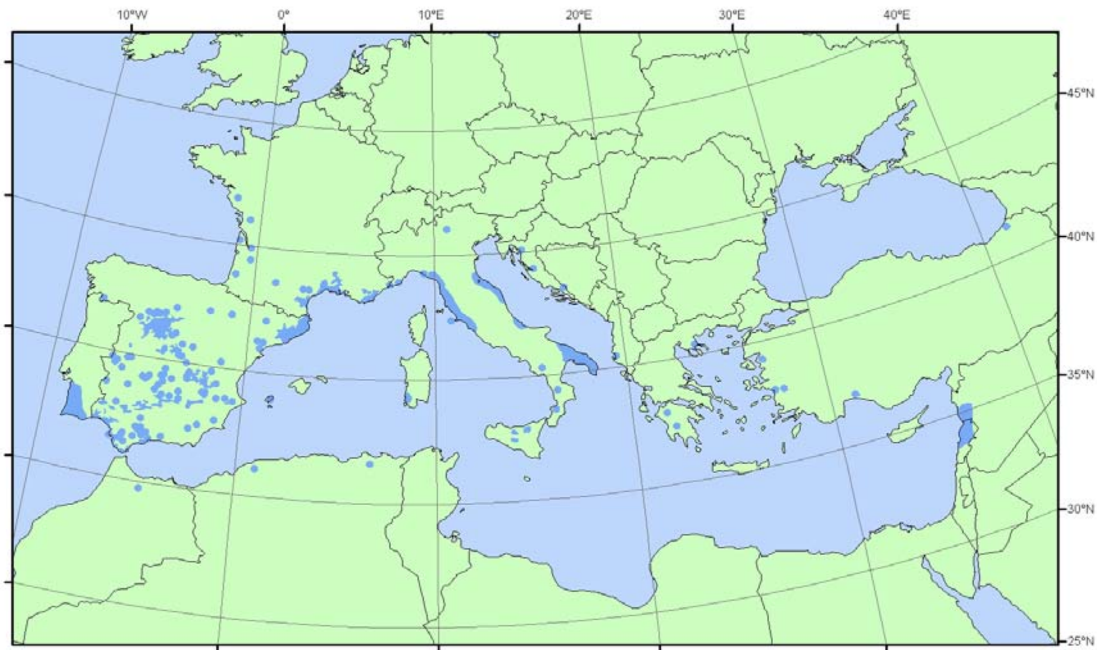

Figura 2. Distribución mundial de *Pinus pinea*. Imagen tomada de Fady *et al.* (2004).

Se puede confirmar la presencia de *P. pinea* en la Península Ibérica desde el Paleolítico (Prada *et al.* 1997), así como su carácter autóctono (Martínez y Montero 2004) a través de los carbones recogidos en los hogares de yacimientos prehistóricos y datos históricos, arqueológicos y paleobiogeográficos (principalmente paleopalinológicos).

En España ocupa una superficie de 391.884 ha (Martín y González 2000), suponiendo dos tercios del área total de su distribución mundial (Mutke *et al.* 2000). En nuestro país, la distribución de esta especie está agrupada en 7 regiones de procedencia, tales como Meseta Norte, Valles del Tiétar y del Alberche, La Mancha, Depresión del Guadalquivir, Sierra Morena, Cataluña Litoral y Cataluña Interior (Prada *et al.* 1997) y 4 procedencias de área restringida (Biar, Sierra de Bogarra, Garrovillas y Marbella) (Figura 3).

A pesar de tratarse de una especie forestal, tiene como aprovechamiento principal sus semillas, puesto que los piñones, de elevado poder energético y nutritivo, poseen un alto valor añadido, aspecto que le

confiere el carácter de árbol "frutal" (Prada *et al.* 1997). España produce el 45% de la cosecha mundial, siendo Estados Unidos el principal importador. Además, en las últimas décadas destaca su papel colonizador y protector del suelo, que se complementa con el aprovechamiento maderable y de piña. En los casos de pinares abiertos constituyen lugares de esparcimiento y recreo de la población urbana (Gordo *et al.* 1999).

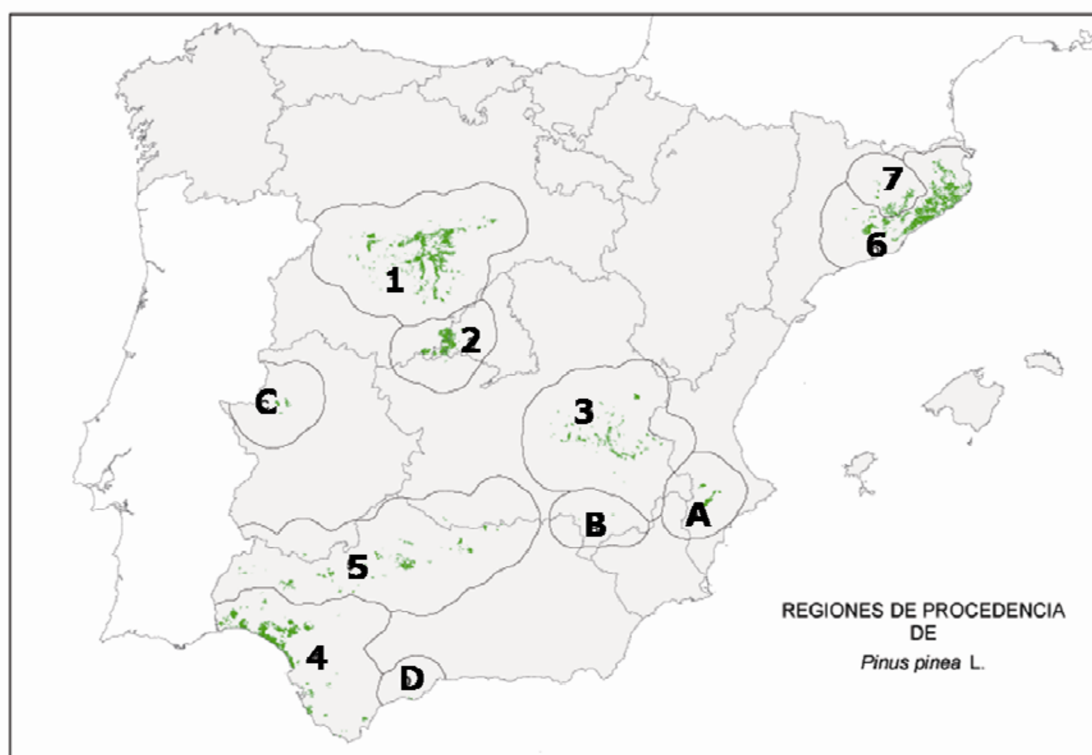

Figura 3. Distribución de *Pinus pinea* en España. Los números indican la región de procedencia: (1) Meseta Norte, (2) Valles del Tiétar y del Alberche, (3) La Mancha, (4) Depresión del Guadalquivir, (5) Sierra Morena, (6) Cataluña Litoral y (7) Cataluña Interior. Las letras indican las procedencias de área restringida: (A) Biar, (B) Sierra de Bogarra, (C) Garrovillas y (D) Marbella. Imagen modificada del Ministerio de Medio Ambiente (http://www.mma.es/images/general/biodiversidad/montes_politica_foresta/pinus_pinea.gif).

Programas de mejora genética y alternativas biotecnológicas

El pino piñonero muestra una gran variación entre individuos para la producción de piña y su rendimiento en piñón (Prada *et al.* 1997). Este aspecto proporciona una interesante oportunidad para el establecimiento de programas de mejora genética con el fin de obtener un incremento en el

rendimiento respecto a estos caracteres. El desarrollo de planes de mejora ha de combinar 3 objetivos generales: (1) Conservar los recursos genéticos en la silvicultura y manejar los bosques regenerados naturales; (2) Mejorar la producción de piña en las áreas más productivas mediante tratamientos específicos; y (3) Definir una línea de reproducción orientada a obtener y propagar genotipos altamente productivos para su uso en nuevas plantaciones y cultivos forestales (Mutke *et al.* 2000).

Dentro del tercer objetivo se ha llevado a cabo el "Programa de Mejora Genética" de Castilla y León, mediante la selección y propagación vegetativa convencional de fenotipos destacados por su alta producción de piña (Catalán 1998), así como promocionando la optimización de un protocolo de micropropagación de árboles seleccionados altamente productivos (Mutke *et al.* 2000). La mejora genética del pino piñonero tiene amplias posibilidades, ya que una vez seleccionados los individuos sobresalientes en lo que se refiere a producción de piña, mediante técnicas de propagación vegetativa podría verse aumentada no sólo su producción, sino también la calidad de la misma. En el marco de los programas de mejora genética, también se han realizado estudios agroforestales que incluyen el desarrollo de modelos fenológicos basados en el análisis de distintos caracteres, tales como elongación de tallo, fenología floral y crecimiento de piña entre otros (Mutke *et al.* 2003), así como el estudio de la relevancia de factores genéticos y ambientales en la producción de piña (Mutke *et al.* 2005).

Sin embargo, debido a la limitación asociada a la propagación vegetativa de las especies forestales, los programas de mejora genética se ven afectados negativamente por los largos tiempos de generación de estas especies y la necesidad de esperar a la fase adulta para poder evaluar la calidad del producto final (Boerjan 2005).

Las tendencias actuales en la mejora genética de especies leñosas están en gran parte dirigidas al desarrollo de métodos y obtención de herramientas que permitan superar estas limitaciones, buscando, por tanto,

alternativas biotecnológicas. El desarrollo de un método de propagación clonal masivo de genotipos seleccionados permitiría el uso adecuado de los recursos genéticos disponibles, incrementando la protección medioambiental y permitiendo la aplicación de métodos de transformación genética para, por ejemplo, obtener árboles resistentes a plagas y enfermedades reduciendo así los costes en pesticidas (López *et al.* 1996).

Micropropagación de pino piñonero: establecimiento del sistema experimental

Multiplicación in vitro*: características y particularidades*

La obtención de individuos genéticamente iguales abre la posibilidad de amplificar progenies derivadas de programas de mejora genética y su comercialización forestal (Miguel *et al.* 2004), realizar ensayos en campo para la evaluación de la influencia ambiental y el genotipo, y establecer un sistema experimental que permita estudiar distintos procesos organogénicos, tales como la inducción caulogénica. La combinación de las técnicas tradicionales de mejora genética con las biotecnológicas más avanzadas permite acelerar el proceso de selección de individuos élite, así como su producción masiva.

Esta multiplicación de individuos *in vitro* se puede realizar mediante la micropropagación, que se subdivide en 3 vías (Thorpe *et al.* 1991): activación y proliferación de yemas axilares, producción de yemas adventicias (organogénesis adventicia) y embriogénesis somática. Se han llevado a cabo diversos intentos de embriogénesis somática en *P. pinea*, aunque sin éxito hasta la fecha (Carneros *et al.* 2007). También se ha abordado la brotación y proliferación de plántulas a partir de meristemos axilares de tallos (Cortizo 2008), punto de partida para el desarrollo de un protocolo de micropropagación de árboles adultos.

El sistema de propagación clonal vía organogénesis adventicia consta de varias etapas (Saborio *et al.* 1997): establecimiento del cultivo e inducción de la formación de yemas, desarrollo y multiplicación de microtallos, enraizamiento de los microtallos obtenidos y aclimatación de las plántulas.

Este sistema se ha empleado en varios trabajos usando como material de partida cotiledones procedentes de semillas de *P. pinea* (García-Férriz *et al.* 1994; Capuana y Giannini 1995; González *et al.* 1998; Sul y Korban 2004); sin embargo, a pesar de las ventajas que este método ofrece, el pino piñonero presenta el inconveniente de ser difícilmente enraizable, lo que limita el éxito de la regeneración de individuos. Además, en cuanto a la selección de los individuos a clonar, existen una serie de dificultades asociadas a la edad de los árboles a propagar, puesto que la transición entre fase de crecimiento vegetativo (juvenil) y reproductivo (adulto) conlleva una pérdida de potencial regenerativo (Greenwood 1995).

Conocimientos previos

El paso previo necesario para cualquier estudio es el establecimiento de un sistema experimental correcto, conociendo y controlando cada una de las variables implicadas. En este sentido, nuestro grupo de investigación ha realizado importantes esfuerzos encaminados a la propagación de esta especie de interés forestal, abordando la multiplicación *in vitro* de *P. pinea* mediante distintas técnicas tales como el microinjerto (Cortizo *et al.* 2004a) y la micropropagación de material juvenil (Valdés *et al.* 2001; Moncaleán *et al.* 2005; Alonso *et al.* 2006) y adulto (Cortizo 2008).

En el caso de la propagación vegetativa a partir de material juvenil, el explanto seleccionado ha sido el cotiledón aislado de embriones maduros de *P. pinea.* Se ensayaron distintos parámetros: (1) El periodo de germinación, demostrándose la relación inversa entre el tiempo de germinación de los embriones y su habilidad para formar tallos (Valdés *et al.* 2001); (2) El

agente inductor de respuesta, siendo la citoquinina (CK) aromática benciladenina (BA) la que permite obtener un mayor número de yemas por cotiledón (Alonso *et al.* 2006); y (3) La concentración y tiempo de exposición a BA aplicada en el medio de cultivo, encontrándose que 44,4 μM de BA durante un periodo de 4-8 d permitía alcanzar niveles de respuesta caulogénica adecuados (Moncaleán *et al.* 2005).

Una vez optimizadas las condiciones de cultivo, se ha establecido un sistema de propagación clonal eficaz para esta especie (Alonso *et al.* 2006), base de los experimentos desarrollados en la presente memoria.

Fidelidad clonal del sistema de propagación

Variación somaclonal y propagación vegetativa

Se define como variación somaclonal a aquella que se observa en plantas regeneradas mediante cultivo *in vitro* (Larkin y Scowcroft 1981). Esta variación puede ser genética (modificaciones en el ADN, heredables e irreversibles), epigenética (variaciones en la expresión del ADN) y fenotípica (cambios en el aspecto fenotípico, reversibles y no necesariamente heredables). Por tanto, la propagación vegetativa de genotipos mediante procesos *in vitro* requiere la evaluación de su estabilidad genética, más aún en coníferas, debido a su largo ciclo de vida, ya que las mutaciones ocasionales pueden detectarse tardíamente durante las distintas etapas del desarrollo del árbol, e incluso durante su floración (Fourré *et al.* 1997; Goto *et al.* 1998).

Dentro de un sistema de propagación *in vitro* existen pasos críticos que incluyen estadíos desdiferenciados tipo callo que pueden derivar en alteraciones tanto fenotípicas como genéticas (Tang y Guo 2001). Además, el empleo de altas concentraciones de reguladores del crecimiento en el medio de cultivo es una fuente de variación genética en plantas micropropagadas (Venkatachalam *et al.* 2007), así como el estrés del

cultivo y la duración del mismo (Larkin 1987; 2004). La confirmación de la estabilidad genética del material clonado es, además, un requisito necesario para el registro legal de variedades vegetales para su uso comercial en sistemas agroforestales (S. Mutke comunicación personal).

Marcadores empleados en la detección de la variación somaclonal

Han sido propuestas varias aproximaciones en la evaluación de la variación somaclonal, tanto a nivel fenotípico como genotípico. Los marcadores fenotípicos requieren exhaustivas observaciones durante tiempos prolongados y dicha variación puede no ser el resultado de una modificación del propio genoma, sino solamente un cambio en su expresión, pudiendo revertir (Fourré *et al.* 1997). Además, hay que considerar que los posibles cambios producidos durante el cultivo *in vitro* pueden no afectar a cambios estructurales del producto génico o a su actividad biológica, con lo que no serán detectados en los caracteres morfológicos y fisiológicos (Palombi y Damiano 2002).

En cuanto a los marcadores genotípicos, las modificaciones cromosómicas (inversiones, deleciones, translocaciones) y génicas (mutaciones) pueden detectarse mediante el uso de marcadores moleculares (Fourré *et al.* 1997). En este sentido, se han desarrollado nuevas y más eficientes herramientas relacionadas con marcadores basados en ADN, tales como ADN Polimórfico Amplificado al Azar (RAPD) (Williams *et al.* 1990), Microsatélites Anclados (ISSR) (Zietkiewicz *et al.* 1994), Microsatélites (SSR) (Tautz 1989), Amplificación de Fragmentos de Longitud Variable (AFLP) (Vos *et al.* 1995) y Amplificación Selectiva de Microsatélites de *Loci* Polimórficos (SAMPL) (Witsenboer *et al.* 1997). Cada marcador molecular posee unas características que los diferencia del resto, constituyendo su selección una de las decisiones más importantes en el diseño experimental (McGregor *et al.* 2000; Bornet y Branchard 2001; Semagn *et al.* 2006).

Los marcadores tipo RAPD son sencillos, rápidos y de bajo coste (Hansen *et al.* 1998), y permiten detectar variación en las secuencias de ADN mediante amplificación al azar de fragmentos cortos del genoma (De Verno *et al.* 1999) (Figura 4).

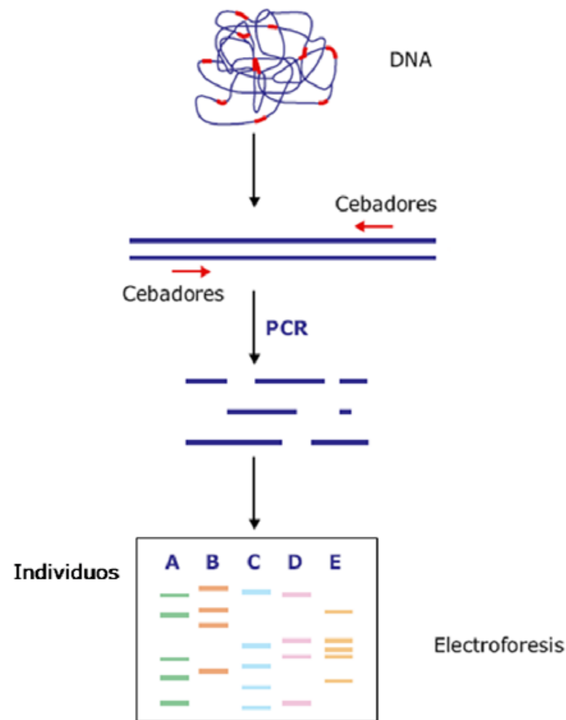

Figura 4. Esquema del marcador RAPD. Tras la extracción del ADN se añaden cebadores cortos que mediante la técnica de la Reacción en Cadena de la Polimerasa (PCR) generan fragmentos de distintos tamaños que se visualizan mediante electroforesis. Imagen modificada de Costa *et al.* (2005).

Estos marcadores han sido muy empleados para verificar la fidelidad genética del material propagado durante cualquier etapa del cultivo, en especies tales como *Pinus thunbergii* (Goto *et al.* 1998), *Picea glauca* (De Verno *et al.* 1999), y *Hagenia abyssinica* (Feyissa *et al.* 2007). Sin embargo, considerando el pequeño tamaño de sus cebadores (10 pares de bases), así como la baja temperatura de alineamiento, son necesarios estrictos controles internos y ensayos previos de reproducibilidad (Semagn *et al.* 2006).

Los marcadores tipo AFLP requieren un mayor número de pasos que los RAPD, siendo una técnica ciertamente laboriosa (Figura 5) pero

altamente fiable y reproducible. Además aporta una mayor información al evaluar una región del genoma mayor y analizar simultáneamente un gran número de *loci* polimórficos (Semagn *et al.* 2006), por lo que han sido empleados para la evaluación de la variación somaclonal en especies como *Quercus suber* (Hornero *et al.* 2001).

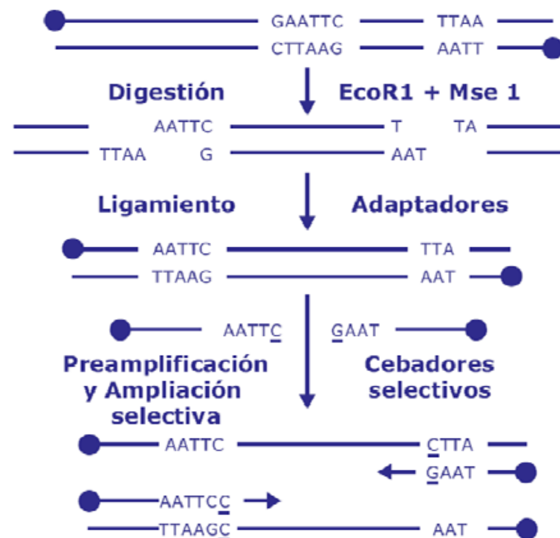

Figura 5. Esquema del marcador AFLP. El ADN aislado es sometido a una digestión con 2 enzimas de restricción y a una ligación con adaptadores de secuencia conocida. Tras 2 procesos de amplificación mediante PCR y empleando cebadores con un número concreto de nucleótidos selectivos, se genera un alto número de fragmentos de distintos tamaños. Imagen tomada de Costa *et al.* (2005).

Los marcadores tipo SSR (Figura 6) son altamente específicos, sin embargo, requieren un conocimiento previo de la secuencia del genoma a amplificar. Por tanto, presentan un uso limitado y aunque cebadores desarrollados para una especie frecuentemente amplifican en especies del mismo género, esto no ocurre en el caso del pino piñonero ya que el uso de marcadores polimórficos descritos para *Pinus taeda* no generan ningún polimorfismo en *P. pinea* (González-Martínez *et al.* 2004).

Basados en este último marcador, se han desarrollado y empleado ampliamente otros que sin requerir información previa del genoma, aprovechan las características hipervariables de las regiones microsatélite (Negi *et al.* 2006). Se trata de los ISSR y SAMPL (Witsenboer *et al.* 1997;

Rakoczy-Trojanowska y Bolibok 2004; Yao *et al.* 2008) que, en realidad, son modificaciones de las técnicas RAPD y AFLP, respectivamente (Negi *et al.* 2006). Ambos marcadores han sido empleados en el estudio de la variación somaclonal (Leroy y Leon 2000; Giménez *et al.* 2005; Thomas *et al.* 2006; Venkatachalam *et al.* 2007), demostrándose que el marcador tipo SAMPL discrimina claramente variantes somaclonales de *Musa* sp. producidas durante el cultivo *in vitro* (Giménez *et al.* 2005).

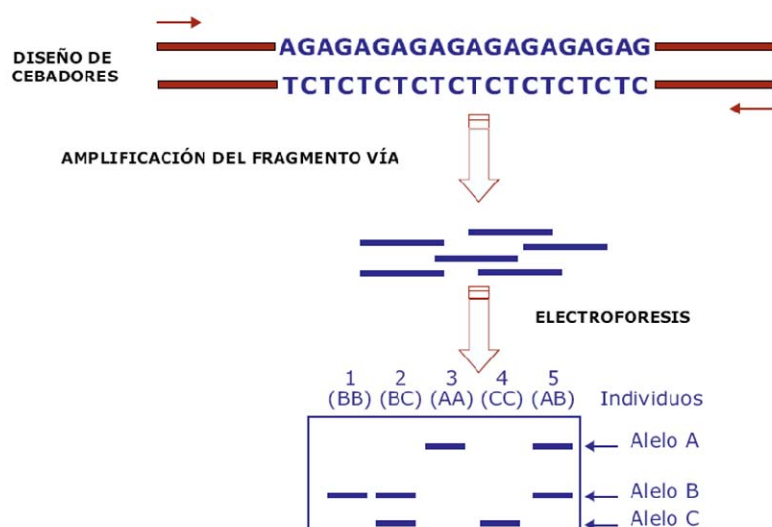

Figura 6. Esquema del marcador SSR. El ADN es amplificado mediante PCR empleando cebadores diseñados en base al conocimiento previo de regiones del genoma. Tras su visualización en electroforesis capilar, gel de agarosa o acrilamida, se obtiene un patrón de bandas para cada muestra, lo que permite identificarlas inequívocamente. Imagen tomada de Costa *et al.* (2005).

Variabilidad genética en coníferas: antecedentes en el pino piñonero

Las coníferas se han caracterizado por una alta variabilidad genética, ya que debido al sistema de reproducción, con tasas de polinización cruzada muy altas y una gran capacidad de dispersión, son especies muy heterozigóticas (Martín y González 2000). A pesar de esta asunción, estudios previos con diversos marcadores en varias especies de pinos como *P. resinosa* (Mosseler *et al.* 1991; 1992) y *P. squamata* (Zhang *et al.* 2005), han mostrado un bajo nivel de variabilidad.

Los escasos estudios acerca de la diversidad genética de la especie *P. pinea* destacan la excepcionalmente baja variabilidad genética asociada, ya sea utilizando isoenzimas (Fallour *et al.* 1997), microsatélites nucleares y cloroplásticos (Martín y González 2000; Gómez *et al.* 2002; González-Martínez *et al.* 2004; Vendramin *et al.* 2008) o marcadores tipo RAPD (Evaristo *et al.* 2002). Este fenómeno se ha explicado como consecuencia de la difusión antropogénica de material reproductivo genéticamente homogéneo (Fallour *et al.* 1997). Mediante microsatélites de cloroplasto se ha comprobado recientemente (Vendramin *et al.* 2008) la baja diversidad genética de esta especie a lo largo de toda su distribución, con casi una total ausencia de variación.

Estudios en genética cuantitativa (Mutke *et al.* 2003; 2005) han determinado los clones más prometedores para propagar masivamente; sin embargo, no se podrá solicitar su reconocimiento legal como materiales de base para producción de material de reproducción o como obtención vegetal (variedad) hasta que no haya marcadores que identifiquen fehacientemente cada genotipo (S. Mutke comunicación personal). No obstante, hasta el momento la estabilidad genética de los individuos clonados de pino piñonero obtenidos mediante distintos sistemas de propagación vegetativa nunca ha sido evaluada.

Bases fisiológicas de la inducción caulogénica

Citoquininas

Las CKs son un grupo de fitohormonas implicadas en el control del desarrollo de las plantas (Novák *et al.* 2008). Desde su descubrimiento como factores que estimulan la división de las células vegetales (Miller *et al.* 1955), se ha demostrado su participación en otros muchos procesos fisiológicos, como la senescencia de la hoja, la movilización de nutrientes, la dominancia apical, la formación y actividad de los meristemos del ápice caulinar, el desarrollo floral, la ruptura de la dormición de las yemas y la

germinación de la semilla. Además, regulan muchos procesos celulares, destacando el control de la división celular (Taiz y Zeiger 2006).

Las CKs naturales conocidas son derivados de la base púrica adenina con un sustituyente, de naturaleza isoprenoide o aromática, en el nitrógeno amínico de la posición 6. Según dicho sustituyente se consideran 2 grandes clases (Segura 2008): las CKs isoprenoídicas, que incluyen las familias de la isopenteniladenina (iP), la zeatina (Z) y la dihidrozeatina (DHZ); y las CKs aromáticas, que comprenden las familias de la BA y las *orto*/*meta*/*para*-hidroxibenciladeninas, comúnmente llamadas topolinas (*o*T, *m*T y *p*T).

Las CKs pueden encontrarse en las plantas como bases libres o formando conjugados con diversos compuestos químicos que se unen al anillo de purina o a la cadena lateral. Las principales formas conjugadas de las CKs son los ribósidos (conjugación con una ribosa en la posición 9 del anillo de purina), los ribótidos (el ácido ortofosfórico se esterifica en posición 5' con el correspondiente ribósido) y los glicósidos (conjugación con un resto de glucosa, ya sea en el anillo (*N*-glucósidos) o en el grupo hidroxilo de la cadena lateral (*O*-glucósidos)) (Segura 2008) (Figura 7).

Dentro de los tejidos vegetales, las bases libres constituyen una pequeña parte del total de estas fitohormonas, ya que son rápidamente metabolizadas y convertidas en sus respectivas formas ribósido y ribótido (Taiz y Zeiger 2006).

Las CKs isoprenoídicas son los componentes mayoritarios descritos en las CKs naturales. Sin embargo, también han sido descritas CKs aromáticas naturales como el ribósido de BA (BAR) en *Pimpinella anisum* (Ernst *et al.* 1983), la BA en agallas de *Lycopersicon esculentum* (Nandi *et al.* 1989), y la *m*T en *Populus*×*canadiensis* (Strnad *et al.* 1992).

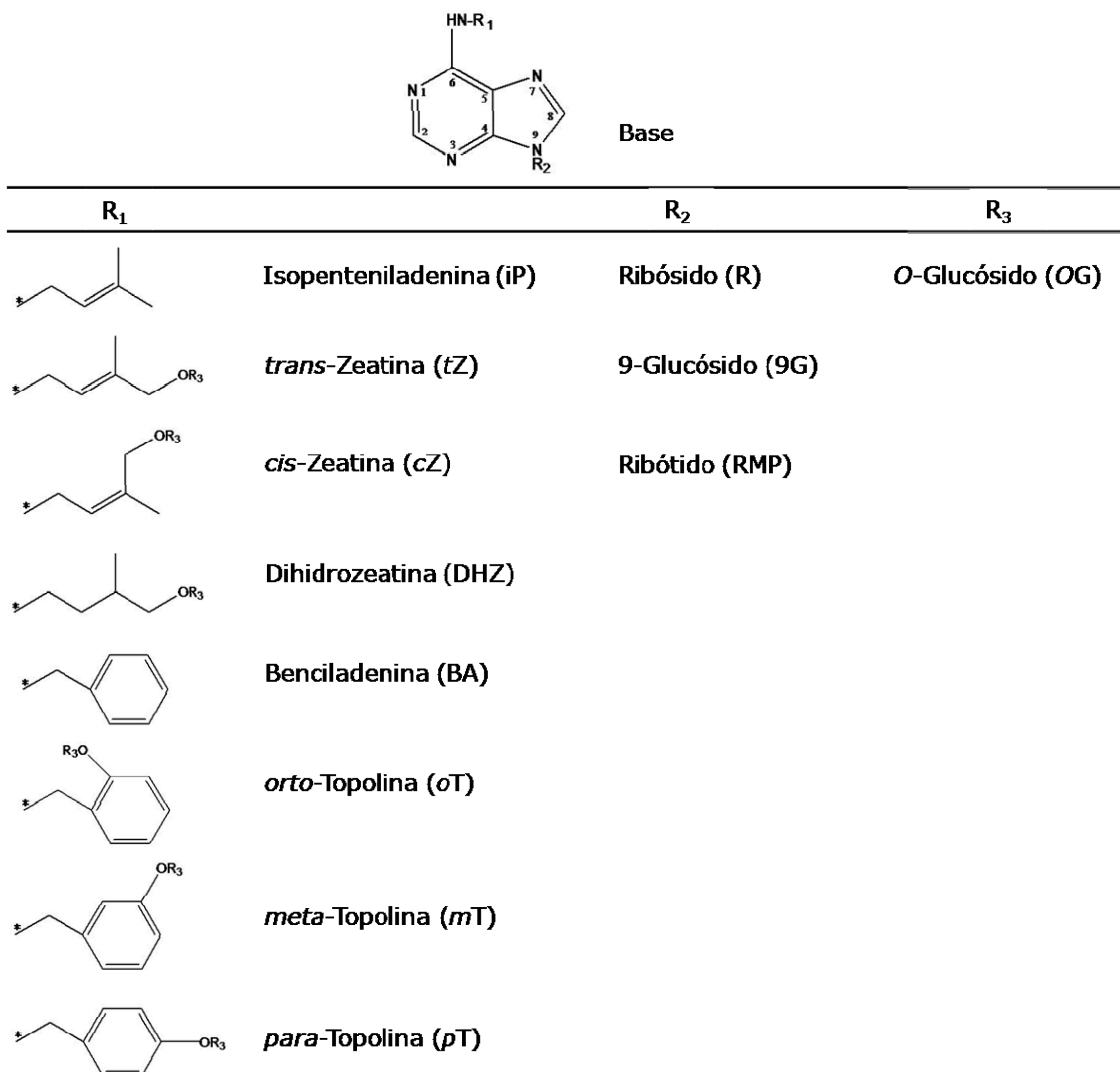

Figura 7. Estructuras, nombres y abreviaturas de las principales CKs naturales.

La mayor parte de los trabajos referidos a la biosíntesis y metabolismo de este grupo de fitohormonas se centran casi exclusivamente en el tipo isoprenoide (Åstot *et al.* 2000; Sakakibara 2006), proponiéndose 2 rutas de biosíntesis independientes (Figura 8).

Una de estas rutas, conocida como isopenteniladenina ribótido (iPRMP)-dependiente, ya que este compuesto sería el primer intermediario de la ruta de síntesis (Kakimoto 2001), comienza con la N^6-prenilación de la adenosina 5'-fosfato (AMP, ADP o ATP) con dimetilalil difosfato (DMAPP), obteniéndose los ribósidos mono, di y trifosfato de iP, y catalizada por el enzima isopenteniltransferasa (IPT). La otra ruta sería iPRMP-

independiente, también catalizada por el enzima IPT e implicaría como primer intermediario el ribótido de *trans*-Zeatina (*t*ZRMP) (Åstot *et al.* 2000).

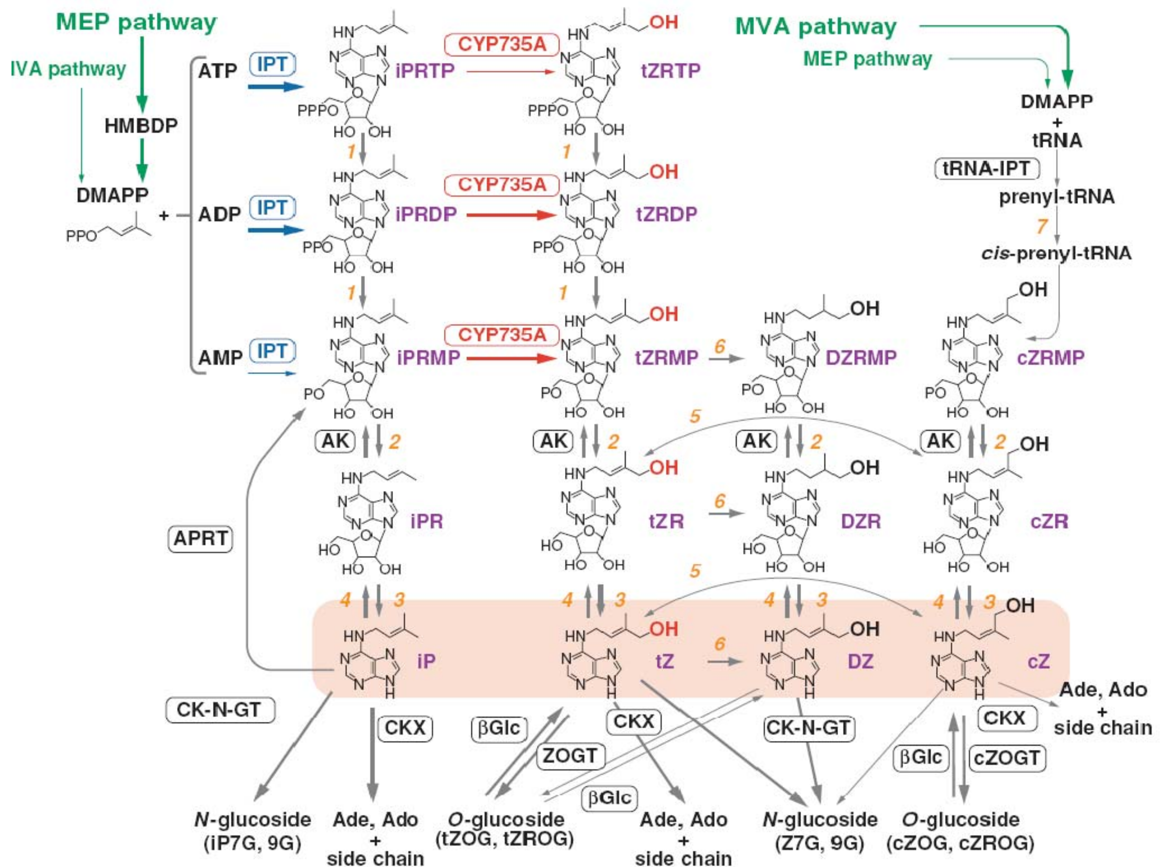

Figura 8. Modelo actual de biosíntesis y metabolismo de las CKs isoprenoídicas en *Arabidopsis thaliana*. En la parte izquierda (MEP: metileritritol) se presenta la ruta iPRMP-dependiente, mientras que en la parte derecha (MVA: mevalonato) se muestra la ruta iPRMP-independiente. Imagen tomada de Sakakibara (2006).

Las cadenas laterales de las CKs isoprenoídicas y aromáticas son muy diferentes entre sí y considerando que los enzimas conocidos implicados en el metabolismo de la BA son comunes al metabolismo de las purinas (Van Staden y Crouch 1996), probablemente la ruta biosintética de las CKs aromáticas sea diferente (Ivanova *et al.* 2006) y más próxima a la de las purinas, habiéndose propuesto como compuesto de partida el aminoácido fenilalanina (Zažímalová *et al*. 1999). Recientemente Taylor *et al.* (2003)

han propuesto como precursor compuestos monoterpenoides, lo que supondría entonces que las topolinas serían precursores de la BA.

En cuanto a los mecanismos moleculares básicos de la recepción y transducción de señal de CKs, ha sido en los últimos años cuando han comenzado a dilucidarse (Fernández *et al.* 2006), centrándose casi exclusivamente en las CKs isoprenoídicas (Brenner *et al.* 2005; Ferreira y Kieber 2005; Hwang y Sakakibara 2006; To y Kieber 2008) (Figura 9). Estos estudios indican que el modelo de percepción y transducción de las señales es similar a los sistemas de señalización de dos componentes de las bacterias.

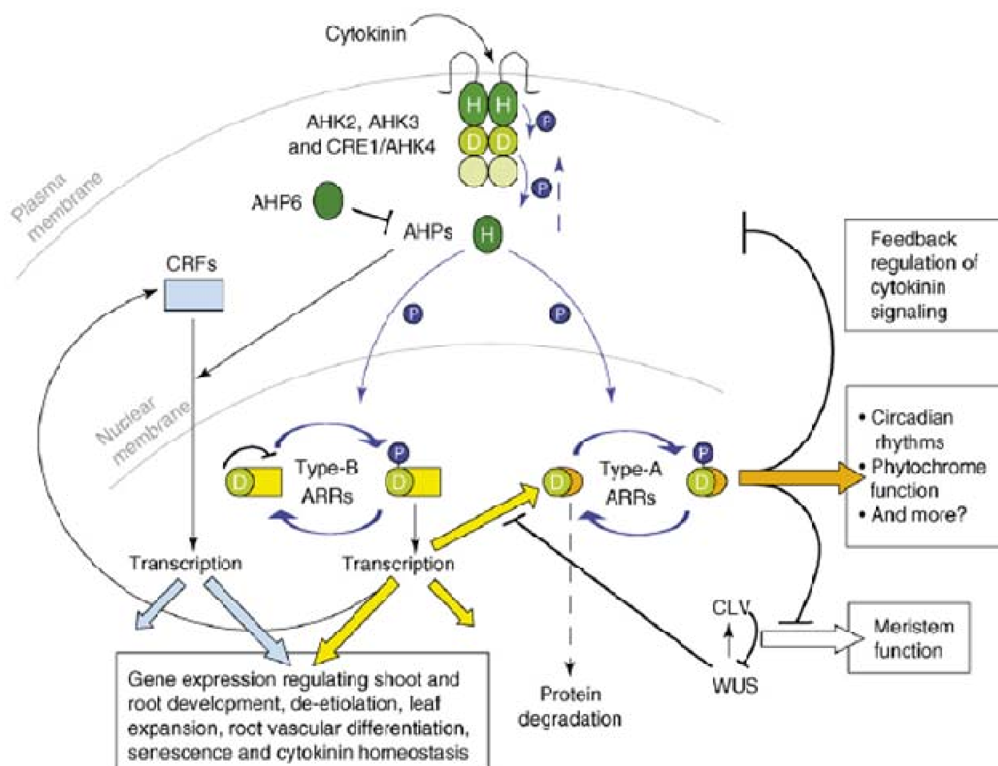

Figura 9. Modelo de recepción y transducción de señales de 2 componentes en CKs. AHK: Histidin-quinasa de *A. thaliana*, AHP: Proteína Fosfotransportadora de *A. thaliana*, ARR: Reguladores de Respuesta de *A. thaliana.* Imagen tomada de To y Kieber (2008).

De modo general, el receptor histidin-quinasa (AHK) situado en la membrana plasmática recibe la señal de la CK. La unión de dicha CK induce la autofosforilación del receptor, transfiriendo el fosfato a unas proteínas de

transmisión de fosfatos (AHP). Estas proteínas fosforiladas son importadas al núcleo, donde transfieren el fosfato a los genes reguladores de respuesta (ARR), responsables de la activación de las proteínas y los genes implicados en la respuesta a las CKs.

Sin embargo, aún queda por conocer el mecanismo preciso de la percepción y transducción de señales de las CKs aromáticas y su relación con las CKs isoprenoídicas. De hecho, ya en 1997 Strnad apunta que los receptores correspondientes a las CKs aromáticas no son simples formas alternativas de una misma señal.

Citoquininas e inducción caulogénica en el pino piñonero

La aplicación exógena de CKs en tejidos cultivados *in vitro* ha sido ampliamente estudiada, al desempeñar un papel crucial en la regeneración de tallos adventicios (Krikorian 1995). En este sentido, la BA es una de las más empleadas, apoyado por distintos estudios que demuestran su alta capacidad de inducción organogénica (Caboni *et al.* 2002; Alonso *et al.* 2006).

La respuesta organogénica está relacionada con la aplicación exógena de BA, influyendo en la intensidad de respuesta tanto su concentración como el tiempo de exposición (Lampugnani *et al.* 1981; Valdés *et al.* 2001; Centeno *et al.* 2003). Además, la caulogénesis *in vitro* incluye complejos y dinámicos mecanismos de regulación, que implican tanto a las CKs exógenamente aplicadas como a las presentes en los tejidos (Auer *et al.* 1999), habiéndose estudiado la absorción y metabolismo de BA y su influencia sobre los niveles endógenos de otras CKs y auxinas (Centeno *et al.* 1996; Auer *et al.* 1999; Charrière *et al.* 1999). Por ello, la respuesta obtenida es una mezcla del estímulo recibido, su efecto *per se* y su influencia sobre los componentes endógenos del propio tejido (Krikorian 1995).

El establecimiento de un sistema de micropropagación vegetativa en cotiledones maduros de *P. pinea* ha sido la base de estudios relacionados con el periodo de inducción caulogénica. Se caracterizó la dinámica del agente inductor de respuesta, BA, al aplicarlo exógenamente en el medio de cultivo a una concentración de 4,4 µM durante 35 d (Moncaleán *et al.* 2005). Los resultados obtenidos apuntaban que la organogénesis seguía el modelo propuesto por Christianson y Warnick (1983; 1984), distinguiéndose 3 fases: adquisición de competencia, determinación caulogénica y diferenciación.

También se conoce la relación entre la absorción de BA, el periodo de germinación y la pérdida de competencia organogénica por parte de los cotiledones pregerminados (Cortizo 2004b). Tras pregerminar embriones maduros de *P. pinea* durante 2 y 4 d, se cultivaron en medio de inducción caulogénica, analizando la absorción y metabolismo de BA. En dichos materiales no se observó el pico de absorción detectado durante el periodo de cultivo para material no pregerminado (Moncaleán *et al.* 2005), y sí niveles endógenos de BA mucho menores (de 2 a 4 veces).

Tras el estudio de las CKs naturales isoprenoídicas en las porciones apicales y basales de los cotiledones pregerminados (Valdés *et al.* 2001), se ha asociado la pérdida de capacidad de formación de tallos adventicios con un descenso en las CKs endógenas tipo Z. Además, en el proceso de inducción caulogénica estudiado por Moncaleán *et al.* (2005) se han relacionado cantidades elevadas de Z con una mayor respuesta organogénica.

En cuanto a la percepción y transducción de señales de CKs, Alonso *et al.* (2007), mediante la realización de una librería sustractiva de cDNA, han identificado al menos 30 genes candidatos de expresión temprana. Recientemente Cortizo (2008) ha estudiado la expresión diferencial de 2 genes implicados en la respuesta temprana a CKs, uno de los cuales está involucrado en la formación de meristemos.

Planteamiento y objetivos del trabajo

En el área de Fisiología Vegetal del Departamento de Biología de Organismos y Sistemas de la Universidad de Oviedo, bajo el marco del proyecto de investigación "Clonación *in vitro* de *Pinus pinea* L. como aplicación biotecnológica para sus programas de mejora genética" (AGL2002-00867), se planteó el establecimiento de cadenas de propagación vegetativa mediante alternativas biotecnológicas que permitiesen llevar a cabo programas de mejora genética del pino piñonero, proponiendo como vías de regeneración la embriogénesis somática (llevada a cabo en el Instituto Madrileño de Investigación Agraria y Alimentaria –IMIA); el desarrollo de microinjertos (Cortizo *et al.* 2004a) y la organogénesis adventicia. Dentro de esta última vía, se desarrolló un protocolo de clonación *in vitro* a partir de material juvenil de *P. pinea*, fijándose el medio de cultivo más adecuado y la concentración y tiempo de aplicación óptimos de la citoquinina BA durante la fase de inducción caulogénica (Moncaleán *et al.* 2005; Alonso *et al.* 2006).

Considerando los antecedentes previamente expuestos, esta tesis presenta 2 objetivos generales, uno de carácter aplicado y otro básico:

1.- Optimizar un protocolo de micropropagación para las familias seleccionadas de *P. pinea* (entendiendo por "familia" el conjunto de semillas que comparten parental femenino, es decir, que tienen un origen genético materno común), evaluando la estabilidad genética de los individuos obtenidos tras la multiplicación *in vitro*. Dicho protocolo permitirá amplificar progenies derivadas de programas de mejora genética para su comercialización forestal y realizar ensayos de campo para la evaluación de la interacción genotipo-ambiente.

2.- Estudiar las bases fisiológicas del proceso caulogénico que tiene lugar durante la micropropagación de dichas familias de *P. pinea*, centrándonos en aquellas que ante los mismos tratamientos manifiestan respuestas morfogénicas de diferente magnitud. El tratamiento de los datos obtenidos

facilitará el avance en el conocimiento de las bases fisiológicas y bioquímicas de los procesos organogénicos inducidos en esta especie, lo que permitirá ahondar en los mecanismos implicados en dichos procesos y establecer una relación entre el contenido hormonal, su base genética y la respuesta organogénica inducida *in vitro*.

Los objetivos parciales de este trabajo son los siguientes:

Clonar *in vitro* 6 familias seleccionadas de pino piñonero, optimizando las condiciones de cultivo y seleccionando aquellas que manifiesten una mayor y menor respuesta caulogénica y/o rizogénica.

Caracterizar el material clonal obtenido mediante el empleo de distintos marcadores moleculares.

Establecer el periodo mínimo de inducción requerido por el material vegetal empleado para obtener una respuesta organogénica.

Estudiar la absorción y metabolismo del agente inductor de respuesta BA durante el periodo de cultivo, posible base de las diferencias detectadas entre las familias seleccionadas de *P. pinea*.

Analizar los niveles endógenos de CKs (isoprenoides y aromáticas) durante el periodo de inducción, relacionándolos con las diferentes capacidades organogénicas de las familias de *P. pinea*.

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CAPÍTULO 1

Clonal micropropagation of six selected half-sibling families of *Pinus pinea* L. and somaclonal variation analysis

Imagen modificada de "Lambert, Aylmer Bourke (1803-1807). *A description of the genus* Pinus. *Vol. 1*. Londres (Reino Unido)".

Clonal micropropagation of six selected half-sibling families of *Pinus pinea* L. and somaclonal variation analysis

Summary

Organogenic response of six selected half-sibling families of stone pine (*Pinus pinea* L.) was evaluated, and showed genotype-dependent behaviour. The caulogenic phase was characterized by high values of Survival and Organogenesis, while the rooting phase (the bottleneck of many coniferous species) showed great variability among families. Provenance influence was also studied, and the rhizogenesis protocol was optimized for the selected families. The highest values were obtained with family 36, with 100% of Organogenesis, a Bud Formation Capacity (BFC) Index of 6.54 and 38.44% Rooting Rate; on the other hand, family 61 presented the worst results, with 83.64% Organogenesis, a BFC Index of 3.01 and 29.69% Rooting Rate. As a result of these results, both families were used in further experiments looking for the underlying bases of the different organogenic behaviour of both families under the same culture conditions.

In addition to this, and for the first time in this species, Random Amplified Polymorphism DNA (RAPD) analysis was carried out to determine whether somaclonal variation had occurred. The results suggested an absence of variation during the whole *in vitro* process, although more thorough studies would be required for a conclusive answer.

Este capítulo se corresponde con la publicación "Cuesta, C., Ordás, R.J., Fernández, B., Rodríguez, A. (2008). Clonal micropropagation of six selected half-sibling families of *Pinus pinea* and somaclonal variation analysis. *Plant Cell Tissue and Organ Culture*, 95: 125-130".

Introduction

In vitro clonal propagation is a suitable system with the potential for eventual mass multiplication of superior and genetically engineered forest tree genotypes, in both coniferous and hardwood species (Tang 2001); as long as highly efficient and reproducible vegetative propagation methods can be developed for economically important species (Abdullah *et al.* 1985; Bergmann and Stomp 1992; Kulchetscki *et al.* 1995; Mathur and Nadgauda 1999; Caboni *et al.* 2002). In addition, *in vitro* clonal multiplication of selected families or superior genotypes allows the maximum genetic gain achieved in breeding programmes to be exploited (Miguel *et al.* 2004).

Stone pine (*Pinus pinea* L.) is a typical Mediterranean species occupying an area of about 400,000 ha in Spain (more than 50% of the total worldwide distribution area) (Nanos *et al.* 2003), whose pine nuts are highly appreciated, and is currently used in reforestation programmes. Therefore, a Spanish breeding programme in "Castilla y León" included this species (Mutke *et al.* 2000), focusing on the optimization of a micropropagation procedure for high-producing trees. To date some attempts at somatic embryogenesis have been carried out without, however, any conclusive results (Carneros *et al.* 2007). Several reports describe protocols of micropropagation of this species based on the use of cotyledons as explants (García-Férriz *et al.* 1994; Capuana and Giannini 1995; González *et al.* 1998; Sul and Korban 2004), all of which highlight the major obstacle during its micropropagation as the great difficulty in rooting. Even the best rate described for this species, nearly 70% of rooted plantlets (Alonso *et al.* 2006), is markedly inferior to the 98% achieved by Dumas and Monteuuis (1995) for *P. pinaster*.

It is also necessary to confirm the genetic stability during the whole *in vitro* tissue culture process, as some *in vitro* steps involve a dedifferentiated callus phase, resulting in widespread genetic and phenotypic alterations (Tang and Guo 2001). For this reason the

development of methods to rapidly screen regenerated plantlets for genetic changes by applying molecular markers such as Random Amplified Polymorphism DNA (RAPD) would be valuable (Tang 2001). There are many reports of the use of such markers for detecting somaclonal variation in conifers (Fourré *et al.* 1997), especially Pinaceae (De Verno and Mosseler 1997; Goto *et al.* 1998). A somaclonal variation analysis of regenerated plantlets of stone pine has so far not been carried out, and RAPD analysis would be valuable.

The aim of this chapter was to test the organogenic response (caulogenesis and rooting phases) of six selected half-sibling families of *P. pinea*, establishing an appropriate propagation protocol for the assayed families, and thus developing a suitable experimental system for exploring the causes of differential organogenic responses of families. Furthermore, we attempted to determine if somaclonal variation occurs during the whole *in vitro* period.

Materials and methods

Plant material

Three-year-old stone pine seeds from six half-sibling and productive selected families were used. These families belong to "Meseta Norte" and "Cataluña Litoral" provenances (B23PH4 (families 44; 61; 78; 93) and B23PH1 (families 36; 89), respectively) and were provided by "Centro Nacional de Mejora Genética Forestal, Puerta de Hierro" (Spain).

Micropropagation protocol and culture conditions

Following the Alonso *et al.* (2006) procedure, seeds were surface sterilized by immersion in 7.5% H_2O_2 for 45 min followed by an imbibition

step of 48 h at 4 ºC in darkness to facilitate dissection of embryos. Cotyledons were excised from embryos and placed horizontally for 6 d in bud induction medium consisting of Le Poivre medium as modified by Aitken-Christie *et al.* (1988) with half strength macroelements (½LP) and supplemented with sucrose and 44.4 µM of benzyladenine (BA). One seed, comprised of 10 to 12 cotyledons, was placed in each baby jar. After this culture period (6 d), cotyledons were transferred to a hormone-free medium supplemented with 0.5% (w/v) of activated charcoal (½LPC), promoting the development of bud primordia into shoots with primary needles (Figure 1). Survival and Organogenesis data were estimated per seed after 35 d. Cotyledons were subcultured to fresh medium and, after 70 d, the organogenic response per cotyledon was evaluated.

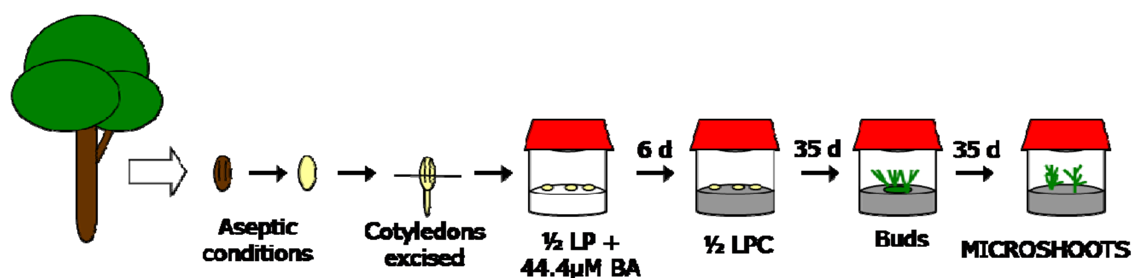

Figure 1. Caulogenic induction of *Pinus pinea* cotyledons, including cotyledon excision, induction culture for 6 d with 44.4 µM of BA, and a 70 d-period of subculturing in ½LPC, obtaining isolated microshoots ready to be rooted.

The caulogenic step took place in baby jars (130 mL) capped with magenta® caps (Sigma, St. Louis, Mo.) containing 10 mL of culture medium, and maintained in a growth chamber at 25 ± 1 ºC with a 16-h photoperiod under a photosynthetic photon flux of 80 ± 5 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TLD 58 W/33, Philips, France).

The indices applied per cotyledon were the percentage of Cotyledons with Caulogenic Response (% CCR= [(% surviving cotyledons) x (% bud-forming cotyledons)]/100), Bud Formation Capacity Index (BFC= [(average number of buds per cotyledon) x (% cotyledons forming buds)]/100) and

Shoot Elongation Capacity Index (SEC= [(average number of shoots >10 mm) / (% cotyledons forming buds)] x 100) according to Capuana and Giannini (1995), and extensively used by many authors (López *et al.* 1996; Saborio *et al.* 1997; Mathur and Nadgauda 1999). These indices allow the presentation of a measurement of the efficiency of a given treatment (in this case "family" treatment) by combining the number of buds or developing shoots formed with the number of responding explants (Kulchetscki *et al.* 1995).

In the next subculture (70 d after induction time), buds were excised from the cotyledonary explants to enhance shoot development, and elongated by subculturing into ½LPC every 2 weeks over a variable period (1-3 subcultures) until they reached 2 cm (Figure 2). Once the microshoots achieved this length, they were ready to be rooted. Alternatively, shoots could be micropropagated by sequential subculturing on ½LPC, according to Alonso *et al.* (2006).

Root growth phase was performed after Alonso *et al.* (2006), applying as plant growth regulator 10 µM of naphtalen acetic acid (NAA) and using glucose instead of sucrose as a carbon source (Figure 2); in addition to this, we retested the macroelement strengths in order to optimize the rooting results.

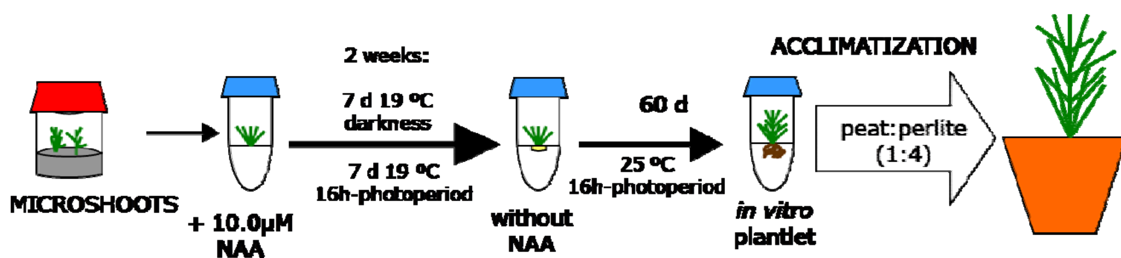

Figure 2. Rhizogenesis protocol of *Pinus pinea* microshoots, including 2 weeks under rooting induction conditions (10.0 µM of NAA), and a culture period without NAA for 60 d to obtain stone pine plantlets and the final acclimatization in greenhouse.

Rooting phase was assayed in culture tubes (25x150 mm, Sigma, St. Louis, Mo.) with 10 mL of medium observing the following environmental conditions: the induction step consisted on one week in the dark at 19 ºC and another week with 16-h photoperiod under a photosynthetic photon flux of 100 ± 5 µmol m^{-2} s^{-1} at the same temperature. The rooting expression step was maintained in a growth chamber at 25 ± 1 ºC with the above-mentioned light conditions.

Three macroelement strengths (1; ½ and ¼) were assayed for induction and expression steps, so nine combinations were applied in at least ten microshoots of each family. After choosing the suitable strength combination, the 6 families were cultured under the same conditions for 2 weeks with the inductor regulator (induction phase). After 60 d in free-hormone medium (expression step), data were collected (% survival, % Microshoots with Calli, % Shoots that Rooted, and Number of Roots per Shoot). Rooting Index (RI) was also calculated (RI= % survival x % rooting, expressed as so much per one), because it combines survival and percentage of rooted shoots data, giving a value close to the real rooting capacity. A suitable way of summarizing the organogenic process and its global efficiency is combining % CCR and RI, so this general index was calculated.

For acclimatization, the rooted shoots were transferred to a peat-perlite mixture (1:4, v/v) and grown under relatively high humidity (95%) in a greenhouse (Figure 2); after 2 weeks plants were transferred to greenhouse humidity conditions. Survival rate of regenerated plants was determined 8 weeks after planting, and plantlets were maintained in the greenhouse.

Statistical analysis of in vitro *data*

A single factor completely randomized experimental design with subsampling was applied according to Compton (1994). A minimum of 7 seeds (10-12 cotyledons per seed) per family and experiment were assayed, allowing comparisons within clone and within- and among families. Experiments were repeated at least 3 times, and data are presented as means ± standard error (SE). The statistical analysis of frequencies (% Survival, % Organogenesis, % CCR, % Calli Presence, % Rooted Shoots, RI) was carried out with the χ^2 and Fisher Exact tests. Quantitative data (BFC, SEC, Buds per Cotyledon, and Number of Roots per Shoot) were analyzed using the parametric tests of Analysis of Variance (ANOVA), *t*-Student and Student-Newman-Keuls. All statistical tests were performed at the 5% confidence level with SIGMASTAT® software.

DNA extraction and RAPD analysis

RAPD analysis was performed on needles from 55 regenerated plantlets that had been maintained in a greenhouse for a period of two years. This involved a number of clonal explants per seed that ranged from 3 (in the case of such low-regenerating families like family 61) to 10 (in high-producing families like family 36).

DNA was isolated applying an extraction method based on Cetyl Trimethylammonium Bromide (CTAB) buffer (Doyle and Doyle 1987) with slight modifications, and parallel extractions from some random selected samples were developed to test reproducibility. DNA concentration was measured on a spectrophotometer (Beckman-Coulter DU800®). Twenty primers were used for RAPD analysis (set A) from Operon Technologies Inc. (Alameda, California). DNA amplification reactions were performed as described in Evaristo *et al.* (2002) with slight modifications, always running

in the same thermocycler (Gene Amp PCR System 9700, Applied Biosystems).

In order to prevent unspecific patterns of bands and to guarantee reproducibility, two different annealing temperatures were assayed (35 and 40 °C). The amplification products were resolved by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and then photographed on a UV transilluminator. Amplified combinations were repeated at least twice, internal controls were included (replicates of the same sample in each combination and different DNA extractions of the same individual), and only the consistently reproducible and distinguished bands were considered. In all cases lambda phage DNA digested with *Eco*RI and *Hin*dIII was used as a size marker.

Results

Bud induction and shoot development

Cotyledon establishment did not show any contamination, and adventitious buds were distributed along the cotyledon surface. Caulogenesis was direct, with no callus formation. Bud induction was synchronized, but their development was asynchronous and no hyperhydricity or plagiotropic growth phenomena were detected.

Different levels of caulogenesis were recorded (Table 1), related not only to the provenance (except for survival results) but also to the family. The provenance results showed significant differences, "Cataluña Litoral" being superior in all cases (e.g. 92.54% CCR of "Cataluña Litoral" provenance vs. 88.96% CCR in "Meseta Norte" individuals).

Regarding the family data (Table 1), the modified method reported here resulted in a high survival and organogenic response, even scoring 100% Organogenesis in family 36. The % CCR was also high, showing

significant differences among the 6 families, mainly between families 36 and 61 (98.07 and 76.92% respectively).

Table 1. Caulogenic response of *Pinus pinea* by family and by provenance. Values are means ± SE (*n*=14). Means followed by the same letters within columns are not significantly different at $P \leq 0.05$ according to χ^2 and Fisher Exact test (% Survival, % Organogenesis, % CCR) and parametric tests ANOVA, *t*-Student and Student-Newman-Keuls (Buds per Cotyledon, BFC).

Families	% Survival ± SE[1]	% Organogenesis ± SE[1]	% CCR ± SE	Buds per Cotyledon ± SE[2]	BFC ± SE[2]
36	98.07 ± 1.06 a	100.00 ± 0.00 a	98.07 ± 1.06 a	6.54 ± 0.36 a	6.54 ± 0.36 a
44	96.43 ± 1.58 ac	96.26 ± 2.17 ac	92.82 ± 2.59 ac	4.71 ± 0.39 b	4.53 ± 0.38 b
61	91.97 ± 2.96 b	83.64 ± 5.65 b	76.92 ± 5.76 b	3.56 ± 0.32 b	3.01 ± 0.35 c
78	89.51 ± 3.87 bd	94.55 ± 1.97 bc	84.63 ± 4.06 bd	3.83 ± 0.41 b	3.63 ± 0.40 b
89	92.84 ± 2.87 cd	94.30 ± 2.57 c	87.55 ± 3.61 cd	4.91 ± 0.48 b	4.72 ± 0.51 b
93	98.76 ± 0.88 a	99.29 ± 0.74 a	98.06 ± 1.14 a	5.02 ± 0.40 b	4.98 ± 0.40 b

Provenances	% Survival ± SE[1]	% Organogenesis ± SE[1]	% CCR ± SE	Buds per Cotyledon ± SE[2]	BFC ± SE[2]
Cataluña Litoral	95.35 ± 1.60 a	97.05 ± 1.40 a	92.54 ± 2.89 a	5.70 ± 0.34 a	5.6 ± 0.37 a
Meseta Norte	94.62 ± 1.20 a	94.02 ± 1.57 b	88.96 ± 2.61 b	4.35 ± 0.21 b	4.1 ± 0.22 b

[1]: data taken at 35 d. [2]: data taken at 70 d.

As Figure 3A-B shows, family 36 reached the highest level of bud formation (BFC value of 6.54), while the lowest was recorded for family 61 (3.01), confirming significant differences (Table 1).

Figure 3. Buds formed by adventitious shoot organogenesis on *Pinus pinea* cotyledons after 70 d of *in vitro* culture. Filter paper radius: 4 cm. **A**. Family 36 showing a high number of Buds per Cotyledon. **B**. Family 61 showing a low number.

On the contrary, SEC Index did not show differences among the families during the whole culture period (data not shown). Excluding family 36, no significant differences were found among families in the number of buds per cotyledon, whereas significant differences were detected for % CCR: families 36 and 93 have the same score but they differ in the number of buds per cotyledon (Table 1). Furthermore, a non-inverse relationship between the number of explants that form buds and bud number was detected. Family 36 data showed the highest values both for Organogenesis (100%) and buds per cotyledon (6.54).

Rooting and Acclimatization

Rooting media were optimized for the assayed families, with best results achieved by ¼ strength macroelements in induction and expression media (Table 2). It is very interesting to note that if we analyze the results focusing exclusively on induction or expression medium, the highest values are also achieved by ¼ macroelement strength.

Once ¼-¼ was chosen as the suitable strength combination, the rhizogenic behaviour was studied. The rooting process was indirect, with callus formation in the shoot base, and non spontaneous rooting detected. After 2 months of rooting expression, provenance results showed significant differences in Survival and RI values (Table 3), those belonging to "Cataluña Litoral" provenance being superior (RI of 0.50 and Survival of 92.25%). Data obtained from the six families assayed were also variable (Table 3). Survival ranged from 55.97% (family 93) to 87.74% (family 36).

Although we have found significant differences in calli presence among families, no relationship was detected between callus formation and rooting rate, as a significant differential rooting efficiency was recorded (RI of 0.34 and 0.25 for families 36 and 78, respectively).

The roots obtained (Figure 4A-B) were morphologically normal, with a well-developed root system and a consistent connection between shoot and roots that has supported normal growth of the micropropagated plantlets for the last 2 years.

Table 2. Rooting culture media assay for the selected families of *Pinus pinea*. Values are means ± SE (*n* ranged from 55 to 113). Data were taken after 60 d in Rooting Expression Medium. Means followed by the same letters within columns are not significantly different at $P \leq 0.05$ according to χ^2 and Fisher Exact test (% survival, % Rooted Shoots, Rooting Index) and parametric tests ANOVA and Student-Newman-Keuls (Number of Roots).

LP Macronutrient strength		% Survival	% Rooted Shoots	Rooting Index	Number of Roots
Induction	Expression	± SE	± SE[1]	± SE	± SE[2]
1	1	65.00 ± 5.93 a	0.00±0.00 a	0.000 ± 0.000 a	0.00 ± 0.00 a
1	½	68.75 ± 6.52 a	15.08±5.61 a	0.104 ± 0.031 ab	1.13 ± 0.18 ab
1	¼	76.04 ± 7.99 a	25.12±9.81 a	0.191 ± 0.087 b	2.78 ± 0.31 ab
½	1	54.29 ± 5.18 a	17.89±2.98 a	0.097 ± 0.030 ab	1.50 ± 0.71 ab
½	½	70.00 ± 4.14 a	28.06±3.96 a	0.196 ± 0.074 b	2.17 ± 1.05 ab
½	¼	82.63 ± 1.94 a	24.36±2.69 a	0.201 ± 0.059 b	2.36 ± 0.51 ab
¼	1	72.92 ± 4.73 a	12.50±7.68 a	0.091 ± 0.024 ab	1.00 ± 0.00 ab
¼	½	69.79 ± 9.15 a	23.06±6.89 a	0.161 ± 0.069 b	2.93 ± 0.10 b
¼	¼	84.38 ± 4.42 a	28.21±2.25 a	0.238 ± 0.085 b	1.88 ± 0.64 ab

[1]: Only survival microshoots are included. [2]: Shoots without roots are excluded.

LP Macronutrient strength Induction	% Survival ± SE	% Rooted Shoots ± SE[1]	Rooting Index ± SE	Number of Roots ± SE[2]
1	69.95±6.78 a	14.16 ± 8.71 a	0.099±0.032 a	2.08±0.11 a
½	68.67±7.56 a	23.24 ± 1.92 a	0.160±0.058 a	2.20±0.99 a
¼	75.69±2.77 a	21.82 ± 3.19 a	0.165±0.047 a	2.17±0.47 a

Expression	% Survival ± SE	% Rooted Shoots ± SE[1]	Rooting Index ± SE	Number of Roots ± SE[2]
1	63.89±5.53 a	9.05 ± 0.37 a	0.058±0.010 a	1.19±0.27 a
½	69.33±2.12 ab	21.95 ± 1.33 b	0.152±0.040 b	2.21±0.27 a
¼	80.98±1.40 b	26.26 ± 1.90 b	0.213±0.064 b	2.30±0.05 a

[1]: Only survival microshoots are included. [2]: Shoots without roots are excluded.

The % of surviving microshoots that rooted ranged from 19.62% (family 93) to 39.40% (family 89), the RI ranged between 0.11 and 0.34 (Table 3), and more than a single root per shoot was detected. After two months in the greenhouse, survival of rooted plantlets (Figure 4C) reached 97.56% (data not shown).

Table 3. Rooting response of *Pinus pinea* by family and provenance. Values are means ± SE (*n* ranged from 55 to 113). Data were taken after 60 d in Rooting Expression Medium. Means followed by the same letters within columns are not significantly different at $P \leq 0.05$ according to χ^2 and Fisher Exact test (% survival, % Rooted Shoots, Rooting Index) and parametric tests ANOVA and Student-Newman-Keuls (Number of Roots).

Families	% Survival ± SE	% Calli Presence ± SE	% Rooted Shoots ± SE[1]	Number of Roots ± SE[2]	RI ± SE
36	87.74±8.28 a	79.89±7.29 a	38.44 ± 5.41 a	2.58±0.29 a	0.34 ± 0.06 a
44	77.78±10.88 bc	83.81±9.58 a	35.34 ± 17.53 a	1.57±0.55 a	0.27 ± 0.14 bc
61	69.62±15.25 cd	85.42±8.59 ab	29.69 ± 14.09 a	1.63±0.63 a	0.21 ± 0.11 cd
78	79.51±9.88 bc	85.37±8.10 ab	31.39 ± 8.40 a	2.00±0.41 a	0.25 ± 0.07 bcd
89	84.17±6.14 ab	84.76±11.00 a	39.40 ± 13.76 a	2.71±0.47 a	0.33 ± 0.12 ab
93	55.97±17.31 d	61.98±17.48 b	19.62 ± 12.46 a	1.30±0.51 a	0.11 ± 0.08 d

Provenances	% Survival ± SE	% Calli Presence ± SE	% Rooted Shoots ± SE[1]	Number of Roots ± SE[2]	RI ± SE
Cataluña	92.25 ± 8.68 a	89.50 ± 12.53 a	54.44 ± 5.12 a	2.67 ± 0.92 a	0.50 ± 0.00 a
Meseta Norte	75.05 ± 23.96 b	94.34 ± 1.86 a	51.52 ± 7.08 a	1.85 ± 0.29 a	0.39 ± 0.18 b

[1]: Only survival microshoots are included. [2]: Shoots without roots are excluded.

In order to establish a measurement of the general efficiency of the organogenic process in each family, a global index was obtained by multiplying the percentage of CCR and the RI. The highest value corresponded to family 36 (33.34%), meanwhile family 61 achieved less than half of this value (16.15%).

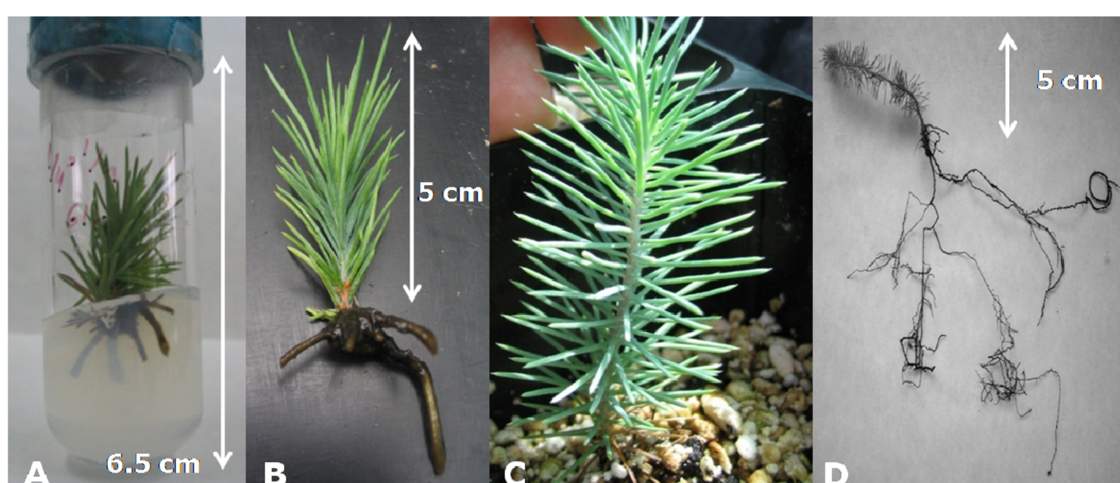

Figure 4. Rooted shoots of *Pinus pinea*. **A**. After 60 d on rooting medium. **B**. Plantlets before acclimatization in the greenhouse. **C**. Plantlets after two months in the greenhouse. **D**. Plants after one year in the greenhouse.

Somaclonal analysis by RAPD

The fisrt step in the RAPD analysis was the selection of the annealing temperature, and the temperature eventually chosen was 40 °C (data not shown). Then, 20 primers were tested, of which 17 amplified correctly, with a total number of 71 scorable bands. However, only 5 primers gave polymorphic and clearly identifiable bands (OPA04; OPA07; OPA10; OPA11; OPA15), which were used in further reactions. The total number of scorable bands of these polymorphic *loci* was 26, with a size between 900 and 3500 bp, but only 9 were polymorphic. The average number of scorable bands per polymorphic primer was 4.18, and the percentage of polymorphic bands (ppb) was 0.29. Polymorphism was only detected among families, always presenting the same pattern within clones.

Discussion

The caulogenic step was direct, decreasing the duration of *in vitro* shoot regeneration, as lack of callus allows a reduction in the shoot induction phase (Sul and Korban 2004). Different levels of caulogenic response were recorded, as has also been described in the production of adventitious shoots in different pine species such as *P. radiata*, *P. taeda* or *P. strobus* (Bergmann and Stomp 1992).

Variable range of Organogenesis data were recorded, with family 36 scoring 100% Organogenesis, improving the previous results for the same species (Sul and Korban 2004; Alonso *et al.* 2006). Considering that our families have been exposed to the same *in vitro* culture conditions, this differential response could depend on the interactions of the embryo genotype with its *in vitro* environment, hence the regeneration efficiency would be influenced by the provenance and/or the genotype (Saborio *et al.* 1997; Liu and Bao 2003; Tereso *et al.* 2006). BFC Index differences can be explained with regard to the size of the cotyledons (López *et al.* 1996): the

bigger the size the less damage caused by excision, so excision size is critical. However, we do not have such great size variation among cotyledons to justify the organogenic response differences observed.

Considering the proved relationship between BA concentration in the culture medium and the organogenic response (Valdés *et al.* 2001; Moncaleán *et al.* 2005), a likely consequence of a differential absorption and/or metabolism of the inductor cytokinin (CK) could explain the different level of response obtained, so a result of this report should be the selection of those families with the highest and the lowest values, in order to compare their BA behaviour.

Rooting culture media were optimized for the assayed families, firstly because optimal treatment varies among species, and secondly, it is common practise to modify some method conditions looking for the most suitable culture media for specific selected families (Tang *et al.* 2001). Microshoots showed a callus phase mediating the rooting induction step in *P. pinea*, as has already been described for difficult-to-root species such as *P. taeda* (Hamann 1998). Analyzing both provenances and families data, the low rooting rates are in agreement with the low rates already described for *P. pinea* for this step (González *et al.* 1998; Sul and Korban 2004), representing the bottleneck of its micropropagation. Rooting data concur with values reported by Capuana and Giannini (1995), but are far from those obtained by Alonso *et al.* (2006). Given that the procedures followed in this work were identical those used by Alonso *et al.* (2006), the authors consider that the differences in results obtained in the two studies must be the result of the plant material source used in each report (families vs open-pollinated (OP) seeds). Similar to our rooting results, significant differences in rooting ability of families of *P. taeda* have been observed (Tang and Ouyang 1999; Tang 2001), implying heritability of the trait (Greenwood and Weir 1994), supporting the genotype-dependent response hypothesis. As was postulated for the caulogenic step, this difference in behaviour could

also be related to the response variation of each family to auxins (Greenwood and Weir 1994).

As a general overview, the global index of organogenic efficiency establishes family 36 as the higher-producing family, supported by the caulogenesis and rhizogenesis data; on the other hand, family 61 represents the lowest caulogenic response as well as a half-value global index compared to family 36, so these two families should be chosen for further basic studies.

Taking into account that we have reported a lack of callus during the caulogenic step, as well as a reduced shoot induction phase in contact with the inductor agent (BA), our data concur with the low somaclonal variation frequency described for regenerated plantlets of *P. taeda* by Tang and Guo (2001). Bearing in mind all our data, and according to Fallour *et al.* (1997), who highlighted the low polymorphism of this species, the common use of clonal micropropagation based on cotyledons could be proposed, as plantlets regenerated are characterized by genetic homogeneity.

In spite of this fact, some reports show the inability of RAPD markers to detect modifications at the DNA level (Fourré *et al.* 1997). Therefore, the lack of polymorphism in our analysis does not necessarily mean an absence of somaclonal variation. It is also necessary to consider that the total number of bands scored is probably not sufficiently high to allow reliable detection of polymorphism, and it cannot be assumed that an extensive genome analysis has been developed, so further exhaustive genome analyses would be required.

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CAPÍTULO 2

PCR-based molecular markers for assessment of somaclonal variation in *Pinus pinea* L.

Imagen modificada de "Reichenbach, Heinrich Gottlieb Ludwig (1849). *Icones florae germanicae et helveticae. Volumen decimum primum.* Leipzig (Alemania)".

PCR-based molecular markers for assessment of somaclonal variation in *Pinus pinea* L.

Summary

Molecular markers based on PCR-techniques were applied for evaluating somaclonal variation of micropropagated families of stone pine (*Pinus pinea* L.). The certification of genetic stability after an *in vitro* process is of paramount importance, so four different markers (RAPD, ISSR, AFLP, and SAMPL) were applied for this species. The total number of primers tested was 130, with 223 combinations assayed. A high number of them amplified successfully (178), representing 79.82% of the total, and the average number of amplified fragments ranged from 2.47 (ISSR) to 65.76 (SAMPL). Based on internal controls, no problems of reproducibility were detected. No somaclonal variation was detected within the clones. Of the tested markers, ISSR, AFLP, and SAMPL showed monomorphic amplification profiles, with only RAPD markers showing some interclonal variation. This is not a greatly surprising result, considering the low genetic diversity attributed to stone pine. To the best of our knowledge, this is the first time that an extensive study has been developed to evaluate the somaclonal variation of stone pine.

Este capítulo se corresponde con la publicación "Cuesta, C., Ordás, R.J., Rodríguez, A., Fernández, B. (enviado). PCR-based molecular markers for assessment of somaclonal variation in *Pinus pinea* L.".

Introduction

In vitro processes for the vegetative propagation of valuable genotypes require an evaluation of genetic stability, especially in forest trees and other woody plants with long rotation times (Ryynänen and Aronen 2005). This type of variability is known as somaclonal variation, and includes those phenomena related to the genetic variations during a whole *in vitro* process (Larkin and Scowcroft 1981). Vegetative propagation of conifers based on tissue culture is a suitable method of clonal production of selected individuals, but the use of high levels of growth regulators (often used to enhance the rate of shoot multiplication) has been found to cause genetic variation in micropropagated plantlets (Venkatachalam *et al.* 2007). However, genetic fidelity of *in vitro*-propagated pines has been scarcely studied, i.e. *Pinus thunbergii* (Goto *et al.* 1998) and *P. taeda* (Tang 2001).

Many approaches have been proposed to evaluate this somaclonal variation phenomenon, and several molecular methods have been developed. A first approach used to be morphological and physiological traits, but these methods require extensive observations of the plants until maturity, and, additionally, the traits may be reversible, disappearing after a few growing seasons. Furthermore, some changes induced by *in vitro* culture cannot be observed because the structural differences in gene product do not always alter its biological activity (Palombi and Damiano 2002). More efficient tools have now been developed using techniques related to DNA-based markers like Random Amplified Polymorphic DNA (RAPD) (Williams *et al.* 1990), Inter Simple Sequence Repeat (ISSR) (Zietkiewicz *et al.* 1994), Simple Sequence Repeat or Microsatellite (SSR) (Tautz 1989), Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.* 1995) and Selective Amplified Microsatellite Polymorphism Length (SAMPL) (Witsenboer *et al.* 1997). The cited techniques have their own specifications, as well as some limitations, that must be taken into account when correctly designing a molecular assessment study, i.e. selecting the marker system and technique used constitute two of the most important

decisions in the experimental design (McGregor *et al.* 2000; Bornet and Branchard 2001).

RAPD markers are very quick and easy to develop (Hansen *et al.* 1998), but if conditions are not strictly controlled (e.g. annealing temperature), their reproducibility could fail, producing artefacts or nonspecific amplifications (Bornet and Branchard 2001). SSR markers however, are very specific and highly polymorphous in genomic and chloroplastic DNA, but they require previous knowledge of the genome sequence. Use of AFLP markers is both laborious and costly but the technique provides a molecular assay that combines the reliability of the Restriction Fragment Length Polymorphism (RFLP) with the power of the Polymerase Chain Reaction (PCR), and is strongly recommended by many authors (Arcade *et al.* 2000; Gómez *et al.* 2001; Hornero *et al.* 2001; Polanco and Ruiz 2002). ISSR and SAMPL markers are actually microsatellite-based modifications of RAPD and AFLP techniques respectively (Negi *et al.* 2006). Hence, both SAMPL and ISSR are characterized by an increased capacity for the detection of polymorphism, due to their association with the hypervariable microsatellite region (Negi *et al.* 2006). The potential for polymorphism detection of these markers, even between closely related genotypes or in species characterized by low genetic diversity, indicates their usefulness (Witsenboer *et al.* 1997; Rakoczy-Trojanowska and Bolibok 2004; Yao *et al.* 2008).

Conifers are characterized by a high level of genetic diversity (Martín and González 2000; González-Martínez *et al.* 2004), but previous studies on several *Pinus* sp. have shown a low level of variability, regardless of the markers used, such as in *P. resinosa* (Mosseler *et al.* 1991; 1992) and *P. squamata* (Zhang *et al.* 2005). Stone pine (*Pinus pinea* L.) is a conifer principally found in the Iberian Peninsula, and the exceptionally low genetic polymorphism of this species has been confirmed in studies applying different markers, such as isozymes (Fallour *et al.* 1997), chloroplastic and nuclear microsatellites (Martín and González 2000; Gómez *et al.* 2002;

González-Martínez *et al.* 2004; Vendramin *et al.* 2008) and RAPD (Evaristo *et al.* 2002). This low genetic variability of stone pine has been explained as a consequence of the anthropogenic diffusion of genetically homogeneous reproductive material (Fallour *et al.* 1997).

A micropropagation technique has successfully been developed for stone pine (Alonso *et al.* 2006), but there is just one published report on the genetic fidelity of the micropropagated plants (Cuesta *et al.* 2008). Therefore, we have undertaken a study of somaclonal variation analysis for stone pine, selecting those markers based on random genome amplification, such as RAPD, ISSR, AFLP and SAMPL, which have hitherto not been applied in this species. We have excluded SSR markers, because as González-Martínez *et al.* (2004) mention, no polymorphism was detected in the transfer of SSR motifs from *P. taeda* to *P. pinea*. Moreover, SSR markers have not been successfully used to examine clonal variation in plants, as they do not always detect major forms of genomic instability (Leroy and Leon 2000).

In summary, the objective of the present chapter was the assessment of genetic stability of micropropagated plantlets from selected families of stone pine, applying 4 different PCR-based molecular markers (RAPD, ISSR, AFLP and SAMPL).

Materials and methods

Plant material and DNA extraction

Plant material used comprised needles of two-year old micropropagated pines from 47 regenerated plantlets, with the number of clones per seed ranging from 1 to 11 (Table 1).

Table 1. Plant material of *Pinus pinea* used in molecular analyses. Nomenclature used was based on the family and the seed i.e. 36.1 means family 36 seed 1.

Family	Seed number	No. clones per seed
36	1	11
	2	4
	5	1
	6	11
	7	1
	8	1
	9	2
	10	1
44	5	8
61	5	2
89	4	1
	7	3
93	3	1
	4	1

The clones were obtained after micropropagation (via adventitious organogenesis) of 6 selected half-sibling families, belonging to two different provenances (Cuesta *et al.* 2008). As Figure 1A shows, this propagation procedure consisted in cotyledon excision, a shoot induction period under 44.4 µM benzyladenine (BA) conditions followed by an expression stage for shoot elongation and finally, root induction with 10.0 µM of naphtalen acetic acid (NAA) and acclimatization of the plantlets obtained.

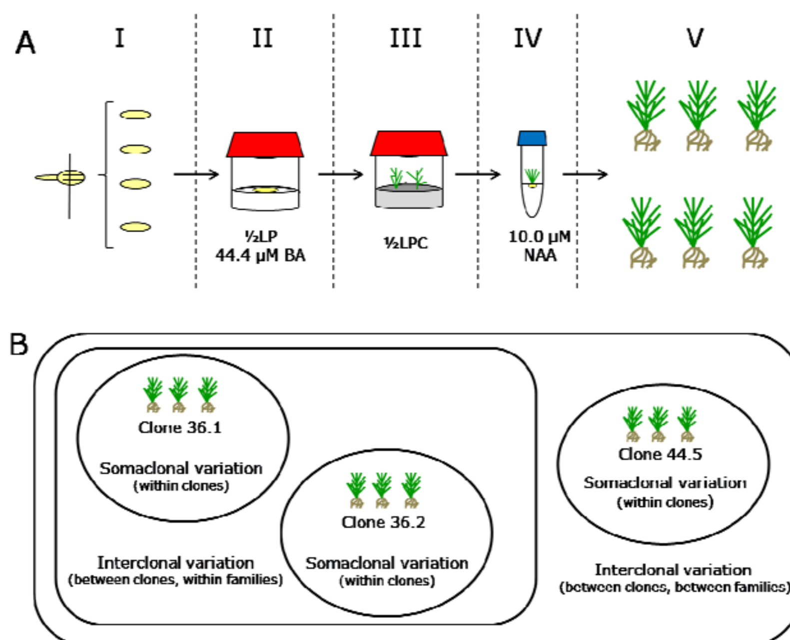

Figure 1. Micropropagation protocol used in *Pinus pinea* cotyledons and levels of comparison performed. **A**. Propagation procedure scheme: excision of cotyledons (I); shoot induction developed by BA application in the culture medium (II); shoot elongation period (III); root induction stage (IV), appearance of roots to obtain regenerated plantlets (clones) (V). **B**. Example of the different levels of variation in regenerated plantlets of stone pine.

Some clones were represented by 8-11 plantlets i.e. 36.1 and 44.5 (Table 1), and these individuals, belonging to different cotyledons of the same seed, enabled the assay of intraclonal variation whilst comparison of seeds from the same family (i.e. 36.1; 36.2 and 36.9) permitted evaluation of the interclonal variation (Figure 1B). Moreover, even those clones from different families represented by just 1-2 plantlets (i.e. 36.5; 61.5; 89.4 and 93.3) allowed comparison between families (Figure 1B).

DNA was isolated applying an extraction method based on Cetyl Trimethylammonium Bromide (CTAB) buffer (Doyle and Doyle 1987) with slight modifications. Parallel DNA extractions on 10 random selected samples were developed to test reproducibility. DNA concentration was measured by spectrophotometer (Beckman-Coulter DU800®).

RAPD and ISSR analyses

Sixty primers were used for the RAPD analysis (sets A; C; H) from Operon Technologies Inc. (Alameda, California), of which 20 had previously been tested (Cuesta *et al.* 2008). For the ISSR analysis, twenty-one primers were assayed: the UBC set (based on *Pinus squamata* (Zhang *et al.* 2005): UBC 807; 808; 811; 812; 813; 818; 820; 825; 828; 834; 840; 842; 844; 855; 857; 864; 886), and the LL set (LL1-LL4,described by Leroy and Leon (2000) (Table 2A), all of them purchased from MWG Laboratories (MWG-Biotech AG, Ebersberg, Germany). DNA amplification reactions and PCR conditions were performed as detailed in Tables 2B and 2C.

Table 2. PCR conditions for the different molecular markers assayed. **A**. Primer sequences of ISSR and SAMPL assays. **B**. PCR reaction compounds. **C**. PCR programmes for each marker (SAMPL conditions are similar to AFLP). Thermocycler used was Gene Amp PCR System 9700 (Applied Biosystems).

A

Primer	Sequence (5'-3')
ISSR: UBC-set	
UBC-807	AGA GAG AGA GAG AGA GT
UBC-808	AGA GAG AGA GAG AGA GC
UBC-811	GAG AGA GAG AGA GAG AC
UBC-812	GAG AGA GAG AGA GAG AA
UBC-813	CTC TCT CTC TCT CTC TT
UBC-818	CAC ACA CAC ACA CAC AG
UBC-820	GTG TGT GTG TGT GTG TC
UBC-825	ACA CAC ACA CAC ACA CT
UBC-828	TGT GTG TGT GTG TGT GA
UBC-834	AGA GAG AGA GAG AGA G(CT) T
UBC-840	GAG AGA GAG AGA GAG A(CT) T
UBC-842	GAG AGA GAG AGA GAG A(CT) G
UBC-844	CTC TCT CTC TCT CTC T(AG) C
UBC-855	ACA CAC ACA CAC ACA C(CT) T
UBC-857	ACA CAC ACA CAC ACA C(CT) G
UBC-864	ATG ATG ATG ATG ATG ATG
UBC-886	(AGC) (AGT) (AGC) CTC TCT CTC TCT CT
ISSR: LL-set	
LL1	CAA CAA CAA CAA CAA
LL2	CAG CAG CAG CAG CAG
LL3	GAT AGA TAG ATA GAT A
LL4	GAC AGA CAG ACA GAC A

Primer	Sequence (5'-3')
SAMPL: CATA/GATA	
CATA	CAC ACA CAC ACA CAC ATA T
GATA	GAG AGA GAG AGA GAG ATA TA
SAMPL: R-SP-SMPL set	
R-SP-SMPL01	CTC TCT CTA ATA TAT ATA TA
R-SP-SMPL02	CAT CAT CAT CAT CGT CAT CAT
R-SP-SMPL03	CAT CAT CGT CCT CCT CAT AT
R-SP-SMPL04	ATC ATC ATC ATA TCA TCA TC
R-SP-SMPL05	ATC ATC ATC ATC AAT ATC ATC
R-SP-SMPL06	TCT CTC TCG TAC ACA CAC ACA C
R-SP-SMPL07	TTG TTG TTA TTC TTC TTC TTA
R-SP-SMPL08	ACA TAT ATG TAT GTA TGT ATG TAT
R-SP-SMPL09	ACA CAC ACA CAT ACA CAC AC
R-SP-SMPL10	CTC TCT CTT TTC TCC TTC TC
R-SP-SMPL11	GAA GAA GGA AGA ATG TGT GTG

B

Molecular marker		DNA	$MgCl_2$	10x Buffer	dNTPs	Primer	Taq polymerase[1]	Total volume
RAPD		10 ng	2.5 mM	2.5 µL	0.2 mM	0.2 µM	1.00 U	25 µL
ISSR	UBC	20 ng	2.5 mM	2.0 µL	0.5 mM	2.5 µM	1.50 U	20 µL
	LL	10 ng	2.5 mM	2.5 µL	2.5 mM	10 µM	1.25 U	25 µL
AFLP	Preamplification	5 µL R/L[2]	2.5 mM	5.0 µL	0.2 mM	0.3 µM	1.25 U	50 µL
	Selective	2 µL Preamp	2.5 mM	1.0 µL	0.2 mM	0.2 µM[3]/0.3 µM	0.75 U	10 µL

[1]: Taq polymerase: Invitrogen. [2]: RL: Restricted/Ligated DNA. [3]: Concentration of IRD-labelled primer.

C

Molecular marker		PCR programme	Detection system
RAPD		1min 95 ºC, [1min 95 ºC/1min 40 ºC/1min 72 ºC] x 44 cycles, 5min 72 ºC	Agarose 2%
ISSR	UBC	5min 94 ºC, [30s 94 ºC/45s 49 ºC/1.5min 72 ºC] x 35 cycles, 7min 72 ºC	Agarose 2%
	LL	1min 94 ºC, [1min 94 ºC/1min Ta[4]/4min 72 ºC] x 27 cycles, 7min 72 ºC	Agarose 2%
AFLP	Preamplification	5min 92 ºC, [1min 92 ºC/45s 60 ºC/1min 72 ºC] x 26 cycles, 5min 72 ºC	Agarose 1.5%
	Selective	5min 92 ºC, [1min 92 ºC/45s 65 ºC/1min 72 ºC][5] x 9 cycles, 1min 72 ºC, [1min 92 ºC/45s 56 ºC/1min 72 ºC] x 24 cycles, 5min 72 ºC	Polyacrylamide 8%

[4]: Ta (Annealing temperature): 54 ºC (LL1); 62 ºC (LL2); 42 ºC (LL3); 52 ºC (LL4). [5]: Touchdown of 1 ºC for each cycle.

Amplification products were resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide, and then photographed on a UV transilluminator (GelLogic 100, Kodak). In all cases lambda phage DNA digested with *Eco*RI (Biolabs® Inc., New England, USA) and *Hin*dIII (Takara Bio Europe S.A.S., France) was used as size marker.

AFLP and SAMPL analyses

Genomic DNA (200 ng) was submitted to enzymatic digestion using 3 U each of *Eco*RI and *Mse*I in their corresponding Restriction/Ligation (RL) buffer, in a final volume of 35 µL at 37 ºC for 2 h. A volume of 10 µL of ligation mixture was made (5 µM of *Mse*I adapter, 0.5 µM of *Eco*RI adapter, 1.2 mM ATP, 1 U of T4 DNA ligase and RL buffer), added to the restriction reaction, and incubated for 5 h at 37 ºC. A template of 5 µL of RL reaction was used for the preamplification, and then verified by electrophoresis in 1.5% agarose. Both amplification reactions and PCR conditions were carried out as indicated in Tables 2B and 2C. Primers used (MWG Laboratories) were those described in Tables 2A and 3; in the case of SAMPL markers, the R-SP-SMPL set was based on Gupta *et al.* (2005), and in the case of CATA and GATA primers, were based on GA and CA motifs, these being the most abundant motifs in conifer genomes (Schmidt *et al.* 2000). The primers were labelled with an infrared dye (IRD 700 and 800), sensitive to the automated sequencer used (Licor Global IR^2 DNA analyzer, Licor Inc., Nebraska, USA). The run was performed in an 8% polyacrylamide gel, and this Automated Li-cor system generated digitized fingerprints (16 Bit TIFF images) during the gel run, which were used in analysis with compatible analysis software.

Data analysis

In order to assure the fidelity of the results obtained, reactions were performed at least twice, including internal controls (replicates of the same sample in each combination, and different DNA extractions of the same individual). Only the consistently reproduced and distinguished bands were considered. The amplified fragments for each genotype and primer combination were scored manually as present or absent. A summary of a comparison between markers were carried out, quantifying the number of primers used, the number of assayed combinations and which of them were amplified, the total number of bands generated, and the average number of bands per molecular marker type. In instances where polymorphism was detected, a dendrogram was generated using the Unweighted Pair-Group Meted with Arithmetic mean method (UPGMA) as well as an Analysis of Molecular Variance statistical analysis (AMOVA), performed with PAST and ARLEQUIN software.

Results

Different molecular methods were used to evaluate the putative variability of *in vitro* clones of stone pine (Figures 2A-D). There was no lack of reproducibility, and though markers applied are sensitive and reliable, there was no somaclonal i.e. intraclonal variation, but some interclonal variation was identified.

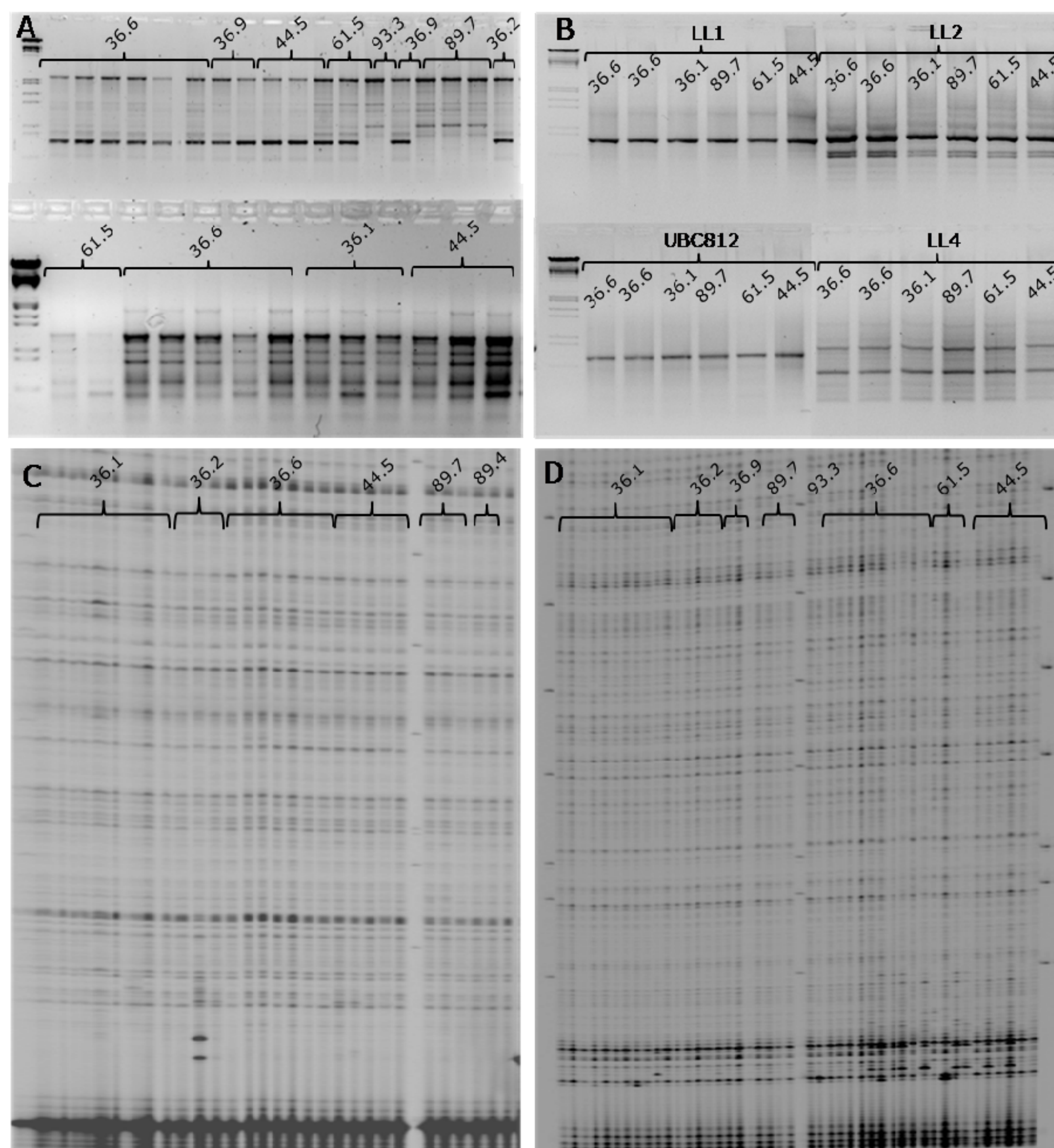

Figure 2. Gel profiles of the different PCR-based markers assayed in micropropagated plantlets of selected families of *Pinus pinea*. **A**. RAPD markers. The first lane shows one of the polymorphic primers (OPA11). The second lane presents a typical monomorphic pattern (OPH13). **B**. ISSR markers. Screening of 4 primers (LL1; LL2; UBC812; LL4). **C**. AFLP profile of a monomorphic combination (Eco+AGCG/Mse+CGTA). **D**. SAMPL monomorphic pattern (GATA/Eco+ACA).

Table 3. Combinations tested and number of bands obtained in AFLP and SAMPL assays of somaclonal variation in selected half-sibling families of *Pinus pinea*. **A**. AFLP results. **B**. SAMPL results.

A

Primer 1	Primer 2: Mse+																			
	CAC	**CAG**	**CAT**	**CCA**	**CCT**	**CGA**	**CCAC**	**CCAG**	**CCCG**	**CCGC**	**CCGT**	**CCTA**	**CCTG**	**CGAC**	**CGAG**	**CGTA**	**CGTC**	**CTTC**	***Average[1]***	***Total***
Eco+AGCG	19	28	43	24	50	31	-	35	20	50	46	68	18	36	45	61	36	-	***38.12***	***610***
Eco+AGCT	67	39	60	79	-	51	24	56	40	72	45	59	33	48	24	27	56	32	***47.76***	***812***
Eco+AAACA	n/t	62	-	38	n/t	42	n/t	n/t	n/t	32	29	n/t	n/t	65	-	32	n/t	-	***42.86***	***300***
Eco+AAACT	10	51	16	-	22	-	53	22	11	-	34	61	14	-	16	-	-	30	***28.33***	***340***

B

Primer 1	Primer 2: Eco +														
	AAC	**AAG**	**AAT**	**ACA**	**ACC**	**ACG**	**ACT**	**AGA**	**AGC**	**AGG**	**AGT**	**ATC**	**M+CTT**	***Average[1]***	***Total***
CATA	88	90	78	87	66	72	69	72	53	71	70	84	44	***72.62***	***944***
GATA	102	128	97	114	57	73	97	84	58	70	81	77	42	***83.08***	***1080***

Primer 1	Primer 2: R-SP-SMPL										
	01	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**11**
Eco+AGA	-	50	44	56	66	16	70	-	76	83	46
Eco+AGC	-	42	55	67	67	50	34	-	70	61	56
Eco+AGG	-	44	82	65	-	-	-	-	60	78	83
Mse+CTT	-	37	39	66	58	18	50	-	65	47	63
Mse+CCTA	21	-	-	n/t	n/t	n/t	57	-	71	84	88
Average[1]	***21***	***43.25***	***55***	***63.5***	***63.67***	***28***	***52.75***	***-***	***68.40***	***70.60***	***67.20***
Total	***21***	***173***	***220***	***254***	***191***	***84***	***211***	***-***	***342***	***353***	***336***

[1]: Average only takes into account the amplified combinations. n/t: non tested; "-": absence of amplification

RAPD and ISSR results

Of the 60 primers from 3 different Operon sets tested, 41 amplified (Figure 2A). The total number of scored bands was 187, with an average of 4.36 bands per primer (Table 4).

Table 4. Summary and comparison of results obtained with the 4 molecular markers assayed in evaluating the genetic stability of micropropagated selected families of *Pinus pinea*.

Molecular marker[2]		No. primers used[1]	No. assayed combinations	No. amplified combinations	No. total bands	Average bands
RAPD	Operon A	20	20	17	102	6.00
	Operon C	20	20	12	42	3.50
	Operon H	20	20	12	43	3.58
	General	***60***	***60***	***41***	***187***	***4.36***
ISSR	UBC	17	17	17	33	1.94
	LL	4	4	4	12	3.00
	General	***21***	***21***	***21***	***45***	***2.47***
AFLP	Mse+3/Eco+4	8	12	11	491	40.90
	Mse+3/Eco+5	8	10	7	241	24.10
	Mse+4/Eco+4	14	24	22	931	38.80
	Mse+4/Eco+5	14	18	12	399	22.20
	General	***22***	***64***	***52***	***2062***	***39.65***
SAMPL	CATA,GATA/Eco+3	14	24	24	1938	80.75
	R-SP-SMPL/Eco+3	14	33	24	1421	59.20
	R-SP-SMPL/Mse+3/4	13	19	14	764	54.57
	General	***27***	***78***	***64***	***4209***	***65.76***
	Total	***130***	***223***	***178***	***6503***	

[1]: Number of primers used does not coincide with the general value, because some primers are common to different combinations. [2]: +3/4/5 indicate the number of selective nucleotides of the corresponding primer.

Only one set (Operon A) presented polymorphism, with a percentage of polymorphic combinations (ppc) of 0.29. Statistical analysis was performed: AMOVA results established that 59.74% of variation was due to intrapopulation variability, which might be due to within-family variation, although some interclonal variability was detected. Though it must be noted that RAPD marker profiles cannot completely distinguish genotypes. Of the twenty-one arbitrary ISSR primers initially screened, all produced clear and scorable bands (Figure 2B). The average bands per primer were 2.47 (Table

4), fewer than in RAPD markers, and every primer tested was monomorphic. Both RAPD and ISSR primers ranged in size from 500 to 3000 bp.

AFLP and SAMPL results

Sixty-four combinations of primers were tested in AFLP analysis, of which 52 showed amplification, but no polymorphism was observed. Results can be classified according to the number of selective nucleotides of the primers used (Table 4); the more selective nucleotides assayed, the lower the number of average bands observed: from 40.90 bands (Mse+3/Eco+4) to 22.20 bands (Mse+4/Eco+5). The total number of scorable bands was 2062, ranging from 75 to 400 bp.

SAMPL assays tested 78 combinations, 64 of them being successfully amplified. No polymorphism was observed, although a total number of 4209 bands were scored. The CATA/GATA primers set presented the highest number of average bands (80.75), meanwhile the R-SP-SMPL set varied between 59.20 and 54.57 (Table 4); both sets ranged from 75-100 to 350-400 bp. The general average of bands for this molecular marker was 65.76, the highest value observed among the 4 PCR-based markers tested. Typical profiles generated by the employment of different markers assayed are shown (Figure 2C-D).

Discussion

Tissue culture techniques may induce stress in regenerated or micropropagated plants. Such stress conditions could also be responsible for the DNA changes observed in these plants, consequently true-to-type clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species (Lakshmanan *et al.* 2007). In this way, PCR-based

techniques would be required to ascertain the genetic fidelity of plants regenerated, testing the specific protocol developed, particularly when high levels of cytokinins (CKs) are used (Venkatachalam *et al.* 2007). Some previous reports have observed that the use of CKs, especially BA, combined with the continuous availability of high levels of nutrients induce morphological alterations (e.g. hyperhydricity), although such changes were not associated with genetic modifications in *P. thunbergii* (Goto *et al.* 1998) and *Musa acuminata* (Lakshmanan *et al.* 2007). As the micropropagation system applied here for the clonal multiplication of selected families of *P. pinea* (Cuesta *et al.* 2008) requires a high concentration of BA (44.4 μM), in order to produce large numbers of shoots, this evaluation of the clonal fidelity of plantlets was essential. In the above mentioned assay, neither morphological alterations nor genetic changes were observed during the whole *in vitro* process. This fact however may be the result of using only one marker (RAPD) and it may be the case that undetected changes may have occurred as a consequence of point mutations occurring outside the priming sites (Lakshmanan *et al.* 2007).

A better analysis of genetic stability of plantlets could be achieved by using more than one DNA amplification technique, allowing increased possibilities for the identification of genetic variation, as different regions of the genome would be targeted (Palombi and Damiano 2002; Lakshmanan *et al.* 2007). The fact that RAPD markers quickly scan the whole genome, whereas AFLP markers check large portions of it (Hornero *et al.* 2001), and microsatellites (and PCR-markers based on them) detect variation at pre-determined sites, such as DNA repetitive regions, lends support to the validity of this area of investigation.

The present study analyzes the possible variability of micropropagated plantlets of stone pine, testing 4 different molecular markers, and applying a high number of combinations (223). Almost 80% of the assayed combinations amplified successfully with 6503 scored bands, thus we can be assured that an extensive screening of the genome was

carried out. Results obtained highlighted the effectiveness of RAPD markers, as this was the only technique to detect interclonal variation, although not all assayed families were uniquely identified. However, it must be acknowledged that some authors have hypothesized that the absence of intraclonal RAPD polymorphism cannot guarantee genetic stability, because important variations like genomic mutations could be missed (Fourré *et al.* 1997; Palombi and Damiano 2002; Polanco and Ruiz 2002). Conversely, several reports have demonstrated the ability of RAPD markers to detect genetic variations in different species (Gómez *et al.* 2001; Tang 2001; Zhang *et al.* 2005), even in closely related organisms (Feyissa *et al.* 2007; Lakshmanan *et al.* 2007). For instance, RAPD results for variation in cork oak have been confirmed with AFLP assays (Gallego *et al.* 1997; Hornero *et al.* 2001). In contrast, ISSR scorable bands (45) did not reveal any polymorphism, with fewer average bands than RAPD. In relation to the AFLP assay, and in order to facilitate the readability of gels, an increasing number of selective nucleotides were screened, obtaining fewer bands per gel, as is recommended in cases of high complex genomes such as conifers (Arcade *et al.* 2000; Cervera *et al.* 2000). Results from the frequently recommended technique SAMPL also showed no variation in amplification profile. This SAMPL technique has been designed to enhance the number of scorable characteristics to enable unequivocal identification of clones (Giménez *et al.* 2005), and to generate more amplified fragments than other techniques, owing to its ability to survey the hypervariable microsatellite region in the genome. One advantage of this marker is its less complex banding pattern compared to that of AFLP (Gupta *et al.* 2005). However, our data does not confirm the improvement of gel readability in conifers (Arcade *et al.* 2000; Cervera *et al.* 2000); indeed, we have tested all the primers proposed by Gupta *et al.* (2005), and though 9 of them amplified correctly, none presented a clearer pattern than AFLP.

An extensive problem associated with molecular markers is related to the reproducibility of banding patterns (Leroy and Leon 2000; McGregor *et*

al. 2000), caused, for instance, by heteroduplex formation of homologous sequences or by competition among different DNA fragments for amplification (Hansen *et al.* 1998). In our case, ISSRs presented high reproducibility, principally because of using longer primers and higher annealing temperatures than those for RAPD (Fang *et al.* 1997; Godwin *et al.* 1997; Zhang *et al.* 2005). AFLP and SAMPL techniques are undoubtedly reliable (Jones *et al.* 1997), and in our study, RAPD assays showed no lack of reproducibility, probably because a higher annealing temperature than usual (40 ºC) was used.

The presence or absence of somaclonal variation depends on the source of explant and the method of regeneration; plantlets derived from tissue-cultured shoots being more resistant to genetic changes than those obtained from unorganized callus (Varhsney *et al.* 2001). Indeed, most of the organized cultures, especially the shoot tips, maintain strict genotypic and phenotypic stability under tissue culture conditions (Goto *et al.* 1998). Our results, which shared a monomorphic pattern, are in accordance with this finding. Therefore we suggest that the present data indicates that no genetic variation is induced during *in vitro* growth culture of stone pine. If a molecular marker had to be recommended for somaclonal assessment in this species, it would be the RAPD marker, as, despite its limitations, it was the only one able to detect variation at interclonal level.

To the best of our knowledge, no extensive study on molecular markers in stone pine has been made before; compiling and optimizing different proved protocols. Powerful techniques have been applied, showing successful amplifications with no intraclonal polymorphism detected. The present results support a view of stone pine as a conifer with an extremely low degree of diversity, confirming previous indications (Fallour *et al.* 1997; Vendramin *et al.* 2008). Moreover, after testing 4 different techniques, some of which are widely recognized as the most powerful (e.g. AFLP and SAMPL), we confirmed an absence of somaclonal variation in the

micropropagation system of the stone pine, i.e. can be carried out without much risk of genetic instability.

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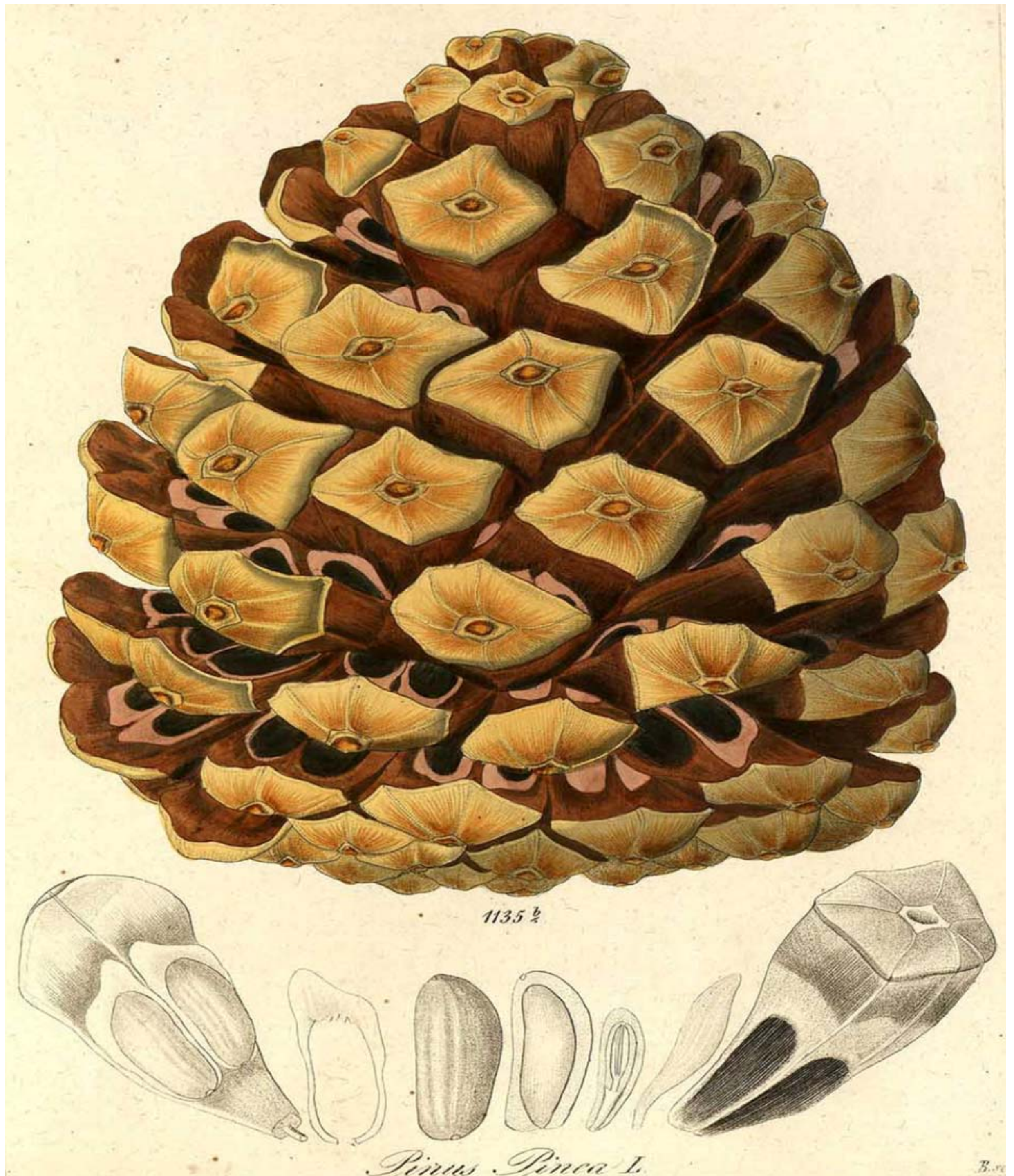

CAPÍTULO 3

Establishment of caulogenic induction in cotyledons of stone pine (*Pinus pinea* L.) and relationship between organogenic response and benzyladenine trends in selected families

Imagen modificada de "Reichenbach, Heinrich Gottlieb Ludwig (1849). *Icones florae germanicae et helveticae. Volumen decimum primum.* Leipzig (Alemania)".

Establishment of caulogenic induction in cotyledons of stone pine (*Pinus pinea* L.) and relationship between organogenic response and benzyladenine trends in selected families

Summary

Adventitious bud formation in stone pine cotyledons cultured in the presence of benzyladenine (BA) has been proposed as a model for the study of *in vitro* shoot-organogenesis in conifers, because of its advantageous characteristics such as the requirement of only one plant growth regulator as BA, the synchronous fashion on its induction and the homogeneity and low degree of differentiation of cotyledons. Although optimal culture conditions have been developed and are in current use, we still lack data about BA dynamics in cotyledons cultured under these conditions, and the morphological description of the early induction stages has not, until now, been approached from a histological perspective. Consequently, this is the focus of the present chapter. Additionally, uptake and metabolism of BA in cotyledons from two selected families, previously characterized by, and selected for, the difference in the magnitude of their organogenic response, were studied.

Media transfer experiments established that cotyledons should be in contact with 44.4 μM of BA for at least 6 h to obtain any caulogenic response (minimum shoot-induction period). Histological observations, carried out here for the first time in this species, determined that meristemoid structures had already begun to appear in explants within 12 h of culture. Moreover, results from the BA uptake and metabolism experiments indicated that the point which explants reached maximum concentration of active forms of BA (276.60 μM at 6 h), and the onset of the determination phase of shoot-organogenesis were directly related.

A direct relationship was also observed between the intensity of the

caulogenic response in cotyledons from families 36 and 61 and the endogenous concentration of BA and its riboside at the start of the induction phase. Hence, family 36, characterized by its higher bud-production, reached concentrations of 251.56 μM whilst family 61, selected for its low bud-producing trait only attained 175.80 μM. Finally, a correlation was observed between benzyladenine 9-glucoside riboside values and the magnitude of shoot-organogenic response.

Este capítulo se corresponde con la publicación "Cuesta, C., Rodríguez, A., Centeno, M.L., Ordás, R.J., Fernández, B. (enviado). Establishment of caulogenic induction in cotyledons of stone pine (*Pinus pinea* L.) and relationship between organogenic response and benzyladenine trends in selected families".

Introduction

A model for studying the control of *in vitro* shoot organogenesis in conifers, especially Pinaceae, has already been proposed for stone pine (*Pinus pinea* L.) (Valdés *et al*. 2001; Moncaleán *et al*. 2005; Alonso *et al*. 2007). The model is based on the induction of adventitious shoot formation (caulogenesis) in cotyledons excised from mature embryos and cultured in the presence of the cytokinin (CK) benzyladenine (BA). The experimental system is characterized by several advantages, such as the need for only one plant growth regulator in the induction medium (BA), the absence of a callus phase as is necessary in other models e.g. *Arabidopsis thaliana* (Cary *et al*. 2002), and the uniformity of the cells in the cotyledonary explants along with the synchronous fashion of their response *in vitro*. Moncaleán *et al*. (2005) identified the first 8-12 d of incubation in 4.4 µM of BA as the period in which shoot induction and determination occurred in stone pine cotyledons. The authors also stated that the organogenic process was initiated by a large (88 µM), early (16 h following culture) and transient increment in the concentration of the active forms of BA in explants. Furthermore, the protocol for direct adventitious shoots formation in stone pine cotyledons was improved by enhancing the exogenously applied BA from 4.4 to 44.4 µM, which reduced the induction phase to 2-4 d (Moncaleán *et al*. 2005; Alonso *et al*. 2006). A subtractive cDNA library was constructed using explants cultured for 16 h in these new conditions and in the absence of BA, in order to identify the genes involved in cell commitment to the caulogenic programme (Alonso *et al*. 2007), and the characterization and differential expression during shoot induction is currently being analyzed by our research group. Nevertheless, data relating to the CK dynamics in cotyledons cultured in 44.4 µM of BA do not exist, and no histological observations have sufficiently explained the morphological switch in non-meristematic cells leading to the formation of buds.

The caulogenic response of *P. pinea* cotyledons depends not only on

the concentration of BA applied, but also on the degree of differentiation of cotyledonary tissues at the time of BA application (Valdés *et al*. 2001), and on the genotype. In this last case, clonal propagation of selected high bud-producing half-sibling families of *P. pinea* seeds was undertaken, and the cotyledons of different families showed different bud-forming capacity under the same culture conditions (Cuesta *et al*. 2008). As reduction of shoot-organogenic competence of cotyledons associated to germination has been shown to be related to the loss of explants' ability to absorb BA from the induction medium (Cortizo 2008), it is possible that genotype-dependent differences in response *in vitro* could also be connected with the efficiency of BA uptake and metabolism by the cotyledons.

In this chapter, we describe the dynamics of BA and its metabolites in cotyledons excised from Open-Pollinated (OP) seeds of *P. pinea* and cultured for 6 d in a medium with 44.4 μM of BA in order to ascertain the concentration of the CK that actually triggers shoot-induction under these conditions. Furthermore, explant incubation times shorter than 2 d were assayed to investigate in more depth changes taking place in the induction stage. To complete our knowledge of the hormonal control of caulogenesis in *P. pinea* cotyledons, a histological approach was taken in looking for the timing of sooth-meristemoid appearance.

Another purpose of the present chapter was to determine the kinetics of uptake, metabolism and concentration of BA in cotyledons isolated from the seeds of two families, 36 and 61, which were selected because they represented respectively the highest and the lowest values of shoot formation in response to BA at 44.4 μM (Cuesta *et al*. 2008). From these results, as well as from those obtained for OP cotyledons, we tried to assess the relationship between the shoot-organogenic competence of *P. pinea* cotyledons associated with genotype, and their ability to absorb and metabolize BA.

Materials and methods

Plant material and tissue culture conditions

Three-year-old mature seeds of *P. pinea* L. were used: from OP trees, and two selected half-sibling families. The selected families belong to the "Cataluña Litoral" and "Meseta Norte" provenances (families 36 and 61, respectively). The families were selected according to their organogenic response in a previous study (Cuesta *et al*. 2008), where they were found to have the highest and lowest values of shoot formation (families 36 and 61 respectively). They were provided by the "Centro Nacional de Mejora Genética Forestal, Puerta de Hierro" from "Dirección General para la Biodiversidad" (Spain).

In accordance with the micropropagation protocol used (Alonso *et al*. 2006), seeds were surface sterilized followed by an imbibition step of 48 h at 4 ºC in darkness. Cotyledons were excised from embryos and placed horizontally in 10 mL of bud induction medium consisting of Le Poivre medium as modified Aitken-Christie *et al*. (1988) with half strength macroelements, and supplemented with a final concentration of 44.4 µM of BA. This step took place in baby jars (130 mL) capped with magenta® caps (Sigma, St. Louis, Mo.) containing 10 mL of culture medium, and maintained in a growth chamber at 25 ± 1 ºC with a 16-h photoperiod under a photosynthetic photon flux of 80 ± 5 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TLD 58W/33, Philips, France). Statistical analysis and experimental design are specified in each section.

The timing of commitment to shoot organogenesis was determined by means of media transfer experiments: cotyledons incubated with 44.4 µM of BA for 6 h; 12 h; 2 d and 6 d were subcultured on a shoot-bud expression medium, consisting of a hormone-free medium with activated charcoal (Alonso *et al*. 2006). Untreated explants were also placed in this medium as a control. Bud appearance was determined after 35 d.

Histology

For microscopical observations, 6 seeds (10 cotyledons per seed) per treatment were collected after 0; 6; and 12 h; 2 and 6 d of culture on the induction medium. Tissue samples were fixed in 5% glutaraldehyde buffered with 25 mM potassium phosphate (pH 6.8) at room temperature for at least 48 h. The samples were then washed in distilled water for 30 min, frozen and sectioned to a thickness of 25-35 µm, using a cryotome (Leica CM1510S). The sections were stained with a toluidine blue solution in 0.1 M acetate buffer (pH 4.4) for 3 min (Ling-Lee *et al.* 1977). Finally, the sections were observed with an optical microscope (Nikon Eclipse E600) coupled with a digital camera for taking microphotographs.

Extraction and measurement of BA uptake

In order to analyze BA uptake and metabolism, explants were harvested after *in vitro* culture on bud induction medium with 44.4 µM of BA, consisting of a mix of 44.1 µM of unlabelled BA (Duchefa, Netherlands) and 0.3 µM of labelled BA (8-[^{14}C]BA, specific activity = 53 mCi mmol^{-1}, Moravek Biochemicals, Brea, USA). Cotyledons were collected at time intervals (0; 1; 2; 6; 16 and 24 h and 2; 4 and 6 d), washed with distilled water and dried with filter paper to determine their fresh weight (FW). Then, they were frozen in liquid N_2 and their dry weight (DW) was estimated. The data were used to calculate the water content per cotyledon. Each experiment was repeated at least 3 times.

A modified protocol based on Moncaleán *et al*. (2005) was followed: 100 mg of lyophilized tissue was extracted at 16 h by repeated inversion at 4 ºC in darkness, using 10 mL of 80% (v/v) methanol containing 45 µM of butylated hydroxytoluene. The procedure was repeated after further 4 h. Both fractions were centrifuged for 30 min at 4 ºC, and supernatants were collected. Three aliquots were taken from the combined extracts, and scintillation fluid was added. Radioactivity was measured with a liquid

scintillation spectrometer (Packard 2500 TR), and total BA uptake (nmol g^{-1} DW) was determined following corrections for extraction volume, sample weight and specific activity of 8-[^{14}C]BA.

Analyses and quantification of BA metabolites

Separation and purification of BA metabolites were achieved as follows: extract volume was reduced to 0.5 mL in a rotary evaporator (R-200 Büchi Labortechnik, Flawil, Switzerland), and resuspended in a final volume of 7 mL of distilled H_2O (adjusted to pH 3 with acetic acid). This solution was eluted twice, firstly with acid water and then with NH_4OH 2M, using a cationic exchange column (Cellulose-Phosphate, Sigma-Aldrich, St. Louis, USA) coupled to a reverse phase column (Sep-Pak C_{18}, Waters, Milford, MA). The resulting eluates were made up of ribotides (the first fraction), and glucosides, ribosides and free bases (the second fraction). Radioactivity was measured in aliquots in order to determine the proportion of each fraction in the initial extract. Both fractions were dried under vacuum conditions (Savant SC-200), in preparation for separation and quantification by High Performance Liquid Chromatography (HPLC). Radioactivity was also measured after each step of the analysis to control recoveries.

Separation of metabolites was achieved using a Kromasil 100 C18 column (5 µm, 15x0.46 cm; Scharlab) in a Waters 600 chromatograph equipped with a photodiode array detector (Waters 996) coupled to a Berthold radioactivity monitor (LB 507 B). Samples from the first fraction (ribotides) were re-suspended in 0.5 mL of methanol:0.2 M acetic acid (pH 3.5 adjusted with triethylamine) (1:19, v/v), and filtered through a 0.45 µm PVDF filter (Millex r-HV, Millipore®) and subjected to HPLC analysis using a linear gradient from 5 to 45% methanol (v/v) over 45 min at a flow rate of 1 mL min^{-1}. Samples from the second fraction (glucosides, ribosides and free bases) were re-suspended in 0.5 mL of acetonitrile:40 mM

triethylamine acetate buffer pH 7 (1:19, v/v), and filtered through a 0.45 µm PVDF filter (Millex r-HV, Millipore®) and subjected to HPLC analysis using a linear gradient from 5 to 20% acetonitrile (v/v) over 20 min at a flow rate of 1.5 mL min^{-1}. Metabolites were putatively identified by co-elution with authentic standards. To verify retention times, standards were always measured after four samples, and each sample was measured twice. Quantification of each metabolite was based on the percentage of each peak area in the radiograph, the radioactivity associated with each fraction, the total radioactivity present in the sample, and recoveries of each step. Once nmol g^{-1}DW was determined, endogenous µM concentration was calculated using water content data.

BA standard and its riboside (BAR) are separated perfectly by HPLC, though the radiograms corresponding to samples showed two peaks that cannot be totally separated, mainly because of the large amount of BA detected. For this reason, all data referred to both molecules were grouped in a unique fraction (BA+BAR). An unidentified metabolite was detected, so fractions were collected, evaporated to dryness, redissolved in 50% methanol in 10 mM of ammonium acetate solution (500 µL) and filtered through a 0.45 µm PVDF filter (Millex r-HV, Millipore®). Fractions were directly injected into a QStar XL QToF mass spectrometer (MS) (Applied Biosystems) equipped with an electrospray interface (capillary voltage +5.5 kV, cone voltage 33 V). Collision-activated dissociation of the protonated molecular ion ($[M+H]^+$) for MS/MS analysis was performed at 20 V using nitrogen as collision gas.

Results

Minimum induction period

Only cotyledons previously incubated in induction medium formed buds after 35 d of culture in expression medium. The caulogenic response was dependent on the previous period of BA exposure since the number of

explants showing buds and the shoots formed per cotyledon were higher as exposure time increased (Figures 1A-B).

Hence it is the view of the authors that the minimum induction period to achieve caulogenic response under 44.4 μM BA conditions can be considered to be 6 h (Figure 1A).

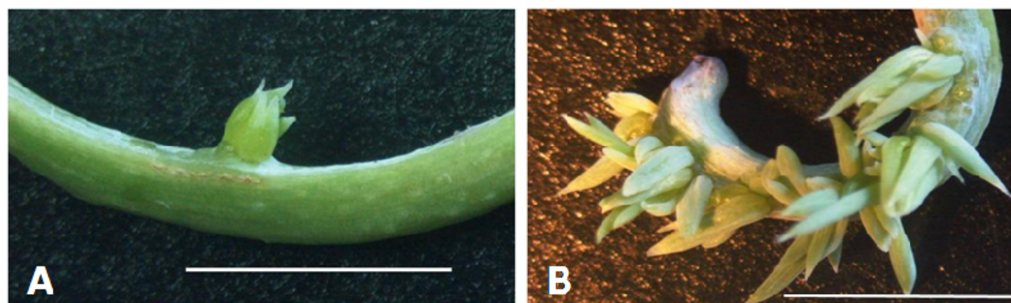

Figure 1. Caulogenic response after 35 d of *in vitro* culture in cotyledons of *Pinus pinea*. Scale bar: 5 mm. **A**. After 6 h under BA exposure (minimum induction period). **B**. After 6 d under BA exposure.

Histology

As Figure 2A shows, at 0 h the *P. pinea* cotyledons presented the classic structure described in other *Pinus* sp. such as *P. radiata* (Villalobos *et al*. 1985) and *P.nigra* (López *et al*. 1996), composed of several epidermal and sub-epidermal layers as well as parenchyma. After 6 h of culture in bud induction medium, epidermal and sub-epidermal cells exhibited mitotic activity (Figure 2B). Meristematic regions appeared at 12 h following culture, structures clearly being visible in the micrograph images (Figures 2C-D). These structures gradually increased in size as a result of periclinal and anticlinal mitotic activity at 2 d (Figure 2E). After 6 d of culture the mitotic activity increased (Figure 2F).

Growth and water content of cotyledons

Dry weight (DW) and water content presented the same trend in the three plant material sources of *P. pinea* studied, although values differed between seed types (Table 1).

The DW of cotyledons increased in all samples during the first 2 h of culture and remained almost constant until day 4, when it increased till the 6th day. A remarkable result was that the DW of cotyledons in family 61 was consistently lower than that of family 36 or OP seeds.

The relative water content of cotyledons decreased by approximately 11-14% from 0 to 16 h after culture, and thereafter increased by 19 to 27% (family 61 and 36, respectively) as a result of water uptake from the medium. In contrast to the effect observed with DW, cotyledons from family 61 showed higher water content than those from family 36 at all time intervals assayed.

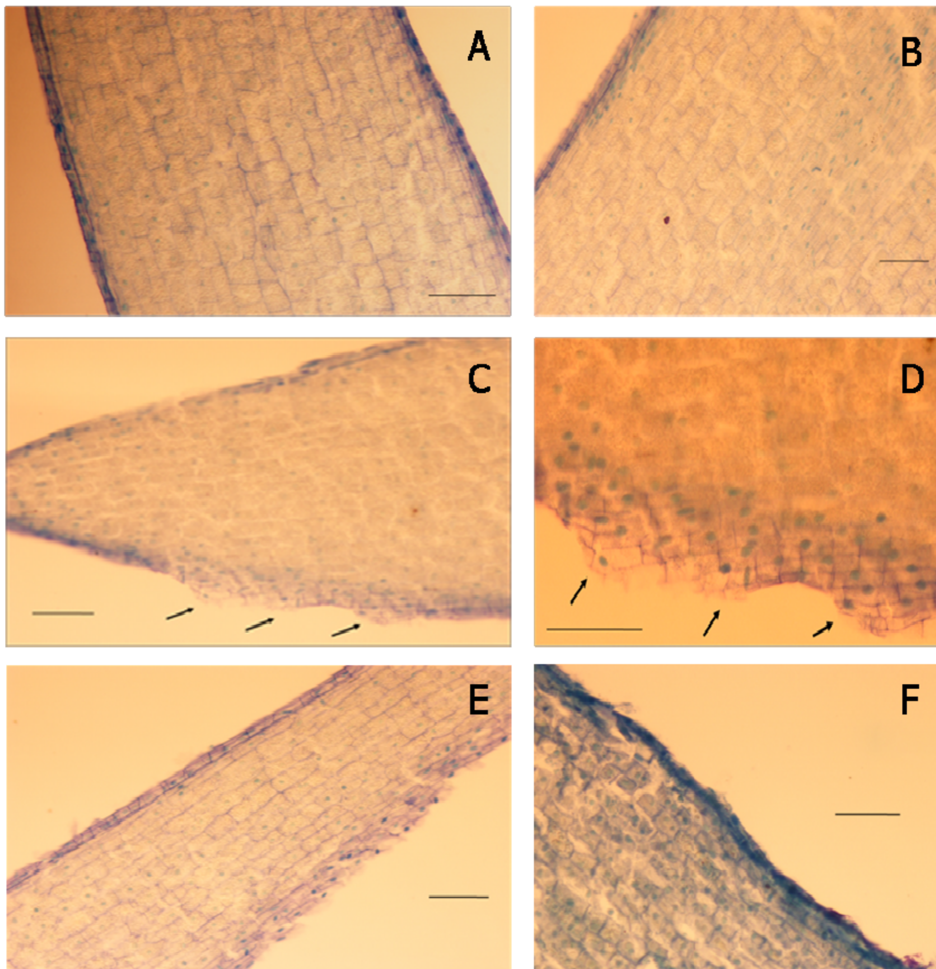

Figure 2. Light micrographs showing the cytological characteristics of cotyledonary cells of *Pinus pinea* in shoot-induction medium. Scale bar: 100 μm. **A**. Micrograph at 0 h, time of cotyledon excision. **B**. Micrograph after 6 h of culture. Mitotic activity is observed. **C-D**. Micrographs after 12 h on induction medium. Meristemoids are indicated by arrows. **E**. Micrograph at 2 d. **F**. Micrograph after 6 d of culture.

Table 1. Dry weight (mg per cotyledon) and water content (%) of *Pinus pinea* cotyledons from families 36 and 61 and Open-Pollinated trees, cultured *in vitro* with 44.4 µM of BA. Values given are means ± SE (each experiment was repeated between 2 and 5 times). Means followed by the same letters within columns are not significantly different at $P \leq 0.05$ according to parametric tests ANOVA and Student-Newman-Keuls.

	Dry weight per cotyledon (mg ± SE)								
Material source	0 h	1 h	2 h	6 h	16 h	24 h	2 d	4 d	6 d
Family 36	1.66 ± 0.08 a	2.02 ± 0.05 a	1.90±0.05 a	1.79±0.06 a	1.68 ± 0.05ab	1.83 ± 0.06 a	1.92 ± 0.05 a	2.04 ± 0.04 a	2.12±0.07 a
Family 61	0.75 ± 0.05 b	0.97 ± 0.05 b	0.96±0.05 b	1.07±0.06 b	1.15 ± 0.05 b	1.03 ± 0.05 b	1.19 ± 0.05 b	1.35 ± 0.05 b	1.49±0.06 b
Open-pollinated	1.84 ± 0.05 c	2.05 ± 0.05 a	1.93±0.06 a	1.97±0.05 c	2.05 ± 0.11 c	2.04 ± 0.09 a	2.21 ± 0.08 c	2.22 ± 0.08 c	2.48±0.16 c

	Water content (% ± SE)								
Material source	0 h	1 h	2 h	6 h	16 h	24 h	2 d	4 d	6 d
Family 36	59.99±2.00 a	52.89±1.01 a	55.30±0.93 a	55.37±1.11 a	53.47 ± 1.75 a	56.66 ± 1.18 a	57.85 ± 0.71 a	62.27±0.97 a	67.82 ± 1.57 a
Family 61	69.20±2.92 b	64.68±0.99 b	61.61 ± 1.42 b	62.05±1.22 b	59.24 ± 0.94 b	61.60 ± 1.44 b	63.20 ± 1.00 b	67.75±0.81 b	70.73 ± 1.01 a
Open-pollinated	59.76±1.30 a	56.19±0.82 c	58.01 ± 1.11 ab	54.62±0.87 a	52.25 ± 2.38 a	59.46 ± 1.36 ab	61.52 ± 0.99 b	66.18±0.69 b	72.20 ± 1.03 a

BA absorption and metabolism

The dynamics of BA uptake and metabolism in OP material cultured in the presence of 44.4 µM of BA is shown in Figure 3. The BA was absorbed eagerly by explants during the first 6 h (56.77 nmol h^{-1} g^{-1}DW), maximum absorption being reached at this time (340.62 nmol g^{-1}DW). From 6 to 24 h BA and/or some BA metabolites were released into the medium, as indicated by the reduced levels of radioactivity in the tissues, and then subsequently BA uptake increased again from 2 to 6 d at a constant rate of 2.405 nmol h^{-1} g^{-1}DW. The BA absorbed by OP cotyledons was metabolized, the metabolites detected being BA, BAR, BA ribotide (BARMP) and a BA glucoside. This last compound was unequivocally identified by mass spectrometry as 6-benzylamino-9-[*O*-glucopyranosyl-(1→3)ribofuranosyl]-purine (BA9GR).

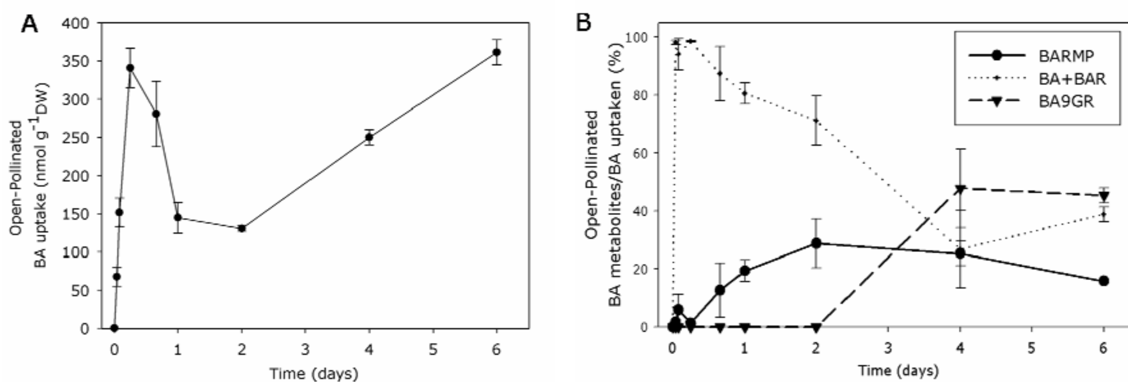

Figure 3. Dynamics of cotyledons from seeds of OP trees of *Pinus pinea* cultured *in vitro* under 44.4 µM BA conditions during 6 d. **A**. BA absorption (nmol g^{-1}DW) of Open-Pollinated material. **B**. Metabolites distribution (%) in Open-Pollinated material.

During the first 6 h, almost all the BA remained in explants as free base and BAR (Table 2 and Figure 3B), but thereafter the CK was quickly transformed into BARMP and BA9GR. The glucoside only appeared after 4 d, becoming at that point the principal BA metabolite present. As a result of the BA absorption and metabolism, the concentration of BA+BAR in OP cotyledons (Table 2) reached a maximum value (276.60 µM) at 6 h, coinciding with the minimum induction period prior to caulogenic response.

Table 2. Metabolites concentration (µM) in each material source of *Pinus pinea* cotyledons from families 36 and 61 and Open-Pollinated trees, cultured *in vitro* with 44.4 µM of BA. Values given are means ± SE (each experiment was repeated between 2 and 5 times).

	BA+BAR (µM±SE)							
Material source	1 h	2 h	6 h	16 h	24 h	2 d	4 d	6 d
Family 36	71.77±9.44	136.63±20.63	251.56±49.32	191.34±34.04	145.97±15.26	130.98 ± 20.73	90.03 ± 12.10	116.84±19.57
Family 61	66.24±14.55	111.50±12.30	175.80±14.47	146.00±28.56	125.70±22.48	75.52 ± 13.23	66.37 ± 14.52	84.97±23.43
Open-pollinated	51.32±6.75	101.70±14.96	276.60±17.60	214.70±43.36	77.37±12.13	57.36 ± 7.39	34.06 ± 17.11	52.91±5.00

	BARMP (µM± SE)							
Material source	1 h	2 h	6 h	16 h	24 h	2 d	4 d	6 d
Family 36	6.68±0.89	4.46±0.70	8.82±3.16	27.43±8.95	13.47±2.54	17.01 ± 3.96	45.29 ± 8.42	31.62±8.42
Family 61	6.51±4.64	8.6±5.51	10.12±2.80	17.09±8.17	23.73±8.53	26.43 ± 5.41	28.96 ± 8.79	14.22±6.04
Open-pollinated	0.89±0.40	6.41±5.96	3.97±0.86	31.01±3.80	18.6±4.49	23.17 ± 6.91	32.17 ± 5.47	21.55±1.81

	BA9GR (µM± SE)							
Material source	1 h	2 h	6 h	16 h	24 h	2 d	4 d	6 d
Family 36	<LOD	<LOD	<LOD	<LOD	<LOD	2.65±2.87	51.30±9.32	94.90±16.11
Family 61	<LOD	<LOD	<LOD	<LOD	<LOD	2.9±0.67	27.42±6.46	33.56±9.39
Open-pollinated	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	60.73±17.51	61.88±5.45

<LOD: under Limit of Detection.

Referring to *P. pinea* family data (Figure 4A), kinetics of BA absorption and metabolism in cotyledons were very similar to those described for OP material (Figure 3A). Nevertheless, some differences were observed between explants from families 36 and 61 which could be related to their caulogenic response.

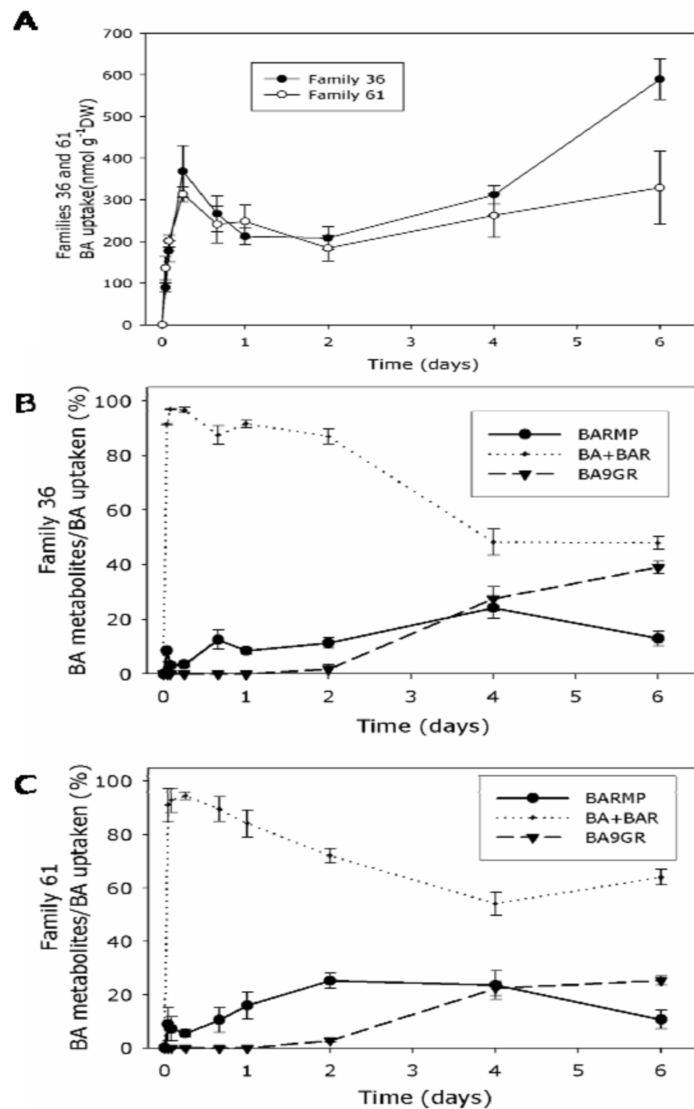

Figure 4. Dynamics of cotyledons from seeds of selected families 36 and 61 of *Pinus pinea* cultured *in vitro* under 44.4 µM BA conditions during 6 d. **A**. BA absorption (nmol g^{-1}DW) of selected families 36 and 61. **B-C**. Metabolites distribution (%) in both families studied.

Specifically, in relation to the total BA absorption at 6 h, family 36 absorbed 8.2 times and family 61 absorbed 7 times more BA than was exogenously applied (368.37 and 313.64 nmol g^{-1}DW respectively), and family 36 also showed a higher rate of BA uptake in the late period of culture (2-6 d) (Figure 4A). Secondly, the concentration of BA+BAR in tissues at 6 h, were 1.4 times higher in family 36 (251.56 and 175.8 µM in families 36 and 61 respectively, Table 2). Thirdly, the relative proportion of BA9GR at 6 d was higher in family 36 (Figures 4B-C), and consequently differential endogenous concentration, showed the same trend (94.90 and 33.56 µM in families 36 and 61 respectively, Table 2).

Discussion

The induction of the caulogenic process in *P. pinea* is controlled by dynamic biochemical pathways, including the dynamics of BA and its metabolites. Using information previously available about BA dynamics, three phases for inducing cotyledons under 4.4 µM BA culture conditions were established (Moncaleán *et al*. 2005): the first constituted by BA absorption reaching peak concentration at around 16 h, indicating the beginning of cell proliferation, the second phase, from 2 to 8 d, in which endogenous BA tended to match the BA concentration in the medium, and finally, the third phase after 8 d of culture, characterized by no significant changes in BA metabolism.

The present chapter has determined the minimum induction period for 44.4 µM BA exposure conditions in OP material: 6 h is the shortest time assayed that results in the appearance of buds on the cotyledonary surface (Figure 1A), rather than the 2 d suggested by Moncaleán *et al*. (2005). Considering that no intermediate callus phase is necessary in stone pine cotyledons, and cotyledons would be determined after 6 h of culture, such explants would probably be competent *per se*.

Histological data agree with findings for *Pinus radiata* (Villalobos *et al*. 1985) and *Abies amabilis* (Kulchetski *et al*. 1995), which showed that promeristemoids were formed in the subepidermal region, later developing into meristemoids and shoot primordia. We found that meristemoid structure is present at 12 h (Figure 2C-D), and is preceeded by intense mitotic activity (Figure 2B).

Furthermore, BA dynamics showed that the peak of BA+BAR appears at 6 h (Figure 3A), leading us to propose three phases under these optimized culture conditions (44.4 μM): from 0 to 6 h, 6 h coinciding with the main peak of BA uptake and the minimum induction period; from 6 h to 2 d, including at 12 h the appearance of meristemoid structures and at 2 d the end of the decrease in the rate of absorption of BA; and from 2 to 6 d, characterized by a renewed increase in BA absorption.

These modified phases clearly explain the shorter period required for obtaining the same response in cotyledons using 44.4 μM of BA rather than 4.4 μM (Moncaleán *et al*. 2005), and are supported by differences in uptake rate, much higher under 44.4 μM conditions: e.g. during the first 2 h 135.74 nmol h^{-1} g^{-1}DW vs. 37 nmol h^{-1} g^{-1}DW (4.4 μM conditions). Gene expression studies also concur with our findings, as a *P. pinea CLAVATA* homologous gene implicated in shoot apical meristem identity is expressed in the early stages of the induction period (Cortizo 2008). Moreover, the timing of the main peak of BA absorption (6 h) leads us to reconsider the timing of the determination of shoot meristem precursors, previously proposed by Alonso *et al*. (2007) to be at 16 h.

Several factors have already been proposed to explain the variable magnitude of organogenic response, such as differential BA uptake or metabolism (Valdés *et al*. 2001), that also have an influence on endogenous auxin and CK levels (Mercier *et al*. 2003). A direct correlation between uptake of exogenous plant growth regulators and shoot development is supported by the present data, as family 36, characterized by the highest

bud-forming values (Cuesta *et al*. 2008) presents higher values of BA absorption. This connection has been previously reported in other species, such as two genetic lines of *Petunia hybrida* cultured *in vitro*, that were characterized by different levels of organogenic response and presented differences in the rate of BA uptake and metabolic inactivation (Auer *et al*. 1992). In addition, two callus lines of kiwi fruit (caulogenic and no caulogenic) also differed in their BA content, it being double in the caulogenic one (Centeno *et al*. 1996).

Uptake of externally applied CK bases into cells has been shown to be associated with rapid formation of the corresponding ribotides, which have a storage function. Ribotides are also thought to play a central role in the regulation of CK levels as they are readily converted to both ribosides and free base forms (Åstot *et al*. 2000), which can be exchanged with the external medium (Strnad 1997), as the riboside form has greater membrane permeability than the ribotide form and is considered an efficient CK transport form (Mader *et al*. 2003). We can thus presume that this interconversion of bases, ribosides and ribotides is an important mechanism in the regulation of the concentration of active compounds (Schmülling 2004).

A simplistic metabolism characterizes cotyledons of *P. pinea*, and with the exception of BA9GR, no other glucoside derived forms were detected, such as the derivative 9-glucoside of BA, a stable form with rooting inhibition effects (Bairu *et al*. 2007). It is interesting to note that none of the traditional *O*-glucoside CKs widely present in other higher order plants were observed. Therefore, we may infer that conifers have a different enzymatic system that conjugates the riboside CKs with hexose (glucose) instead of the side chain, as proposed Zhang *et al*. (2001). It is clear that previous studies did not detect the glucoside attached to the riboside (BA9GR) because of the separation methods used, no radioactivity monitor being coupled. From our results, we may suppose that this glucoside, described for the first time by Auer and Cohen (1993), was also present

under 4.4 µM BA conditions, as it was found in induced cotyledons previously germinated 2 or 4 d under these conditions (Cortizo 2008) in very low concentrations, connecting low levels of BA9GR with a low magnitude of caulogenic response.

Similar glucoside forms to BA9GR have been described in other CKs, in which the hexose moiety is attached directly to the riboside (e.g. glucoside of trans-zeatin riboside (*t*ZR) (Morris *et al*. 1990)). Indeed, it has been postulated that they may increase the pool of interconvertible CKs (Strnad 1997). The function of the novel CK glucosides remains to be determined, but in contrast to *O*-glucoside CKs, they have freely available side chains so could be active forms (Zhang *et al*. 2001; 2003). Once the activity of BA9GR is supposed, and if we consider previous assumptions about its implication in bud morphogenesis (Zhang *et al*. 2001), our results agree with its positive implication in caulogenic processes, at least from a strength magnitude approach. This assertion is supported in the present chapter by the existence of a correspondence between the level of organogenic response and the glucoside concentration: family 36 presents the highest caulogenic response and the highest glucoside concentration at the end of the induction period (6 d), meanwhile family 61 shows the lowest values in both caulogenic response and glucoside concentration.

As we have reported here, minimum induction period under optimized conditions is shorter than previous information has indicated (Moncaleán *et al*. 2005), and these data will help to understand the behaviour of expression profiles of genes related to caulogenic induction (Alonso *et al*. 2007).

In addition, our data support the hypothesis that differences in hormonal contents may provoke physiological responses, connecting the magnitude of the induced response with the concentration of the substance. However, considering that during the caulogenic process an interaction between exogenously applied compounds and natural plant growth

regulators takes place, as well as the fact that BA assimilation modifies natural endogenous auxin and CK levels (Centeno *et al*. 1996), further complementary studies on endogenous natural CKs dynamics would be valuable for exploring the underlying causes of this differential organogenic response.

Acknowledgements

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CAPÍTULO 4

Endogenous cytokinin profile during adventitious caulogenesis in *Pinus pinea* L. cotyledons and its relationship to family organogenetic responses

Imagen modificada de "Lambert, Aylmer Bourke (1803-1807). *A description of the genus* Pinus. *Vol. 1*. Londres (Reino Unido)".

Endogenous cytokinin profiles during adventitious caulogenesis in *Pinus pinea* L. cotyledons and its relationship to family organogenetic responses

Summary

The caulogenic process based on mature cotyledons of stone pine (*Pinus pinea* L.) is promoted by the exogenous application of the cytokinin (CK) benzyladenine (BA), obtaining a variable range of shoots. This experimental system has been used as a model for analyzing CK trends, such as BA uptake and metabolism, connecting its dynamics with the organogenic response. In this way, the endogenous CK profile has been characterized here, taking into consideration not only the CK type (e.g. isoprenoid or aromatic) but also the metabolic groups (e.g. free base, riboside, ribotide or glucoside). Additionally, two selected families which differed in their shoot organogenic response have also been evaluated.

Before BA application, ribotide fraction constituted the main pool, but 1 h after initiating culturing conditions, the results showed that aromatic CKs represented more than 90% of the total pool of endogenous compounds, mainly free bases and ribosides (considered the active forms). BA *N*-glucosides were also observed, as well as the formation of topolins, which at 2 d started increasing till the end of the culture period.

CK profile of pine families also showed differences in values, although general trends were maintained. During the first 6 h the family characterized by the highest magnitude of caulogenic response showed higher levels in BA, BA riboside (BAR) and *meta*-topolin (*m*T), while the least organogenic family presented the highest levels of ribotides and aromatic glucosides.

Finally, and for the first time in this species (and also in conifers), the aromatic BA, its riboside and ribotide and *para*-topolin (*p*T) have been found as naturally occurring in all the materials assayed.

Este capítulo se corresponde con la publicación "Endogenous cytokinin profiles during adventitious caulogenesis in *Pinus pinea* L. cotyledons and its relationship to family organogenetic responses (en preparación)".

Introduction

Clonal micropropagation is a suitable system for obtaining and maintaining desirable genotypes, especially in those species with long rotation times such as the conifer stone pine (*Pinus pinea* L). Therefore great efforts have been focused on its optimization (Capuana and Giannini 1995; Sul and Korban 2004; Moncaleán *et al*. 2005; Alonso *et al*. 2006), as well as family assays developed (Cuesta *et al*. 2008). Furthermore, this vegetative propagation method may be the starting point for exploring the underlying causes of the physiological processes implied, specifically during the shoot-organogenesis induction, as highlighted in recent literature (Valdés *et al*. 2001; Moncaleán *et al*. 2005; Alonso *et al*. 2007; Cortizo 2008).

Cytokinins (CKs) are a group of plant growth regulators involved in many physiological processes, also being widely applied in *in vitro* culture for inducing shoot organogenesis (Krikorian 1995). Referring to stone pine cotyledons, the aromatic CK benzyladenine (BA) has been successfully applied in the regeneration protocol (Alonso *et al*. 2006), and some studies have stated the negative effect of the germination period on the macromorphological response (Valdés *et al*. 2001), to be directly related to a dramatic decrease in BA absorption (Cortizo 2008). Moreover, the dynamics of BA under optimized culture conditions (44.4 μM of BA for 6 d) have been studied, with the discovery of a relevant main peak of absorption after 6 h of culture (see Chapter 3). Similarly, after analyzing the BA dynamics of families characterized by high or low levels of organogenic response (Cuesta *et al*. 2008), it has been found that increased absorption leads to more response intensity (see Chapter 3), suggesting a direct relationship between BA absorption and the caulogenic response, in agreement with Auer *et al*. (1992) in *Petunia hybrida* and Centeno *et al*. (1996) in *Actinidia deliciosa*.

However, it is also necessary to consider that *in vitro* shoot

organogenesis is controlled by dynamic biochemical pathways, involving both exogenous and natural endogenous CKs (Auer *et al*. 1999). Furthermore, tissue response to externally added CKs is usually complicated by the unknown content and physiological activity of endogenous natural hormones (Baroja-Fernández *et al*. 2002), such as the diversity of CK forms (active, translocation, and storage), and the continuous depletion or replenishment of pools creating difficulties in the comparisons of such patterns in order to deduce the physiological effects.

For these reasons, analyses of endogenous CKs were carried out on *P. pinea* tissues (Valdés *et al*. 2001; Moncaleán *et al*. 2005), analyzing some isoprenoid compounds (zeatin, Z; zeatin riboside, ZR; dihydrozeatin, DHZ; dihydrozeatin riboside, DHZR; isopentenyladenine, iP; and isopentenyladenosine, iPR) under 4.4 μM BA culture conditions. Nevertheless, the current culture conditions (44.4 μM) have not been evaluated for none CK metabolic forms. Nothing is known either about the endogenous aromatic CKs and their role in the caulogenic induction.

Methodological improvements have been described recently, simultaneously analyzing isoprenoid and aromatic CKs (Novák *et al*. 2003; 2008). Therefore, using this approach we attempted to evaluate CK dynamics during the induction period in open-pollinated (OP) material. Further to this, the two previously cited families with different magnitude of organogenic response were analyzed to discover possible explanations for their different organogenetic capacity.

The aim of this chapter was to characterize both endogenous isoprenoid and aromatic CK profiles during caulogenic induction of OP cotyledons of stone pine. After that, family analysis was also carried out, allowing the determination of whether differences in magnitude of caulogenic response are correlated with differences in CK levels, and investigate in more depth the shoot formation process that occurs in stone pine cotyledons cultured *in vitro*. To the best of our knowledge this is the

first time that natural endogenous aromatic CKs have been quantified and associated with shoot formation in these materials.

Materials and methods

Plant material and culture conditions

Three-year-old mature seeds from two half-sibling selected families and OP trees of *Pinus pinea* L. were used. The selected families belong to "Cataluña Litoral" and "Meseta Norte" provenances (families 36 and 61 respectively), and all of them were provided by the "Centro Nacional de Mejora Genética Forestal, Puerta de Hierro" from "Dirección General para la Biodiversidad" (Spain). The families were selected according to their caulogenic response (Cuesta *et al*. 2008), representing the highest and the lowest value of shoot formation (families 36 and 61, respectively).

Following the Alonso *et al.* (2006) procedure, seeds were surface sterilized by immersion in 7.5% H_2O_2 for 45 min followed by an imbibition step of 48 h at 4 ºC in darkness to facilitate dissection of embryos. Cotyledons were excised from embryos and placed horizontally for 6 d in bud induction medium consisting of Le Poivre medium as modified by Aitken-Christie *et al.* (1988) with half strength macroelements (½LP) and supplemented with sucrose and 44.4 µM of BA. Explants were cultured under induction conditions for 6 d, and samples were collected at different periods (0; 1; 2; 6; 16 and 24 h and 2; 4 and 6 d).

Cotyledons taken from each time interval were washed with distilled water and dried with filter paper, and their fresh weight (FW) determined. Then, they were frozen in liquid N_2, lyophilized and their dry weight (DW) determined, calculating the water content per cotyledon. A completely randomized experimental design was applied, repeating each experiment at least 3 times.

CK extraction and purification

CK nomenclature follows Novák *et al*. (2008) (Table 1), and the purification method was based on Novák *et al*. (2003), modified as follows: freeze–dried plant material was homogenized in liquid N_2 and extracted in 15 mL Bielesky buffer (60% methanol, 25% $CHCl_3$, 10% HCOOH and 5% H_2O). After overnight extraction at -20 ºC, the homogenate was centrifuged (15000 *g* at 4 °C) and the pellets were re-extracted. The combined supernatants were purified using a cationic exchange SCX column (Varian Inc., CA, USA). Then, the samples were purified coupling Sephadex (Sigma-Aldrich, St. Louis, MA, USA) and reverse-phase (C_{18}) columns (Waters, Milford, MA, USA), obtaining two fractions (bases/ribosides/glycosides, and ribotides). No enzymatic cleavage treatment was applied and the metabolic eluates were evaporated to dryness.

Table 1. CK nomenclature according to Novák *et al.* (2008).

Isoprenoid CKs	**Abbreviation**	**Aromatic CKs**	**Abbreviation**
cis-zeatin	*c*Z	benzyladenine	BA
cis-zeatin riboside	*c*ZR	benzyladenine riboside	BAR
cis-zeatin riboside-5′-monophosphate	*c*ZRMP	benzyladenosine-5′-monophosphate	BARMP
trans-zeatin	*t*Z	benzyladenine 3-glucoside	BA3G
trans-zeatin riboside	*t*ZR	benzyladenine 7-glucoside	BA7G
trans-zeatin riboside-5′-monophosphate	*t*ZRMP	benzyladenine 9-glucoside	BA9G
trans-zeatin 7-glucoside	*t*Z7G	benzyladenine 9-glucoside riboside	BA9GR
trans-zeatin 9-glucoside	*t*Z9G	*meta*-topolin	*m*T
trans-zeatin *O*-glucoside	*t*ZOG	*meta*-topolin riboside	*m*TR
trans-zeatin riboside *O*-glucoside	*t*ZROG	*meta*-topolin 7-glucoside	*m*T7G
dihydrozeatin	DHZ	*meta*-topolin 9-glucoside	*m*T9G
dihydrozeatin riboside	DHZR	*ortho*-topolin	*o*T
dihydrozeatin riboside-5′-monophosphate	DHZRMP	*ortho*-topolin riboside	*o*TR
dihydrozeatin 9-glucoside	DHZ9G	*ortho*-topolin 7-glucoside	*o*T7G
dihydrozeatin *O*-glucoside	DHZOG	*ortho*-topolin 9-glucoside	*o*T9G
isopentenyladenine	iP	*para*-topolin	*p*T
isopentenyladenosine	iPR	*para*-topolin riboside	*p*TR
isopentenyladenosine-5′-monophosphate	iPRMP		
isopentenyladenine 7-glucoside	iP7G		
isopentenyladenine 9-glucoside	iP9G		

To check the recovery during purification and to validate the determination, deuterium-labelled CK internal standards (Olchemim Ltd.,

Czech Republic) were added to each sample. The used standards were [^{13}H$_5$]*c*Z, [^{2}H$_5$]*t*Z, [^{2}H$_5$]*t*ZR, [^{2}H$_5$]*t*ZRMP, [^{2}H$_5$]*t*Z7G, [^{2}H$_5$]*t*Z9G, [^{2}H$_5$]*t*ZOG, [^{2}H$_5$]*t*ZROG, [^{2}H$_3$]DHZ, [^{2}H$_3$]DHZR, [^{2}H$_3$]DHZRMP, [^{2}H$_3$]DHZ9G, [^{2}H$_7$]DHZOG, [^{2}H$_6$]iP, [^{2}H$_6$]iPR, [^{2}H$_6$]iPRMP, [^{2}H$_6$]iP7G, [^{2}H$_6$]iP9G, [^{2}H$_7$]BA, [^{2}H$_7$]BAR, [^{2}H$_7$]BARMP, [^{2}H$_7$]BA9G, [^{15}H$_4$]*m*T, and [^{13}H$_5$]*o*T.

CK quantification

The CK fractions were analyzed by ultra-performance liquid chromatography (UPLC) (ACQUITY UPLC™; Waters, Milford, MA, USA) linked to a Quattro micro™ API triple quadrupole mass spectrometer equipped with an electrospray interface (Waters MS Technologies, Manchester, UK) following Novák *et al*. (2008).

A reverse-phase column (BEH C18; 1.7 µm; 2.1 x 150 mm; Waters, Milford, MA, USA) was used, applying a linear gradient of 15 mM ammonium formate (pH 4) and methanol, with retention times for the monitored compounds ranging from 2.50 to 6.50 min. The gradient consisted of 10 to 50% methanol over 8 min at flow rate of 0.25 mL min^{-1} and a column temperature of 40 °C. Quantification was obtained by multireaction monitoring (MRM) of $[M+H]^+$ and the appropriate product ion. For selective MRM experiments, optimal mass spectrometric conditions, cone voltage, and collision energy in collision cell corresponding to exact diagnostic transition were optimized for each CK (Novák *et al*. 2008).

Data analysis

Levels and concentrations of CKs were expressed as nmol g^{-1}DW and nM, by applying FW, DW and water content data.

The method applied here allows the analysis of 46 different CKs. In order to simplify the interpretation of the large amount of data generated,

several categories have been considered. The first one establishes two main groups of CKs: the aromatic (also divided in the two classes BA and topolins) and the isoprenoid types. In addition, 4 different groups were considered according to their metabolic function: free bases, ribosides, ribotides and *N*-glucosides.

Results

Endogenous CK trends in Open-Pollinated cotyledons of stone pine

Plant material of OP cotyledons of *P. pinea* do not present all the CKs analyzed, such as the *O*-glucosides, *t*ZRMP, DHZRMP and the 7/9-glucoside of isoprenoid fraction.

The main pool of endogenous CKs in the OP material assayed was characterized by aromatic CKs. At 6 d of culture, 99.6% was presented in the form of BA, BAR and BARMP, these being an order of magnitude higher as a result of exogenous BA application. Therefore, these compounds were considered separately from the rest of the CKs measured. Relative percentage of each group occurring in stone pine cotyledons are presented in Table 2.

At the beginning of the culture (0 h), isoprenoid CKs were predominant (86.4%), but at 1 h topolins reached very high levels (90.5%), which were maintained till the end of the culture period (76.0% at 6 d), highlighting their putative relevance in the process. Moreover, at the end of the culture period (Table 2A) BA glucosides also increased, scoring 22.4%.

Table 2. Endogenous CKs distribution in OP material of *Pinus pinea* cotyledons. CKs are sequestered to several classes, presented as nmol g^{-1}DW with relative percentage in brackets. BA, BAR and BARMP have not been considered because of exogenous BA application. **A**. CKs classified according to specific group: BA glucosides, topolins and isoprenoids. **B**. CKs classified according to their metabolic function: free bases, glucosides, ribosides and ribotides.

Cytokinin type	**Incubation time**								
A. Cytokinin group nmol g^{-1}DW (relative %)	**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
BA glucosides	0.0 (0.0%)	0.1 (0.1%)	0.4 (0.4%)	1.1 (0.5%)	10.6 (4.4%)	19.1 (11.1%)	58.3 (25.1%)	271.7 (21.5%)	514.4 (22.4%)
Topolins	1.6 (13.6%)	89.7 (90.5%)	176.9 (93.4%)	230.9 (95.2%)	221.5 (92.5%)	140.6 (82.0%)	170.8 (73.6%)	979.2 (77.6%)	1743.0 (76.0%)
Isoprenoids	10.2 (86.4%)	9.3 (9.4%)	11.9 (6.2%)	10.4 (4.3%)	7.5 (3.1%)	11.8 (6.9%)	3.1 (1.3%)	10.7 (0.9%)	35.8 (1.6%)
B. Metabolic group nmol g^{-1}DW (relative %)	**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
Free bases (Topolins, Isoprenoids)	2.0 (16.8%)	89.9 (90.7%)	176.2 (93.1%)	228.4 (94.2%)	211.4 (88.2%)	120.8 (70.5%)	145.5 (62.6%)	863.8 (68.5%)	1508.0 (65.8%)
Glucosides (BA and Topolin glucosides)	0.0 (0.0%)	0.1 (0.1%)	0.4 (0.2%)	1.1 (0.5%)	11.7 (4.9%)	20.8 (12.1%)	64.5 (27.8%)	358.7 (28.4%)	634.6 (27.7%)
Ribosides (Topolins, Isoprenoids)	2.1 (17.7%)	1.5 (1.5%)	3.3 (1.8%)	4.5 (1.8%)	10.5 (4.4%)	21.0 (12.2%)	19.4 (8.4%)	31.7 (2.5%)	131.3 (5.7%)
Ribotides (Isoprenoids)	7.8 (65.5%)	7.6 (7.7%)	9.3 (4.9%)	8.4 (3.5%)	6.0 (2.5%)	8.9 (5.2%)	2.8 (1.2%)	7.4 (0.6%)	19.3 (0.8%)

Another way of grouping data independently of its type, is following their metabolic function (Table 2B): at the beginning of the induction, the ribotide fraction constituted the main pool (65.5%), but at 1 h free bases constituted the major proportion (90.7%).

Twenty-four hours after starting the induction period, free bases constituted 70.4% of the total pool, whole glucosides as well as ribosides each accounted for 12%. Finally, at 6 d free bases maintained their predominance (65.8%), followed by glucosides (27.7%), ribosides (5.7%) and ribotides (0.8%).

BA and pT as naturally occurring CKs in Open-Pollinated material

The presence of BA as a natural CK in stone pine cotyledons from OP material has been observed for the first time, scoring 209.70 pmol g^{-1}DW at 0 h (Table 3A). BA derivatives were also detected, with values of 0.13 (BAR) and 1.45 pmol g^{-1}DW (BARMP). *p*T was also recorded, with 1.6 pmol g^{-1}DW (Table 3C).

Aromatic CKs: BA and metabolites

BA profile in OP material of *P. pinea* during the 6-d culture period is shown in Figure 1A. The BA dynamic showed an initial increase from 0 to 6 h, reaching a smooth peak between 6 and 16 h, which corresponds to the period of meristemoid appearance (see Chapter 3). From this point till 2 d, BA cytokinin levels reduced to $196.1x10^3$ pmol g^{-1}DW, with BAR and BARMP starting to increase (Table 3A). In the last step the three compounds increased again, scoring $441.3x10^3$ (BA), $73.0x10^3$ (BAR) and $32.6x10^3$ (BARMP) pmol g^{-1}DW at 6 d. The corresponding molar concentrations are shown in Table 4A, which highlights the 16-h BA value ($514.2x10^3$ nM),

which might originate from the combination of natural BA and BA uptake from the medium (Figures 1A-B).

BA glucosides were found after 1 h of culture (BA9G, Table 3B), increasing at 16 h. After 2 d the three *N*-glucosides were detected (BA3G, BA7G, BA9G), and their levels increased till the end of the culture period with BA7G being the highest with a concentration of 96.9 nM (Table 4B).

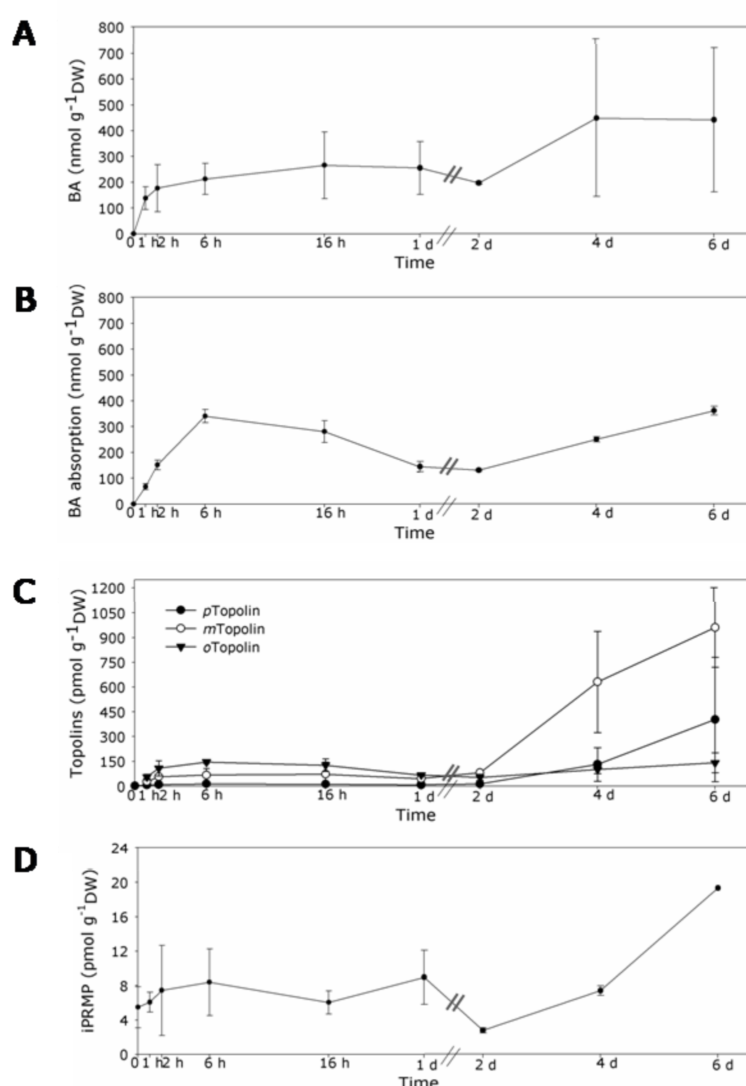

Figure 1. Profile of several endogenous CKs in OP material of *Pinus pinea*. **A**. BA dynamics (nmol g^{-1}DW). **B**. Data belonging to BA absorption report (see Chapter 3). **C**. Dynamics of free bases of topolins (pmol g^{-1}DW). **D**. iPRMP profile during the culture period (pmol g^{-1}DW).

Aromatic CKs: topolins and metabolites

Topolins of stone pine cotyledons from OP material showed a progressive increase in the three structural forms detected (free bases, ribosides and glucosides) (Table 4C), with a total molar concentration of 589.7 nM, most of this belonging to free bases (508.6 nM) (Table 4C). Considering free bases, *m*T reached the highest values, up to 900 pmol g^{-1} DW (Figure 1C).

Table 3. Endogenous CKs in OP material of *Pinus pinea* according to their metabolic function (free base, riboside, ribotide and glucoside) expressed as pmol g^{-1}DW. **A**. BA, BAR and BARMP. **B**. BA glucosides (BA3G, BA7G, BA9G). **C**. Topolin derivatives: free bases (*p*T, *m*T, *o*T), ribosides (*p*TR, *m*TR, *o*TR) and glucosides (*m*T7G, *m*T9G, *o*T7G, *o*T9G). **D**. Isoprenoid cytokinins: free bases (*t*Z, *c*Z, DHZ, iP), ribosides (*t*ZR, *c*ZR, DHZR, iPR) and ribotides (*c*ZRMP, iPRMP).

Cytokinin type	Incubation time								
A.BA, BAR, BARMP (pmol g^{-1}DW)	**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
BA	209.70	137.8 x10^{3}	176.8 x10^{3}	212.5 x10^{3}	265.7 x10^{3}	254.9 x10^{3}	196.1 x10^{3}	448.5 x10^{3}	441.3 x10^{3}
BAR	0.13	59.6	168.9	1.4 x10^{3}	3.1 x10^{3}	6.1 x10^{3}	7.8 x10^{3}	28.0 x10^{3}	73.0 x10^{3}
BARMP	1.45	608.5	1.3 x10^{3}	5.9 x10^{3}	6.9 x10^{3}	18.4 x10^{3}	23.8 x10^{3}	20.8 x10^{3}	32.6 x10^{3}
Total	211.28	138.5 x10^{3}	178.3 x10^{3}	219.8 x10^{3}	275.7 x10^{3}	279.4 x10^{3}	227.7 x10^{3}	497.3 x10^{3}	546.9 x10^{3}
B.BA glucosides (pmol g^{-1}DW)	**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
BA3G	<LOD	<LOD	<LOD	<LOD	3.0	8.2	30.3	87.9	145.1
BA7G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	10.0	138.5	286.3
BA9G	<LOD	0.1	0.4	1.1	7.6	11.0	18.0	45.2	83.0
Total	0.0	0.1	0.4	1.1	10.6	19.2	58.3	271.6	514.4
C.Topolins (pmol g^{-1}DW)	**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
Free bases	1.6	89.1	175.0	227.8	211.0	118.9	145.3	861.6	1503.3
Ribosides	<LOD	0.6	1.9	3.1	9.4	20.0	19.3	30.6	119.5
Glucosides	<LOD	<LOD	<LOD	<LOD	1.1	1.7	6.2	87.0	120.2
Total	1.6	89.7	176.9	230.9	221.5	140.6	170.8	979.2	1743.0
D.Isoprenoids (pmol g^{-1}DW)	**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
Free bases	0.4	0.8	1.2	0.6	0.4	1.9	0.2	2.2	4.7
Ribosides	2.1	0.9	1.4	1.4	1.1	1.0	0.1	1.1	11.8
Ribotides	7.8	7.6	9.3	8.4	6.0	8.9	2.8	7.4	19.3
Total	10.3	9.3	11.7	10.4	7.5	11.8	3.1	10.7	35.8

<LOD: Under Limit of Detection.

Isoprenoid CKs

The isoprenoid CK fraction was composed of free bases, ribosides and ribotides, with the last being the most abundant (Table 3D). Their profile was quite constant throughout the culture period; only the ribotide fraction oscillated, with a dramatic 3-fold decrease at 2 d, but with a second increase till the end of the culture period (Table 3D). Most notably isoprenoid CK was the iPRMP, whose dynamics (Figure 1D) were similar to BA case.

Table 4. Endogenous CKs in OP material of *Pinus pinea* according to their metabolic function (free base, riboside, ribotide and glucoside) expressed as nM. **A**. BA, BAR and BARMP. **B**. BA glucosides (BA3G, BA7G, BA9G). **C**. Topolin derivatives: free bases (*p*T, *m*T, *o*T), ribosides (*p*TR, *m*TR, *o*TR) and glucosides (*m*T7G, *m*T9G, *o*T7G, *o*T9G). **D**. Isoprenoid compounds: free bases (*t*Z, *c*Z, DHZ, iP), ribosides (*t*ZR, *c*ZR, DHZR, iPR) and ribotides (*c*ZRMP, iPRMP).

Cytokinin type	**Incubation time**							
A.BA, BAR, BARMP (nM)	**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
BA	153.7	152.9 x10^{3}	172.3 x10^{3}	210.5 x10^{3}	514.2 x10^{3}	245.1 x10^{3}	151.8 x10^{3}	247.3 x10^{3}	149.3 x10^{3}
BAR	0.098	66.1	164.7	1.3 x10^{3}	6.1 x10^{3}	5.9 x10^{3}	6.0 x10^{3}	15.5 x10^{3}	24.7 x10^{3}
BARMP	1.06	675.3	1.3 x10^{3}	5.8 x10^{3}	13.4 x10^{3}	17.7 x10^{3}	18.4 x10^{3}	11.5 x10^{3}	11.0 x10^{3}
Total	154.86	153.6 x10^{3}	173.8 x10^{3}	217.6 x10^{3}	533.7 x10^{3}	268.7 x10^{3}	176.2 x10^{3}	274.3 x10^{3}	185.0 x10^{3}
B.BA glucosides (nM)	**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
BA3G	<LOD	<LOD	<LOD	<LOD	5.7	7.8	23.4	48.5	49.1
BA7G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	7.7	76.4	96.9
BA9G	<LOD	0.2	0.4	1.1	14.7	10.6	13.9	24.9	28.1
Total	0.0	0.2	0.4	1.1	20.4	18.4	45.0	149.8	174.1
C.Topolins (nM)	**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
Free bases	1.1	98.9	170.6	225.6	408.4	114.3	112.5	475.1	508.6
Ribosides	<LOD	0.6	1.9	3.1	18.2	19.3	14.9	16.8	40.4
Glucosides	<LOD	<LOD	<LOD	<LOD	2.1	1.6	4.8	48.0	40.7
Total	1.1	99.5	172.4	228.7	428.6	135.2	132.2	539.9	589.7
D.Isoprenoids (nM)	**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
Free bases	0.3	0.9	1.2	0.6	0.8	1.8	0.1	1.2	1.6
Ribosides	1.5	1.0	1.4	1.3	2.1	0.9	0.1	0.6	4.0
Ribotides	5.7	8.4	9.0	8.3	11.7	8.6	2.1	4.1	6.5
Total	7.5	10.3	11.6	10.2	14.6	11.4	2.4	5.9	12.1

<LOD: Under Limit of Detection.

Comparison of CK dynamics in P. pinea cotyledon families

Above-cited profiles of the different CKs detected were similar in family materials, though different levels were reported for some CKs, as shown in Tables 5-6 and Figures 2A-D.

Aromatic CKs: BA and metabolites

As occurred in OP material, BA was also found as naturally occurring CK, being 2-fold higher in the less organogenic family 61 (Tables 5A-6A).

BA levels expressed as pmol g^{-1}DW were not very different between families (Table 5A) e.g. 6-h time point showed $241.5x10^3$ and $237.0x10^3$ pmol g^{-1}DW (families 36 and 61, respectively). However, if we focus on molar concentration (Table 6A), these differences increased e.g. 6-h samples showed 1.6-fold higher concentration in family 36 than 61 (298.1 and 177.8 μM respectively). Analyzing other BA metabolites such as BARMP, after 6 h, levels expressed as nM (Table 6A) were 2.1-fold higher in family 61. At 16 h this difference rapidly increased to 9.2-fold (Table 6A). In contrast BAR at 6 d was 2.1-fold higher in family 36.

BA glucosides (Tables 5B-6B) also appeared in both families after 2 h of culture. Family 61 showed higher initial concentration (1.5 nM at 6 h), becoming 6.6-fold higher than family 36 at 16 h (Table 6B), probably as a consequence of the high amount of BA present at the same time. Subsequently, this relationship was reversed, showing levels 1.4-fold higher in family 36 at 6 d (205.6 nM).

Aromatic CKs: topolins and metabolites

*p*T was also recorded as naturally occurring CK in both families (Table 5C). The general trend of topolin free bases was of a progressive increase, becoming more pronounced after 2 d of culture. At that time (2 d)

differences between both families increased (Figure 2D), although if total molar concentration was observed (Table 6C), values were similar between families.

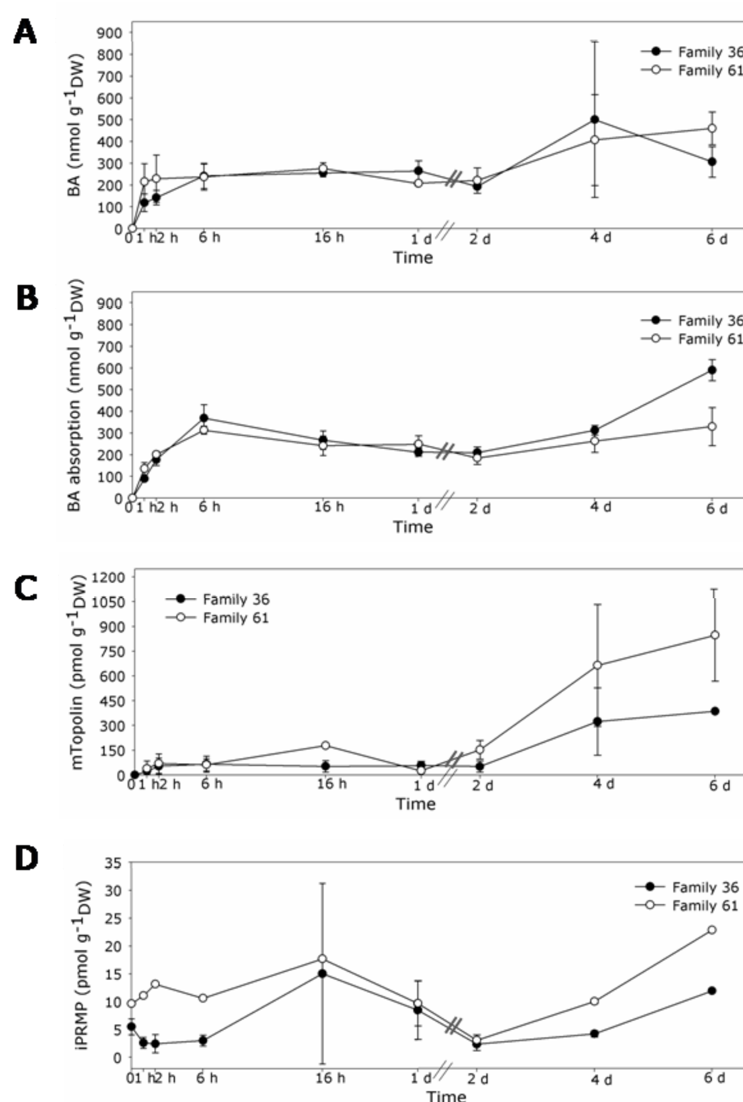

Figure 2. Profile of several endogenous CKs in family materials of *Pinus pinea*. **A**. BA dynamics (nmol g^{-1}DW). **B**. Data belonging to BA absorption report (see Chapter 3). **C**. Dynamics of *m*T in both families (pmol g^{-1}DW). **D**. iPRMP profile during the culture period (pmol g^{-1}DW).

Table 5. Endogenous CKs in two families assayed (36 and 61) of *Pinus pinea* according to their metabolic function (free base, riboside, ribotide and glucoside) expressed as pmol g^{-1} DW. **A**. BA, BAR and BARMP. **B**. BA glucosides (BA3G, BA7G, BA9G). **C**. Topolin derivatives: free bases (*p*T, *m*T, *o*T), ribosides (*p*TR, *m*TR, *o*TR) and glucosides (*m*T7G, *m*T9G, *o*T7G, *o*T9G). **D**. Isoprenoid compounds: free bases (*t*Z, *c*Z, DHZ, iP), ribosides (*t*ZR, *c*ZR, DHZR, iPR) and ribotides (*c*ZRMP, iPRMP).

Cytokinin type	Family	Incubation time								
A.BA, BAR, BARMP (pmol g^{-1}DW)		**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
BA	36	76.89	118.3 x10^3	142.2 x10^3	241.5 x10^3	255.5 x10^3	264.8 x10^3	192.5 x10^3	500.1 x10^3	305.7 x10^3
	61	169.80	215.1 x10^3	229.3 x10^3	237.0 x10^3	276.1 x10^3	207.2 x10^3	220.1 x10^3	406.1 x10^3	459.9 x10^3
BAR	36	0.29	18.89	52.82	399.5	1.3 x10^3	3.1 x10^3	8.9 x10^3	23.7 x10^3	76.0 x10^3
	61	0.52	98.36	252.39	1.9 x10^3	30.0 x10^3	4.8 x10^3	6.9 x10^3	32.7 x10^3	58.4 x10^3
BARMP	36	1.13	442.63	707.79	3.2 x10^3	10.3 x10^3	11.8 x10^3	17.9 x10^3	20.6 x10^3	26.7 x10^3
	61	1.20	1.6 x10^3	2.3 x10^3	11.1 x10^3	52.8 x10^3	18.9 x10^3	19.4 x10^3	30.9 x10^3	33.7 x10^3
Total	36	78.31	118.8 x10^3	142.9 x10^3	245.1 x10^3	267.1 x10^3	279.7 x10^3	219.3 x10^3	544.4 x10^3	408.4 x10^3
	61	171.52	216.8 x10^3	231.8 x10^3	250.0 x10^3	358.9 x10^3	230.9 x10^3	246.4 x10^3	469.7 x10^3	552.0 x10^3
B.BA glucosides (pmol g^{-1}DW)		**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
BA3G	36	<LOD	<LOD	<LOD	<LOD	1.3	4.3	27.1	130.8	140.7
	61	<LOD	<LOD	<LOD	<LOD	9.2	4.4	31.1	98.1	120.4
BA7G	36	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	4.4	206.8	226.6
	61	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	25.7	257.5	319.5
BA9G	36	<LOD	<LOD	0.1	0.5	6.9	12.2	24.5	48.4	75.4
	61	<LOD	0.3	0.5	2.0	21.4	9.2	16.7	57.7	69.8
Total	36	0.0	0.0	0.1	0.5	8.2	16.4	56.0	386.0	442.8
	61	0.0	0.3	0.5	2.0	30.6	13.6	73.6	413.2	509.6
C. Topolins (pmol g^{-1}DW)		**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
Free bases	36	2.9	76.3	165.3	214.3	193.4	190.3	120.5	445.4	612.6
(*m*T, *p*T, *o*T)	61	1.0	125.8	205.1	207.1	697.9	74.2	245.8	938.5	1258.3
Ribosides	36	<LOD	<LOD	1.2	2.1	6.7	26.1	21.9	21.7	81.3
(*p*TR, *m*TR, *o*TR)	61	<LOD	<LOD	4.5	5.3	116.0	11.4	14.9	43.9	107.3
Glucosides	36	<LOD	<LOD	<LOD	<LOD	1.0	1.0	3.9	49.4	72.8
(*m*T7G,*m*T9G,*o*T7G,*o*T9G)	61	<LOD	<LOD	<LOD	<LOD	4.0	1.4	11.2	97.0	132.6
Total	36	2.9	76.3	166.5	216.4	201.0	217.4	146.3	516.4	766.8
	61	1.0	125.8	209.6	212.3	817.9	87.0	271.9	1079.4	1498.2
D. Isoprenoids (pmol g^{-1}DW)		**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
Free bases	36	1.7	0.5	0.8	1.1	1.0	0.5	0.0	2.7	6.0
(*t*Z, *c*Z, DHZ, iP)	61	3.4	2.7	1.2	0.8	0.5	0.3	0.5	2.7	13.7
Ribosides	36	2.2	0.4	0.5	0.5	1.4	0.9	0.2	0.5	5.5
(*t*ZR, *c*ZR, DHZR, iPR)	61	1.9	2.2	1.7	2.3	2.4	1.6	0.3	2.2	14.1
Ribotides	36	5.5	4.3	2.5	4.3	15.0	8.5	2.3	4.2	11.9
(*c*ZRMP, iPRMP)	61	13.5	15.4	13.2	14.6	19.3	9.7	5.0	10.0	27.1
Total	36	9.4	5.1	3.7	5.9	17.5	9.9	2.6	7.4	23.5
	61	18.8	20.3	16.1	17.7	22.2	11.6	5.8	15.0	54.9

<LOD: Under Limit of Detection.

Table 6. Endogenous CKs in two families assayed (36 and 61) of *Pinus pinea* according to their metabolic function (free base, riboside, ribotide and glucoside) expressed as nM. **A**. BA, BAR and BARMP. **B**. BA glucosides (BA3G, BA7G, BA9G). **C**. Topolin derivatives: free bases (*p*T, *m*T, *o*T), ribosides (*p*TR, *m*TR, *o*TR) and glucosides (*m*T7G, *m*T9G, *o*T7G, *o*T9G). **D**. Isoprenoid compounds: free bases (*t*Z, *c*Z, DHZ, iP), ribosides (*t*ZR, *c*ZR, DHZR, iPR) and ribotides (*c*ZRMP, iPRMP).

Cytokinin type	Family	Incubation time								
A.BA, BAR, BARMP (nM)		**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
BA	36	42.15	111.8 x10^3	160.4 x10^3	298.1 x10^3	136.3 x10^3	267.8 x10^3	193.5 x10^3	347.1 x10^3	142.0 x10^3
	61	94.64	156.1 x10^3	203.5 x10^3	177.8 x10^3	264.5 x10^3	146.3 x10^3	144.1 x10^3	192.2 x10^3	131.9 x10^3
BAR	36	0.16	17.86	59.58	493.0	689.2	3.2 x10^3	9.0 x10^3	16.4 x10^3	35.3 x10^3
	61	0.29	71.39	22.39	1.4 x10^3	28.7 x10^3	3.4 x10^3	4.5 x10^3	15.5 x10^3	16.7 x10^3
BARMP	36	0.62	418.42	798.5	3.9 x10^3	5.5 x10^3	12.0 x10^3	18.0 x10^3	14.3 x10^3	12.4 x10^3
	61	0.67	1.2 x10^3	2.0 x10^3	8.3 x10^3	50.6 x10^3	13.4 x10^3	12.7 x10^3	14.6 x10^3	9.7 x10^3
Total	36	42.93	112.2 x10^3	161.2 x10^3	302.5 x10^3	142.5 x10^3	283.0 x10^3	220.5 x10^3	377.8 x10^3	189.7 x10^3
	61	95.60	157.4 x10^3	205.5 x10^3	187.5 x10^3	343.8 x10^3	163.1 x10^3	161.3 x10^3	222.3 x10^3	158.3 x10^3
B.BA glucosides (nM)		**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
BA3G	36	<LOD	<LOD	<LOD	<LOD	0.7	4.3	27.3	90.8	65.3
	61	<LOD	<LOD	<LOD	<LOD	8.8	3.1	20.3	46.4	34.5
BA7G	36	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	4.4	143.5	105.2
	61	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	16.8	121.9	91.6
BA9G	36	<LOD	<LOD	0.1	0.6	3.7	12.3	24.7	33.6	35.0
	61	<LOD	0.2	0.5	1.5	20.5	6.5	11.0	27.3	20.0
Total	36	0.0	0.0	0.1	0.6	4.4	16.6	56.3	267.9	205.6
	61	0.0	0.2	0.5	1.5	29.3	9.6	48.2	195.6	146.2
C. Topolins (nM)		**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
Free bases	36	1.6	72.1	186.4	264.4	103.1	192.4	121.1	309.1	284.5
(*m*T, *p*T, *o*T)	61	0.5	91.3	182.0	155.3	668.7	52.4	160.9	444.3	360.9
Ribosides	36	<LOD	<LOD	1.3	2.6	3.6	26.4	22.1	15.1	37.7
(*p*TR, *m*TR, *o*TR)	61	<LOD	<LOD	4.0	3.9	111.1	8.1	9.8	20.8	30.8
Glucosides	36	<LOD	<LOD	<LOD	<LOD	0.5	1.0	3.9	34.3	33.8
(*m*T7G,*m*T9G,*o*T7G,*o*T9G)	61	<LOD	<LOD	<LOD	<LOD	3.8	1.0	7.3	45.9	38.0
Total	36	1.6	72.1	187.8	267.0	107.2	219.9	147.1	358.4	356.0
	61	0.5	91.3	186.0	159.2	783.7	61.5	178.0	510.9	429.7
D. Isoprenoids (nM)		**0 h**	**1 h**	**2 h**	**6 h**	**16 h**	**24 h**	**2 d**	**4 d**	**6 d**
Free bases	36	1.0	0.5	0.9	1.4	0.6	0.5	0.0	1.9	2.8
(*t*Z, *c*Z, DHZ, iP)	61	1.9	2.0	1.1	0.6	0.4	0.2	0.3	1.3	3.9
Ribosides	36	1.2	0.3	0.5	0.6	0.7	0.9	0.2	0.4	2.6
(*t*ZR, *c*ZR, DHZR, iPR)	61	1.0	1.6	1.5	1.7	2.3	1.1	0.2	1.1	4.0
Ribotides	36	3.0	4.0	2.8	5.4	8.0	8.6	2.3	2.9	5.5
(*c*ZRMP, iPRMP)	61	7.5	11.1	11.7	11.0	18.5	6.8	3.3	4.7	7.8
Total	36	5.2	4.8	4.2	7.3	9.3	10.0	2.6	5.2	10.9
	61	10.5	14.7	14.3	13.3	21.3	8.2	3.8	7.1	15.8

<LOD: Under Limit of Detection.

Isoprenoid CKs

At the beginning of the culture period, most isoprenoid compounds belonged to ribotides (Tables 5D and 6D), and endogenous concentration of these CK metabolites remained constant throughout the whole culture period (Table 6D). Only iPRMP presented a dynamic with an increase after 2 d (Figure 2D).

Discussion

Caulogenic response is triggered by exogenous stimuli, and how this organogenic process takes place may be explained at least partially by dynamics of endogenous CKs. In this way, the regulation of particular physiological processes is effectively governed by changes in levels of endogenous CK metabolites (Kamínek *et al*. 1997).

As BA is the exogenously applied growth regulator used for shoot induction in stone pine cotyledons, it is interesting to note the confirmation of BA, BAR, BARMP and *p*T as naturally occurring CKs, for the first time not only in *P. pinea* but also in conifers. BA was found for the first time as naturally occurring by Nandi *et al*. (1989) in primary crown gall tumors of tomato, and BAR in *Pimpinella anisum* by Ernst *et al*. (1983). The topolin metabolites were identified in *Populus*×*canadiensis* by Strnad *et al*. (1992; 1997). More recently, some other research groups have found these naturally occuring CKs in different plant species (e.g. *Cocos nucifera* (Sáenz *et al*. 2003), *Tagetes minuta* (Stirk *et al*. 2005)). Indeed, aromatic CKs were assumed to appear only sporadically in a few plant species, but presumably will occur in many more plant species as reviewed by Taylor *et al*. (2003).

The different trends of various CK groups analyzed in OP material during caulogenic induction period of *P. pinea* cotyledons support the view that particular CKs have specific roles in the various physiological processes

in plants, as *in vitro* responses are the result of a wide variety of factors, e.g. the uptake and metabolism of the exogenously applied growth regulator and its effect on endogenous CK levels (Valdés *et al*. 2001; Moncaleán *et al*. 2003). In accordance with this, at 0 h most CKs detected were isoprenoid, but after just 1 h in contact with exogenous BA in medium, 94% of the total CK pool was constituted by BA, BAR and BARMP. The rest of the pool (Table 2A) was mainly composed of hydroxylated BA forms (topolins) (relative percentage of 90.5%). Concurring with these data, aromatic CKs represented more than 90% of the total endogenous CK content in *in vitro Solanum tuberosum* plantlets (Baroja-Fernández *et al*. 2002), but this contrasts with non hormone-treated *Cocos nucifera* material, where isoprenoid forms were the most abundant CK types (Sáenz *et al*. 2003). Cotyledons represent specific reserve organs whose major physiological function is to ensure the development of the growing seedling (Ananieva *et al*. 2004), and metabolic CK group most detected at 0 h was ribotides (Table 2B). After 24 h, when high cellular division is assumed, free bases were predominant. Additionally, CKs exhibited a progressive increase during the culture period, related to the enhancement of shoot regeneration.

It is known that BA uptake triggers the signalling cascade for meristemoid appearance, influencing endogenous 3-indolylacetic acid (IAA) and iP levels (Mercier *et al*. 2003). Charrière *et al*. (1999) observed that endogenous CK levels rise in response to increased BA levels in *Helianthus annuus* material; and after comparing changes in iP and iPR concentrations, it was suggested that iPR might replace BA as the inductor of morphogenic responses. Concurring with this idea, the present report shows that the main isoprenoid form detected in these materials was iPR (Figure 1D). This form is also the precursor of the rest of isoprenoid CKs in *de novo* biosynthetic CK iPRMP-dependent pathway (Sakakibara 2006). Nevertheless, total concentration of isoprenoid CKs was very low (Table 3D). The absence of highly active free base isoprenoid forms has been

explained in two ways: either they do not play a role in early stages of caulogenic induction, or they are rapidly utilized and are under tight regulation and thus cannot accumulate at high levels (Stirk *et al*. 2005). This theory is supported by the data reported here, although Moncaleán *et al*. (2005) detected, in the same species, high levels of isoprenoid CKs, mainly Z, in cotyledons of OP material under 4.4 µM BA treatment.

To a better understand of CK dynamics, the previous Chapter analyzed trends of BA cytokinins under 44.4 µM BA conditions (Figure 1B), considering there to be three phases in caulogenic induction period: the first (0 to 6 h) coinciding with the main peak of BA uptake and the minimum induction period required for shoot formation (6 h); the second (6 h to 2 d) includes the moment of meristemoid appearance (12 h) and at 2 d the end of the decrease in the rate of absorption of BA; and the last comprised of 2 to 6 d, characterized by a renewed increase in BA absorption and the appearance of BA9GR (see Chapter 3). Associating these stages with endogenous CK data (Tables 3 and 4), we can properly establish the implication of these compounds in bud appearance: a first stage corresponding to the minimum induction period (0 to 6 h) revealed high topolin levels (Tables 3C and 4C), and a second stage, from 6 h to 2 d, where slight fluctuations took place. In the final stage (2 to 6 d) a high increase of aromatic CK levels was seen (Tables 3A-B and 4A-B), mainly interconvert compounds (ribotides), and storage and inactive forms (aromatic CK *N*-glucosides). The detection of high levels of inactive products of metabolism like 9-glucosides has been cited as evidence of high CK turnover (Sáenz *et al*. 2003).

Present results relating to cotyledons of OP material from *P. pinea* do not show a constant hormonal level during the 6-d period, maybe because this culture period only includes the initiation of major developmental processes, requiring rapid and significant changes in the concentration of a particular hormone, and homeostasis will be required later for further development of the initiated events (Kamínek *et al*. 1997). This assumption

is supported by morphological data, as at least 35 d are necessary for detection of a clear bud structure (Cuesta *et al*. 2008).

Comparison of family regeneration capacity is connected to CK profiles with the differential magnitude of caulogenic response reported. As with OP material, the two selected families also contained BA, its metabolites (BAR and BARMP) and *p*T as naturally endogenous, but differing in their levels, with the less organogenic family having 2-fold higher values than the more organogenic family (Tables 5A and 6A). At the 6-h time point the endogenous concentration of active BA forms was higher for the more organogenic family, matching the minimum induction period. Regarding the ribotides, endogenous CK values of the less organogenic family were almost 10-fold higher after 16 h. An association between high BA uptake and an increase in isoprenoid CKs in relation to family influence has previously been reported in *Petunia hybrida* by Auer *et al*. (1999). From a general overview, from 0 to 6 h, matching the minimum induction period previously reported at 6 h, it could be assumed that higher levels in BA, BAR and *m*T in family 36 is associated with a higher magnitude of caulogenic response (Table 6). In this respect we can note that *m*T has been proved to be a CK with high morphogenetic activity (Bairu *et al*. 2007). Meanwhile, less organogenic material (family 61) showed the highest levels of ribotides and aromatic CK glucosides. After 2 d (coinciding with the third step of the induction period), the opposite trend takes place (Table 6), with values referring to family 61 being higher than family 36. These tendencies support the importance of establishing discrete steps during the induction culture period in order to formulate the correct interpretation of hormonal levels.

As we found here, Baroja-Fernández *et al*. (2002) found different response of genotypes to the *in vitro* conditions, partially attributed to a different endogenous CK pattern, including a strong correlation between survival and CK levels. Other works on *Ulmus glabra* (Malá *et al*. 2005) also considered that higher contents of iP were related to better *in vitro* morphogenic responses of explants.

For the first time, the present chapter reports qualitative and quantitative analysis of endogenous isoprenoid and aromatic CKs of stone pine. The data presented here reveal the complexity of CK regulation in a relatively simple tissue culture system. In this study, changes in endogenous CK concentrations during the shoot induction culture period in the two stone pine families have been characterized, providing a general picture of their dynamics during *in vitro* organogenesis. The results suggest that aromatic CKs play an active role in the caulogenic process of *in vitro* cultured cotyledons of stone pine. These data should provide a useful basis for further investigation of the function of CKs in caulogenic processes.

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DISCUSIÓN

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La micropropagación mediante organogénesis adventicia a partir de cotiledones maduros de *Pinus pinea* es un modelo experimental adecuado para el estudio de las bases fisiológicas y moleculares de los procesos organogénicos inducidos en coníferas, y más concretamente en estudios relacionados con la inducción caulogénica, tales como la dinámica de absorción del agente inductor de respuesta (Moncaleán *et al.* 2005) o la identificación de genes implicados en el proceso (Alonso *et al.* 2007). En esta línea de investigación se enmarca la presente Tesis Doctoral, que plantea si, al igual que se ha visto en otras especies como *Actinidia deliciosa* (Centeno *et al.* 1996; 2003) y *Petunia hybrida* (Auer *et al.* 1992; 1999), existe una relación entre la respuesta organogénica observada *in vitro*, la absorción y metabolismo del agente inductor de respuesta y los niveles endógenos de citoquininas (CKs).

En el trabajo presentado en esta tesis se aborda la micropropagación de 6 familias seleccionadas de *P. pinea* procedentes del Banco Clonal del Centro Nacional de Mejora Genética "Puerta de Hierro". Según la respuesta organogénica obtenida, se escogieron aquellas familias que presentaron la mayor y menor intensidad de respuesta *in vitro*, lo que nos permitió estudiar la relación entre el estado fisiológico de los explantos y la respuesta morfogénica *in vitro*.

El protocolo de regeneración de plántulas de pino piñonero debe ser adecuado y aplicable a las familias seleccionadas, además de repetitivo y fiable, es decir, sin fenómenos de variación genética, indeseables en la multiplicación clonal de individuos sobresalientes. La micropropagación mediante organogénesis adventicia a partir de cotiledones maduros de *P. pinea* ha sido descrita y mejorada en diversos trabajos: inicialmente el método se caracterizaba por la aplicación de la CK benciladenina (BA) a una concentración de 4,4 μM durante 30 d (Valdés *et al.* 2001), y posteriores ensayos redujeron a 4-8 d el tiempo de exposición al regulador de crecimiento, aumentando su concentración 10 veces (44,4 μM) (Moncaleán

et al. 2005; Alonso *et al.* 2006). Los primeros resultados publicados referentes al enraizamiento de los tallos adventicios fueron muy bajos, nunca superiores al 15; 20 y 34,4% (González *et al.* 1998; Sul y Korban 2004 y Capuana y Giannini 1995, respectivamente), lo que confirma lo crítico de esta etapa, considerada el cuello de botella de la micropropagación de numerosas coníferas, tales como *Abies amabilis* (Kulchetscki *et al.* 1995) y *Pinus taeda* (Hamann 1998). Por ello, la mejora de la etapa de enraizamiento ha sido ampliamente estudiada por diversos autores, considerando factores tales como las condiciones ambientales (temperatura, fotoperiodo), el medio de cultivo (cantidad de auxina aplicada al medio, contenido en azúcar) (Potes comunicación personal), y la concentración de macronutrientes (Sriskandarajah *et al.* 1990).

Siguiendo el protocolo descrito por Alonso *et al.* (2006) el periodo de inducción caulogénica escogido fue de 6 d con una concentración de 44,4 µM de BA en el medio de cultivo. Las familias ensayadas presentaron una supervivencia muy elevada, con una Capacidad de Formación de Yemas (BFC) variable, lo que refleja la diferente capacidad de respuesta de las mismas. Este comportamiento asociado al genotipo ensayado ya había sido observado en otras especies como *Pinus ayacahuite* (Saborio *et al.* 1997), *Platanus acerifolia* (Liu y Bao 2003) y *Pinus pinaster* (Tereso *et al.* 2006). En nuestro caso, las familias de *P. pinea* con una mayor y menor intensidad de respuesta fueron la 36 y 61 respectivamente.

En algunas pináceas como *P. contorta* (Flygh *et al.* 1993) y *P. taeda* (Tang *et al.* 2001) se observó que el medio de cultivo óptimo variaba según el genotipo ensayado. Para adecuar el protocolo de enraizamiento a las familias de *P. pinea* seleccionadas, se re-evaluó la concentración de macronutrientes del medio de cultivo. Tras realizar el ensayo, los mejores resultados se correspondieron con la combinación de medios ½LP-½LP, distintos a los propuestos por Alonso *et al*. (2006) para material de polinización abierta (¼LP-¼LP). Durante el proceso de inducción rizogénica, las bases de los tallos adventicios suelen producir masas de callo y

compuestos fenólicos que frecuentemente conducen a la formación de tejidos necróticos (Kulchetscki *et al.* 1995), por lo que al evaluar la capacidad rizogénica de las familias seleccionadas de *P. pinea* se ha de considerar la supervivencia de los explantos como un factor crítico, e incluir este parámetro en el cálculo de la eficiencia del proceso. Este Índice de Enraizamiento (RI) combina supervivencia y porcentaje de enraizamiento, reflejando en esta fase la capacidad real de producir microplantas por parte de los microtallos de una familia. Así, la familia 93, cuyo porcentaje de enraizamiento se sitúa en los valores medios de rizogénesis obtenidos (19,64%), presenta una supervivencia muy baja (55,97%), lo que reduce la eficiencia del proceso a un 11%.

Los valores obtenidos durante la etapa de enraizamiento concuerdan con aquellos descritos por varios autores para la misma especie (Capuana y Giannini 1995; González *et al.* 1998; Sul y Korban 2004), pero difieren del éxito obtenido en material de polinización abierta por Alonso *et al.* (2006), que llega a alcanzar el 70% de enraizamiento. Distintos niveles de rizogénesis asociados a genotipo y heredables ya han sido reflejados por distintos autores para *P. taeda* (Greenwood y Weir 1994; Tang y Ouyang 1999; Tang 2001), e igualmente se ha observado una respuesta dependiente de genotipo en la capacidad rizogénica de las 6 familias de *P. pinea* seleccionadas para este estudio, siendo, de nuevo, la familia 36 la que alcanzó valores más elevados, mientras que para la familia 61 se obtuvieron valores más discretos.

En la elección de las familias más adecuadas para los posteriores estudios fisiológicos, se buscó un parámetro global, el Indice General de Eficiencia Organogénica, que combinando el porcentaje de Cotiledones con Respuesta Caulogénica (CCR) y el Índice de Enraizamiento (RI) nos confirmó que la familia 36 presentó la mayor respuesta organogénica (33,34%), mientras que la familia 61 únicamente alcanzó la mitad de dicho valor (16,15%). Por tanto, estas familias fueron las seleccionadas para

tratar de establecer una relación entre la absorción y metabolismo de BA, los niveles de CKs endógenas y la respuesta organogénica obtenida.

La fidelidad clonal es un requisito indispensable en la micropropagación de cualquier especie (Lakshmanan *et al.* 2007). Para corroborar que los individuos obtenidos son genéticamente idénticos (clones), es necesario realizar estudios de variación somaclonal, como ya se han realizado en distintas especies de interés comercial (*Asparagus officinalis* (Pontaroli y Camadro 2005), *Dieffenbachia* sp. (Shen *et al.* 2007) o *Musa acuminata* (Lakshmanan *et al.* 2007)).

Aunque la técnica de micropropagación empleada en cotiledones maduros de pino piñonero presenta una caulogénesis directa y una rizogénesis indirecta sin fases prolongadas de callo, el estrés derivado del cultivo *in vitro* y el empleo de altas concentraciones de CKs pueden inducir cambios en el ADN (Venkatachalam *et al.* 2007). La ausencia de estudios de variación somaclonal en esta especie hizo que planteásemos una primera aproximación mediante la técnica RAPD, apoyándose en su sencillez y éxito en otras pináceas como *P. thunbergii* (Goto *et al.* 1998) y *P. taeda* (Tang 2001). Tras utilizarse 20 cebadores diferentes, se encontraron diferencias a nivel de familia (interclonal), aunque no se pudieron distinguir inequívocamente todas ellas. Considerando las limitaciones inherentes a la técnica citada (Lakshmanan *et al.* 2007) podrían no detectarse mutaciones genómicas existentes (Fourré *et al.* 1997; Hornero *et al.* 2001; Palombi y Damiano 2002; Polanco y Ruiz 2002). Además, esta especie ha sido descrita por su baja variabilidad genética mediante isoenzimas (Fallour *et al.* 1997) y marcadores moleculares tipo microsatélite (Martín y González 2000; Gómez *et al.* 2002; González-Martínez *et al.* 2004).

Numerosos trabajos abordan el estudio de la posible variación somaclonal durante el proceso de micropropagación *in vitro* empleando para ello un número muy reducido de combinaciones (6 AFLP en *Quercus suber* (Hornero *et al.* 2001), 10 RAPD en *Picea glauca* (De Verno *et al.* 1999), 8

SAMPL en *Musa acuminata* (Giménez *et al.* 2005)) e incluso un único marcador molecular (RAPD en *Pinus thunbergii* (Goto *et al.* 1998), ISSR en *Camellia sinensis* (Thomas *et al.* 2006)). En nuestro caso se realizó un análisis exhaustivo del genoma, empleando un total de 223 combinaciones distintas, de las cuales el 80% (178) amplificaron, e incluían marcadores tipo RAPD, ISSR, AFLP y SAMPL, estos últimos capaces de detectar regiones hipervariables (Giménez *et al.* 2005). Los resultados obtenidos reflejan, además, las características inherentes de cada uno de los marcadores; así se produce un descenso en el número medio de bandas obtenidas por AFLP y SAMPL a medida que aumenta el número de nucleótidos selectivos, estrategia recomendada para el análisis de genomas altamente complejos como es el caso de las coníferas (Arcade *et al.* 2000; Cervera *et al.* 2000). A la vista de los resultados mostrados, asumimos que en *P. pinea* no tienen lugar fenómenos de variación somaclonal durante el proceso de micropropagación utilizado y, pese a sus limitaciones, la técnica RAPD fue la única que nos permitió detectar variación interclonal en esta especie.

La excepcionalmente baja variabilidad genética descrita para esta especie, evaluada recientemente por Vendramin *et al.* (2008) a lo largo de su distribución mundial, y ya apuntada en trabajos previos (Fallour *et al.* 1997; Martín y González 2000; Gómez *et al.* 2002; González-Martínez *et al.* 2004) se confirma con la ausencia de polimorfismo detectada mediante marcadores ISSR, AFLP y SAMPL en los clones obtenidos por micropropagación *in vitro*. Estos resultados avalan la hipótesis de que el éxito de adaptación de *P. pinea* no se debe a su variabilidad genética medida mediante marcadores moleculares (Vendramin *et al.* 2008), sino a su alta plasticidad fenotípica y a la variación existente en caracteres cuantitativos (Mutke *et al.* 2005).

En especies con genomas complejos se han recomendado marcadores moleculares alternativos como los TE-AFLP (Van der Wurff *et al*. 2000), ya que al ser más restrictivos (la amplificación selectiva está precedida por una digestión con 3 enzimas y una ligación selectiva) ofrecen patrones más

claros y, por tanto, facilitan la búsqueda de polimorfismos. Sin embargo, el gran número de marcadores empleados en esta memoria apunta la baja probabilidad de detectar dicha variación. Los estudios cariológicos publicados para otras especies como *Picea abies* (Fourré *et al*. 1997) muestran una asociación entre alteraciones en el número de cromosomas y anomalías morfológicas. En esta línea, Pichot y Maataoui (1997) llevaron a cabo estudios de citometría de flujo en 4 especies de pino (*Pinus halepensis*, *P. nigra, P. pinaster* y *P. pinea*), destacando el mayor contenido de ADN nuclear de esta última.

En trabajos previos realizados por nuestro grupo de investigación orientados al estudio de la absorción y metabolismo de BA en material de *P. pinea* micropropagado, se emplearon semillas procedentes de polinización abierta, cuyos cotiledones fueron cultivados en medio con una concentración de 4,4 µM de BA. Por tanto, el primer paso antes de analizar la relación entre la respuesta caulogénica y los niveles de CKs en las familias seleccionadas (36 y 61), fue caracterizar el material de polinización abierta en las condiciones de cultivo optimizadas (44,4 µM de BA), incluyendo el establecimiento del periodo mínimo de inducción, el momento de la aparición de los meristemoides, la dinámica de absorción de BA y su posterior metabolización, y los niveles de CKs endógenas, para así ahondar en las bases fisiológicas del proceso.

La inducción caulogénica ha sido dividida en etapas discretas por Christianson y Warnick (1983; 1984; 1988), incluyendo una primera etapa de adquisición de competencia (pre-requisito necesario para poder responder a estímulos hormonales externos), una fase siguiente de predeterminación (periodo durante el cual el explanto no sería capaz de desarrollar un órgano adventicio si se suprime el estímulo externo), la determinación (punto a partir del cual el explanto ya es capaz de regenerar un órgano adventicio sin la presencia del agente inductor de respuesta) y finalmente la postdeterminación (ya aparecerían de modo visible los órganos adventicios inducidos). Una vez que el explanto está determinado,

se situaría un periodo de sensibilidad a los distintos agentes inductores (periodo de determinación), que modularía la intensidad de respuesta y cuya duración sería variable, dependiendo del explanto y del balance de reguladores de crecimiento aplicados.

Conocimientos previos en condiciones de cultivo de 4,4 µM de BA (Moncaleán *et al.* 2005) establecieron que la inducción y determinación de los cotiledones de *P. pinea* ocurría durante los primeros 8-12 d, indicando que si se aumentaba la concentración de BA a 44,4 µM, el periodo mínimo de inducción se reducía a 2 d. En el caso de los cotiledones de *P. pinea* no se necesita de un tratamiento previo para la adquisición de competencia, por lo que, al igual que Alonso (2006), proponemos que los cotiledones procedentes de semillas no pregerminadas son competentes *per se*. Durante esta memoria se ha descrito cómo el número de yemas producidas varía según el tiempo de exposición a BA, confirmando que entre las 6 h y los 6 d los cotiledones son sensibles al estímulo aplicado, por lo que el explanto se encuentra en el periodo de deteminación. Ensayos anteriores de tiempos de inducción superiores bajo las mismas condiciones de cultivo (Moncaleán *et al.* 2005) describen un aumento progresivo en el número de yemas por cotiledón, luego el periodo de determinación se prolonga más allá de los 6 d de cultivo ensayados en la presente memoria.

El análisis histológico del proceso de inducción de yemas realizado en el presente trabajo confirma que la acumulación de almidón está asociada con la inducción por CKs de la formación de yemas adventicias, como describen López-Escamilla *et al*. (2000) en *Picea chihuahuana*. Además, tras 12 h de cultivo, muestra que los promeristemoides se forman en la región subepidérmica de la parte del cotiledón que está en contacto con el medio de cultivo; éstos están constituidos por grupos de células pequeñas y de citoplasma denso, de acuerdo con resultados observados en *Abies amabilis* por Kulchetscki *et al.* (1995). Estas estructuras promeristemáticas, origen de los órganos adventicios, han sido descritas por Flinn *et al*. (1987) como unidades morfogénicamente competentes en *Pinus strobus*. Por ello, futuros

análisis histológicos en las familias seleccionadas podrían ayudar a dilucidar el por qué de la diferente respuesta caulogénica. Otro modo de abordar la respuesta diferencial entre familias sería la tinción de histonas, descrita por Patel y Thorpe (1984) en *P. radiata* como indicadores de intesidad de respuesta.

En cuanto a la absorción y metabolismo del agente inductor de respuesta bajo las condiciones de cultivo empleadas en este trabajo (44,4 µM de BA), se han podido establecer 3 etapas, diferentes a las descritas por Moncaleán *et al.* (2005) en material de polinización abierta de *P. pinea* en condiciones de cultivo de 4,4 µM de BA. Las etapas ahora establecidas constan de un primer periodo de 0 a 6 h, siendo a las 6 h el momento de máxima absorción de BA y coincidente con el periodo mínimo de inducción; la siguiente etapa, de 6 h a 2 d, incluye la aparición de los meristemoides a las 12 h y un descenso del contenido endógeno de BA; y, finalmente, de 2 a 6 d, donde se observa de nuevo un aumento en la absorción de BA. Los metabolitos detectados fueron BA y sus correspondientes ribósido (BAR), ribótido (BARMP) y ribósido del 9-glucósido (BA9GR). Durante las primeras 6 h predominaron BA y su ribósido, siendo a partir de las 6 h cuando el nucleótido cobra importancia, probablemente como mecanismo de detoxificación de la gran cantidad de BA más su ribósido que se encuentra en el cotiledón (276,60 µM a las 6 h). En cuanto al BA9GR, su aparición es tardía (2 d), aumentando rápidamente hasta el final del cultivo.

La aplicación exógena de BA provoca una respuesta organogénica en los cotiledones de pino piñonero. Sin embargo, no se debe olvidar que una vez asimilada, esta BA absorbida así como sus metabolitos probablemente tengan algún efecto sobre el resto de reguladores presentes en el tejido, y más concretamente sobre las CKs naturales (Valdés *et al.* 2001; Moncaleán *et al.* 2003). Así pues el estudio de la dinámica de las CKs endógenas se hace necesario para la comprensión del proceso de inducción caulogénica, puesto que los cambios en sus niveles regulan los procesos fisiológicos (Kamínek *et al.* 1997).

La presente memoria supone el primer trabajo que describe la presencia de las CKs aromáticas BA, BAR, BARMP y *para*-topolina (*p*T) en cantidades relevantes y de modo natural en coníferas. De hecho, su hallazgo confirma la idea de que las CKs aromáticas no son hormonas de presencia esporádica en las plantas, sino que están presentes en gran número de especies vegetales (Strnad 1997).

Antes de que los cotiledones estuvieran en contacto con la BA, el componente mayoritario de las CKs naturales fueron las CKs isoprenoídicas, siendo la forma ribótido la más abundante. Esto concuerda con la función de almacenamiento y reserva descrito para los cotiledones (Ananieva *et al*. 2004). Nuestros resultados apoyan la propuesta de la BA como precursora de sus formas hidroxiladas, descritas por primera vez por Strnad en 1997 como topolinas, puesto que tras 1 h en medio de cultivo, la BA y sus formas ribosilada y ribotilada conformaron más del 94% del total de las CKs; y en tiempos posteriores aparecieron las topolinas. Por el contrario, no corroboran que las topolinas sean las precursoras de la BA, como proponen Taylor *et al.* (2003).

La tendencia general observada en todas las CKs estudiadas es un aumento de sus niveles endógenos, asociado a la regeneración de tallos mediante cultivo *in vitro* y de acuerdo con Perrin *et al.* (1997). La dinámica de las CKs se correlaciona con las etapas discretas establecidas durante el periodo de inducción, ya que la información de ambas se complementa. Es decir, la interpretación de ambas clarifica, por ejemplo, que el ribósido del 9-glucósido de BA no participa en la determinación del cotiledón, puesto que aparece en el periodo tardío (2 d), mientras que los niveles de las CKs aromáticas como BA, BAR y *m*T ya en etapas tempranas (0-6 h) son elevados y dinámicos. En cuanto a las CKs isoprenoídicas, los altos niveles de ribótido de isopenteniladenina (iPRMP) sugieren que la ruta biosintética iPMRP-dependiente está presente en este material, puesto que este compuesto sería el precursor del resto de CKs isoprenoídicas (Sakakibara 2006). Otra idea derivada de los resultados obtenidos es la importancia de

los primeros estadios de la inducción en la posterior obtención de respuesta, y, por tanto, es en esas etapas iniciales donde se ha de incidir de un modo más profundo y exhaustivo, como demuestran los resultados obtenidos por Cortizo (2008) en la expresión diferencial de genes clave en la caulogénesis, como el gen de *P. pinea* homólogo al gen *CLAVATA* de *Arabidopsis thaliana*.

No se puede obviar que los valores obtenidos para el contenido en las distintas CKs pueden no reflejar las concentraciones endógenas dentro de la célula, ya que al igual que sucede en los cloroplastos de *Nicotiana tabacum* (Lexa *et al*. 2003), puede haber una distribución diferencial de las CKs en los distintos compartimentos celulares.

Una vez establecidas las distintas fases de la inducción caulogénica, se procedió a analizar la dinámica de este grupo de compuestos en las familias seleccionadas, buscando las posibles diferencias entre ambas, tanto en la absorción y metabolismo de la BA, como en los perfiles de las CKs endógenas.

Los niveles de absorción de BA en las familias 36 y 61 fueron diferentes, siendo superiores los correspondientes a la familia más organogénica (familia 36). Estos distintos niveles de BA registrados pueden ser claves en la magnitud de la respuesta obtenida, puesto que durante el periodo comprendido entre 6 h y 6 d se incluye el periodo de determinación, etapa sensible a la influencia de reguladores aplicados al medio de cultivo.

Las familias 36 y 61 también difirieron en las concentraciones endógenas de los metabolitos de BA detectados en los cotiledones durante los 6 d de cultivo en medio de inducción. Las mayores diferencias se encontraron en el ribósido del 9-glucósido de BA, descrito en *Petunia hybrida* como un metabolito implicado positivamente en la respuesta caulogénica (Auer y Cohen 1993). De hecho, la familia 36 (más organogénica) alcanza niveles 3 veces superiores a los de la familia 61 (menos organogénica), lo que denota la relevancia de esta CK en el proceso caulogénico.

En cuanto a las CKs endógenas, también en las familias seleccionadas de *P. pinea* se ha descrito la presencia de BA, BAR, BARMP y *p*T como compuestos naturales, siendo mayores los niveles detectados en la familia menos organogénica (familia 61). Durante las primeras 6 horas de cultivo se comprobó que el metabolismo de las CKs es diferente en ambas familias, así la familia con mayor respuesta caulogénica (familia 36) presentó mayores niveles de las formas activas BA, BAR y *m*T, mientras que la menos caulogénica (familia 61) mostró valores mayores de las formas de reserva ribótidos y glucósidos aromáticos. A las 16 h los ribótidos fueron 10 veces superiores en la familia 61, y tras 2 d en medio de inducción, los valores más altos también se encontraron en dicha familia 61. Estos resultados indican que la diferente intensidad de respuesta está íntimamente ligada al comportamiento de las CKs en los tejidos estudiados, así como revelan la alta complejidad de estos procesos en sistemas experimentales relativamente sencillos.

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CONCLUSIONES

1.- El protocolo de micropropagación *in vitro* a partir de cotiledones maduros de *Pinus pinea* ha sido optimizado para 6 familias seleccionadas, certificando la estabilidad genética del material clonado mediante el uso de marcadores moleculares (AFLP, ISSR, RAPD y SAMPL). Además, la respuesta organogénica (caulogénica y rizogénica), mostró una dependencia del genotipo ensayado.

2.- El periodo mínimo de inducción caulogénica necesario para la aparición de yemas en cotiledones maduros de *P. pinea*, en contacto con una concentración de benciladenina de 44,4 µM, es de 6 h. Por primera vez en esta especie análisis histológicos han mostrado estructuras promeristemáticas en medio de inducción caulogénica tras 12 h de cultivo.

3.- La distinta magnitud de respuesta caulogénica en las familias seleccionadas de *P. pinea* se relaciona con la absorción de benciladenina aplicada al medio de cultivo tras 6 h de cultivo en medio de inducción, siendo dicha absorción mayor cuanto mayor es la intensidad de respuesta caulogénica.

4.- Por primera vez en coníferas se confirman como naturales las citoquininas aromáticas benciladenina, ribósido de benciladenina, ribótido de benciladenina y *para*-Topolina.

5.- El ribósido del 9-glucósido de benciladenina es el metabolito de benciladenina que se asocia con una mayor intensidad de respuesta caulogénica en las familias estudiadas, apareciendo en etapas tardías del cultivo en medio de inducción.

6.- En las primeras etapas de cultivo, existe una relación entre mayor magnitud de respuesta caulogénica y mayores niveles endógenos de benciladenina, ribósido de benciladenina y *meta*-Topolina, mientras que la menor intensidad de respuesta caulogénica se asocia con mayores niveles endógenos de ribótidos y glucósidos aromáticos.