Universidad del País Vasco
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Desarrollo y optimización de herramientas biotecnológicas para la obtención de material clonal de *Pinus radiata* D. Don

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Abreviaturas

ABA: Ácido abscísico (abscisic acid).

AC: Carbón activo (activated charcoal).

ANOVA: Análisis de la varianza (analysis of variance).

BA: N^6-benziladenina (N^6-benzyladenine).

BA9G: 9-Glucósido de benciladenina (N^6-benzyladenine 9-glucoside).

BAR: Ribósido de benciladenina (benzyladenine riboside).

BARMP: Ribonucleótido de benciladenina (benzyladenine ribonucleotide).

BFS: Capacidad formadora de tallos en yemas (buds forming shoots).

C: Control (control).

CG: Mezcla de aminoácidos compuesta por 1 g L^{-1} de hidrolizado de caseína y 500 mg L^{-1} de L-glutamina (amino acid mixture: 1 g L^{-1} casein hydrolysate and 500 mg L^{-1} L-glutamine).

CGM: Medio de cultivo EDM con la mezcla CG de aminoácidos (EDM medium supplemented with CG amino acid mixture).

CK: Citoquinina (cytokinin).

CKs: Citoquininas (cytokinins).

cZ: *cis*-zeatina (*cis*-zeatin).

cZ9G: 9-Glucósido de *cis*-zeatina (*cis*-zeatin 9-glucoside).

cZOG: O-Glucósido de *cis*-zeatina (*cis*-zeatin O-glucoside).

cZR: Ribósido de *cis*-zeatina (*cis*-zeatin riboside).

cZRMP: Ribonucleótido de *cis*-zeatina (*cis*-zeatin ribonucleotide).

cZROG: O-Glucósido de ribósido de *cis*-zeatina (*cis*-zeatin riboside O-glucoside).

2,4-D: Ácido 2,4-diclorofenoxiacético (2,4-dichlorophenoxyacetic acid).

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: Ribonucleótido de dihidrozeatina (dihydrozeatin ribonucleotide).

DHZROG: O-glucósido de ribósido de dihidrozeatina (dihydrozeatin riboside O-glucoside).

DW: Peso seco (dry weight).

ECLs: Líneas celulares establecidas (established cell lines).

ED: Mezcla de aminoácidos del medio EDM (EDM amino acid mixture).

EDM: Medio de cultivo para el desarrollo del embrión (embryo development medium).

EFS: capacidad formadora de tallos en embriones (embryos forming shoots).

ET: Tejido embriogénico (embryogenic tissue).

ETs: Tejidos embriogénicos (embryogenic tissues).

Fig.: Figura (figure).

Figs.: Figuras (figures).

FW: Peso fresco (fresh weight).

G: Germinados (germinated).

HPLC: Cromatografía líquida de alta resolución (high performance liquid chromatography).

IAA: Ácido indol-3-acético (indole-3-acetic acid).

IBA: Ácido indol-3-butírico (indole-3-butyric acid).

iP: N^6-isopenteniladenina (N^6-isopentenyladenine).

iP9G: 9-Glucósido de isopenteniladenina (N^6-isopentenyladenine 9-glucoside).

iPR: Ribósido de isopenteniladenina (N^6-isopentenyladenine riboside).

iPRMP: Ribonucleótido de isopenteniladenina (N^6-isopentenyladenine ribonucleotide).

LP: Medio Quorin Lepoivre (Quorin and Lepoivre medium).

1/2LP: Medio LP con macroelementos diluidos a la mitad a excepción del hierro (half strength macronutrients except for the iron and complete micronutrients and vitamins LP medium).

1/4LP: Medio LP con macroelementos diluidos a un cuarto a excepción del hierro, diluido a la mitad, al igual que las vitaminas y los micronutrientes (quarter strength macronutrients except for the iron, and half strength iron, micronutrients and vitamins LP medium micronutrients).

LPAC: Medio LP con AC (LP medium supplemented with AC).

LPE: 1/2LP con carbón activo (1/2LP medium supplemented with AC).

M: Media (mean).

mT: *meta*-topolina (*meta*-topolin).

mT9G: 9-Glucósido de *meta*-topolina (*meta*-topolin 9-glucoside).

mTOG: O-glucósido de *meta*-topolina (*meta*-topolin O-glucoside).

mTR: Ribósido de *meta*-topolina (*meta*-topolin riboside).

mTRMP: Ribonucleótido de *meta*-topolina (*meta*-topolin ribonucleotide).

mTROG: O-glucósido de ribósido de *meta*-topolina (*meta*-topolin riboside O-glucoside).

NAA: Ácido 1-naftalenacético (1-naphthalene acetic acid).

NG: No germinados (non germinated).

OP: Polinización abierta (open pollinated).

oT: *orto*-topolina (*orto*-topolin).

oT9G: 9-Glucósido de *orto*-topolina (*orto*-topolin 9-glucoside).

oTOG: O-glucósido de *orto*-topolina (*orto*-topolin O-glucoside).

oTR: Ribósido de *orto*-topolina (*orto*-topolin riboside).

oTRMP: Ribonucleótido de *orto*-topolina (*orto*-topolin ribonucleotide).

oTROG: O-glucósido de ribósido de *orto*-topolina (*orto*-topolin riboside O-glucoside).

pT: *para*-topolina (*para*-topolin).

pTOG: O-glucósido de *para*-topolina (*para*-topolin O-glucoside).

pTR: Ribósido de *para*-topolina (*para*-topolin riboside).

pTRMP: Ribonucleótido de *para*-topolina (*para*-topolin ribonucleotide).

pTROG: O-glucósido de ribósido de *para*-topolina (*para*-topolin riboside O-glucoside).

SE: Embriogénesis somática (somatic embryogenesis).

S.E.: Error estándar (standard error).

SEC: Capacidad de elongación de tallo (shoot elongation capacity).

T: Tidiazurón (thidiazuron).

tZ: *trans*-zeatina (*trans*-zeatin).

tZ9G: 9-Glucósido de *trans*-zeatina (*trans*-zeatin 9-glucoside).

tZOG: O-Glucósido de *trans*-zeatina (*trans*-zeatin O-glucoside).

tZR: Ribósido de *trans*-zeatina (*trans*-zeatin riboside).

tZRMP: Ribonucleótido de *trans*-zeatina (*trans*-zeatin ribonucleotide).

tZROG: O-glucósido de ribósido de *trans*-zeatina (*trans*-zeatin ribose O-glucoside).

Z: Zeatina (zeatin).

Índice

INTRODUCCIÓN

Introducción

1. LOS BOSQUES: PERSPECTIVA ACTUAL

Los bosques son componentes esenciales de los paisajes naturales y juegan un papel fundamental en la regulación del ciclo global del carbono, la respuesta planetaria al cambio climático, el control de la erosión y el mantenimiento de la biodiversidad (Campbell et al. 2003). Por otra parte, los bosques proveen el material de partida para innumerables industrias con dividendos multimillonarios, entre las que se incluyen aquellas implicadas en el procesamiento de la madera, en la conversión de la biomasa en energía y otras proveedoras de productos no madereros. El consumo medio por habitante y año de productos de todo tipo que emplean materias primas de origen forestal ha crecido de forma continuada durante las últimas décadas en todos los países del mundo, particularmente en los más desarrollados (Celestino et al. 2005).

La propiedad de los bosques en Europa está muy repartida, existen más de 10 millones de propietarios forestales que, junto con innumerables trabajadores de zonas rurales y de las industrias de transformación, dependen del manejo sostenible de los bosques para asegurar su renta. En los últimos años tanto el reciclado de papel, como el establecimiento de plantaciones forestales intensivas que puedan dar respuesta a las necesidades crecientes de madera y otros productos, se están considerando como la mejor defensa de los ecosistemas amenazados por deforestación (Sutton 1999). A este respecto, uno de los objetivos principales del Convenio Marco sobre el Cambio Climático de Naciones Unidas es la estabilización de las emisiones de CO_2 y otros gases de efecto invernadero a los niveles de 1990. Además, desde el protocolo de Kyoto (http://unfccc.int/kyoto_protocol/status_of_ratification/items/2613.php) se insta a los gobiernos tanto a incrementar la superficie forestal como a mejorar la eficiencia de los sistemas forestales. Así, adaptando el protocolo de Kyoto al escenario español, se han diseñado las siguientes acciones [Plan Forestal Español (http://www.mma.es/ secciones/biodiversidad/montes_politica_forestal/estrategia_monte/pfe.htm)] para contribuir a la consecución de los objetivos del citado protocolo:

- El incremento de la superficie forestal a través de los procesos de reforestación (restauración de sistemas forestales en los terrenos que han sido tradicionalmente dedicados a este uso) y forestación (instauración de bosques en terrenos agrícolas abandonados o sin uso definido).

- La conservación y mejora de la superficie forestal mediante la aplicación, entre otras, de técnicas apropiadas de restauración hidrológico-forestal y la lucha contra la desertificación, además de la defensa del monte contra los incendios forestales, las plagas y las enfermedades.

- La mejora de la capacidad de captación de CO_2 de los sistemas forestales mediante la mejora de su eficiencia en términos de biomasa, a través de actuaciones silvícolas, siempre cumpliendo el principio de una gestión forestal sostenible.

Asimismo, el Plan Forestal Español refleja la necesidad de aumentar los terrenos forestales con el fin de evitar/detener los procesos de desertización. Con las nuevas planificaciones, progresivamente se están abandonando superficies dedicadas a prácticas agrícolas, con lo que, cada vez existe más superficie disponible con fines silvícolas. Pudiendo aspirar a una explotación sostenible y duradera de tales superficies excedentes, sobre todo en zonas rurales donde las oportunidades de empleo son muy limitadas, la actuación forestal se encuentra muy justificada.

La superficie forestal española es de 26 millones de hectáreas (ha), lo que corresponde al 52% del territorio nacional. En este contexto las coníferas tienen un papel decisivo tanto desde el punto de vista ecológico como forestal. Los pinos están representados en España por siete especies autóctonas (Martín y González 2000): pino negro *(Pinus uncinata)*, pino silvestre o albar (*P. sylvestris),* pino laricio o salgareño (*P. nigra*), pino marítimo, negral, rodeno o resinero (*P. pinaster*), pino piñonero (*P. pinea*), pino carrasco (*P. halepensis*), y pino canario (*P. canariensis*). Existen otras Pináceas introducidas con fines de producción maderera en repoblaciones forestales, o con carácter ornamental. De ellas, sólo el pino radiata, insigne o de Monterrey (*P. radiata*) alcanza una notable significación territorial (Ferreras y Arozena 1987).

Las especies del género *Pinus* citadas ocupan en España una superficie de 6 millones de ha (Inventario Forestal Nacional, 1997-2000, http://www.mma.es/portal/secciones/biodiversidad/inventarios/ifn/index.htm), cifra que tenderá a incrementarse en el futuro, ya que uno de los objetivos del Plan Forestal Español es la plantación de 1.000 millones de nuevos árboles en la próxima década. En el Plan Forestal también se contempla la necesidad de utilizar planta de calidad en los programas silvícolas, en respuesta al aumento de la demanda de madera de

coníferas, a los incendios y enfermedades que sufren nuestros bosques y a la creciente desertización que afecta al país.

2. LA ESPECIE (*Pinus radiata* D. Don)

El pino radiata (*Pinus radiata* D. Don) es originario de California, hallándose su hábitat natural reducido a cinco poblaciones nativas entre las latitudes 28º N y 37º N, tres de ellas en la costa de California (USA) y otras dos en islas de la costa oeste de México (Cedros y Guadalupe). Estas poblaciones ocupan 5300 ha (Burdon 2001), todas ellas comparten un clima mediterráneo, con lluvias en invierno y una corriente oceánica de aire húmedo durante la época de sequía estival (Libby 1997). En contraste, las plantaciones de pino radiata ocupan en el mundo 4 millones de ha. Actualmente, en el Hemisferio Sur las plantaciones de esta especie ocupan 1400000 ha en Chile, 1250000 ha en Nueva Zelanda, 750000 ha en Australia y 300000 ha en Sudáfrica (Eldridge 1997). En el Hemisferio Norte, además de su área natural californiana y una reducida presencia en Calabria (Italia), en España el pino radiata ocupa 280000 ha, y su distribución se encuentra principalmente localizada en la Cornisa Cantábrica. El 56% de esta superficie corresponde a la Comunidad Autónoma del País Vasco (Michel 2003) (Fig. 1a).

Es una especie de crecimiento rápido, monoica, de talla grande a muy grande, en España puede sobrepasar los 30 m, alcanzando en otros países hasta 50 m. Las acículas con una longitud entre 7 y 15 cm, son persistentes, simples, flexibles y de color verde brillante, se hallan reunidas en grupos de tres (salvo la variedad "binata" de las islas Guadalupe y Cedros que lo hacen predominantemente en fascículos de dos) (Fig. 1b).

Las piñas muy asimétricas y subsentadas, poseen un tamaño de 7-14 x 5-8 cm, son serótinas, su peso medio es de 100 g y contiene entre 70 y 120 semillas. Las semillas miden de 5 a 8 mm, de aspecto negruzco, con ala estrecha de 15 a 20 mm y cabeza lignificada, maduran en otoño del año siguiente al de la floración, y la producción de semillas es máxima cuando el árbol alcanza entre 15-20 años de edad.

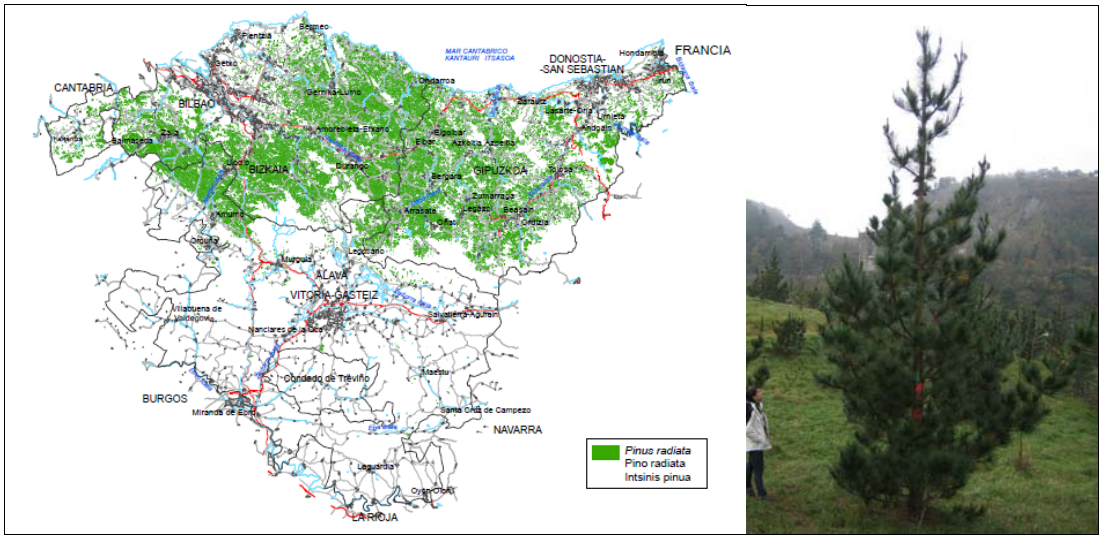

Fig. 1 (a) Distribución de *Pinus radiata* en el País Vasco, imagen tomada del inventario forestal de la Comunidad Autónoma del País Vasco (2005); (b) porte de un ejemplar de *Pinus radiata* de 15 años (huerto semillero, Deba, España).

El pino radiata se cultiva principalmente a baja altitud, por debajo de los 800 m, en zonas de clima suave y húmedo. En su hábitat de origen es resistente a la sequía ya que apenas percibe precipitaciones, sin embargo en las repoblaciones donde las recibe, crece muy rápidamente. Esta especie proporciona una madera homogénea que la hace muy apreciada para la industria de carpintería y mueble, de estructuras de madera y de embalaje. Debido a su rapidez de crecimiento, es manejado en la península en turnos de corta en torno a 30 años. Scott (1961; 1962) cifraba su crecimiento en 9-20 m^3 ha^{-1} $año^{-1}$, aunque tras los programas de mejora genética desarrollados en algunos países, se ha aumentado esta cifra hasta alcanzar 25-30 m^3 ha^{-1} $año^{-1}$ (Burdon y Moore 1997).

3. BIOTECNOLOGÍA FORESTAL

La biotecnología forestal tiene como principales objetivos: la selección de marcadores moleculares para caracteres de interés, el desarrollo de tecnologías de transformación genética que posibilitan la integración de genes que confieran resistencia a enfermedades, resistencia a estreses abióticos o que aumenten la productividad (Walter 2004), y la multiplicación clonal *in vitro* que posibilite la multiplicación ilimitada de estos genotipos élite (Menzies y Aimers-Halliday 2004).

En el último decenio se han puesto en ejecución numerosos proyectos de conservación y mejora forestal que, por lo general, persiguen un fin económico: mejorar cuantitativa y cualitativamente la producción forestal, en particular en coníferas (http://www.serida.org/boletin.php).

Estos proyectos se enmarcaron dentro de las prioridades del Plan Nacional de Investigación (2004-2007) (http://www.micinn.es/portal/site/MICINN/). La primera prioridad temática del citado plan se refería a "la mejora y optimización de las empresas agroalimentarias mediante la incorporación de aquellas técnicas que aseguren una producción sostenible con tecnologías no contaminantes y que contribuyan a incrementar y/o aprovechar la diversidad biológica de los sistemas forestales, así como a la conservación del medioambiente y el uso integral del territorio". A este respecto, figuran entre las líneas principales de actuación:

- Análisis y mejora de los sistemas de producción agrícola y forestal, actuando sobre la mejora genética y la biotecnología de especies vegetales, la fisiología vegetal y el manejo de cultivos y la mecanización.

- La investigación sobre protección vegetal orientada a un mejor diagnóstico y caracterización de los agentes causantes de daños, su biología y ecología.

Para desarrollar las líneas de actuación descritas, es necesario trabajar intensamente en los programas de mejora forestal. Estos programas se enfrentan a numerosas dificultades:

- Los caracteres de selección sólo pueden ser evaluados en la fase madura de los árboles, por lo que el proceso es muy lento teniendo en cuenta los largos ciclos generacionales de las coníferas (Von Aderkas y Bonga 2000).

- La alta heterozigosidad de las especies forestales, unida al alto componente genético de tipo no aditivo que controla diversos caracteres de interés (Tang et al. 2007).

- El cambio de fase implica una serie de cambios fisiológicos y morfológicos (Greenwood 1995) que suponen la pérdida brusca de su potencial de regeneración por vías asexuales tradicionales como mediante estaquillado, acodado o injerto (Menzies et al. 2000).

- En los programas silvícolas de especies alógamas, como es el caso, sólo es posible la utilización de semillas procedentes de polinización abierta y/o controlada para obtener

cierta ganancia genética, lo cual limita el aprovechamiento total de las cualidades genéticas de los parentales.

Por todos los motivos expuestos, los países avanzados en el sector forestal combinan las técnicas tradicionales de propagación (injerto, estaquillado, etc.) con nuevas herramientas biotecnológicas como el cultivo *in vitro*.

3.1. Micropropagación o cultivo *in vitro*

El cultivo *in vitro* se define como una técnica de cultivo de tejidos en la que se regenera planta a partir de pequeñas porciones de tejidos, órganos o células vegetales, en condiciones asépticas y controladas (Davis y Becwar 2007). Según Villalobos y Thorpe (1991), la micropropagación se fundamenta en dos principios elementales: la totipotencia celular entendida como la aptitud de las células para expresar la totalidad de las potencialidades del genoma (Haberlandt 1902) y la hipótesis del balance hormonal propuesta por Skoog y Miller (1957). Las células vegetales en cultivo pueden manifestar su totipotencia (regenerando plantas completas) siguiendo dos rutas alternativas (Segura 1993):

- Organogénesis, que conduce a la diferenciación de meristemos caulinares y/o radiculares (que originan tallos y raíces, respectivamente) (Martínez Pulido et al. 1990; Thorpe et al. 1991).

- Embriogénesis somática, que lleva a la formación de embriones somáticos siguiendo las fases de desarrollo del embrión zigótico, aunque sin fusión gamética (Dodeman et al. 1997; Ramage y Williams 2002).

3.1.1. Organogénesis

Desde un punto de vista aplicado, cabe resaltar que los primeros árboles generados mediante técnicas de cultivo *in vitro* se obtuvieron en el inicio de los años 60 utilizando la vía organogénica (Celestino et al. 2005).

La organogénesis puede desarrollarse a partir de la activación y proliferación de yemas axilares preexistentes, o a través de la generación de yemas adventicias (formadas *de novo*). Una vez desarrolladas esas yemas, se forman tallos que tras elongarse y en su caso re-multiplicarse, son sometidos a un proceso de enraizamiento; tras la inducción de raíces adventicias se procede a la aclimatación *ex vitro* de los explantos (Saborio et al. 1997).

En *P. radiata*, Aitken-Christie et al. (1988) desarrolló un protocolo de organogénesis a partir de embriones zigóticos. Más adelante, los conocimientos en esta materia han sido ampliados por Hargreaves et al. (2005) desarrollándose protocolos de transformación (Charity et al. 2002) y crio-conservación (Hargreaves et al. 2004) en cotiledones de esta especie. Esta metodología permite la obtención de material clonal para experimentación, y la multiplicación clonal de las progenies obtenidas mediante cruzamientos seleccionados en los huertos semilleros, estableciendo cadenas micropropagativas a partir de los explantos cotiledonares (Hargreaves et al. 2005). Una de las ventajas de este sistema es que puede iniciarse en cualquier momento del año, dado que el material de partida son semillas maduras. La mayor desventaja consistiría en la necesidad de evaluar y seleccionar estas progenies en base a los caracteres de interés que se persigan, ya que los caracteres de selección solo pueden ser evaluados en la fase madura de los árboles (Malabadi y Van Staden 2005).

Los cuellos de botella descritos más habitualmente en el proceso organogénico son: bajos porcentajes de caulogénesis en determinadas especies, procedencias y/o genotipos (Von Arnold y Eriksson 1981; Tereso et al. 2006), bajos porcentajes de enraizamiento (Gómez y Segura 1995; Cortizo et al. 2009) y/o comportamiento *ex vitro* irregular (plagiotropismo, baja supervivencia) (Niemi et al. 2004).

Las dificultades enumeradas suelen ser más severas cuando se trata de micropropagar individuos adultos, por lo mencionado anteriormente respecto al cambio de fase y porque en ocasiones las características juveniles de los explantos regenerados son transitorias. Cuando se trata de multiplicar el material adulto, se habla de un proceso de revigorización. Esta revigorización *in vitro* puede lograrse mediante el uso de reguladores de crecimiento (citoquininas) e implica un aumento temporal del vigor que posibilita la recuperación de ciertas capacidades organogénicas (Valdés et al. 2003). Pero esta recuperación de competencia morfogénica es transitoria, y los microtallos generados pueden mostrar características adultas haciendo más difícil su enraizamiento y aclimatación *ex vitro* (Cortizo et al. 2009).

3.1.2. Embriogénesis somática

En los últimos años, se está apostando por la embriogénesis somática, ya que presenta ventajas como un elevado potencial de multiplicación, la posibilidad de cultivo en biorreactores (Etienne and Berthouly 2004), la opción de aplicar técnicas de encapsulación

para fabricar semillas sintéticas (Aquea et al. 2008) y la utilización de los cultivos embriogénicos como dianas para la transformación genética (Charity et al. 2005). Además, el tejido embriogénico puede ser crio-conservado, manteniendo su potencialidad mientras se realizan los ensayos de progenie (Park 2002). La combinación de estas dos técnicas (crio-conservación y embriogénesis somática) es la que ha posibilitado el desarrollo de la silvicultura clonal de alto valor (Weng et al. 2010).

La embriogénesis somática se pueden dividir en las siguientes fases: iniciación, proliferación y maduración del tejido embriogénico, germinación de los embriones somáticos y aclimatación *ex vitro* de los explantos. Las ventajas anteriormente descritas sólo son aplicables cuando todos y cada unos de los pasos de este proceso han sido optimizados, haciendo más sencilla y económica la aplicación práctica de esta vía de propagación vegetativa (Stasolla y Yeung 2003).

Pero esta vía de propagación aplicada a coníferas también presenta ciertos problemas, siendo los más comunes: los bajos porcentajes de iniciación y la baja regeneración de planta a partir de tejido embriogénico. Las principales causas de los bajos porcentajes de iniciación son la baja captura genotípica en muchas especies y/o genotipos (Davis y Becwar 2007), y la dependencia de la estacionalidad a la hora de iniciar el tejido embriogénico (Yildirim et al. 2006). Esta estacionalidad se debe a que, en coníferas la embriogénesis somática sólo es posible a partir de semillas inmaduras (Stasolla y Yeung 2003). Así, la ventana de competencia durante la que es posible iniciar tejido embriogénico se reduce a unas 4 semanas (MacKay et al. 2006). En los casos en los que se ha intentado iniciar tejido embriogénico a partir de semillas maduras (Garin et al. 1998; Tang et al. 2001), el porcentaje de iniciación logrado ha sido demasiado bajo como para considerarse como una alternativa. En este sentido, en coníferas no ha sido posible desarrollar tejido embriogénico a partir de material adulto, salvo en los estudios descritos para *Pinus kesiya* (Malabadi et al. 2004), *P. patula* (Malabadi y Van Staden 2005) y *P. roxburghii* (Malabadi y Nataraja 2006).

En cuanto a la conversión del tejido embriogénico en plántulas somáticas, existen dos factores limitantes que cobran especial importancia en el género *Pinus*, por un lado el bajo número de embriones somáticos de buena calidad obtenido a partir de tejido embriogénico (Choudhury et al. 2008) y por otro la baja conversión de estos embriones en plántulas somáticas (Lin y Leung 2002).

En diversas especies de coníferas es posible regenerar planta por la vía organogénica a partir de individuos adultos (Chang et al. 2001, De Diego et al. 2008), pero si fuera posible producir tejido embriogénico a partir de material adulto y generar plántulas somáticas, por un lado se evitarían efectos indeseados como la transitoriedad de las características juveniles y por otro se eliminaría la incertidumbre previa a los ensayos de campo (Bonga 2004).

La combinación de ambas técnicas (organogénesis y embriogénesis somática) ayudaría a mejorar la rentabilidad de determinados procesos como en el caso de la multiplicación organogénica de embriones o plántulas somáticas para aumentar el número de clones generados (Kim et al. 2009; Siva et al. 2009). Asimismo, la combinación de vías de propagación *in vitro* puede posibilitar el desarrollo de procesos de otra manera inviables. Un ejemplo en este sentido sería la aplicación de tratamientos organogénicos para revigorizar el material vegetal como paso previo a la inducción embriogénica en tejido adulto de angiospermas (Conde et al. 2004; San-José et al. 2010).

Desde hace décadas, diversas empresas multinacionales como ArborGen, Genfor, Mininco, o Weyerhaeuser y empresas nacionales como Agromillora catalana, Cultesa o Vitrotech Biotecnología Vegetal han aplicado y desarrollado herramientas biotecnológicas para la obtención del material clonal seleccionado que demanda el mercado. Como consecuencia de la aplicación de éstos nuevos métodos, estas empresas distribuyen diversos tipos de materiales como: semillas genéticamente mejoradas de *Pseudotsuga menziesii* (Weyerhaeuser), huertos semilleros clonales de *Eucalyptus globulu*s o *Pinus radiata* (Mininco) y clones seleccionados de *Juglans regia* (Vitrotech), *Musa x paradisiaca* (Cultesa) o *Pinus taeda* (ArborGen).

Además, el desarrollo y la optimización de estas técnicas han posibilitado que empresas multinacionales como las anteriormente citadas (http://www.weyerhaeuser.com/Businesses/SustainableForestry) apliquen el modelo de gestión forestal sostenible a nivel mundial (Fenning et al. 2008).

3.2. Micropropagación y reguladores de crecimiento

La respuesta morfogénica en cultivo *in vitro* (regeneración de tallos, raíces o embriones) viene determinada por la interacción de numerosos factores: genotipo de la planta donadora, tipo de explanto y estado fisiológico, composición química del medio de cultivo y ambiente físico del cultivo (Giri et al. 2004). De todos los factores implicados, los reguladores de

crecimiento desempeñan un papel fundamental en el control de la morfogénesis (Segura 1993).

Hasta el momento, la hipótesis más aceptada para explicar el control hormonal de la organogénesis postula que dicho proceso está regulado por cambios en los niveles endógenos de auxinas y citoquininas (CKs). En este proceso, la diferenciación de yemas vegetativas (caulogénesis) es promovida por balances auxina/citoquinina favorables a las citoquininas, mientras que los balances favorables a las auxinas inducen la formación de raíces (rizogénesis) (Segura 2000).

En la embriogénesis somática la aplicación de reguladores de crecimiento al medio de cultivo también juega un papel esencial. Para inducir una "reprogramación" hacía la vía embriogénica de las células somáticas en la planta donante se suelen aplicar altas concentraciones de auxinas y citoquininas; durante la fase de proliferación este contenido hormonal se mantiene o disminuye y en la fase de maduración se sustituye por acido abscísico (Stasolla et al. 2002).

3.2.1 Auxinas

Las auxinas modulan diversos procesos del crecimiento y desarrollo de la planta, tales como: respuestas trópicas a la luz y a la gravedad, arquitectura del tallo y de la raíz, desarrollo de órganos y sistema vascular y determinación del eje apical-basal en las células y en la totalidad de la planta (Normanly et al. 2005).

Los ensayos sobre la actividad de estos compuestos se hallan entre los más antiguos dentro del campo de la fisiología vegetal (Woodward y Bartel 2005). Fruto de estos ensayos se descubrió el ácido indol-3-acético (IAA). Este compuesto es ubicuo en los tejidos vegetales, y activo a concentraciones nanomolares (Pěnčik et al. 2009). Se han descubierto otros compuestos naturales con actividad auxínica, aunque parece que esta depende de la posibilidad de que sean transformados por el tejido en IAA (Barceló et al. 2005).

El ácido indol-3-butírico (IBA), es también una auxina indólica. Pese a que en un principio se creyó que era un compuesto sintético, posteriormente ha sido hallado en tejidos vegetales (Bartel et al. 2001). Esta auxina ha demostrado ser más efectiva en la inducción de raíces laterales tanto *in vitro* como *ex vitro*, y es empleada a nivel comercial con este propósito (Zolman et al. 2000).

En este sentido, se han comercializado y se usan habitualmente en el cultivo *in vitro* de tejidos vegetales dos compuestos sintéticos con gran actividad auxínica: el ácido 1-naftalenacético (NAA) y el ácido 2,4-diclorofenoxiacético (2,4-D) (Woodward y Bartel 2005).

3.2.2. Citoquininas

Las CKs son un grupo de reguladores de crecimiento implicadas en el control del desarrollo de las plantas (Nóvak et al. 2008). Desde su descubrimiento (Miller et al. 1955) se ha demostrado su participación en otros muchos procesos fisiológicos como la senescencia foliar, 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, el control de los estomas, la formación de cloroplastos y la germinación de semillas (Mok et al. 2000; Rivero et al. 2009). Además, regulan muchos procesos celulares, destacando el control de la división celular (Kyozuka 2007).

Las CKs naturales son derivados de la adenina con un sustituyente de naturaleza isoprenoide o aromática en la posición N^6 (Mok y Mok 2001). Según dicho sustituyente, las CKs se pueden clasificar en dos grandes grupos: las CKs isoprenoídicas y las CKs aromáticas (Segura 2000). La *trans*-zeatina (tZ) una CK isoprenoídica, es la CK más abundante en los tejidos vegetales. La abundancia de otras CKs isoprenoídicas como la N^6-isopenteniladenina (iP), la *cis*-zeatina (cZ) o la dihidrozeatina (DHZ) varía según la especie (Voller et al. 2010). Mientras que las CKs isoprenoídicas son ubicuas en las plantas, las CKs aromáticas representadas por la N^6-benziladenina (BA), y sus derivadas hidroxiladas, las Topolinas solo han sido identificadas en un limitado grupo de taxones (Strnad et al. 1997) (Fig. 2).

Las CKs pueden encontrarse en las plantas como bases libre o formando conjugados con diversos compuestos químicos que se unen al anillo de purina o a la cadena lateral. Las principales formas conjugadas son los ribósidos, los ribonucleótidos y los glicósidos (Sakakibara 2006) (Fig. 2).

La aplicación exógena de CKs solas o en combinación con auxinas para la regeneración *in vitro* de tallos adventicios ha sido ampliamente descrita tanto en angiospermas (Moncaleán et al. 2001; Lu 2002) como en gimnospermas (Mata Rosas et al. 2001; Sul y Korban 2005). Pero la morfogénesis *in vitro* implica complejos mecanismos de regulación, que incluyen tanto a los reguladores de crecimiento aplicados exógenamente como a los presentes en los propios tejidos vegetales (Auer et al. 1999; Fehér et al. 2003).

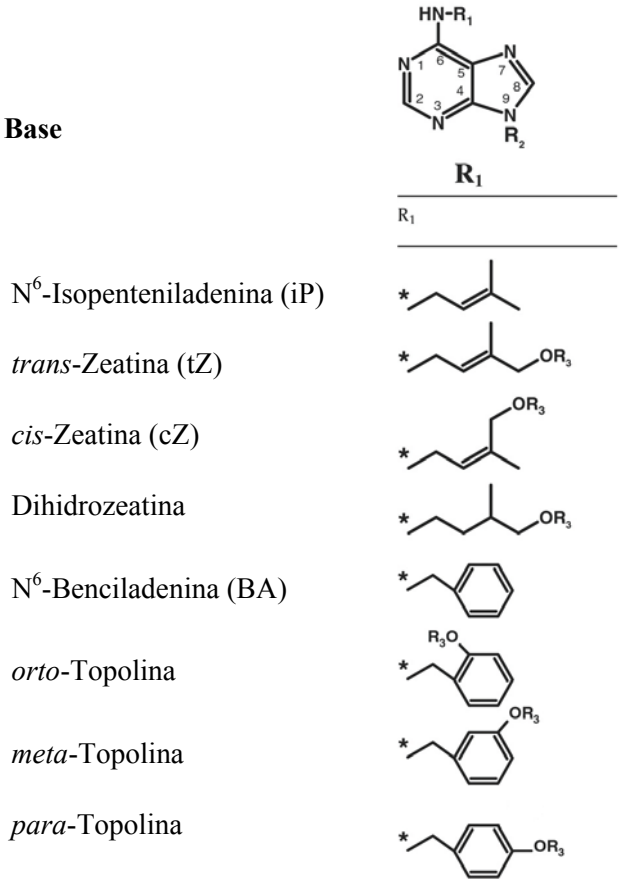

Fig. 2 Estructuras, nombres y abreviaturas de las principales citoquininas naturales.

4. PLANTEAMIENTO Y OBJETIVOS

Anteriormente se ha señalado la importancia de las plantaciones de *Pinus radiata* a nivel mundial y especialmente en nuestra comunidad autónoma. Así, la razón principal para plantear esta investigación surge de la necesidad de desarrollar y optimizar herramientas biotecnológicas para la micropropagación de *Pinus radiata* que, combinadas con los programas de mejora y conservación, permitan el logro de objetivos difícilmente abordables mediante el uso de técnicas de propagación tradicionales.

En nuestro equipo de investigación, dentro del Departamento de Biotecnología de NEIKER-TECNALIA, se contaba con una experiencia previa en el cultivo *in vitro* de *Pinus radiata* y de otras especies forestales como *P. pinaster* (De Diego et al. 2008), *P. pinea* (Cortizo et al. 2009) o *P. sylvestris* (De Diego et al. 2010). Fruto de estos estudios, se ha constatado la importancia de la selección del explanto y del medio de cultivo, y en particular, de los reguladores de crecimiento aplicados al mismo a la hora de desencadenar una respuesta morfogénica satisfactoria.

En *Pinus radiata*, pese a haber sido descritos protocolos de regeneración de planta mediante organogénesis (Horgan 1987; Aitken-Christie et al. 1988) y embriogénesis somática (Walter et al. 1998), estos protocolos se limitaban a testar un reducido número de condiciones físico-químicas. Las líneas prioritarias del Plan Forestal, de los planes de investigación, la importancia del *Pinus radiata* en el sector forestal vasco así como la experiencia de nuestro grupo de investigación en el desarrollo de técnicas de micropropagación de especies pináceas, nos llevó a plantearnos la necesidad de estudiar en profundidad los mecanismos necesarios para la optimización de diversas técnicas de cultivo *in vitro* en esta especie.

En este contexto, durante los últimos años hemos trabajado en el marco de los siguientes proyectos de investigación:

- "Desarrollo y optimización de técnicas biotecnológicas para la clonación de individuos del género *Pinus* spp." (CLONCITOII, DAPA: VED2007014).
- "Clonación de individuos adultos del género *Pinus* spp. Identificación de genes implicados en los procesos organogénicos" (CLONCITO, MEC: AGL2005-08214-CO2-02; DAPA: VEC2005021).

- "Multiplicación asexual de genotipos élite de *Pinus radiata* D. Don. como aplicación biotecnológica para sus programas de mejora genética" (CLONAPIN, DAPA: VED2003017).

El objetivo general de esta tesis fue el desarrollo y optimización de herramientas biotecnológicas para la obtención de material clonal de *Pinus radiata* D. Don. Para lograr este objetivo general nos propusimos abordar los siguientes objetivos parciales:

1. Estudiar las respuestas caulogénicas y rizogénicas de embriones zigóticos tras su cultivo *in vitro* en diferentes ambientes químicos y evaluar la influencia de los mismos en la viabilidad de las microplantas tras su aclimatación *ex vitro*.

2. Establecer el estadío óptimo de desarrollo del embrión zigótico y las condiciones de cultivo más adecuadas para iniciar y proliferar tejido embriogénico.

3. Determinar la influencia de distintos componentes del medio de cultivo en la conversión de tejido embriogénico en plantas somáticas.

4. Estudiar la viabilidad de la inducción caulogénica y rizogénica de embriones somáticos cultivados *in vitro*.

5. Analizar el proceso de organogénesis *in vitro* en yemas vegetativas procedentes de individuos adultos. Determinar los niveles de citoquininas y ácido indol-3-acético endógenos a lo largo del proceso y definir posibles marcadores de potencial organogénico.

6. Evaluar la influencia del medio de cultivo y el tipo de explanto inicial en la obtención de tejido embriogénico a partir de yemas vegetativas procedentes de individuos adultos.

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CAPÍTULO 1

Could the induction periods with different cytokinins determine the *in vitro* response and the *ex vitro* behaviour of *Pinus radiata* shoots?

Este capítulo se corresponde con el artículo: Could the induction periods with different cytokinins determine the *in vitro* response and the *ex vitro* behaviour of *Pinus radiata* shoots? (*Forestry*, en revisión).

Capítulo 1: Could the induction periods with different cytokinins determine the *in vitro* response and the *ex vitro* behaviour of *Pinus radiata* shoots?

1. INTRODUCTION

The *in vitro* regeneration of plantlets in conifers can proceed through two pathways, somatic embryogenesis and shoot organogenesis. Somatic embryogenesis in conifers has been widely reported in the past few years (Von Arnold et al. 2005; Klimaszewska et al. 2007). Some problems associated to this technology are: a narrow competence window for initiation of the embryogenic tissue (MacKay et al. 2006), and sometimes limited results in the maturation of embryogenic tissue into cotyledonary somatic embryos (Nehra et al. 2005). For these reasons, the production of clonal plants from seeds via organogenesis has been used for several conifers in the last 30 years (Von Arnold and Hawes 1989; Nugent et al. 2001; Tang and Newton 2005).

In 1957, Skoog and Miller proposed that a balance between auxin and cytokinin (CK) determines the morphogenic competence of an explant in *in vitro* culture. Manipulation of the composition and ratio of these plant growth regulators inside the tissue is often the primary empirical approach to the optimization of *in vitro* culture. Through this process of plant growth regulator optimization, abnormal or unusual organ development is often observed and attributed to plant growth regulator imbalance (Ramage and Williams 2004).

As we have pointed above, the type, concentration and exposure time to plant growth regulators are among the most important factors influencing morphogenic response in *in vitro* cultured tissues (Martinez Pulido et al. 1992).

Seed organogenesis involves a four-step process: a) induction and development of adventitious buds, b) elongation of shoot buds, c) multiplication of shoots, and d) rooting of shoots and plantlet transfer to *ex vitro* conditions (Thorpe et al. 1991). In *Pinus radiata*, Aitken-Christie et al. (1988) developed an organogenesis protocol to generate large numbers of meristematic nodules from zygotic embryos. Later on, Hargreaves et al. (2004; 2005) developed cryopreservation and *in vitro* protocols for plantlet regeneration in *P. radiata*. In

the aforementioned protocols, shoot induction was achieved in radiata pine with 22 µM benzyladenine (BA) for 3 weeks. In this sense, Stange et al. (1999) studied the effect of different shoot induction treatments in embryos of the same species; they assayed different BA and thidiazuron (T) concentrations to test their effect on the number and quality of the shoots obtained. Currently, BA is the most widely used CK in plant micropropagation due to its effectiveness and affordability, but it has been reported to provoke hyperhydricity in some species (Bairu et al. 2007).

Although zeatin (Z) and *meta*-topolin (mT) have been studied as alternative phytohormones for micropropagation of other *Pinus* species (De Diego et al. 2008; Cortizo et al. 2009; De Diego et al. 2010), till date there is no report on *Pinus radiata* organogenesis where the effect of these CKs has been studied.

Once the induction and elongation of shoots has been achieved, rooting these shoots is attained through the application of exogenous auxins to the culture medium. In radiata pine, a combination of a naturally occurring auxin [indole-3-butyric acid (IBA)] with a synthetic auxin [1-naphtalene acetic acid (NAA)] has been used for root induction in the past few years (Horgan and Holland 1989; Hargreaves et al. 2005). We have studied the effect of this auxin combination (Hargreaves et al. 2005) and the effect of each auxin separately on rooting of shoots from three different shoot induction treatments.

It has been described in angiosperm species that plants "remember" the treatments (auxins and CKs) to which they were exposed during the initial phases of their growth, thus conditioning their future development (Moncaleán et al. 2001). Till date, these effects have not been evaluated in *P. radiata* organogenesis protocols.

The global aim of the present study was to determine the influence of *in vitro* culture conditions in the explant response and *ex vitro* behaviour of *Pinus radiata* shoots. In particular, we focused on:

-Testing previously assayed CKs as BA and T, and others not tested before such as mT and Z in order to improve the shoot induction stage.

-Determining the best induction period to produce adventitious shoots.

-Establishing the best basal medium and auxin type to induce roots in adventitious shoots.

-Analysing the effect of shoot and root induction treatments on *ex vitro* development of the regenerated plants.

2. MATERIAL AND METHODS

In 2006, *Pinus radiata* D. Don seeds were obtained from open-pollinated (OP) trees grown in natural stands of the Basque coastline, Spain (batch number: 2530). They were provided by Servicio de Material Genético of Ministerio de Medio Ambiente (Spain).

In 2008, *P. radiata* seeds were obtained from selected trees from Kangairoa, New Zealand (batch number: 06/513B). They were provided by Proseed (New Zealand).

Seeds were washed in sterile distilled water plus two drops of Tween 20® for 5 min, followed by three rinses in sterile distilled water. Then, they were immersed in a fungicide solution (0.1 g L^{-1} Benlate®) for 15 min, followed by three rinses in sterile distilled water. Next steps were carried out in sterile conditions in the laminar flow unit; seeds were immersed in 10% commercial bleach for 10 min, followed by three rinses in sterile distilled water. After this, seeds were immersed in 70% ethanol for 2 min, followed by three rinses in sterile distilled water. Seeds were stored in moistened sterile paper for 48 h at 4 ºC in darkness.

Prior to dissection, seeds were immersed in 7.5 % (v/v) H_2O_2 for 10 min, followed by three rinses in sterile distilled water. Seed coats and megagametophytes were removed aseptically and embryos were cultured in an inverted position with the cotyledons immersed in the induction medium.

The cultures were maintained at 21±1°C under a 16 h photoperiod of 120 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France).

2.1. Shoot induction and development

2.1.1. Experiment 1

In 2006, mature zygotic embryos of radiata pine were cultured on half strength macronutrients except for the iron and complete micronutrients and vitamins LP medium (Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988) supplemented with 30 g L^{-1} sucrose,

4 g L^{-1} Gelrite® and 0.2 g L^{-1} Difco Agar® (1/2LP). The pH was adjusted to 5.8 before autoclaving at 121ºC for 20 min.

The effect of different CKs at different concentrations in 1/2LP medium was assayed. Four CKs: BA, mT, T or Z; each at five concentrations: 0.4, 1, 4.4, 10 or 40 µM were tested separately. In order to determine the optimum induction period, different induction periods were assayed: 2, 3 or 4 weeks. The experiments comprised four CKs, at five concentrations and three induction periods, a total of sixty treatments (Fig. 1a). As controls, embryos were cultured in the absence of CK for the three induction periods. The following codes were used to identify the induction media: 2, 3 or 4 for the induction periods, followed by the abbreviated name of the hormone assayed and its concentration (for the controls only C letter). Thus, 3BA4.4 is an induction treatment with BA at 4.4 µM for 3 weeks.

Ten embryos were cultured on each 90 x 15 mm Petri dish, three Petri dishes per CK type, concentration and induction period were laid out randomly on the shelves of the growth chamber. Cultures were kept at 21±1ºC under a 16 h photoperiod of 120 mmol m^{-2} s^{-1}.

After the induction period, explants were transferred to glass jars with the same basal medium but lacking growth regulators for 3 weeks (Fig. 1c). Then, embryos were monthly subcultured to elongation medium (LPE); this medium was 1/2LP supplemented with 0.2% (w/v) activated charcoal (AC).

2.1.2. Experiment 2

In 2008, mature zygotic embryos of radiata pine were cultured on the same basal medium described in Experiment 1. The best three treatments from experiment 1 were assayed for shoot organogenesis. Ten embryos were cultured on each 90 x 15 mm Petri dish, twelve Petri dishes per CK type, concentration and induction period were laid out randomly on the shelves of the growth chamber.

After the induction treatments, explants were transferred to glass jars with 1/2LP medium lacking growth regulators for 3 weeks. Then, embryos were monthly subcultured to LPE.

2.2. Root induction, development and acclimatization of plants

Elongated shoots (>15 mm) from Experiment 2 were transferred to glass jars with rooting medium. Two basal media were assayed: 1/2LP (Quoirin and Lepoivre 1977, modified by

Aitken-Christie et al. 1988), and quarter strength macronutrients except for the iron, and half strength iron, micronutrients and vitamins (1/4LP). Both media were supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} Difco Agar® granulated.

Basal media were supplemented with three different auxin treatments: 1.5 mg L^{-1} 1-naphthalene acetic acid (NAA), 1.5 mg L^{-1} indole-3-butyric acid (IBA), and 1 mg L^{-1} IBA with 0.5 mg L^{-1} NAA (MIX). The root induction experiments comprised two basal media and three auxin treatments. The following codes were used to identify the induction media: 2 for 1/2LP basal medium and 4 for 1/4LP medium followed by the name of the rooting treatment assayed. Thus, 4NAA is 1/4LP supplemented with 1.5 mg L^{-1} NAA.

Four shoots were cultured on each culture vessel and five glass jars per treatment were used. After 4 weeks in root induction medium, the shoots were transferred to LPE medium and kept under the same conditions described above.

After a month on LPE medium explants with and without visible roots were transferred to a wet sterile peat:perlite mixture (3:1) and acclimatized in the greenhouse under controlled conditions at 21±2ºC and decreasing humidity progressively. Two months later the explants were transplanted to 5 x 5 x 20 cm pots filled with the same mixture.

2.3. Data collection and statistical analyses

After shoot induction period in Experiment 1, the percentage of contaminated embryos was recorded. After 4 months on elongation medium, the percentage of necrosed, hyperhydric (Fig. 1b) and embryos forming shoots (EFS) was recorded. Moreover, the height of shoots was measured, and the percentage of shoots higher or shorter than 1.5 cm was calculated (Fig. 1d).

The shoot elongation capacity (SEC) index was determined as described by Lambardi et al. (1993):

SEC= (average number of shoots> 3mm per embryo) x (EFS)

Normality was evaluated by Shapiro–Wilk normality test (Shapiro and Wilk 1965) without transformation. The equal variance of the data was analysed by Levene's test (Brown and Forsythe 1974). Statistical analyses were performed using the SPSS© version 15.0 software package.

Analysis of variance (ANOVA) was carried out to determine differences among treatments for the EFS percentage, the number of shoots per embryo and SEC index. Data for the number of shoots per embryo and the SEC index were subjected to log (x) transformation. Multiple comparisons were made using Duncan´s post hoc test ($p \leq 0.05$).

After shoot induction period in Experiment 2, the percentage of contaminated embryos was analysed. After 4 months on elongation medium, the percentage of necrosed, hyperhydric and EFS was recorded. The number of shoots > 3mm per embryo and the SEC index (Lambardi et al. 1993) were also recorded as mentioned above.

ANOVA was carried out to determine differences between the treatments for the EFS, the number of shoots per embryo and the SEC index. Data for the number of shoots per embryo and for SEC index were subjected to log (x) transformation. Multiple comparisons were made using Duncan´s post hoc test ($p \leq 0.05$).

After root induction and a month on elongation medium, the percentage of shoots with roots and the number of roots per shoot were recorded (Figs. 3a, 3b and 3c). ANOVA was carried out to determine differences between shoot induction treatments and root induction treatments, for the percentage of shoots with roots and the number of roots per shoot. Multiple comparisons were made using Duncan´s post hoc test ($p \leq 0.05$).

After 2 months in the greenhouse, the survival of the explants rooted *in vitro* was recorded. The number of explants non-rooted *in vitro* that developed roots *ex vitro* was also recorded (Fig. 3d). The total acclimatized explants was determined as the percentage of explants rooted *in vitro* that survived plus the percentage of explants that developed roots under *ex vitro* conditions (Fig. 3e).

ANOVA was carried out to determine differences between root induction treatments, for the survival percentage of the shoots rooted *in vitro*, the percentage of non-rooted shoots that developed roots *ex vitro*, and the percentage of acclimatized explants. Multiple comparisons were made using Duncan´s post hoc test ($p \leq 0.05$).

3. RESULTS

3.1. Shoot induction and development

3.1.1. Experiment 1

Contamination percentages were below 7% for all CK concentrations and induction periods assayed.

The percentage of necrosed embryos reached values higher than 50% when no CK was applied or when it was applied for 2 weeks (Table 1). In explants cultured with BA for 4 weeks, percentages of necrosed explants higher than 50% were also observed. The lowest percentages of necrosed embryos were achieved when T or Z was applied to the induction medium for 3 or 4 weeks (Table 1).

Hyperhydricity percentages (Fig. 1b, Table 2) remained low (under 7%) when no CK was applied or when it was applied at 0.4 μM for no more than 3 weeks. When the CK used was Z, no clear tendency could be observed. Induction medium supplemented with BA for 4 weeks produced a higher number of hyperhydric explants than the same media with CK applied for 2 or 3 weeks. The percentage of hyperhydric explants increased with CK concentration when mT or T was assayed, particularly for the longest induction period (Table 2). After recording hyperhydricity percentages for the different treatments, hyperhydric explants were discarded.

The percentages of EFS for the different treatments assayed are shown in Tables 3. When the induction period was analysed, independently of the CK type and concentration used in the culture media, explants induced for 3 weeks produced the highest percentages of EFS. In the same way, when CKs were analysed independently of the concentration and the induction period, explants cultured on Z supplemented medium showed the highest values in the mentioned parameter. This hormone induced the highest number of EFS (54.6%) when the induction period was 3 weeks. Independently of the induction period and concentration, the 48.2% of the embryos cultured with Z showed an organogenic response. Embryos treated with BA, mT, T and controls showed a significantly lower response.

Table 1 Necrosed explants (%) of *Pinus radiata* zygotic embryos after *in vitro* culture for 2, 3 o 4 weeks on 1/2LP medium supplemented with different cytokinins (BA, mT, T or Z) at 5 concentrations (0.4, 1, 4.4, 10, 40 μM) (M).

NECROSED EXPLANTS (%)			
Cytokinin type and concentration	**Induction period**		
	2 weeks	**3 weeks**	**4 weeks**
Control	58.0	52.5	46.1
BA0.4	50.0	30.0	40.0
BA1	30.0	46.7	46.0
BA4.4	30.0	36.7	58.0
BA10	26.7	33.3	50.0
BA40	33.3	46.7	52.0
mT0.4	40.0	30.0	28.7
mT1	46.7	40.0	42.5
mT4.4	46.7	43.3	30.0
mT10	30.0	40.0	38.7
mT40	70.0	43.3	37.5
T0.4	50.0	50.0	34.0
T1	63.3	16.7	24.0
T4.4	26.7	60.0	26.0
T10	30.0	36.7	26.0
T40	33.3	20.0	10.0
Z0.4	40.0	23.3	12.0
Z1	53.3	20.0	30.0
Z4.4	46.7	40.0	30.0
Z10	36.7	40.0	32.0
Z40	60.0	20.0	36.0

Table 2 Hyperhydric explants (%) of *Pinus radiata* zygotic embryos after *in vitro* culture for 2, 3 or 4 weeks on 1/2LP medium supplemented with different cytokinins (BA, mT, T or Z) at 5 concentrations (0.4, 1, 4.4, 10, 40 μM) (M).

HYPERHYDRIC EXPLANTS (%)			
Cytokinin type and concentration	**Induction period**		
	2 weeks	**3 weeks**	**4 weeks**
Control	4.0	0.0	6.9
BA0.4	0.0	10.0	16.0
BA1	20.0	10.0	22.0
BA4.4	20.0	23.3	10.0
BA10	43.3	56.7	22.0
BA40	33.3	23.3	20.0
mT0.4	6.7	3.3	18.7
mT1	10.0	10.0	18.7
mT4.4	13.3	13.3	26.2
mT10	20.0	10.0	28.7
mT40	13.3	30.0	26.2
T0.4	13.3	3.3	12.0
T1	13.3	43.3	18.0
T4.4	20.0	6.7	30.0
T10	26.7	23.3	50.0
T40	40.0	46.7	34.0
Z0.4	6.7	20.0	16.0
Z1	10.0	13.3	6.0
Z4.4	30.0	23.3	20.0
Z10	40.0	6.7	14.0
Z40	16.7	16.7	22.0

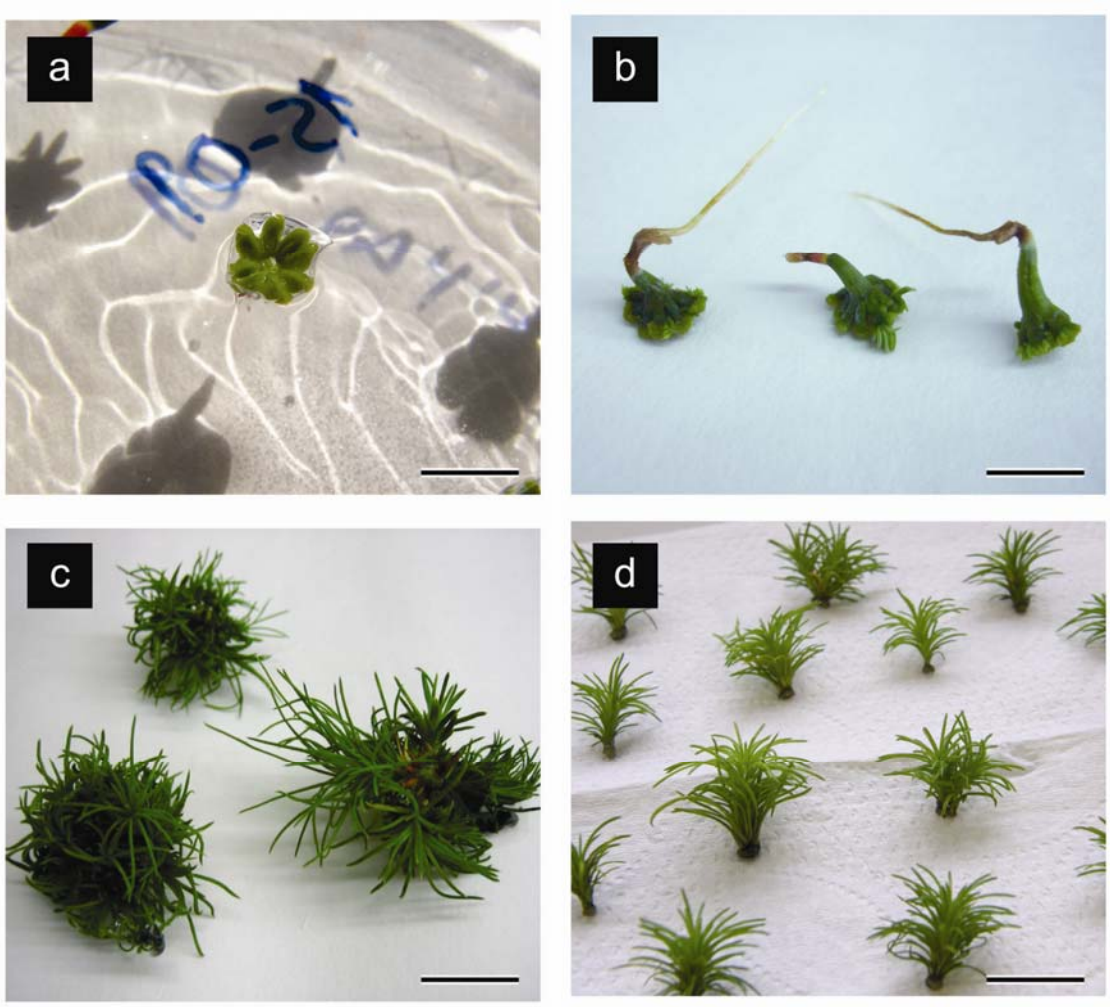

Fig. 1 Shoot induction proccess in *Pinus radiata* zygotic embryos: (a) zygotic embryo cultured for 2 weeks on 1/2LP medium supplemented with 1µM BA (bar=7 mm); (b) hyperhydric explants cultured for 3 weeks on 1/2LP medium supplemented with 4.4 µM BA (bar=10 mm); (c) explants cultured for 3 weeks on 1/2LP medium supplemented with 1 µM Z, after 3 weeks on the same medium lacking growth regulators and 4 weeks on LPE medium (bar=15 mm); (d) explants induced with 4.4 µM BA for 3 weeks after 4 months on LPE medium (bar=25 mm).

The EFS percentage analysed after treatments with different CK types, independently of the concentration, showed that explants cultured with BA had a higher response when it was applied for 2 weeks. On the contrary, T produced a higher number of EFS when it was applied for 4 weeks (Table 3). The results obtained in embryos cultured with mT were statistically non significant for induction periods of 2 and 3 weeks. BA induced significantly lower number of EFS when compared with the other CKs tested or the control (Table 3).

Table 3 Embryos forming shoots [EFS (%)] in *Pinus radiata* zygotic embryos after *in vitro* culture for 2, 3 or 4 weeks on 1/2LP medium supplemented with different cytokinins (BA, mT, T or Z) at 5 concentrations (0.4, 1, 4.4, 10, 40 μM) (M±S.E.). Different letters within column and different numbers within a row show significant differences by Duncan´s post hoc test ($p \leq 0.05$).

EFS (%)				
Cytokinin type and concentration	**Induction period**			**Mean**
	2 weeks	**3 weeks**	**4 weeks**	
Control	38.0 ± 10.2 c 2	47.5 b ± 8.5 1	40.7 ± 6.1 c 2	41.4 ± 4.4 b
BA0.4	50.0 ± 5.8	30.0 ± 14.3	42.0 ± 8.0	
BA1	50.0 ± 15.3	43.3 ± 17.6	32.0 ± 7.3	
BA4.4	46.7 ± 8.8	30.0 ± 15.3	24.0 ± 8.7	35.9 ± 2.6 c
BA10	30.0 ± 0.0	10.0 ± 5.8	28.0 ± 8.6	
BA40	33.3 ± 12.0	30.0 ± 10.0	20.0 ± 4.5	
Mean	43.7 ± 4.4 ab 1	32.6 ± 6.0 c 2	31.1 ± 3.5 e 2	
mT0.4	53.3 ± 6.7	60.0 ± 10.0	45.0 ± 9.8	
mT1	43.3 ± 12.0	36.7 ± 6.7	31.2 ± 8.7	
mT4.4	40.0 ± 0.0	43.3 ± 14.5	33.7 ± 8.4	39.7 ± 2.7 b
mT10	50.0 ± 15.3	50.0 ± 15.3	31.2 ± 8.3	
mT40	16.7 ± 8.8	26.7 ± 14.5	25.0 ± 6.5	
Mean	44.7 ± 5.1 a 1	46.3 ± 5.7 b 1	34.5 ± 3.7 d 2	
T0.4	36.7 ± 8.8	36.7 ± 8.8	54.0 ± 5.1	
T1	23.3± 3.3	30.0 ± 10.0	40.0 ± 12.6	
T4.4	53.3 ± 12.0	33.3 ± 6.7	34.0 ± 5.1	41.1 ± 2.7 b
T10	43.3 ± 8.8	36.7 ± 8.8	24.0 ± 10.3	
T40	26.7 ± 3.3	33.3 ± 12.3	56.0 ± 12.9	
Mean	39.9 ± 4.2 bc 2	34.2 ± 4.5 c 3	45.1 ± 4.7 b 1	
Z0.4	36.7 ± 5.8	56.7 ± 6.7	58.0 ± 9.3	
Z1	36.7 ± 14.5	66.7 ± 8.8	60.0 ± 8.9	
Z4.4	13.3 ± 8.8	36.7 ± 6.6	36.0 ± 10.2	48.2 ± 3.1 a
Z10	23.3 ± 3.3	53.3 ± 8.7	52.0 ± 9.7	
Z40	16.7 ± 6.7	50.0 ± 13.3	22.0 ± 10.2	
Mean	28.7 ± 4.0 d 3	54.6 ± 4.4 a 1	50.0 ± 4.9 a 2	
Total	40.3 ± 2.3 2	44.1 ± 2.7 1	40.7 ± 2.0 2	

When the EFS percentage was studied independently of the CK concentration, for induction periods of 3 or 4 weeks, Z induced the highest organogenic response (54.6% and 50%, respectively); whereas embryos cultured with BA or mT for 2 weeks showed the best EFS percentages (43.7 and 44.7%, respectively) (Table 3).

Analysing the induction treatments individually (CK type, concentration and induction period), EFS percentages decreased when mT was applied at 40 μM in all the induction periods assayed. The same effect was observed when embryos were cultured for 2 or 4 weeks with BA or Z. When explants were treated with T, no clear trend was found for the EFS percentage. This parameter achieved the highest values when Z was applied at low concentrations (0.4 μM and 1 μM) for 3 or 4 weeks; a similar value was recorded in explants cultured with mT at 0.4 μM for 3 weeks. Considering all shoot induction treatments assayed, the best organogenic response (66.7) was obtained in explants cultured in the presence of Z at 1 μM for 3 weeks (Table 3).

As shown in Table 4, considering all CK types and concentrations together, the highest number of shoots per embryo was obtained when explants were cultured in induction medium for 3 weeks. When the results for the different CKs were analysed, independently of the concentration, Z and T produced the highest number of shoots per embryo when they were applied for 3 weeks, although the latter produced similar results when it was applied for 2 weeks (Table 4). BA was the best CK for the number of shoots per embryo (14.5) and no significant differences were observed among induction periods for this plant growth regulator (Table 4).

There were no significant differences among treatments for the number of shoots per embryo when the induction period was 3 or 4 weeks, except in explants cultured in CK absence. When the induction period was 2 weeks, BA produced more shoots per embryo than mT and the control (Table 4).

The explants cultured in the absence of CK always produced a lower number of shoots per embryo (less than 5) than explants cultured with CKs (more than 6). The highest number of shoots per embryo (31.1) was achieved when explants were cultured with BA at 4.4 μM for 3 weeks (Fig. 1d).

Table 4 Shoots per *Pinus radiata* zygotic embryo (No.) after *in vitro* culture for 2, 3 or 4 weeks on 1/2LP medium supplemented with different cytokinins (BA, mT, T or Z) at 5 concentrations (0.4, 1, 4.4, 10, 40) (M±S.E.). Different letters within column and different numbers within a row show significant differences by Duncan´s post hoc test ($p \leq 0.05$) (n.s.: non significant).

No. of shoots per embryo				
Cytokinin type and concentration	**Induction period**			**Mean**
	2 weeks	**3 weeks**	**4 weeks**	
Control	4.0 ± 0.6 c n.s.	4.3 ± 0.9 b n.s.	4.8 ± 0.5 b n.s.	4.5 ± 0.4 c
BA0.4	8.1 ± 1.2	12.8 ± 2.5	13.0 ± 3.1	14.5 ± 0.5 a
BA1	19.5 ± 3.5	11.6 ± 2.7	12.1 ± 2.4	
BA4.4	14.0 ± 3.3	31.1 ± 5.6	10.5 ± 2.3	
BA10	18.8 ± 5.0	29.3 ± 9.8	12.6 ± 1.9	
BA40	13.4 ± 1.7	9.7 ± 1.7	17.2 ± 3.6	
Mean	14.5 ± 1.4 a n.s.	16.8 ± 2.1 a n.s.	12.9 ± 1.2 a n.s.	
mT0.4	6.4 ± 1.1	8.6 ± 1.5	8.3 ± 0.8	10.5 ± 0.5 b
mT1	9.8 ± 1.1	20.4 ± 3.4	10.4 ± 1.9	
mT4.4	10.3 ± 1.6	9.5 ± 2.5	14.9 ± 2.0	
mT10	7.5 ± 1.0	11.5 ± 1.5	8.3 ± 1.3	
mT40	13.4 ± 4.8	14.1 ± 1.9	11.2 ± 2.0	
Mean	8.7 ± 0.7 b n.s.	12.1 ± 1.1 a n.s.	10.5 ± 0.7 a n.s.	
T0.4	8.4 ± 2.4	9.2 ± 2.6	7.8 ± 1.4	11.2 ± 0.6 b
T1	7.9 ± 3.4	14.4 ± 3.7	11.0 ± 2.0	
T4.4	12.7 ± 2.1	12.1 ± 1.5	9.9 ± 2.0	
T10	16.5 ± 3.3	17.6 ± 3.0	9.4 ± 1.7	
T40	17.6 ± 3.9	16.2 ± 2.1	7.8 ± 1.3	
Mean	12.8 ± 1.4 ab 1	13.9 ± 1.2 a 1	9.0 ± 0.7 a 2	
Z0.4	7.1 ± 1.6	12.3 ± 1.4	6.8 ± 1.0	11.5 ± 0.6 b
Z1	8.4 ± 1.7	18.3 ± 3.2	9.9 ± 1.4	
Z4.4	14.5 ± 3.0	13.1 ± 2.0	11.3 ± 2.0	
Z10	16.9 ± 3.5	13.6 ± 2.3	11.7 ± 1.8	
Z40	9.2 ± 3.5	16.3 ± 2.7	6.9 ± 2.0	
Mean	10.5 ± 1.2 ab 2	14.9 ± 1.1 a 1	9.5 ± 0.7 a 2	
Total	11.1 ± 0.6 2	13.5 ± 0.6 1	9.6 ± 0.4 3	

The SEC index combines EFS percentage and the number of shoots> 3mm per embryo. In the longest induction period the SEC indices achieved were lower than in the 2 or 3 weeks induction periods. Considering all CK types and concentrations together, embryos cultured for 3 weeks achieved the highest SEC index. When each CK type was analysed separately, this induction period was also the best for explants treated with Z and mT. Embryos cultured for 2 and 3 weeks with BA presented similar SEC indices in the 2 weeks induction period. The explants induced with T did not show significant differences among induction periods for the SEC index (Table 5).

Gathering all concentrations and periods together, explants induced with Z showed the highest SEC index, but this value only differed statistically with values obtained in the explants cultured in presence of mT and the controls (Table 5).

When the induction periods were analysed separately, the explants cultured with BA or T gave the highest SEC indices in the shortest induction period. In the longest induction period non significant differences were observed in the SEC indices obtained for the different CK types assayed, only the SEC index in the control explants was significantly lower. As pointed above, Z produced the highest SEC index, this was especially patent in the 3 weeks induction period, with an overall SEC index for this period of 8.3 (Table 5). The highest SEC was achieved in explants cultured with Z at 1 μM for 3 weeks. SEC indices higher than 9 were also obtained in explants after treatments with BA at 1 μM for 2 weeks and BA at 4.4 μM for 3 weeks (Table 5). These three treatments were selected for the next experiments.

As it was described previously, the height of all shoots induced in the different induction treatments assayed was measured, and explants were classified into 2 categories: <1.5 cm and ≥1.5 cm.

In embryos cultured with Z, the percentages of shoots shorter than 1.5 cm tended to increased with CK concentration when induction periods of 2 or 4 weeks were assayed. Explants incubated for 4 weeks with BA also showed this trend (Figs. 2a and 2c). When zygotic embryos were cultured for 2 weeks with mT or T, the concentration of these CKs that produced a higher number of shoots per embryo (40 μM) also produced higher percentages of short shoots (Fig. 2a). Embryos cultured for 3 weeks showed this trend in treatments with BA and mT, where the concentrations producing the highest number of shoots per embryo (BA at 4.4 μM and mT at 1 μM) led to higher percentages of short shoots (<1.5 cm) (Fig. 2b).

Explants treated with T for 3 weeks had similar percentages of shoots higher than 1.5 cm regardless of the concentration used (Fig. 2b).

Table 5 Shoot elongation capacity (SEC) in *Pinus radiata* zygotic embryos after *in vitro* culture for 2, 3 or 4 weeks on 1/2LP medium supplemented with different cytokinins (BA, mT, T or Z) at 5 concentrations (0.4, 1, 4.4, 10, 40) (M±S.E). Different letters within column and different numbers within a row show significant differences by Duncan´s post hoc test ($p \leq 0.05$).

SEC				
Cytokinin type and concentration	**Induction period**			**Mean**
	2 weeks	**3 weeks**	**4 weeks**	
Control	1.5 ± 0.2 d n.s.	2.0 ± 0.4 c n.s.	1.9 ± 0.2 b n.s.	1.9 ± 0.2 c
BA0.4	4.0 ± 0.6	3.8 ± 0.7	5.5 ± 1.3	5.0 ± 0.3 ab
BA1	9.7 ± 1.7	5.0 ± 1.1	3.9 ± 0.8	
BA4.4	6.6 ± 1.6	9.3 ± 1.7	2.5 ± 0.5	
BA10	5.6 ± 1.5	2.9 ± 1.0	3.5 ± 0.5	
BA40	4.4 ± 0.6	2.9 ± 0.5	3.4 ± 0.7	
Mean	6.2 ± 0.7 a 1	5.1 ± 0.6 b 1	4.0 ± 0.4 a 2	
mT0.4	3.4 ± 0.6	5.2 ± 0.9	3.7 ± 0.3	4.0 ± 0.2 b
mT1	4.2 ± 0.5	7.6 ± 1.3	3.2 ± 0.6	
mT4.4	4.1 ± 0.6	4.1 ± 1.1	5.1 ± 0.7	
mT10	3.8 ± 0.5	5.7 ± 0.7	2.6 ± 0.4	
mT40	2.3 ± 0.8	3.8 ± 0.5	2.8 ± 0.5	
Mean	3.7 ± 0.3 bc 2	5.3 ± 0.4 b 1	3.5 ± 0.2 a 2	
T0.4	3.1 ± 0.9	3.4 ± 0.9	4.2 ± 0.7	4.4 ± 0.2 ab
T1	1.8 ± 0.8	4.3 ± 1.1	4.4 ± 0.8	
T4.4	6.7 ± 1.1	4.0 ± 0.5	3.4 ± 0.7	
T10	7.1 ± 1.4	6.5 ± 1.1	2.3 ± 0.4	
T40	4.7 ± 1.0	5.3 ± 0.7	4.4 ± 0.7	
Mean	5.2 ±0.6 ab n.s.	4.7 ± 0.4 b n.s.	3.9 ± 0.3 a n.s.	
Z0.4	2.6 ± 0.6	7.0 ± 0.8	4.0 ± 0.6	5.7 ± 0.3 a
Z1	3.1 ± 0.6	12.3 ± 2.1	5.9 ± 0.8	
Z4.4	1.9 ± 0.4	4.8 ± 0.7	4.0 ± 0.7	
Z10	3.9 ± 0.8	7.2 ± 1.2	6.1 ± 0.9	
Z40	1.6 ± 0.6	8.3 ± 1.4	1.5 ± 0.4	
Mean	2.8 ± 0.3 c 3	8.3 ± 0.7 a 1	4.7 ± 0.4 a 2	
Total	4.4 ± 0.2 2	5.8 ± 0.3 1	3.8 ± 0.1 3	

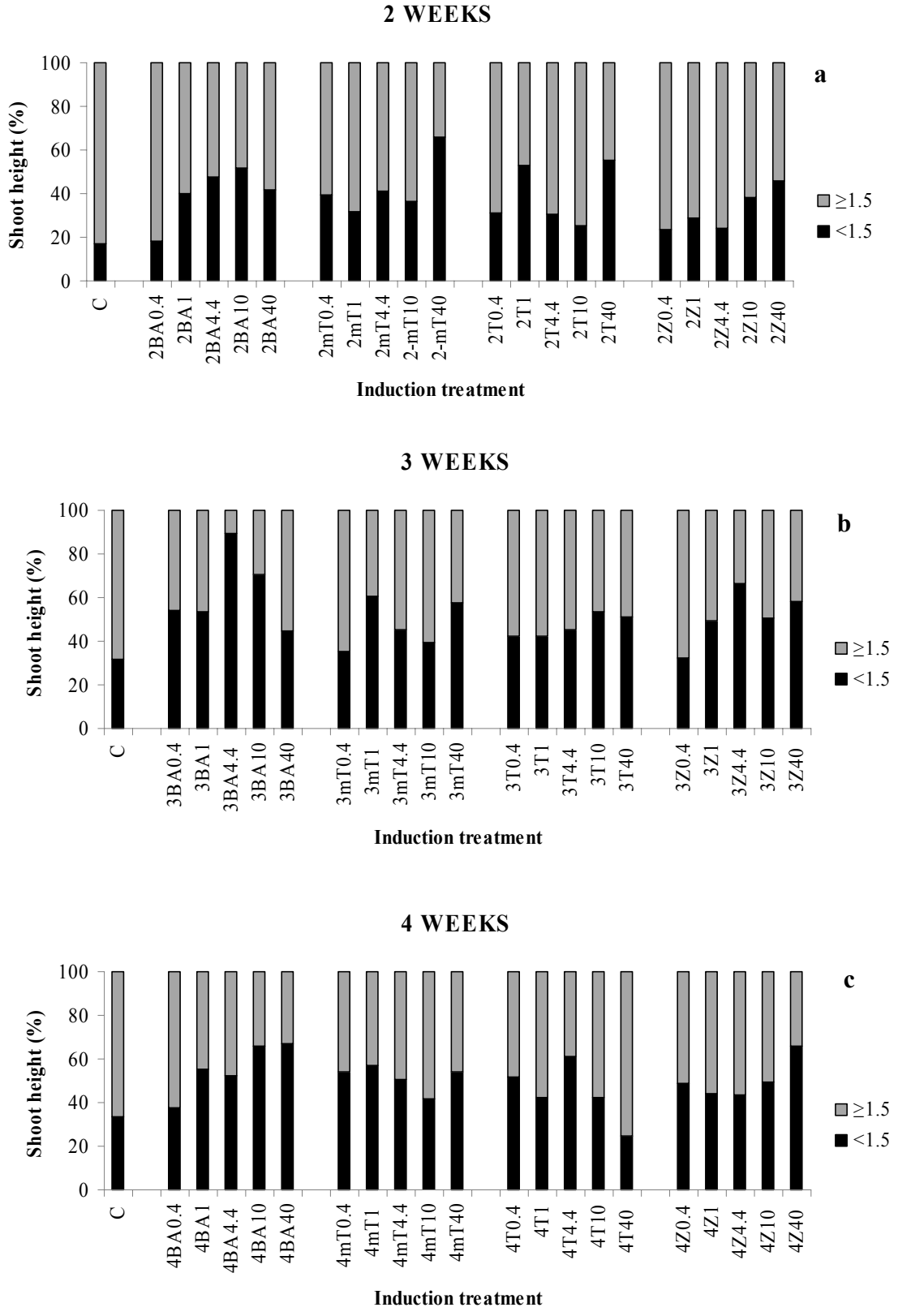

Fig. 2 Shoots higher and shorter than 1.5 cm (%) obtained in *Pinus radiata* zygotic embryos cultured on 1/2LP medium supplemented with different cytokinins (BA, mT, T or Z) at 5 concentrations (0.4, 1, 4.4, 10, 40 µM) for (a) 2 weeks, (b) 3 weeks and (c) 4 weeks.

In explants cultured for 4 weeks with Z, as observed in the 2 weeks induction period, the percentage of short shoots increased with CK concentration. When explants were cultured in the presence of BA for 4 weeks, the highest percentages of short shoots were also obtained after treatments with 10 and 40 μM BA. In this induction period, explants induced with mT showed similar percentages of shoots higher than 1.5 cm regardless of the concentration used (Fig. 2c).

The controls showed high percentages of shoots higher than 1.5 cm. In the induction periods corresponding to 2 and 4 weeks, this percentage was similar to that obtained in shoots after applying BA at 0.4 μM (Figs. 2a and 2c).

3.1.2. Experiment 2

After shoot induction period, the contamination percentages were under 5% for all treatments assayed. After elongation period, the percentage of necrosed embryos was below 5%. The percentage of hyperhydric embryos was similar in treatments with 1 μM Z for 3 weeks and 1 μM BA for 2 weeks (13.3 and 14.2, respectively) (Table 6). When BA concentration and incubation period was increased, the percentage of hyperhydric embryos slightly augmented (17.5) (Table 6).

Table 6 Contaminated, necrosed or hyperhydric explants (%) of *Pinus radiata* zygotic embryos after *in vitro* culture on 1/2LP medium supplemented with BA at 1 μM for 2 weeks (2BA1), BA at 4.4 μM for 3 weeks (3BA4.4), and Z at 1 μM for 3 weeks (3Z1) (M). Different letters within a column show significant differences by Duncan´s post hoc test ($p \leq 0.05$) (n.s.: non significant).

Induction Treatment	Contaminated embryos (%)	Necrosed embryos (%)	Hyperhydric embryos (%)
2BA1	0 n.s.	1.7 n.s.	14.2 n.s.
3BA4.4	0 n.s.	4.2 n.s.	17.5 n.s.
3Z1	2.5 n.s.	5.0 n.s.	13.3 n.s.

There were no significant differences between shoot induction treatments for the percentage of EFS. These percentages ranged from 78.3% to 84.2% (Table 7). The number of shoots per embryo obtained after 3BA4.4 treatment (41.1) was significantly higher than the number of shoots per embryo in treatments 3Z1 and 2BA1 (28.6 and 25.6, respectively) (Table 7). Embryos cultured with 4.4 μM BA for 3 weeks also showed a significantly higher SEC value

(32.4) when compared with embryos induced with 1 µM Z for 3 weeks (22.6) and with 1 µM BA for 2 weeks (21.9) (Table 7).

Table 7 Explants forming shoots [EFS (%)], number of shoots per embryo and shoot elongation capacity index (SEC) of *Pinus radiata* zygotic embryos after *in vitro* culture on 1/2LP medium supplemented with BA at 1 µM for 2 weeks (2BA1), BA at 4.4 µM for 3 weeks (3BA4.4) and Z at 1 µM for 3 weeks (3Z1) (M±S.E.). Different letters within a column show significant differences by Duncan´s post hoc test ($p \leq 0.05$) (n.s.: non significant).

Induction Treatment	EFS (%)	No. of shoots per embryo	SEC
2BA1	84.2 ± 4.3 n.s.	25.6 ± 1.7 b	21.9 ± 1.4 b
3BA4.4	78.3 ± 3.4 n.s.	41.1 ± 3.3 a	32.4 ± 2.6 a
3Z1	79.2 ± 3.4 n.s.	28.6 ± 2.1 b	22.6 ± 1.7 b

3.2. Root induction, development and acclimatization of plants

As shown in Table 8, the shoot induction treatment applied previously did not seem to influence the rooting process in radiata pine shoots. These rooting percentages ranged from 21.7% to 38.3% in shoots cultured previously with 3Z1 and 3BA4.4, respectively.

Nevertheless, there were significant differences for the percentage of shoots with roots among root induction treatments. NAA was the most efficient auxin in terms of rooting percentages, with 60% and 48% of explants developing roots after 2NAA and 4NAA treatments (Fig. 3b). The latter treatment did not show significant differences with the percentages obtained when IBA and NAA were applied together (2MIX and 4MIX treatments) (Table 8) (Fig. 3c). Significantly lower rooting percentages were achieved when rooting medium was supplemented only with IBA (Fig. 3a, Table 8). There was not an interaction between the shoot induction and the root induction treatments applied to the explants.

When shoots presented roots, there were no significant differences in the number of roots per shoot neither for the shoot induction treatments nor for the root induction treatments. The lowest number of roots per shoot was observed in shoots obtained after 2BA1 treatment and rooted in 4IBA medium (1.5). In contrast, the highest number of roots per shoot was achieved in explants obtained after the aforementioned shoot induction treatment, but rooted in 2MIX medium (Table 9).

Table 8 *Pinus radiata* rooted shoots (%) after different root induction treatments: shoots were obtained from different induction treatments: (2BA1, 3Z1, and 3BA4.4) (M±S.E.). Different letters within a row show significant differences by Duncan´s post hoc test ($p \leq 0.05$) (n.s.: non significant).

Rooted shoots (%)			
Rooting treatment	**Induction treatment**		
	2BA1	3Z1	3BA4.4
4IBA	10.0 ± 6.1 n.s	10.0 ± 6.1 n.s	0 n.s
4NAA	35.0 ± 12.7 b	25.0 ± 9.7 b	85.0 ± 6.1 a
4MIX	35.0 ± 15.0 n.s	25.0 ± 8.8 n.s	45.0 ± 12.7 n.s
2IBA	15.0 ± 9.1 n.s	0 n.s	0 n.s
2NAA	55.0 ± 9.3 n.s	50.0 ± 7.9 n.s	75.0 ± 13.7 n.s
2MIX	45.0 ± 12.2 n.s	20.0 ± 5.0 n.s	25.0 ± 0.0 n.s
Mean	32.5 ± 5.4 n.s.	21.7 ± 4.6 n.s.	37.5 ± 6.8 n.s.

Table 9 Number of roots per shoot in *Pinus radiata* after different root induction treatments: shoots were obtained from different induction treatments (2BA1, 3Z1 and 3BA4.4) (M±S.E.). Different letters within a row show significant differences by Duncan´s post hoc test ($p \leq 0.05$) (n.s.: non significant).

No. of roots per shoot			
Rooting treatment	**Shoot Induction treatment**		
	2BA1	3Z1	3BA4.4
4IBA	1.5 ± 0.5 n.s.	2.0 ± 1.0 n.s.	0.0 ± 0.0 n.s.
4NAA	4.4 ± 1.6 n.s.	5.4 ± 1.9 n.s.	7.5 ± 1.7 n.s.
4MIX	6.0 ± 2.4 n.s.	5.8 ± 3.6 n.s.	6.0 ± 2.2 n.s.
2IBA	3.7 ± 2.7 n.s.	0.0 ± 0.0 n.s.	0.0 ± 0.0 n.s.
2NAA	4.2 ± 1.1 n.s.	6.2 ± 2.0 n.s.	4.9 ± 1.1 n.s.
2MIX	6.9 ± 2.7 n.s.	5.7 ± 2.1 n.s.	3.4 ± 1.7 n.s.

After a month on LPE medium, rooted and non-rooted explants were transferred to the greenhouse. After 2 months growing under *ex vitro* conditions, the survival of the explants rooted *in vitro* was recorded (Table 10). All the explants rooted with IBA alone survived *ex vitro* (Fig. 3b), the survival of explants rooted with the auxin mixture and with treatment 4NAA was significantly lower. The worst survival rate (58%) was observed in explants rooted with 2NAA (Table 10).

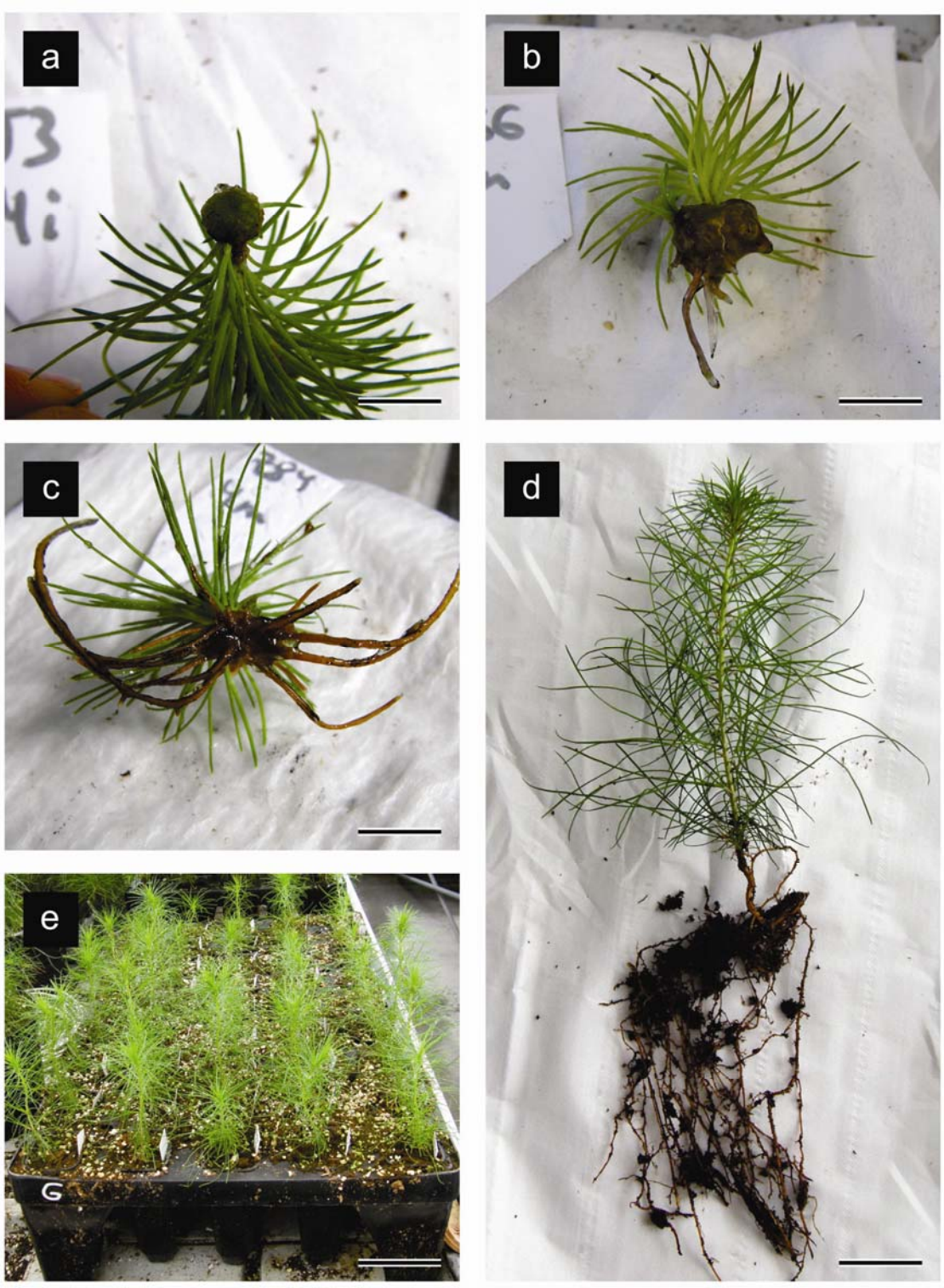

Fig. 3 Root induction in *Pinus radiata* zygotic embryos: (a) explant cultured for 4 weeks on 1/4LP medium supplemented with 1.5 mg L^{-1} IBA after 4 weeks on LPE medium (bar=6 mm); (b) explant cultured for 4 weeks on 1/4LP medium supplemented with 1.5 mg L^{-1} NAA after 4 weeks on LPE medium (bar=12 mm); (c) explant cultured for 4 weeks on 1/4LP medium supplemented with 1 mg L^{-1} IBA and 0.5 mg L^{-1} NAA after 4 weeks on LPE medium (bar=12 mm); (d) plantlet rooted on 1/4LP medium supplemented with 1.5 mg L^{-1} IBA after 2 months in the greenhouse (bar=40 mm); (e) plantlets after 4 months in the greenhouse (bar=95 mm).

The percentage of non-rooted explants that developed roots *ex vitro* was also significantly higher for treatments with IBA. Only a 3% of non-rooted explants treated with NAA developed roots *ex vitro* (Table 10). Thus, as a result of a bad *ex vitro* performance of the explants rooted in media with NAA, and the *ex vitro* development of roots in non-rooted explants treated with IBA, there were no significant differences among rooting treatments for the percentage of acclimatized explants after 2 months in the greenhouse (Table 10).

Table 10 *Pinus radiata* shoots rooted *in vitro* (%) and survival (%) after 2 months in the greenhouse. Shoots that developed roots *ex vitro* (%), and acclimatization (%) after 2 months in the greenhouse. Shoots were rooted in 4NAA, 4IBA, 4MIX, 2NAA, 2IBA and 2MIX (M±S.E.). Different letters within a column show significant differences by Duncan´s post hoc test ($p \leq 0.05$) (n.s.: non significant).

Rooting treatment	**Shoots rooted *in vitro* (%)**	**Survival (%)**	**Shoots rooted *ex vitro* (%)**	**Acclimatized plants (%)**
4IBA	6.7±2.9 c	100.0±0.0 a	40.0±7.2 a	46.7±7.3 n.s.
4NAA	48.3±9.3 ab	80±5.0 b	3.3±2.2 c	31.7±8.6 n.s.
4MIX	33.3±8.0 b	85±4.7 b	15.0±4.1 bc	31.7±6.7 n.s.
2IBA	5.0±3.0 c	100.0±0.0 a	28.3±7.3 ab	33.3±7.6 n.s.
2NAA	60.0±6.4 a	58.3±7.6 c	3.3±2.2 c	21.7±5.4 n.s.
2MIX	30.0±5.0 b	85.0±3.3 b	6.7±3.8 c	21.7±5.9 n.s.

4. DISCUSSION

In 2006, the experiments were carried out with seeds from the Basque coastline. Hyperhydricity percentages for these embryos were under 10% in controls and when a low concentration of CK was applied (2BA0.4, 2mT0.4, 3mT0.4, 3T0.4 or 2Z0.4). The highest hyperhydricity percentages (over 40%) were observed in some treatments with BA or T at 10 or 40 μM. Regarding this, Debergh et al. (1992) stated that the CK type and its concentration were related to the development of hyperhydricity.

The embryos induced with mT for 2 or 3 weeks exhibited lower hyperhydricity percentages than those induced with the same CK for 4 weeks. These results are in accordance with the findings in *Pinus pinea* by Capuana and Giannini (1995); these authors reported that high CK concentration or long culture periods on induction medium caused the appearance of hyperhydricity in conifers.

In 2008, the origin of the seeds was Kangairoa (New Zealand); these seeds, when treated with BA, showed a decreased of a 6% in the percentages of hyperhydricity; whereas when they were cultured with Z the percentage of hyperhydric explants was the same of that observed in 2006 (13.3%). One factor responsible of these differences could be the different sensitivity to CKs of the seeds used in the experiments.

Hyperhydricity is a critic factor as hyperhydric explants present several morphological and physiological disorders (Hazarika 2006). These explants are difficult to root, more susceptible to infections, and present low survival rates when transferred to the greenhouse (Von Arnold and Eriksson 1984).

In the first shoot induction experiment, the EFS percentage decreased when mT was applied to the induction medium at 40 µM in all the induction periods assayed. The same effect was observed in experiments when BA or Z was applied for 2 or 4 weeks. This decline was also observed in *P. pinaster* cotyledons by David et al. (1982) when they raised BA concentration from 0.8 µM to 5 µM. Contrary to what was reported by Moncaleán et al. (2005) in *P. pinea* cotyledons, the explants treated with BA for 2 or 3 weeks showed better percentages than those induced for 4 weeks. The highest EFS percentage was achieved after treatment 3Z1 (66.7%); Lambardi et al. (1993) obtained similar results with Z in *P. halepensis* embryos but at a higher concentration and longer exposure time.

In the second shoot induction experiment, the percentages of EFS were higher than those obtained in 2006; this could be due to the different origin of the seeds. The percentages in the second experiment were similar to the results obtained in New Zealand by Hargreaves et al. (2005) in the same species.

Pérez-Bermúdez and Sommer (1987) reported in *P. elliottii* that embryos on hormone-free medium did not produce adventitious shoots and developed as normal seedlings. On the contrary, our radiata pine explants when cultured in the absence of CK produced an average of 4 shoots per embryo. In this sense, we have to point out that all shoot induction treatments tested in these experiments produced a higher number of shoots per embryo than the controls.

In the experiments carried out in 2006, all the CKs tested produced the highest number of shoots per embryo when they were applied to the induction medium for 3 weeks. The best treatment was 3BA4.4 with an average of 31 shoots per embryo. Stange et al. (1999) increased two fold the number of shoots with this treatment in the same species, but the

hyperhydricity percentages in their experiments were also three times higher than ours. Aitken-Christie and Thorpe (1984) obtained 180 shoots per embryo in *P. radiata* embryos applying 22.2 µM BA for 3 weeks; in this work a distinction was made between hyperhydric and normal explants, but no data on the percentage of hyperhydric explants was reported. In our experiments after the induction treatments, explants were transferred to 1/2LP lacking growth regulators for 3 weeks and then, subcultured to elongation medium (LPE). AC in elongation medium, as shown in other micropropagation protocols for *Pinus* spp. (Mathur and Nadgauda 1999; Sul and Korban 2004), was beneficial and promoted the elongation of the shoots produced. The favourable effects of AC on morphogenesis may be mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing the toxic metabolites, phenolic exudation and brown exudate accumulation (Thomas 2008).

In general, when the induction period lasted 2 or 4 weeks a decreased in the number of shoots per embryo was observed. Capuana and Giannini (1995) reported a 3 weeks induction period with BA at 5 µM as the best in *P. pinea* cotyledons, although Moncaleán et al. (2005) obtained better values when they applied 4.4 or 10 µM BA to cotyledons of the same species for 5 weeks. Stojičić et al. (1999) also stated as optimum 4.4 µM BA for induction periods lasting 2 to 4 weeks in *P. heldreichii* embryos.

In experiment 2, when BA was applied (treatments 3BA4.4 and 2BA1), the number of shoots per embryo obtained was the same as in experiment 1 (31 and 20 shoots per embryo, respectively). On the other hand, when 1 µM Z was applied for 3 weeks the number of shoots obtained was higher (27 shoots per embryo). Lambardi et al. (1993) reported similar results in *P. halepensis* embryos after a treatment with 5 µM Z for 4 weeks, but they observed high hyperhydricity percentages.

The SEC index combines the EFS percentage and the number of shoots> 3mm per embryo, giving a more realistic determination of the efficacy of a treatment. In experiment 1, the best SEC index was achieved after 3Z1 treatment, although this value did not significantly differ from the values obtained in treatments 2BA1 and 3BA4.4. In the second shoot induction experiment, the SEC indices were higher, particularly in embryos induced with 4.4 µM BA for 3 weeks. The fact that seeds from experiment 1 came from OP trees and seeds from experiment 2 came from selected and progeny tested breeding stock could have impacted on the *in vitro* performance of the explants.

Our results in *P. radiata* embryos for the SEC index after the abovementioned treatments are three-fold higher than those reported by Lambardi et al. (1993) in *P. halepensis* embryos and Álvarez et al. (2009) in *P. pinaster* cotyledons.

The height of the shoots produced was considered as another important factor, shoots higher than 1.5 cm are ready to be subjected to a rooting treatment, thus the treatments that produce higher percentages of shoots ≥1.5 cm after the elongation period can be considered more effective in operational and economical terms.

When BA or Z was applied for 2 or 4 weeks, higher CK concentrations led to higher percentages of shoots shorter than 1.5 cm. This trend was also observed by Pérez-Bermúdez and Sommer (1987) in *P. elliottii* and by Tereso et al. (2006) in *P. pinaster*. As we have pointed above, embryos cultured for 3 weeks produced the highest number of shoots per embryo for all tested CKs; but these treatments, except for Z, produced high percentages of shoots <1.5 cm. The highest percentage of shoots ≥1.5 cm was achieved in controls and in treatments at low concentrations of BA or Z for 2 weeks. These is in agreement with the results reported by Moncaleán et al. (2001) in angiosperms, where explants cultured in the absence of BA or in the presence of BA for 1 day produced longer shoots. Saborio et al. (1997) concluded in *P. ayacahuite* that exposure to CK was essential for shoot formation, but that the time of BA removal was important both to allow bud development and to prevent undesirable effects (such as hyperhydricity or callus formation) in the next culture stages.

In addition, a short bud induction period could lead to a higher rooting capacity as a result of a minor CK inhibitory influence in the rooting process (Alonso et al. 2006). In our experiments the best SEC indices were obtained at low CK concentrations, although shoot production was lower, hyperhydricity was also lower. With treatments 3Z1 and 2BA1, 50% and 60% of the shoots produced were rootable after 4 months.

The shoot induction treatment had no significant effect on the mean number of rooted shoots, as observed by Martinez Pulido et al. (1994) in *P. canariensis*. Rooting media 2NAA produced a higher percentage of rooted shoots than the medium with the mixture of IBA and NAA commonly used in *P. radiata* (Hargreaves et al. 2005). But after 2 months in the greenhouse, there were no significant differences among rooting treatments; this was due to a higher mortality of explants treated with NAA alone and to the *ex vitro* development of roots in explants treated with IBA. The shoots induced with IBA did not show roots but a callus on

their base at the end of *in vitro* phase; these explants had a healthier aspect and developed roots after transferring them to the greenhouse. *In vitro* rooting with IBA was also reported to be better than with NAA or a mixture of both auxins in *Cunninghamia lanceolata* (Zhu et al. 2007). Rooting in *P. radiata* cuttings has been currently performed with IBA alone (Smith and Thorpe 1976).

In conclusion, shoot induction treatments with BA or Z at low concentrations for 3 weeks are more effective in terms of SEC index and growth of the shoots. These treatments had not a significant effect on subsequent plant behaviour. *In vitro* rooting of the shoots was significantly higher in rooting media supplemented with NAA, but the *ex vitro* survival of these plantlets was significantly lower. The use of IBA alone can be an alternative for a more efficient plant production due to high survival rates and better quality of the plantlets obtained.

Our results widen the existing knowledge on *Pinus radiata* seed organogenesis. The fact that we have adjusted the CK culture conditions in different seed provenances, and the auxin treatment, could imply saving time and money in the production of plants from *P. radiata* seeds.

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CAPÍTULO 2

Enhancing initiation and proliferation in radiata pine somatic embryogenesis through seed family screening, zygotic embryo staging and media adjustments

Este capítulo se corresponde con el artículo: Enhancing initiation and proliferation in radiata pine somatic embryogenesis through seed family screening, zygotic embryo staging and media adjustments (*Acta Physiologiae Plantarum*, en revisión).

Capítulo 2: Enhancing initiation and proliferation in radiata pine somatic embryogenesis through seed family screening, zygotic embryo staging and media adjustments

1. INTRODUCTION

Somatic embryogenesis (SE) is the developmental pathway by which somatic cells develop into structures that resemble zygotic embryos through an orderly series of characteristic embryological stages without fusion of gametes. Since SE was described for the first time almost 50 years ago (Steward et al. 1958), and due to the importance shown, it has been the subject of many studies in different especies. SE is an adequate system for mass-propagation of plants as well as a good tool to investigate zygotic embryogenesis (Jiménez 2005).

SE in *P. radiata* was first described by Smith et al. (1994). Lately, there have been improvements in different aspects of the process such as initiation (Hargreaves et al. 2009), cryopreservation (Hargreaves et al. 2002), expression of genes during the process (Bishop-Hurley et al. 2003; Aquea and Arce-Johnson 2008; Aquea et al. 2008) and genetic transformation (Walter et al. 1998b; Cerda et al. 2002; Charity et al. 2005).

Explant selection in the induction of ET in conifers is dependent on the species studied. This is a critical step because the competence window is narrow only lasting around 4 weeks (MacKay et al. 2006; Yildirim et al. 2006). Then, the initiation decreases to very low rates. There has been limited recent work on initiation from mature zygotic embryo explants of *Pinus* spp. (Radojevic et al. 1999; Tang et al. 2001). Even though mature seed explants provide an advantage of being able to initiate embryogenic cultures at any time of the year, the success rate is usually low. Moreover, for many economically important pine species, the initiation frequency of SE is insufficient for commercial application. The improvement in SE initiation is important for developing varietal lines as well as managing genetic diversity (Park et al. 2006).

When considering initiation media, Pullman et al. (2003a; 2003b; 2004; 2005) published advances specifically for SE initiation in *P. taeda* and *P. elliottii*. It is likely that adjustments of tissue culture media can result in higher success of SE initiation frequencies (Park et al.

2006); in this sense some studies have focused on improving initiation and proliferation through testing different media (Zhang et al. 2007), plant growth regulator concentrations (Choudhury et al. 2008), sugar types (Salajova and Salaj 2005) or gelling agent concentrations (Li et al. 1998).

The current success of initiation is a problem and limits the ability to capture cell lines (Hargreaves et al. 2009). A tree's genetic background has great influence on cloning efficiency, which ultimately impacts the cost of clonal production. It is generally accepted that all genotypes or families within a species are not equivalent in their ease of propagation, and this reflects the interaction of genetic factors with the particular propagation system being used (Davis and Becwar 2007). SE initiation is under strong genetic control, indicating that this is the phase that can be manipulated most effectively by breeding due to the large amount of genetic variation existing within zygotic embryos (Park 2002). Improving the rate of success for SE initiation is not only important for developing varietal lines, but is critical for the management of genetic diversity, genetic engineering and molecular tree breeding (Park et al. 2006).

Due to the aforementioned reasons, the objectives of this research were to determine the best immature zygotic embryo developmental stage for initiation in two consecutive years, and to test the effect of different sources of organic nitrogen in initiation and proliferation steps in *Pinus radiata* SE.

2. MATERIAL AND METHODS

2.1. Plant material

One-year-old green female cones, enclosing immature zygotic embryos of *Pinus radiata* D. Don, were collected from open-pollinated (OP) trees in a clonal seed orchard established by Neiker-Tecnalia in Deba-Spain (latitude: 43º16'59''N, longitude: 2º17'59''W, elevation: 50 m).

In 2007, two cones were sampled weekly during the months of June and July, and stored for a maximum of a week in paper bags at 4ºC. These cones were collected from trees: 12, 14, 22, 28, 42, 50 and 67.

In 2008, based on 2007 results, two cones were sampled weekly during the month of June and the first week of July, and stored for 2 to 3 weeks in paper bags at 4ºC. These cones were collected from trees: 12, 14 and 67.

Intact cones were sprayed with 70% (v/v) ethanol, split into quarters and all megagametophytes extracted. Seeds were surface sterilised in 10% (v/v) H_2O_2 plus two drops of Tween 20® for 8 min, then they were rinsed three times in sterile distilled H_2O under sterile conditions in the laminar flow unit. Seed coats were removed and whole megagametophytes containing immature embryos were excised out aseptically and placed horizontally onto medium.

2.2. Initiation and proliferation of embryogenic tissue

2.2.1. Experiment 1

In 2007 green cones from seven open pollinated (OP) families were collected weekly for 7 weeks, from the 15th of June to the 26th of July. Embryo development medium (EDM) (Walter et al. 1998a) with 30 g L^{-1} sucrose and a combination of 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 µM benzyladenine (BA) was used for culture initiation. Before autoclaving, the pH of the medium was adjusted to 5.7 and then 3 g L^{-1} gellan gum (Gelrite®) was added. Medium was autoclaved at 121ºC for 20 min. After autoclaving, filter-sterilized solutions with the pH adjusted to 5.7 containing 550 mg L^{-1} L-glutamine, 525 mg L^{-1} asparagine, 175 mg L^{-1} arginine, 19.75 mg L^{-1} L-citrulline, 19 mg L^{-1} L-ornithine, 13.75 mg L^{-1} L-lysine, 10 mg L^{-1} L-alanine and 8.75 mg L^{-1} L-proline were added to the cooled medium, prior to dispensing into Petri dishes (90 x 20 mm).

Ten to twelve megagametophytes were cultured on each Petri dish. The experiments were replicated two times, in each replication three Petri dishes per treatment were laid out randomly on the shelves of the growth chamber. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks.

In each collection date, 10 megagametophytes per seed family were destructively sampled, and zygotic embryo development was classified on an eight-stage scale under a stereoscopic microscope (LEICA MZ95). At stage 1, pro-embryos had formed (visible suspensor cells, but no pronounced cleavage of the primary embryo) (Fig. 1a). Stage 2 showed early cleavage polyembryony (no clearly dominant embryo, but often four or more embryo initials visible,

some separation of suspensors) (Fig. 1b). Stages 3–6 are ‘‘bullet’’ stages with clearly dominant (bullet shaped) embryos of increasing development, with the numeric score assigned to the dominant embryo (at this stage the dominant embryo has formed a bundle of suspensor tissue) (Figs. 1c, 1d, 1e and 1f). Stage 7 has clearly developed epicotyls (Fig. 1g) and at stage 8, a clear whorl of cotyledons is present (Fig. 1h) (Hargreaves et al. 2009).

After 4–8 weeks, proliferating ET with a size around 3-5 mm in diameter, was separated from the megagametophyte; at this moment, the initiation (Fig. 2a).

ET was subcultured to maintenance medium every 2 weeks; maintenance medium had the same composition of the initiation media, but a higher concentration of Gelrite®, 5.5 g L^{-1}. The concentration of gellan gum in the media was increased to maintain the spiky morphotype of the embryogenic cell lines (Breton et al. 2005).

Following 4 subculture periods, actively growing ETs were recorded as established cell lines (ECLs) (Fig. 2b). Contamination percentages were recorded along the process.

2.2.2. Experiment 2

In 2008 two initiation media were tested, the previously described EDM medium (Walter et al. 1998a) and CGM medium; this second medium only differed from EDM in the amino acid composition. Its amino acid composition consisted of 1 g L^{-1} casein hydrolysate added to the medium prior to sterilization and 500 mg L^{-1} L-glutamine added after autoclaving in a filter-sterilized solution.

Three OP families were processed weekly for 4 weeks, from the 16th of June to the 7th of July. Eight explants were cultured on each 90 x 20 mm Petri dish. The experiments were replicated two times, in each replication four Petri dishes per treatment were laid out randomly on the shelves of the growth chamber. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks.

In each collection date 10 megagametophytes per seed family were destructively sampled, and zygotic embryo development was classified on an eight-stage scale (Hargreaves et al. 2009).

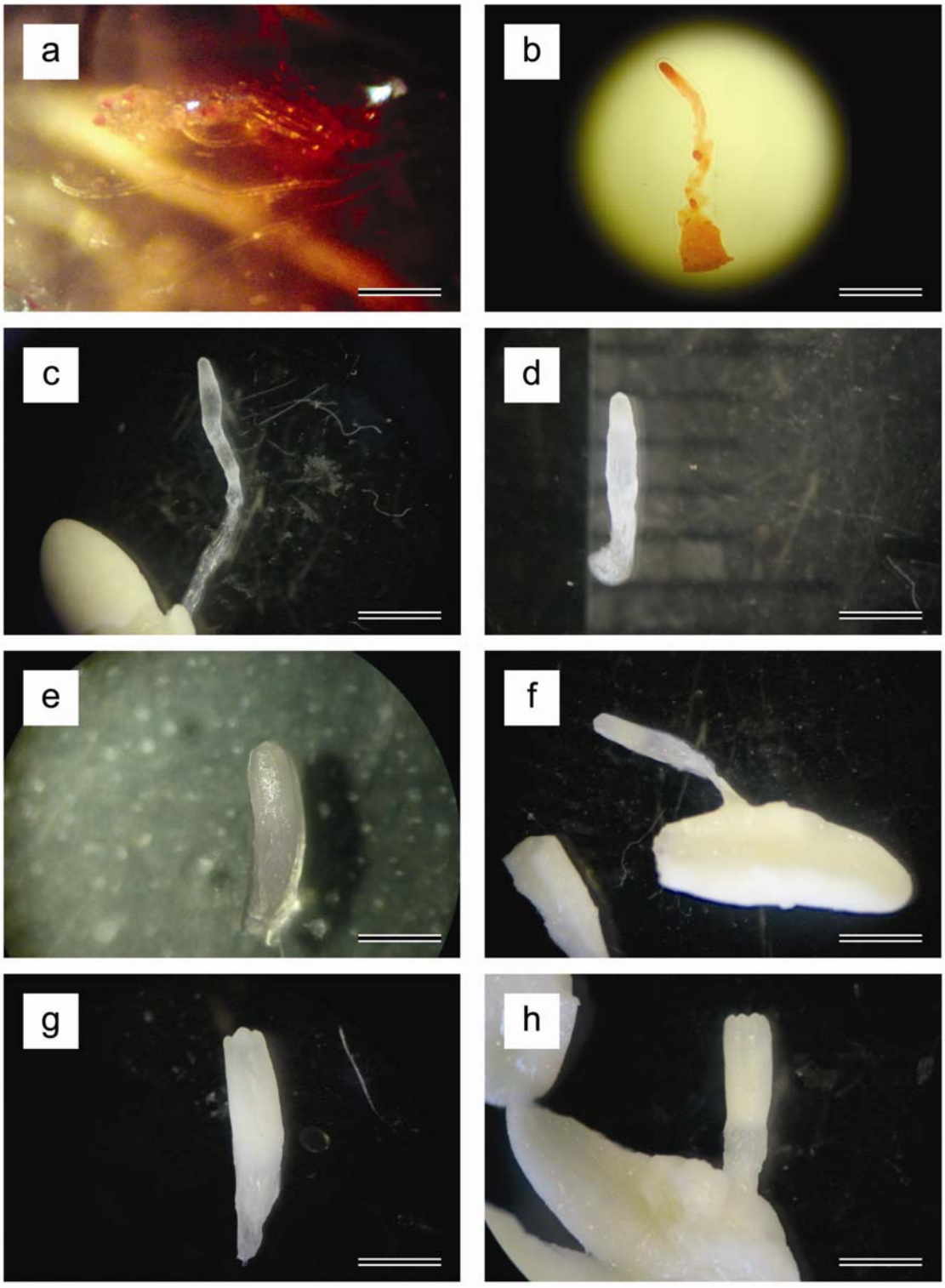

Fig. 1 Zygotic embryo developmental stages: (a) at stage 1, pro-embryos had formed (stained with 2% acetocarmine, visible suspensor cells, but no pronounced cleavage of the primary embryo (bar=2 mm); (b) stage 2 showed early cleavage polyembryony (stained with 2% acetocarmine, bar=2 mm); (c, d, e, f) stages 3–6 are ‘‘bullet’’ stages with clearly dominant (bullet shaped) embryos of increasing development, with the numeric score assigned to the dominant embryo [(bars=2mm for (c), (d) and (e), bar=4 mm for (f)]; (g) stage 7 has clearly developed epicotyls (bar=2 mm); (h) at stage 8, a clear whorl of cotyledons is present (bar=2 mm).

After 4–8 weeks, proliferating ETs with a size around 3-5 mm in diameter were recorded as initiated cell lines. These ETs were separated from the megagametophyte and subcultured to the maintenance medium every 2 weeks. Maintenance media had the same composition of the initiation media, EDM or CGM, but a higher concentration of Gelrite®, 5.5 g L^{-1}. Following 4 subculture periods, actively growing ETs were recorded as established cell lines.

Actively growing ET samples from the two maintenance media were stained with 2% (w/v) acetocarmine directly on glass slides for 4 min. Then, samples were rinsed with distilled sterile water and stained with 0.5% (w/v) Evan´s blue for 2 min. Finally, ET samples were mounted with a cover slide. Samples were sealed with commercial nail varnish to allow preservation in the medium term (Gupta and Durzan 1987). Samples were observed with an inverted microscope (LEICA DM4500) using a 40-fold magnification. Cellular organization analysis especially focused on the proembryo and early embryo development stages in the embryogenic tissues.

2.3. Data collection and statistical analysis

All statistical models were simple generalized linear models (MacCullagh and Nelder 1989). Normality was evaluated by Shapiro–Wilk normality test (Shapiro and Wilk 1965) without transformation. The equal variance of the data was analysed by Levene's test (Brown and Forsythe 1974). The analyses were performed using the SPSS© version 15.0 software package.

2.3.1. Experiment 1

In 2007, proliferation percentages were calculated per Petri dish for different collection dates and seed families.

The data followed normal distribution and were directly subjected to ANOVA using model:

$$Y_{ijr} = \mu + S_i + W_j + SW_{ij} + e_{ij}$$

Where Y_{ijr} is the percentage of *r*th ECLs per Petri dish from the *i*th seed family that was collected in the *j*th week; μ is the experimental mean, S_i is the effect of the *i*th seed family, W_j is the effect of the *j*th week, SW_{ij} is the interaction between the *i*th seed family and the *j*th week, and e_{ij} is the random error component. All effects were considered as fixed effects.

Multiple comparisons were made using Tamhane post hoc test ($p \leq 0.05$).

2.3.2. Experiment 2

In 2008, initiation and proliferation percentages were calculated per Petri dish for different collection dates and seed families and media.

The data followed normal distribution and were directly subjected to ANOVA using model:

$$Y_{ijkr} = \mu + S_i + W_j + M_k + SW_{ij} + SM_{ik} + WM_{jk} + SWM_{ijk} + e_{ijk}$$

Where Y_{ijkr} is the percentage of *r*th ECLs per Petri dish from the *i*th seed family that was collected in the *j*th week, and cultured on the *k*th medium, μ is the experimental mean, S_i is the effect of the *i*th seed family, W_j is the effect of the *j*th week, M_k is the effect of the *k*th medium, SW_{ij} is the interaction between the *i*th seed family and the *j*th week, SM_{ik} is the interaction between the *i*th seed family and the *k*th medium, WM_{jk} is the interaction between the *j*th week and the *k*th medium, SWM_{ijk} is the interaction between the *i*th seed family, the *j*th week and the *k*th medium and e_{ij} is the random error component. All effects were considered as fixed effects.

Multiple comparisons were made using Tamhane post hoc test ($p \leq 0.05$).

3. RESULTS

3.1. Experiment 1

When considering all the families together, the highest proliferation percentages were obtained at collections 3 and 4 (19 and 20%, respectively), although they did not present significant differences with the results at collection 2. Collection 2 did not show significant differences with collection 5 and 6, the values for these dates ranged from 7% to 13% (Tables 1 and 2).

Table 1 Embryogenic tissue proliferation (%) and embryo development for seven seed families of *Pinus radiata* cultured *in vitro* on EDM medium at seven collection dates in 2007 (M±S.E.). Different letters within a row or a column show significant differences by Tamhane´s post hoc test ($p \leq 0.05$).

Proliferation per seed family and week (%)								
Collection Date / **Seed family**	**14-June 1**	**21-June 2**	**28-June 3**	**5-July 4**	**12-July 5**	**19-July 6**	**26-July 7**	**Mean**
12	4.67±1.69	27.93±7.91	42.53±3.79	27.45±3.97	11.16±3.44	9.90±1.98	0.0	20.61±2.69a
14	0	37.98±6.91	26.40±4.10	28.20±4.84	16.18±5.20	11.99±2.55	0.83±0.80	20.13±2.66a
22	0	0.17±0.16	0	0	0	0	0.0	0.03±0.02c
28	0	1.19±.1.10	0	23.82±7.94	4.17±3.09	4.04±2.56	0.0	5.53±1.98bc
42	1.70±0.90	12.52±5.02	18.06±8.37	18.26±5.24	10.95±3.38	8.99±4.31	0.0	11.75±2.13ab
50	1.89±1.38	0	7.53±2.72	8.55±2.30	6.25±2.61	5.20±2.18	0.0	4.90±0.94b
67	12.43±5.00	11.97±4.84	38.67±9.30	34.18±4.69	18.92±5.26	9.10±2.41	0.0	20.61±2.69a
Mean	2.96±0.98de	13.11±2.65abc	19.03±3.14ab	20.07±2.40a	9.66±1.60bc	7.03±1.08cd	0.12±0.11e	
Embryo stage	1.5	2	3	4	5	6	7.5	

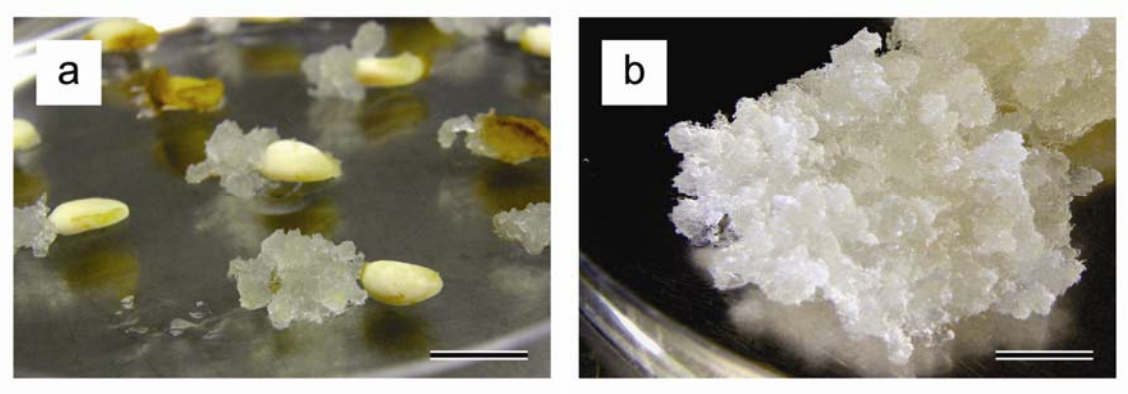

Fig. 2 *Pinus radiata* megagametophytes cultured on EDM medium: (a) embryogenic tissue initiation (bar=10mm); (b) embryogenic tissue proliferation (bar=7mm).

The lowest proliferation percentages were achieved at collections 1 and 7, in the latter collection date the proliferation percentage was close to 0. The average embryo stage was similar for all families at any given date, the best collection dates corresponded to embryo stages between 2 to 4 (Figs. 1b, 1c and 1d). There was a significant interaction between seed families and collection dates (Table 2).

Table 2 Analysis of variance for proliferation (%) in *Pinus radiata* megagametophytes cultured *in vitro* on EDM medium at six collection dates in 2007.

Source	df	Mean square	F value	Significance
Seed Family (SF)	4	2349.21	28.92	p<0.001
Collection Date (CD)	5	2436.86	30.00	p<0.001
SF * CD	20	364.50	4.49	p<0.001
Error	150	81.23		

*n.s.: non significant ($p \leq 0.05$)

Studying the families separately, at collection 3 (28 June) families 67 and 12 achieved their highest proliferation percentages, 39% and 42%, respectively. Family 67 also displayed similar proliferation percentage (34%) at collection 4. Family 14 presented its highest proliferation percentage (38 %) at collection 2 (21 June). Whereas at collections 3 and 4, the proliferation percentages for this family were lower (27 and 28%); the same results were obtained in family 12 at collections 2 and 4 (28 and 27%) (Table 1).

Families 67, 14 and 12 produced a significantly higher number of ECLs than family 50. While family 42 only showed significant differences with 22, this family did not reach proliferation percentages higher than 18% (collections 3 and 4). Families 28 and 50 presented overall percentages around 5%; these families produced their highest proliferation rates at collection 4. No embryogenic tissue proliferation was observed in family 22 except at collection 2 (0.17%) (Table 1). Contamination percentages were always below 1%.

3.2. Experiment 2

Based on 2007 proliferation results, the best three families (12, 14 and 67 seed families) were chosen for 2008 initiation and proliferation experiments.

When analysing the second initiation experiment, there were no significant differences for the initiation percentages of the three seed families studied and the two media assayed (EDM and CGM) (Table 3).

Table 3 Analysis of variance for initiation (%) in *Pinus radiata* megagametophytes cultured *in vitro* on EDM or CGM at four collection dates in 2008.

Source	df	Mean square	F value	Significance
Seed Family (SF)	2	613.63	2.27	n.s.
Medium (M)	1	827.01	3.06	n.s.
Collection Date (CD)	3	19754.40	73.14	p<0.001
SF * M	2	47.35	0.17	n.s.
SF * CD	6	2068.69	7.66	p<0.001
M * CD	3	149.70	0.55	n.s.
SF * M * CD	6	381.86	1.41	n.s.
Error	168	270.09		

*n.s.: non significant ($p \leq 0.05$)

The initiation rates only showed significant differences when different collection dates were considered. The highest values, between 59 and 67%, were observed in the first collection date, corresponding to an embryo developmental stage between 2 and 3, overall mean initiation percentage diminished approximately 15% in every collection date reaching 16% in the last collection date when zygotic embryos were in stage 6 (Table 4).

There were no significant differences among seed families, initiation rates in family 12 decreased progressively along zygotic embryo development. Family 14 showed remarkable results in the second collection date and then decrease was observed in the following weeks. Family 67 maintained initiation percentages around 30% in the collection dates 2, 3 and 4. There was a significant interaction between seed families and collection dates (Table 3).

In proliferation experiments, there were no significant differences on proliferation percentages for the three seed families studied. On CGM medium, a significantly higher number of ECLs proliferated than on EDM medium. However, there was neither an interaction between the medium and the other factors considered (seed family and collection date) nor between the seed family and the collection date (Table 5). As observed for initiation, a significantly higher percentage of ECLs proliferated at collection 1 (43%), corresponding to zygotic embryo developmental stages between 2 and 3 (Table 6). Again, there was a significant decrease in the proliferation percentages observed between collection dates, along with zygotic embryo development (Table 6). There was a significant interaction between seed families, media and collection dates (Table 5). Contamination percentages were always below 1%.

Table 4 Embryogenic tissue initiation (%) and embryo development for three seed families of *Pinus radiata* cultured *in vitro* on EDM or CGM at four collection dates in 2008 (M±S.E.). Different letters within a row show significant differences by Tamhane´s post hoc test ($p \leq 0.05$) (n.s.: non significant).

Initiation per seed family, medium and week (%)										
Collection	**16-June 1**		**23-June 2**		**30-June 3**		**7-July 4**		**Mean**	
Medium / Family	**EDM**	**CGM**	**EDM**	**CGM**	**EDM**	**CGM**	**EDM**	**CGM**	**EDM**	**CGM**
12	61.85±3.16	57.93±4.08	43.46±8.14	52.34±9.87	25.00±3.54	30.47±5.47	7.81±3.29	17.19±4.05	34.53±4.33	39.48±4.81
14	64.63±4.01	76.56±6.86	61.46±6.37	64.58±5.63	28.12±6.02	30.47±5.34	7.03±2.19	10.94±3.49	40.31±4.90	45.64±5.38
67	49.22±8.22	67.97±5.96	33.59±3.53	27.34±4.56	36.72±5.07	42.97±6.41	30.36±4.28	20.31±4.05	37.47±2.94	39.65±4.15
Mean	58.75±3.38	67.49±3.55	46.17±4.23	48.09±5.94	29.95±2.94	34.63±3.41	15.07±2.92	16.14±2.28	37.44±2.38 n.s.	41.59±2.76 n.s.
Total Mean	63.03±2.51 a		47.13±3.61 b		32.29±2.25 c		15.61±1.84 d			
Embryo stage	2.5		3.5		5		6			

Table 5 Analysis of variance for proliferation (%) in *Pinus radiata* megagametophytes cultured on EDM or CGM at three collection dates.

Source	Df	Mean square	F value	Significance
Seed Family (SF)	2	502.10	1.94	n.s.
Medium (M)	1	3115.19	12.01	p<0.001
Collection Date (CD)	3	12778.25	49.28	p<0.001
SF * M	2	140.89	0.54	n.s.
SF * CD	6	774.73	2.14	n.s.
M * CD	3	282.78	2.99	n.s.
SF * M * CD	6	249.79	0.96	p<0.008
Error	168	259.28		

*n.s.: non significant ($p \leq 0.05$)

Considering the macro-morphological morphotype of the ET, no differences were observed between the ET initiated and proliferated on EDM and CGM (Figs. 2a and 2b). Squashes of ETs established on EDM presented a well organized structure where proembryos could be observed (Fig. 3a). ETs proliferating on CGM showed a more unorganized structure, where it was not possible to distinguish early embryos (Fig. 3b) and these lines stopped proliferating after 4 months on CGM proliferation medium. Embryogenic lines proliferating on EDM medium showed healthy morphology and continue proliferating after 6 months.

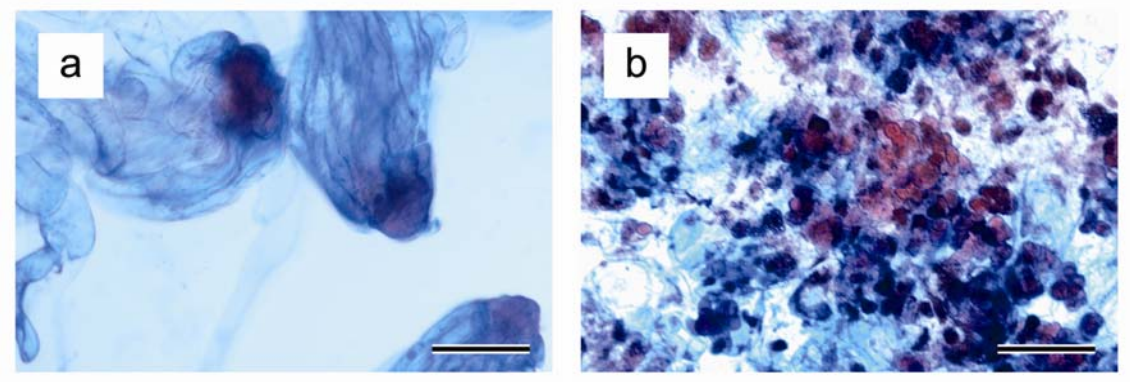

Fig. 3 Microscopic details of *Pinus radiata* embryogenic tissue stained with 2% acetocarmine and 0.5% Evan´s blue: (a) embryogenic tissue proliferating on EDM medium where proembryos can be observed (bar=100µm); (b) embryogenic tissue proliferating on CGM medium showing an unorganized structure (bar =200µm).

Table 6 Embryogenic tissue proliferation (%) and embryo development for three seed families of *Pinus radiata* cultured on EDM or CGM medium at four collection dates (M±S.E.). Different letters within a row show significant differences by Tamhane´s post hoc test ($p \leq 0.05$).

Proliferation per seed family, medium and week (%)										
Collection	**16-June 1**		**23-June 2**		**30-June 3**		**7-July 4**		**Mean**	
Medium / Family	**EDM**	**CGM**	**EDM**	**CGM**	**EDM**	**CGM**	**EDM**	**CGM**	**EDM**	**CGM**
12	42.27±3.92	37.31±4.20	32.98±9.37	35.94±9.40	10.16±4.86	22.66±4.08	2.34±1.64	10.94±3.69	21,94±3.98	26,71±4.07
14	43.44±4.40	60.94±5.38	35.49±6.01	42.45±7.04	10.16±2.88	20.31±6.76	1.56±1.02	2.34±1.14	22.66±3.65	31.51±4.79
67	28.91±7.18	46.88±7.47	20.31±4.98	19.53±3.00	9.38±2.89	28.13±8.01	6.25±2.36	12.50±3.34	16.21±2.78	26.76±3.64
Mean	38.20±3.26	48.37±3.80	29.59±4.12	32.64±5.31	9.89±2.02	23.70±3.65	3.38±1.06	8.59±1.87	20.27±2.02b	28.33±2.41a
Total Mean	43.29±2.59 a		31.12±3.33 b		16.80±2.29 c		6.00±1.13 d			
Embryo stage	2.5		3.5		5		6			

4. DISCUSSION

It has been established in several studies that initiation of SE is influenced by the developmental stage of immature embryos, the genotype of the parent trees and the formulation of tissue culture medium (Klimaszewska et al. 2001; Pullman et al. 2004; Park et al. 2006). Optimizing these factors can lead to improve initiation response. But in some instances, SE initiation may not result in establishment of an embryogenic line because the ensuing ET ceases to proliferate. Thus, when assessing the success rate, it is important to distinguish between the initial outgrowth from an explant and continuous growth (Klimaszewska et al. 2007). In this sense, this is the first report where proliferation of *Pinus radiata* ET has been studied for two consecutive years.

Unlike most spruce species (Fourré et al. 1997; Tremblay et al. 1999), SE in pines is often initiated from megagametophytes bearing immature zygotic embryos at pre-cotyledonary stage (Nagmani et al. 1993; Maruyama et al. 2007). Analysing our initiation results in 2008 (60% on EDM medium and 67% on CGM medium), these were similar to those obtained by Hargreaves et al. (2009) in the same species (70%); although these authors used different initial explants (isolated immature zygotic embryos) and different culture conditions (Hargreaves et al. 2009). However, the procedure of excising the zygotic embryos from megagametophytes is time consuming and requires sophisticated technical skill in order not to damage or contaminate the immature zygotic embryo. Our initial explants were whole megagametophytes; this explant type is more amenable to *in vitro* manipulation and contamination percentages were always below 1%. Moreover, the presence of the megagametophyte may extend the influence of the mother tree into culture to a greater degree and thus account for the importance of the maternal effects (MacKay et al. 2006).

Studies performed in *P. strobus* (Klimaszewska et al. 2001) indicated that staging zygotic embryo development is essential to obtain maximum initiation. As a result from our experiments on proliferation in two consecutive years, we have identified the zygotic developmental stages ranging from 2 to 4 as the most responsive for ET initiation and proliferation. These stages correspond to early cleavage polyembryony (no clearly dominant embryo, but often four or more embryo initials visible, some separation of suspensors) and the first ‘‘bullet’’ stages with dominant (bullet shaped) embryo. This optimum phase lasted from 2 to 3 weeks and before and after reaching these embryo stages, initiation and proliferation

decreased to extremely low frequencies; this trend was also observed in *P. pinea* (Carneros et al. 2009) and *P. radiata* (Hargreaves et al. 2009).

In this sense, in an attempt to overcome the narrow competence window, SE has been also obtained from mature embryos in *P. lambertiana* (Gupta and Durzan 1986), *P. strobus* (Garin et al. 1998) and *P. taeda* (Tang et al. 2001) but at low frequencies. From our point of view, another approach to partly solve this problem could be to store the cones at the most responsive stage at cold temperatures. Park (2002) showed that *P. strobus* cones could be stored for at least 40 days at 3ºC without reducing embryogenic capacity. In 2008 we stored the cones at 4ºC for 2 to 3 weeks and, confirming this hypothesis, the proliferation percentages obtained were similar to those obtained in 2007 for the families studied.

We have also observed an interaction between the seed families and collection dates in 2007 proliferation experiments and 2008 initiation experiments. On the contrary, there was no interaction in 2008 proliferation experiments, these differences could be due to the fact that there was not a single week when all the families were responsive but two or three, and some of these families showed similar percentages for two consecutive weeks whereas others peak percentages only lasted one collection date. A similar trend was observed in *P. strobus* initiation (Klimaszewska et al. 2001) where wide-ranging differences among families at a given collection date contributed to significant interactions between families and collection dates. In 2008 initiation and proliferation results there was no interaction between the seed families and the media, in accordance with the initiation results in *P. strobus* (Park et al. 2006) and *P. pinea* (Carneros et al. 2009).

Initiation of conifer SE is known to be under strong genetic control (Park et al. 1993; Lelu et al. 1999; Niskanen et al. 2004). In our experiments carried out in 2007 we found different performances depending on the mother tree, some authors have pointed that the heritability of SE initiation is large when compared with other quantitative traits (Klimaszewska et al. 2001). In our experiments the megagametophytes came from open-pollinated mothers but interestingly, the good results on proliferation observed in the first year for the most responsive families (between 36 and 42%) where corroborated the second year (38% for EDM and 48% for CGM). Although it is important to ratify the most adequate zygotic embryo developmental stage; there are not many studies apart from those carried out by Park et al. (2006) in which the staging is verified through various sampling years.

In accordance with our results, moderately high maternal effects, in addition to strong additive variance has been found in several *Pinus* species such as *P. pinaster* (Lelu et al. 1999), *P. sylvestris* (Niskanen et al. 2004) or *P. taeda* (MacKay et al. 2006). These studies also indicated that practical improvement in SE culture initiation could be achieved in a predictable manner by selecting the most favourable female parent, or in some cases, a favourable male parent. The experiments by MacKay et al. (2006) pointed out that the initiation from a given mother tree in a control-pollinated cross could be predicted based on the response of the open-pollinated seed, which thus might help planning controlled pollinations in order to optimize initiation frequency.

As mentioned above, the third key issue involved in SE initiation and proliferation process, is the culture medium. In 2007, our cultures were initiated and proliferated on EDM basal medium (Walter et al. 1998a) a medium currently used in *P. radiata* SE but also in other *Pinus* species such as *P. strobus* (Klimaszewska and Smith 1997). The proliferation medium used in our experiments contained 5.5 g L^{-1} Gelrite®, because we observed in previous experiments that increasing gellan gum concentration was beneficial to maintain the spiky morphotype of ETs in accordance with Choudhury et al. (2008) results in *P. kesiya*. These authors pointed out that enhancing the gellam gum concentration in the proliferation medium increased gel strength and decreased water availability. This fact triggered a shift in the development programme of the cultures from proliferation of embryogenic cells with cleavage polyembryony to the development of proembryos and early-stage embryos in the maintenance medium.

In the second year, we tested two media, EDM medium and CGM medium with the same basal composition but differing from EDM on the amino acid mixture. The CG amino acid mixture has been employed in several *Pinus* species (Klimaszewska et al. 2001; Lelu-Walter et al. 2006; Carneros et al. 2009); when using this amino acid combination we obtained higher proliferation rates (48%) but coupled with negative effects on the organization of the ET at the microscopic level (Fig. 3). This lack of organization in the tissue has been associated with ET aging and worse maturation yields in *P. pinaster* (Breton et al. 2005; 2006). These authors pointed out the importance of selecting lines with high early embryogenic ability which could be more effective using micro-morphological criteria instead of macro-morphological criteria. Breton et al. (2005) also noted that a gradual increase in growth rate was associated to a decrease in cellular organization.

The role of organic nitrogen and carbon source in different SE steps has been described by many authors recently (Morcillo et al. 1999; Pérez-Rodríguez et al. 2006). Robinson et al. (2009) found that the major sources of carbon and organic nitrogen (sucrose and glutamine) had a positive effect on the physiological state of proliferating cultures of *P. taeda* prior to exposure to maturation conditions. In this case, although EDM and CGM medium had the same concentration of sucrose, glutamine concentration was slightly higher on EDM medium than in CGM medium. Casein hydrolysate in CGM medium also may have had a negative influence on proliferation when compared with EDM mixture of amino acids. Amino acids have been widely studied in several plants for their importance in SE. In this sense, Pérez-Rodríguez et al. (2006) suggested that the composition of the culture medium during SE, particularly the carbohydrate to nitrogen ratio, may represent a key factor responsible for the expression of certain glutamine synthetase-related and photosynthesis-related genes. It could be one reason why in our experiments amino acids on EDM medium played a key role in the proliferation process.

Rational manipulation of the culture medium is critical for successful regeneration of embryos and subsequent conversion into somatic seedlings (Stasolla et al. 2002). Furthermore, a certain degree of morphological organization in the proliferating ET must be present prior to maturation treatment, as maturation is not induced if the embryogenic heads of the early filamentous embryos are too small (Jalonen and Von Arnold 1991).

Robinson et al. (2009) observed that ET cultures that were developmentally more advanced at the time of subculture to maturation medium were more productive. Thus, the physiological state of proliferating cultures prior to exposure to maturation conditions clearly impacted the responsiveness of the culture once exposed.

In conclusion, we have determined zygotic developmental stages ranging from 2 to 4 as the best for ET initiation and proliferation, and we have identified the most responsive seed families demonstrating their embryogenic potential for two consecutive years. Besides, EDM medium with higher gellan gum content during ET proliferation maintained the tissue in a better micro-morphological arrangement for a longer time. This ET state has implications on aging and subsequent maturation ability of the ET and the quality and quantity of the somatic embryos obtained. These findings could imply great labour savings in the future enhancing not only the results in initiation and proliferation steps but also the maturation and conversion yield.

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CAPÍTULO 3

Bottlenecks in *Pinus radiata* somatic embryogenesis: improving maturation and germination

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Capítulo 3: Bottlenecks in *Pinus radiata* somatic embryogenesis: improving maturation and germination

1. INTRODUCTION

Utility of *in vitro* organogenesis has been proven for clonal propagation of *Pinus radiata* (Aitken-Christie 1984), a limitation of this method for mass production on a commercial scale is the high cost of the process. Propagation via somatic embryogenesis (SE) has many advantages; this method is effective in propagating elite plants when it is combined with other technologies, such as cryopreservation of the embryogenic tissue (ET) during selection of elite clones in field tests. This system offers the capability to produce unlimited numbers of somatic embryo derived plantlets (Attree et al. 1994; Park 2002) and artificial seeds (Aquea et al. 2008). Furthermore, somatic embryogenesis is an ideal system for genetic transformation because somatic embryos initiate from single cells (Zhang et al. 2007), and has been already used in pines (Charity et al. 2005).

As it has been mentioned in Chapter 2, SE in *P. radiata* was first described by Smith et al. (1994) and then, many studies have been carried out in order to improve different aspects of SE such as initiation, cryopreservation, expression of genes and genetic transformation (Klimaszewska et al. 2007). Although ET initiation protocols in some pine species are now fairly well established, maturation of the ET into cotyledonary, normal somatic embryos is not always successful. Problems such as low or asynchronous embryo production (Breton et al. 2005; Yildirim et al. 2006), abnormal morphology or poor root development have been also reported for *P. pinea* (Carneros et al. 2009) and *P. kesiya* (Choudhury et al. 2008).

In conifer SE, the development of mature somatic embryos into plantlets occurs without the benefit of the megagametophyte, which is a major organ for storage of both lipids and proteins. For this reason, the composition of the culture medium takes on special importance in this phase, as it must be a substitute for the megagametophyte to supply adequate amounts of nitrogen and carbon (Lin and Leung 2002). However, there are no studies in *Pinus* genus where the effect of different media on germination was tested, although some authors have studied the influence of pre-germination treatments (Jones and Van Staden 2001), light

quality (Merkle et al. 2006) or explants position on the media (Percy et al. 2000; Aronen et al. 2009).

Considering the abovementioned aspects, the objectives of this study were to optimize the *Pinus radiata* ET maturation process by improving the quality and number of the somatic embryos formed, and to test the influence of germination media on their conversion into plantlets.

2. MATERIALS AND METHODS

2.1. Plant material

One-year-old green, female cones, enclosing immature seeds of *Pinus radiata* D. Don, were collected from open pollinated (OP) trees in a seed orchard established by Neiker-Tecnalia in Deba-Spain (latitude: 43º16'59''N, longitude: 2º17'59''W, elevation: 50 m).

In 2008 and 2009, one cone was collected fortnightly during the month of May and two cones weekly during the month of June. The cones were stored for a maximum of a week in paper bags at 4ºC. Intact cones were sprayed with 70% (v/v) ethanol, split into quarters and all immature seeds extracted.

Seeds were surface sterilised in 10% (v/v) H_2O_2 plus two drops of Tween 20® for 8 min, then they were rinsed three times in sterile distilled H_2O in aseptic conditions. Finally, seed coats were removed, and megagametophytes were excised out aseptically and placed horizontally onto culture media.

2.2. Initiation and proliferation of ET

Embryo development medium (EDM) (Walter et al. 1998a) was used for initiation. This medium was supplemented with 30 g L^{-1} sucrose as well as a combination of 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 µM benzyladenine (BA). Before sterilization, the pH of the medium was adjusted to 5.7 and then 3 g L^{-1} gellan gum (Gelrite®) were added. Medium was autoclaved at 121ºC for 20 min. Afterwards, filter-sterilized solutions, of 550 mg L^{-1} L-glutamine, 525 mg L^{-1} L-asparagine, 175 mg L^{-1} L-arginine, 19.75 mg L^{-1} L-citrulline, 19 mg L^{-1} L-ornithine, 13.75 mg L^{-1} L-lysine, 10 mg L^{-1} L-alanine and 8.75 mg L^{-1}

L-proline were added to the cooled medium; the pH of these solutions was also adjusted to 5.7. Eight explants were cultured in each 90 x 20 mm Petri dish. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks.

After 4–8 weeks on initiation medium, proliferating ET was separated from the megagametophyte and subcultured onto maintenance medium every 2 weeks. Maintenance medium had the same composition as initiation medium but a higher concentration of gellan gum, 5.5 g L^{-1}. Following 4 subculture periods, actively growing ETs were recorded as established cell lines.

2.3. Maturation of somatic embryos

In 2008, a maturation experiment was designed to assess the effect of the initial amount of ET required for maturation and the influence of the presence of AC in the ET suspension.

ET was first suspended in liquid growth regulators-free EDM medium, with or without 0.5 % (w/v) activated charcoal (AC) in 50 mL centrifuge tubes; and was vigorously shaken by hand for a few seconds. Thereafter, a 5 mL aliquot containing either 100 or 150 mg fresh mass of suspended ET was poured onto a filter paper disc (Whatman nº2, 7 cm) in a Büchner funnel. A vacuum pulse was applied for 10 seconds, and the filter paper with the attached ET was transferred to maturation medium. The maturation medium designated as A9S3ED had the salt formulation of EDM, but a higher concentration of gellan gum, 9 g L^{-1}, and was supplemented with 90 µM abscisic acid (ABA), 30 g L^{-1} sucrose (88 mM, S3) and the amino acid mixture used for initiation and maintenance of the ET (ED).

Twelve embryogenic cell lines from 4 OP trees were tested**,** six embryogenic cell lines for each initial amount of ET (100 and 150 mg). The experiments were replicated two times, in each replication two to three Petri dishes per embryogenic cell line were laid out randomly on the shelves of the growth chamber.

Also in 2008, once the initial amount of ET for maturation was established, a second maturation experiment was performed to assess the joint effects on somatic embryo production of the presence or absence of AC in the ET suspension, and of ABA and sucrose at two concentrations and of two amino acid mixtures in the maturation medium.

Two liquid media were tested, EDM with 5 g L^{-1} of AC and EDM without AC. The maturation media had the salt formulation of EDM medium, but a higher concentration of gellan gum, 9 g L^{-1}. The following codes were used to identify the EDM media supplemented with: 60 μM (A6) or 90 μM (A9) ABA; 30 g L^{-1} sucrose (88 mM, S3) or 60 g L^{-1} sucrose (175 mM, S6); the amino acid mixture used for initiation and maintenance of the ET (ED) or a second amino acid mixture composed of 1 g L^{-1} of casein hydrolysate and 500 mg L^{-1} of L-glutamine (CG). Thus, A6S6ED was a medium containing ABA at 60 μM, 60 g L^{-1} sucrose and the amino acid mixture used for initiation and maintenance.

The maturation experiments comprised two liquid media and eight semi-solid media, a total of sixteen combinations. A single cell line, 2162 was used for maturation experiments; this line was 4 months old at the start of the maturation process. The experiments were replicated two times, in each replication two to three Petri dishes per treatment were laid out randomly on the shelves of the growth chamber.

In 2009, 24 embryogenic cell lines were established from 4 OP trees. These lines were tested on the best maturation medium selected from 2008 experiments. The experiments were replicated two times, in each replication two to three Petri dishes per embryogenic cell line were laid out randomly on the shelves of the growth chamber. Cultures were kept in darkness at 21±1ºC.

2.4. Osmolality and water availability of the maturation media

The osmolality (mosm kg^{-1}) of all maturation media was measured at the onset of the experiment using a Micro-Osmometer Automatic according to the manufacturer's instructions. Briefly, each maturation medium without gellan gum was measured. Then, the gelled maturation media were diluted in distilled water at a ratio 1:10 (w/v) and measured. The measurements were replicated three times.

The water availability of the maturation media was determined on three Petri dishes per medium by placing one autoclaved filter paper disc (Whatman nº 2, 7 cm) on the surface of the medium of each Petri dish. First, each filter paper disc was weighed in a sterile Petri dish and then immediately placed on the medium. The Petri dishes were sealed with clingfilm and incubated for 48 h under the same conditions as for maturation of the ET. Filter paper discs were subsequently weighed and the amount of water (mg) absorbed by the filter paper was calculated. The measurements were replicated three times.

2.5. Germination of somatic embryos

In 2008, somatic embryos were transferred to three different germination media. The somatic embryos were obtained from ET dispersed in liquid EDM without AC and plated on A6S6ED maturation medium; they were placed on germination media without any pre-germination treatment.

Germination media were EDM with 0.2% (w/v) AC, half strength macronutrients modified LP medium (Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988) (1/2LP), and 1/2LP with 0.2% (w/v) AC (LPE). All germination media were solidified with 5.5 g L^{-1} gellan gum.

The experiments were replicated two times, in each replication two to three Petri dishes per treatment with eight somatic embryos per Petri dish, with the embryonal root caps pointing downwards, were tilted vertically at an angle of approximately 45º–50º C, and placed under dim light for 7 days (Klimaszewska et al. 2001). Cultures were maintained at 21±1°C under a 16 h photoperiod at 120 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France). The plantlets were subcultured once onto fresh medium of the same composition. After 14–16 weeks on germination medium the plantlets were transferred to sterile peat:perlite (3:1) and acclimatized in a greenhouse.

The pH of maturation and germination media was verified at the start of the experiments in order to prevent any change of the conditions caused by medium components such as AC. The measurements were carried out on three Petri dishes for each medium.

In 2009, the somatic embryos obtained from the tested lines were germinated on the best germination medium from the 2008 experiments.

2.6. Data collection and statistical analysis

In 2008, after 12 weeks on maturation medium, mature somatic embryos were counted and classified as normal or abnormal. Normal somatic embryos were white to yellowish, non-germinating, with a distinct hypocotyl region, and at least three cotyledons. Any somatic embryo germinating precociously, with less than three cotyledons or bearing abnormally shaped cotyledons was classified as abnormal (Garin et al. 2000).

In 2008, a linear model was designed to test the effects of ABA concentration, sucrose concentration and amino acid mixture; these variables were considered as fixed effects. The number of mature somatic embryos per 100 mg fresh mass of ET per filter paper disc was recorded for each maturation media. Analysis of variance (ANOVA) was carried out to determine differences between the treatments that produced cotyledonary somatic embryos. The data for osmolality and water availability in the maturation media were subjected to ANOVA to determine differences among maturation treatments. Multiple comparisons were made using Duncan´s post hoc test ($p \leq 0.05$).

Normality was evaluated by Shapiro–Wilk normality test (Shapiro and Wilk 1965) without transformation. The equal variance of the data was analysed by Levene's test (Brown and Forsythe 1974). The analyses were performed using the SPSS© version 15.0 software package.

Also in 2008, after 14–16 weeks on different germination media, the number and morphology of the somatic seedlings was recorded. After 10 months in the greenhouse, the survival percentage and height of the somatic plantlets was assessed.

In 2009, after 12 weeks on maturation medium, the number of mature somatic embryos per 100 mg fresh mass of ET per Petri dish was recorded for the tested embryogenic cell lines. ANOVA was carried out to determine differences between the mother trees. Multiple comparisons were made using Duncan´s post hoc test ($p \leq 0.05$).

In 2009, after 14–16 weeks on germination medium the number and morphology of the somatic seedlings was recorded.

3. RESULTS

3.1. Maturation of somatic embryos

In 2008, when 150 mg per filter paper disc was chosen as initial amount of ET for maturation experiments, there was an overgrowth of the ET that hindered the development of somatic embryos, and only a few of them were obtained (Fig. 1a). In experiments with 100 mg of ET per filter paper, a higher number of SE was obtained. But when the ET was suspended in liquid medium with AC, these embryos showed an abnormal morphology (Table 1).

Table 1 Mature somatic embryos per 150 mg or 100 mg fresh mass of embryogenic tissue (No.). The embryos were obtained from embryogenic tissue suspended in liquid EDM with or without AC and placed on EDM supplemented with 90 µM ABA, 30 g L^{-1} sucrose and ED amino acid mixture, and solidified with 9 g L^{-1} gellan gum (M±S.E.). Values followed by letter A are somatic embryos featuring abnormal morphology and values followed by letter N are somatic embryos featuring normal morphology.

Initial ET amount (mg) per filter paper disc	Embryogenic Line	No. of somatic embryos (EDM with AC)	No. of somatic embryos (EDM without AC)
100	E2-184	1 ± 0.2 A	1 ± 0.2 N
	11-162	7 ± 1.7 A	1 ± 0.2 N
	E1-144	17 ± 1.9 A	3 ± 1.2 N
	E1-195	23 ± 5.8 A	55 ±9.1 N
	E9-164	54 ± 9.3 A	1 ± 0.2 N
	20-162	57 ± 7.4 A	129 ± 4.1 N
150	E9-264	0	0
	E15-144	0	0
	E4-145	0	0
	E11-184	0	0
	1-193	1 ± 0.2 A	0
	E1-295	14 ± 3.1 A	10 ± 2.8 N

On the basis of these results, the initial amount of ET was set to 100 mg per filter paper for the next set of maturation experiments. Sixteen combinations of media tested with 2162 embryogenic line produced somatic embryos. But those formed when the ET was dispersed in EDM with 0.5% AC displayed abnormal morphology, manifested by precocious germination and in some by a lack of cotyledons (Fig. 1b). Mature cotyledonary somatic embryos were collected from ET suspended in EDM without AC on the eight maturation media tested (Fig. 1c). Analysis of variance indicated that there were significant effects among treatments for the number of embryos produced (Fig. 2).

There was a significant effect of the sucrose concentration together with the amino acid mixture. CG amino acid mixture and 30 g L^{-1} sucrose in the maturation media produced the lowest number of somatic embryos. Fourteen and 16 somatic embryos per 100 mg of ET were produced from A6S3CG and A9S3CG, respectively. The number of somatic embryos collected from media containing ED amino acid mixture and 30 g L^{-1} sucrose (A6S3ED and A9S3ED) was similar to that recorded from media containing CG amino acid mixture and 6%

sucrose (A6S6CG and A9S6CG), ranging from 31 to 42 somatic embryos per 100 mg of ET (Fig. 2).

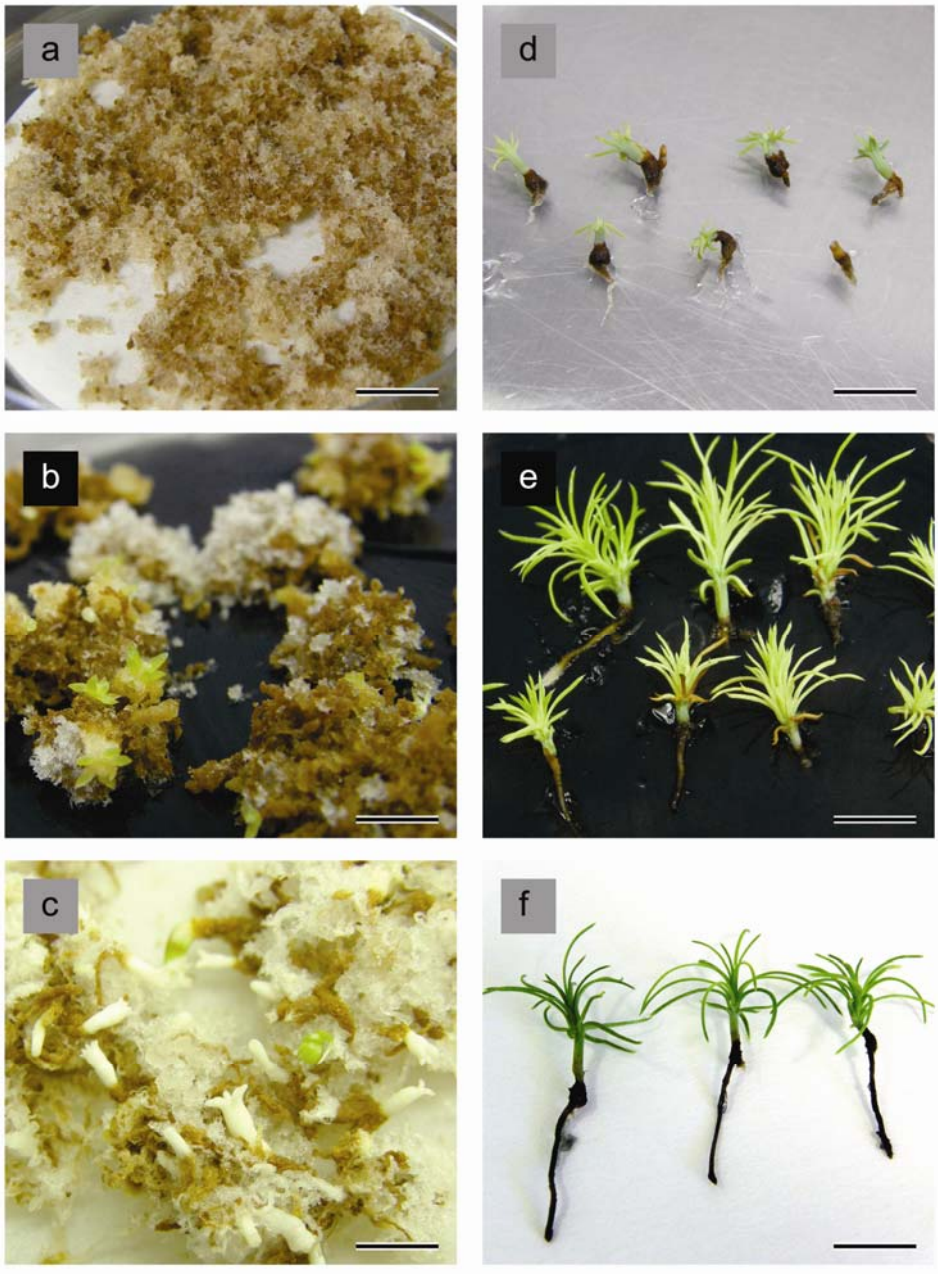

Fig. 1 *Pinus radiata* somatic embryo maturation and germination: (a) tissue overgrowth after 12 weeks on EDM supplemented with 90 µM ABA, 30 g L^{-1} sucrose and ED amino acid mixture (A9S3ED) when 150 mg of embryogenic tissue was used (bar=10 mm); (b) abnormal somatic embryos from 100 mg of embryogenic tissue suspended in liquid EDM with AC and cultured on EDM supplemented with 60 µM ABA, 60 g L^{-1} sucrose and the ED amino acid mixture (A6S6ED) for 12 weeks (bar=5 mm); (c) mature cotyledonary somatic embryos obtained from 100 mg of embryogenic tissue suspended in liquid EDM without AC and cultured A6S6ED for 12 weeks (bar=10 mm); (d, e, f) somatic plantlets after 14 weeks: on 1/2LP (d); on EDM with 0.2% AC (e); on LPE, prior to transfer to the greenhouse (f) [bar=5 mm for (d), bars=10 mm for (e) and (f)].

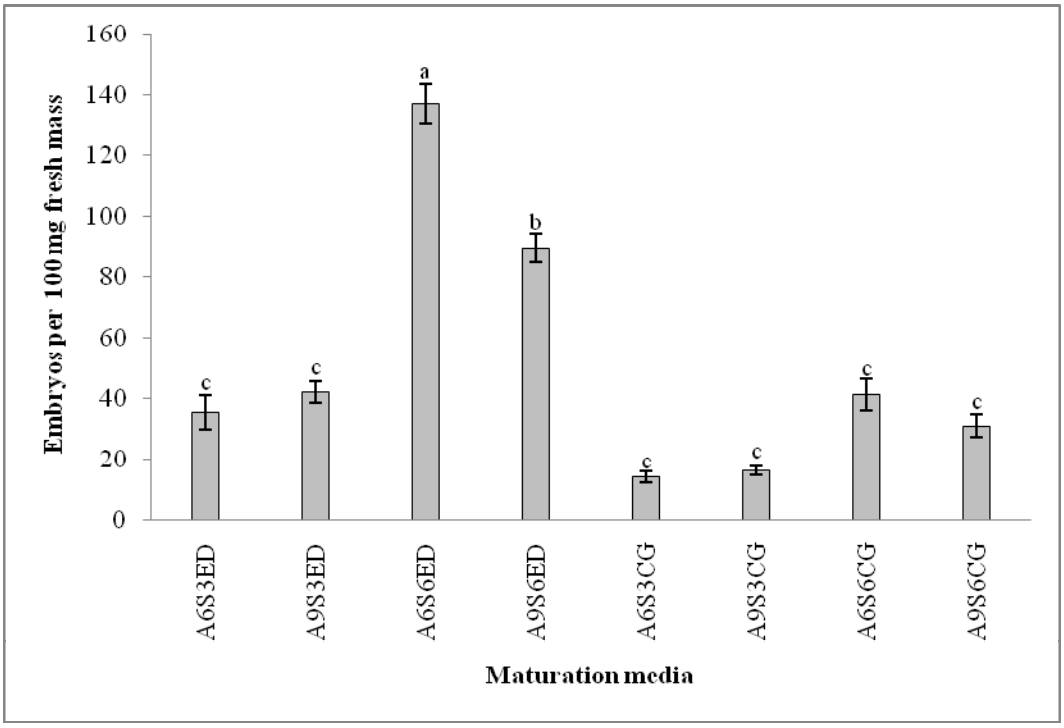

Fig. 2 Number of mature somatic embryos per 100 mg fresh mass of embryogenic tissue. The embryogenic tissue was suspended in liquid EDM without AC and placed on 8 different maturation media for 12 weeks. Maturation medium was EDM supplemented with 60 µM (A6) or 90 µM (A9) ABA, 30 g L^{-1} sucrose (S3) or 60 g L^{-1} sucrose (S6), and ED amino acid mixture or CG amino acid mixture; solidified with 9 g L^{-1} gellan gum (M±S.E.). Values followed by different letters are significantly different according to Duncan´s test ($p \leq 0.05$).

The number of somatic embryos did not increase with ABA concentration. A9S6ED produced a significantly higher number of somatic embryos than the other media tested (90 somatic embryos per 100 mg of ET) except for A6S6ED, the most effective medium. In A6S6ED the number of somatic embryos recorded was 137 per 100 mg of ET (Fig. 2).

In 2009, 24 embryogenic lines were suspended in liquid EDM medium without AC and plated on A6S6ED medium. All embryogenic cell lines produced cotyledonary somatic embryos. Eleven lines produced more than 60 somatic embryos per 100 mg of ET, and over 100 somatic embryos per 100 mg of ET were obtained in 5 of these lines (Table 2). No significant differences were found among mother trees for the number of somatic embryos obtained.

Table 2 Number of mature somatic embryos per 100 mg fresh mass of embryogenic tissue obtained from 24 embryogenic cell lines (M±S.E.).

Mother Tree	Embryogenic Line	No. of somatic embryos per 100 mg fresh mass
14	1(14-2)	22 ± 0.3
	19(14-5)	53 ± 11.2
	4(14-4)	105 ± 4.8
	10(14-5)	153 ± 6.3
16	3(16-3)	1 ± 0.3
	1(16-2)	5 ± 0.3
	12(16-3)	11 ± 3.0
	13(16-3)	21 ± 0.3
	2(16-6)	22 ± 1.2
	11(16-6)	35 ± 5.7
	15(16-4)	60 ± 3.8
	11(16-4)	65 ± 6.3
	17(16-6)	81 ± 1.2
	25(16-6)	90 ± 11.2
	12(16-4)	155 ± 13.1
18	4(18-3)	38 ± 5.7
	6(18-4)	43 ± 1.5
	8(18-4)	63 ± 15.7
	1(18-3)	121 ± 8.3
19	1(19-4)	1 ± 0.3
	7(19-4)	26 ± 5.2
	6(19-3)	39 ± 0.7
	7(19-2)	70 ± 2.9
	13(19-4)	103 ± 3.1

3.2. Osmolality and water availability of the maturation media

The osmolality of the maturation media was similar with or without gellan gum. The osmolalities of the media were significantly higher when they contained 60 g L^{-1} sucrose compared with media with 30 g L^{-1} sucrose. CG amino acid mixture in the media led to significantly lower values when compared with media with the same ABA and sucrose concentration, but with the ED amino acid mixture. The concentration of ABA in the media did not alter the osmolality (Table 3).

The change in filter paper weight after incubation on the medium surface was measured to verify whether maturation medium composition had any effect on the amount of water to which ET was exposed during culture on the surface of the filter paper. The amount of water absorbed by the filter papers was not statistically different for any maturation treatment, being 572 mg and 580 mg the lowest and the highest values respectively (Table 3).

Table 3 Osmolality (mosm kg^{-1} water) of the maturation media and water availability (mg) in the maturation media (M± S.E.). Different letters within a column show significant differences by Duncan´s post hoc test ($p \leq 0.05$) (n.s.: non significant).

Maturation media	Osmolality (mosm kg^{-1})	Water availability (mg)
A6S3ED	166 ± 0.3 c	577.8 ± 1.5 n.s.
A9S3ED	167 ± 0.7 c	572.5 ± 2.2 n.s.
A6S6ED	278 ± 0.7 a	580.9 ± 6.0 n.s.
A9S6ED	280 ± 0.1 a	575.1 ± 2.7 n.s.
A6S3CG	151 ± 0.1 d	573.1 ± 3.4 n.s.
A9S3CG	154 ± 0.1 d	574.1 ± 2.4 n.s.
A6S6CG	266 ± 0.1 b	577.5 ± 1.8 n.s.
A9S6CG	262 ± 0.7 b	577.1 ± 1.4 n.s.

3.3. Germination of somatic embryos

In 2008, seven weeks after the initiation of the germination experiments, 80 to 90% of the somatic embryos obtained from A6S6ED medium germinated in the three tested media. After subculturing the explants onto fresh medium of the same composition, the seedlings on 1/2LP without AC did not develop further, and died shortly after (Fig. 1d). The somatic seedlings germinating on EDM with AC became yellow (Fig. 1e), which in turn had a detrimental effect on their survival when transferred to the greenhouse. Somatic seedlings on LPE elongated and were characterized by normal morphology (Fig. 1f).

These somatic seedlings were transplanted to a potting mix in the greenhouse. After 10 months, the survival percentage of the plantlets was close to 90% for the somatic seedlings that were germinated on LPE, the height of these plantlets ranged from 15 to 25 cm (Fig. 3). The survival percentage of the somatic plantlets that were germinated in EDM with AC was under 50%, the height of these plantlets also ranged from 15 to 25 cm.

In 2009, 95% of the somatic embryos obtained germinated on LPE, these explants were transferred to the greenhouse in May 2010.

Fig. 3 Somatic seedlings germinated on LPE medium, after 10 months in the greenhouse.

4. DISCUSSION

In the past it was a common practice to culture the clumps of embryogenic tissue directly on the maturation medium instead plating suspended ET; the former practice has been carried out in several conifer genera such as *Abies* (Salajova et al. 1996), *Larix* (Gutmann et al. 1996), *Picea* (Iraqui and Tremblay 2001) and *Pinus* (Keinonen-Mettälä et al. 1996); but this method required a large amount of embryogenic tissue.

For our maturation experiments, the ET was suspended in growth regulators-free liquid medium; this technique is currently used in *Pinus* genus to maximize embryo production (Klimaszewska et al. 2007). The initial amount of embryogenic tissue usually ranges from 500 mg (Maruyama et al. 2007) to 50 mg (Carneros et al. 2009) per filter paper disc (7 cm).

We found that 150 mg of ET versus 100 mg of ET did not lead to a higher production of mature somatic embryos as a general trend although we tested different embryogenic cell lines. Lelu-Walter et al. (2006) reported similar results for *P. pinaster*. In our case, this could have been due to an overgrowth of the tissue that hindered maturation. Although the aforementioned authors pointed out that coating the ET with AC enhanced the process, this was not the case in our experiments with radiata pine. When the ET was dispersed in liquid EDM with AC, the somatic embryos obtained displayed abnormal morphology; these cultures underwent an overgrowth and somatic embryos tended to germinate precociously.

Van Winkle and Pullman (2003) reported that when AC was added to the culture medium, it had an impact on the pH and on the availability of different metallic species. We did not observe any pH variation when liquid EDM supplemented with AC was used to suspend the ET; hence, we cannot attribute our results to pH changes. Although AC plays an essential role in *Pinus* micropropagation for its capacity of adsorbing phenolics and residual plant growth regulators (De Diego et al. 2008; Thomas 2008); Pan and Van Staden (1998) stated that AC can promote or inhibit *in vitro* growth depending on the species and tissues used.

In order to stimulate a shift in the developmental program of the ET from proliferation of cells and early embryos to production of cotyledonary somatic embryos, it is a common practice to restrict water availability by exposing the ET to maturation media with high gellam gum concentration or high osmolality. In this way, sucrose at relatively high concentrations enhanced somatic embryo maturation in *P. pinaster* (Ramarosandratana et al. 2001), *P. monticola* (Percy et al. 2000) and different spruce species (Tremblay and Tremblay 1995; Iraqi and Tremblay 2001). Sucrose functions as both a carbon source and an osmoticum. We observed that 60 g L^{-1} sucrose and ED amino acid mixture in the maturation medium significantly increased the osmolality. These media with higher osmotilality (A6S6ED and A9S6ED) produced the highest number of somatic embryos in the tested lines of radiata pine.

Glutamine concentration was higher in ED amino acid mixture than in CG amino acid mixture. Previous studies have shown that an increase in the concentration of glutamine could be critical for somatic embryo maturation (Garin et al. 2000). But, as in our experiments, Garin et al. (2000) raised medium osmolality when they increased the glutamine concentration. This fact in turn may have had an effect on the maturation response. It is therefore difficult to separate the effects the ED amino acid mixture itself, from that of the change in osmolality of the medium.

Some amino acids present in ED mixture could have played a key role not only in the number but in the quality of the somatic embryos obtained. In peanut, proline and glutamine reduced the total number of embryos formed, but the resulting embryos were larger, greener and had a more synchronous development (Murch et al. 1999) and in carrot, glutamine and alanine strongly stimulated somatic embryo formation (Higashi et al. 1996). In this species, during somatic embryo development, glutamine continued to increase in concentration, whereas arginine and its related metabolites, as ornithine, were biphasic; increasing in globular and torpedo stage embryos and decreasing in germinating embryos (Joy et al. 1996). In oil palm SE, when arginine was added to the maturation medium, it improved embryo maturation and plant conversion favouring the accumulation of the dominant storage protein content per dry weight (Morcillo et al. 1999). Furthermore Pérez-Rodríguez et al. (2006) suggested that the composition of the culture medium during pine somatic embryogenesis, particularly the carbohydrate to nitrogen ratio, may represent a key factor responsible for the expression of certain glutamine synthetase-related and photosynthesis-related genes.

Our results showed the importance of sucrose and amino acids in the maturation media; in many coniferous species, somatic embryo maturation could only be achieved by supplementation of exogenous ABA to the maturation medium (Stasolla and Yeung 2003). ABA is known to function effectively in switching embryo developmental pathway from proliferation to maturation. In radiata pine, concentrations of ABA ranging from 57 to 90 μM have been used in maturation (Cerda et al. 2002; Bishop-Hurley et al. 2003). Similar and higher concentrations have been used in other pine species such as *P. bungeana* (Zhang et al. 2007), *P. rigida* x *P. taeda* (Kim and Moon 2007), *P. brutia* (Yildirim et al. 2006), *P. pinaster* (Lelu-Walter et al. 2006), and *P. strobus* (Klimaszewska et al. 2001). Kong and Von Aderkas (2007) reported that the ability to consume exogenous ABA by ET of interior spruce depended on a genotype; genotypes with a higher capability of embryo maturation demonstrated greater utilization of exogenous ABA. Although our results are only based on a single cell line, the highest number of somatic embryos was obtained in medium with 60 μM ABA (A6S6ED). Despite A6S6ED was the best maturation treatment, the number of somatic embryos in A9S6ED was also significantly higher than the results obtained in the other media tested. These results indicate that more critical is the amount of sucrose in combination with the organic nitrogen source than the concentration of ABA. This is in agreement with findings in maritime pine; where there was no apparent effect of ABA concentration on maturation of somatic embryos (Lelu-Walter et al. 2006).

The gellan gum concentration in the culture medium had a critical effect in our experiments, as it has been described in the maturation of *P. strobus* ET (Klimaszewska and Smith 1997) and *P. pinaster* ET (Ramarosandratana et al. 2001; Klimaszewska et al. 2007). Klimaszewska et al. (2000) found out that a higher agar and gellan gum concentration in the maturation medium increased medium strength and decreased water availability. The somatic embryos of *P. strobus* obtained from media with a higher solidifying agent concentration showed the highest number of morphologically normal germinants. In accordance with these authors we observed that a high concentration of gellan gum in the medium delayed the maturation process. On medium with 9 g L^{-1} gellan gum after 12 weeks we obtained somatic embryos; whereas Walter et al. (1998a) and Cerda et al. (2002) used maturation media gelled with a lower concentration of gellan gum, between 4.5 and 6 g L^{-1}, and obtained somatic embryos after 6 to 8 weeks of maturation. Although our maturation process lasted longer, it was not necessary to apply any pre-germination treatment to the somatic embryos.

Conversion of somatic embryos into plants was successful when somatic embryos were placed on 1/2LP with AC (LPE). In some pine species such as *P. strobus* (Klimaszewska et al. 2001) and *P. pinaster* (Lelu-Walter et al. 2006) somatic embryos have been germinated on media without AC, whereas in other pine species such as *P. armandii* (Maruyama et al. 2007), *P. densiflora* (Maruyama et al. 2005), and *P. nigra* (Salajova and Salaj 2005) the germination medium of choice contained AC. Usually, the germination medium has the same composition as initiation and proliferation medium, but is devoid of plant growth regulators. In our experiments, we tested the medium used for initiation and proliferation (EDM) but also another medium often used for shoot organogenesis and somatic embryogenesis in radiata pine (1/2LP) (Cerda et al. 2002; Hargreaves et al. 2005).

The best germination results were obtained on LPE. Somatic embryos germinating in 1/2LP without AC did not grow and died. The beneficial effect of AC in the germination medium could be attributed to its property of adsorbing residual plant growth regulators (Ebert et al. 1993; Von Aderkas et al. 2002). But AC alone was not sufficient for germination; although somatic embryos germinating on EDM with AC elongated, they became yellow, whereas on LP the germinated embryos were green and had normal morphology. This could be due to the higher content of calcium in LP medium when compared with EDM medium. Although reduced calcium concentration in maturation medium improved the cotyledonary embryo yield in *P. taeda* (Pullman et al. 2003), it did not improve germination. However, calcium has

been found to improve germination when used for encapsulation of somatic embryos in *Centella asiatica* (Joshee et al. 2007) and *Selinum tenuifolium* (Joshi et al. 2006).

It is remarkable that somatic embryos germinated without any pretreatment, this could be due to the maturation treatment itself. High concentration of gellan gum leads to an increase in the dry weight of the embryos; this could have implications in the germination ability of the embryos produced according with the results of Klimaszewska et al. (2000).

The results obtained on maturation and germination of a single line in 2008 were confirmed the next year. In 2009, only the best maturation medium from 2008 was used for maturation experiments. Among the 24 embryogenic cell lines tested, 11 lines produced more than 60 SE per 100 mg ET and in 2 of these lines a higher number of somatic embryos than in 2008 experiments was obtained. Further testing with this maturation medium will be done in the future to confirm its superiority.

The 95% of the somatic embryos obtained in these maturation experiments germinated successfully on LPE. This result improves those reported by Walter et al. (1998b) with germination percentages ranging from 50% to 80% and Aquea et al. (2008) with germination percentages ranging from 34% to 57%.

Based on these results, we recommend the following protocol: 100 mg FW of ET, no subcultures during the entire maturation period, no pre-germination treatment needed due to the high quality of the somatic embryos obtained, and high conversion of somatic embryos into plants, resulting in a significant saving of cost and labour. We believe that this simplified maturation and germination protocol offers an efficient tool for the production of *P. radiata* clonal plants through somatic embryogenesis.

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CAPÍTULO 4

A combined pathway of somatic embryogenesis and organogenesis to regenerate radiata pine plants

Este capítulo se corresponde con el artículo: A combined pathway of somatic embryogenesis and organogenesis to regenerate radiata pine plants (*Plant Biotechnology Reports*, en revisión).

Capítulo 4: A combined pathway of somatic embryogenesis and organogenesis to regenerate radiata pine plants

1. INTRODUCTION

The level of domestication in forest trees is significantly lower than for agricultural plants. However, there is a great potential for improvement through genetic breeding. Using this type of technologies, considerable genetic gain has been achieved in plantation forest trees such as *Pinus radiata*. Originating from California, this species shows superior growth rates and many improvements to its genetic value have been made over the past 50 years (Walter 2004). Although conventional seed orchards provide genetically improved seeds, traditional breeding strategies combined with *in vitro* vegetative propagation have showed advantages such as: additional genetic gain achieved by capturing non-additive genetic variation, as well as the speed which clones may be introduced to meet market goals and the ability to program diversity into a clonal plantation (Park et al. 1998).

In vitro vegetative propagation from physiologically mature tissue (even as young as one year old in some species) tends to be quite difficult and can result in changes in the attributes of the resulting plants. In Neiker-Tecnalia, our research group has achieved adult clonal propagation in *Pinus* spp. (De Diego et al. 2008; Cortizo et al. 2009; De Diego et al. 2010) but sometimes changes in the attributes of the resulting plants have been observed and the reinvigoration of the material has been transient in *in vitro* conditions (Cortizo et al. 2009).

On the other hand, vegetative propagation from physiologically juvenile tissue has been successful in a number of conifer species (Bergmann and Stomp 1992; Klimaszewska et al. 2007). This phenomenon has led many organizations to focus on production of elite families through juvenile tissue propagation in the near term, either for operational use or for clonal tests, while they continue to research methods of selected mature trees propagation (Talbert et al. 1993).

The two main *in vitro* techniques used routinely for plant micropropagation including somatic embryogenesis (SE) and organogenesis (Giri et al. 2004). As for *in vitro* embryogenesis,

organogenesis has been used as a model system to study the structural, physiological, and molecular bases of development. Studies on organogenesis and SE have shown the fundamental role of plant growth regulators in *in vitro* culture (Jimenez 2005). This basic understanding has greatly contributed to the extension of tissue culture for commercial applications (Stasolla and Thorpe 2010).

In this sense, propagation via SE is an effective method in propagating elite plants when it is combined with other technologies, such as cryopreserving the embryogenic tissue (ET) and selecting elite clones in field tests (Park 2002). Due to the importance of this technology, SE in pines has been widely reported in the past few years (Klimaszewska et al. 2007; Lelu-Walter et al. 2008); in the majority of these reports, the induction of ET is carried out from immature seeds. The problem is that the competence window for this type of explant is narrow, lasting around 4 weeks (MacKay et al. 2006; Yildirim et al. 2006). After these weeks, the initiation decreases to very low rates. In this sense, in an attempt to overcome the narrow competence window, ET initiation has been also obtained from mature embryos, but at low frequencies in *P. lambertiana* (Gupta and Durzan 1986), *P. strobus* (Garin et al. 1998) and *P. taeda* (Tang et al. 2001). Even though mature seed explants overcome the problem of the competence window of immature seeds, the low success rate makes this approach unfeasible for large-scale production (Klimaszewska et al. 2007).

But the major bottleneck in *Pinus* SE is maturation of the ET. In several pine species the somatic embryos obtained feature abnormal morphology, and when they resemble their zygotic counterparts, they appear in a very low number (Choudhury et al. 2007; Carneros et al. 2009). In fact, during the maturation phase of SE, the embryos attain physiological and biochemical attributes that enable subsequent germination and conversion. Thus, cotyledonary mature somatic embryos germinate with vigour and establish plants at a high frequency. Nowadays, due to the aforementioned problems, the large-scale production of some genotypes is too expensive and the number of genotypes that can be candidates for clonal forestry decreases (Davis and Becwar 2007).

On the other hand, the production of clonal plants from seeds via organogenesis has been thoroughly studied in several pine species such as *Pinus pinaster* (Álvarez et al. 2009)*, P. strobus* (Webb et al. 1988), *P. wallichiana* (Mathur and Nadgauda 1999), *P. canariensis* (Martínez Pulido et al. 1994) or *P. pinea* (Moncaleán et al. 2005). Some years ago, a breakthrough in organogenesis automation potential was made in radiata pine with the

development of a protocol to generate large numbers of meristematic nodules from embryos (Aitken-Christie et al. 1988). In an attempt to improve the output of this technique some protocols have been developed in the last decade in this species (Stange et al. 1999; Hargreaves et al. 2005) and in those protocols different initial explants (whole embryos or cotyledons) and environmental conditions have been assayed.

In vitro organogenesis is not under restricted to a certain moment of the year as SE initiation, and the material obtained can be multiply and rooted when demanded, with no detrimental effect on the explants produced.

This study presents novel approach to overcome some problems associated with SE such as the aforementioned seasonality of ET initiation or low embryo production due to limited success in maturation and germination steps.

The approach presented here consist on the development of a combined SE and organogenesis protocol; for this purpose the effect of different initial explants, BA concentrations and induction periods and culture conditions were assayed in somatic embryos of *Pinus radiata* D. Don.

2. MATERIALS AND METHODS

2.1. Plant material

Pinus radiata ET was obtained from immature megagametophytes cultured on EDM basal medium (Walter et al. 1998) with a combination of 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 µM benzyladenine (BA). Before autoclaving, the pH of the medium was adjusted to 5.7 and then 3 g L^{-1} Gelrite® were added. Medium was autoclaved at 121ºC for 20 min. After autoclaving, filter-sterilized solutions of 550 mg L^{-1} L-glutamine, 525 mg L^{-1} L-asparagine, 175 mg L^{-1} L-arginine, 19.75 mg L^{-1} L-citrulline, 19 mg L^{-1} L-ornithine, 13.75 mg L^{-1} L-lysine, 10 mg L^{-1} L-alanine and 8.75 mg L^{-1} L-proline were added to the cooled medium. The pH of these solutions was also adjusted to 5.7.

Somatic embryos were obtained from embryogenic line 2162 according to the maturation method described by Montalbán et al. (2010). Briefly, ET was first suspended in liquid growth regulators-free EDM medium; and was vigorously shaken by hand for a few seconds.

Thereafter, a 5 mL aliquot containing either 100 mg fresh mass of suspended ET was poured onto a filter paper disc (Whatman nº2, 7 cm) in a Büchner funnel. A vacuum pulse was applied for 10 seconds and the filter paper with the attached ET was transferred to maturation medium. The maturation medium had the salt formulation of EDM medium, but a higher concentration of Gelrite®, 9 g L^{-1}, and was supplemented with 60 µM abscisic acid (ABA), 60 g L^{-1} sucrose and the amino acid mixture used for initiation and maintenance of the ET. Cultures were kept in darkness at 21±1ºC. After 12 weeks on maturation medium, mature somatic embryos were selected and isolated from ET. The somatic embryos collected were white to yellowish, non-germinating, with a distinct hypocotyl region, and at least three cotyledons. Two types of explants were used for induction experiments: freshly collected somatic (NG) embryos (Fig. 1a) and germinated for one week somatic (G) embryos (Fig. 1b).

Germination medium was half strength macronutrients except for the iron and complete micronutrients and vitamins from LP medium (Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988) (LPE), supplemented with 30 g L^{-1} sucrose, 0.2% (w/v) activated charcoal (AC) and 10 g L^{-1} Difco Agar® granulated. Cultures were kept under dim light at 21±1ºC.

2.2. Shoot induction

Induction media was half strength LP medium (Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988) (1/2LP), supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} Difco Agar® granulated and BA. The pH was adjusted to 5.8 before autoclaving at 121ºC for 20 min.

When NG embryos were used as initial explants, different induction periods and BA concentrations were assayed: 1 µM BA for 2 weeks (2BA1), 1 or 4.4 µM BA for 3 weeks (3BA1 and 3BA4.4) and 1, 4.4 or 22 µM BA for 4 weeks (4BA1, 4BA4.4 and 4BA22). When G embryos were used as initial explants the induction treatments assayed were 1 or 4.4 µM BA for 3 weeks (Fig. 1c).

Embryos were placed on 90 x 15 mm Petri dishes containing 15 mL induction medium. Six to eight embryos per Petri dish were cultured in an inverted position with the cotyledons immersed in the induction medium (Aitken-Christie et al. 1988).

After induction treatments, the explants were transferred to glass jars with the previously described 1/2LP medium but lacking BA for 4 weeks. Then, they were monthly subcultured on elongation medium (LPE) (Figs. 1d and 1e).

The experiments were replicated two times, in each replication two to three Petri dishes per treatment were laid out randomly on the shelves of the growth chamber. Cultures were kept at 21±1°C under a 16 h photoperiod at 120 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France)

2.3. Root induction

Elongated shoots (>15 mm) were transferred to glass jars with rooting medium. The basal medium chosen for rooting was quarter strength macronutrients except for the iron, and half strength iron, micronutrients and vitamins LP medium (Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988), supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} Difco Agar® granulated (1/4LP). This basal medium was supplemented with three different auxin treatments: 1.5 mg L^{-1} 1-naphthalene acetic acid (NAA), 1.5 mg L^{-1} indole-3-butyric acid (IBA) and 1 mg L^{-1} IBA with 0.5 mg L^{-1} NAA (MIX). The effect of the photon flux (120 mmol m^{-2} s^{-1} and darkness) in the first week of the explants in the rooting media was also tested; the root induction experiments comprised three auxin treatments and two light regimes, a total of 6 combinations. The following codes were used to identify the induction media: the rooting treatment assayed (IBA, MIX or NAA) followed by the light treatment tested the first week of the root induction period: light (L) or darkness (D). Thus, NAA-D is 1/4LP supplemented with 1.5 mg L^{-1} NAA and kept in the darkness the first week.

Four shoots were cultured on each culture vessel and five glass jars per treatment were used. After 3 weeks in root induction medium, the shoots were transferred to LPE medium and kept under the same conditions described above. After a month on LPE medium (Fig. 1f) explants with and without visible roots were transferred to a wet sterile peat:perlite mixture (3:1) and acclimatized in the greenhouse under controlled conditions at 21±2ºC and decreasing humidity progressively.

2.4. Data collection and statistical analyses

After 3 months on LPE the percentage of necrosed, hyperhydric embryos and embryos forming shoots (EFS) was recorded. After 6 months on LPE, the number of shoots > 3mm per

embryo and the shoot elongation capacity (SEC) index were recorded. SEC was calculated as described by Lambardi et al. (1993):

SEC= (average number of shoots > 3mm per embryo) x (% explants forming shoots)

Normality was evaluated by Shapiro–Wilk normality test (Shapiro and Wilk 1965) and the equal variance of the data was analysed by Levene's test (Brown and Forsythe 1974). The analyses were performed using the SPSS© version 15.0 software package.

Analysis of variance (ANOVA) was carried out to determine differences between the shoot induction treatments for the EFS percentage, the number of shoots per embryo and the SEC index. Data for the number of shoots per embryo and the SEC index were subjected to log (x+1) transformation. Multiple comparisons were made using Duncan´s post hoc test ($p \leq 0.05$).

After a month on LPE, the percentage of shoots with roots was recorded. ANOVA was carried out to determine differences between root induction treatments and for the percentage of shoots with roots. Multiple comparisons were made using Duncan´s post hoc test ($p \leq 0.05$).

3. RESULTS

3.2. Shoot induction

In NG embryos the contamination percentages were below 3.2%, except in explants treated with 1 μM BA for 2 weeks (16.7%). No contamination was observed in G embryos (Table 1).

Increasing BA concentration or induction period decreased the number of necrosed embryos. The lowest percentages for this parameter were obtained in G embryos cultured in induction medium with 1 or 4.4 μM BA for 3 weeks (11.1 and 27.8%, respectively). The highest percentage of embryos that did not produced shoots and died shortly after the induction period was obtained in NG embryos cultured in induction medium with 1 μM BA for 2 weeks (70.8%) (Table 1).

Although high percentages of hyperhydricity were observed when NG embryos were exposed to 1 and 22 μM BA for 4 weeks (20 and 22.5%, respectively), this was not the case for NG

embryos treated with 4.4 μM BA for the same induction period (5%). Lower exposure periods did not produce hyperhydric explants (Table 1).

Table 1 Contaminated, necrosed, or hyperhydric explants (%) in non-germinated (NG) and germinated (G) *Pinus radiata* somatic embryos cultured on 1/2LP supplemented with 1 μM BA for 2 weeks (2BA1), with 1 or 4.4 μM BA for 3 weeks (3BA1, 3BA4.4) and with 1, 4.4 or 22 μM BA for 4 weeks (4BA1, 4BA4.4, 4BA22) (M).

Treatment	Contaminated (%)	Necrosed (%)	Hyperhydric (%)
2B1 (NG)	16.7	70.8	0
3B1 (NG)	3.1	59.4	0
3B4,4 (NG)	0	53.1	0
4B1 (NG)	2.5	52.5	20.0
4B4,4 (NG)	2.5	37.5	5.0
4B22 (NG)	0	35.0	22.5
3B1 (G)	0	11.1	0
3B4,4 (G)	0	27.8	0

All surviving explants showed an organogenic response and produced shoots. There were significant differences for the EFS percentage between NG and G embryos (Fig. 2). When NG embryos were used as initial explants, treatments with 1 μM BA for 2 and 4 weeks presented the lowest induction percentages (12.5 and 25.0%, respectively). On the contrary, treatments with 4.4 μM BA for 3 and 4 weeks showed the highest organogenic response (46.9 and 55.0%, respectively). Neither the induction time nor the concentration of BA showed a clear trend for caulogenic response (Fig. 2).

When G embryos were used as initial explants the EFS percentages obtained did not differed significantly (88.9% for treatment 3BA1 and 72.2% for treatment 3BA4.4). Comparing NG with G explants subjected to the same induction treatments (1 and 4.4 μM BA for 3 weeks), significantly better results for G embryos were observed (Fig. 2).

The lowest number of shoots in NG embryos was obtained when 1 μM BA was applied for 2 and 3 weeks to the induction medium (Fig. 3). Longer induction periods (3 or 4 weeks) and higher BA concentrations (4.4 or 22 μM) produced a significantly higher number of shoots per NG embryo. When NG and G embryos were compared, the number of shoots per embryo in NG explants after treatment 3BA4.4 (19.3 shoots per embryo) was significantly better than the number of shoots obtained in NG embryos after treatment 3BA1 and in G embryos after treatments 3BA1and 3BA4.4 (Fig. 3).

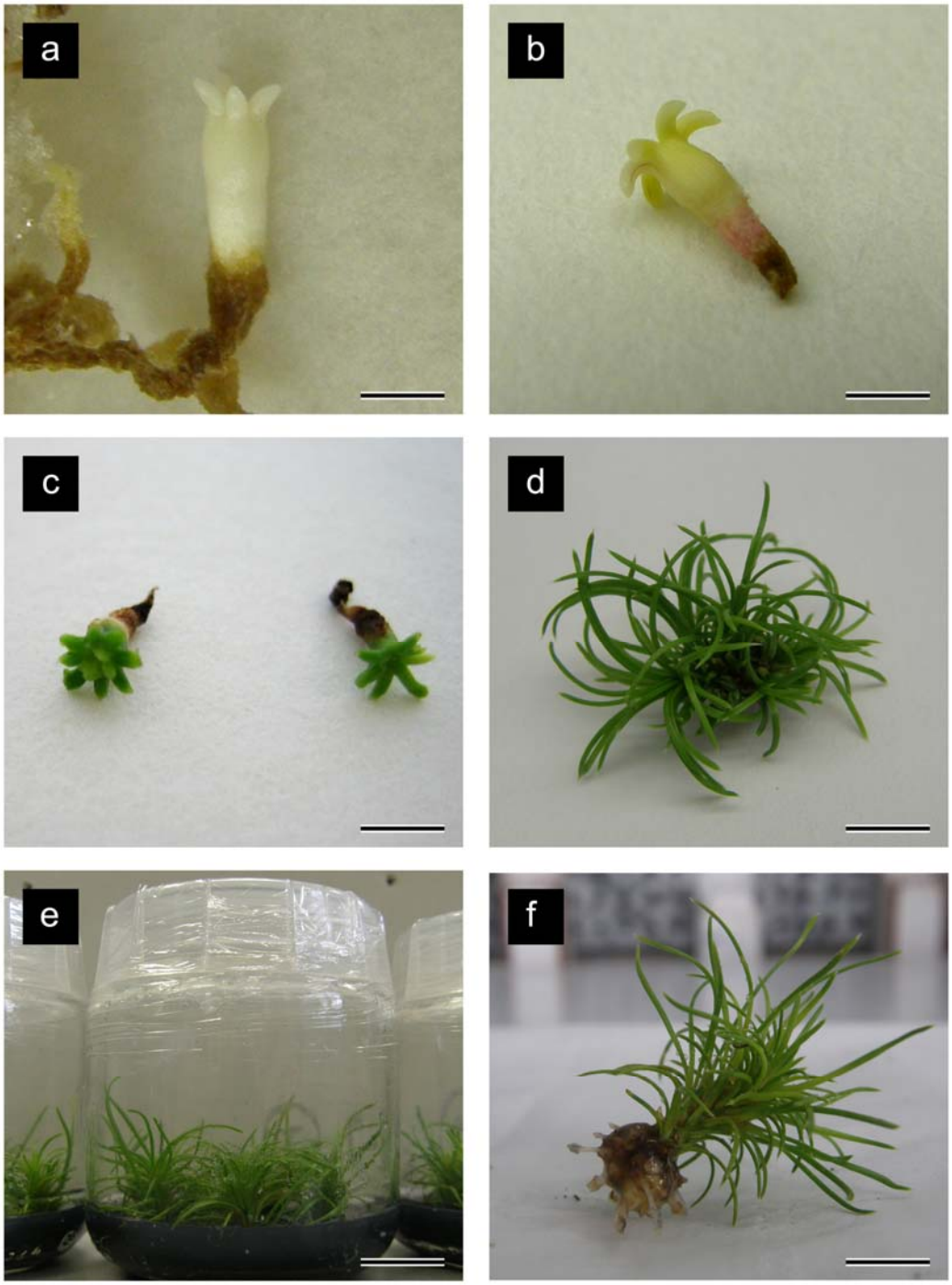

Fig. 1 Shoot induction in *Pinus radiata* somatic embryos: (a) non-germinated mature somatic embryos (bar=3 mm); (b) somatic embryo germinated for 1 week under dim light on LPE (bar=4 mm); (c) germinated somatic embryos cultured for 3 weeks on 1/2LP supplemented with 4.4 µM BA (bar=8 mm); (d) non-germinated somatic embryos cultured for 3 weeks on 1/2LP supplemented with 4.4 µM BA, after 4 weeks on the same basal medium lacking growth regulators, and 8 weeks on LPE (bar=11 mm); (e) rootable explants obtained from non-germinated somatic embryos cultured for 3 weeks on 1/2LP supplemented with 4.4 µM BA, after 4 weeks on 1/2LP without growth regulators, and 5 months on LPE (bar=22 mm); (f) explant cultured for 3 weeks on 1/4LP medium supplemented with 1 mg L^{-1} IBA and 0.5 mg L^{-1} NAA (MIX), after 4 weeks on LPE (bar=14 mm).

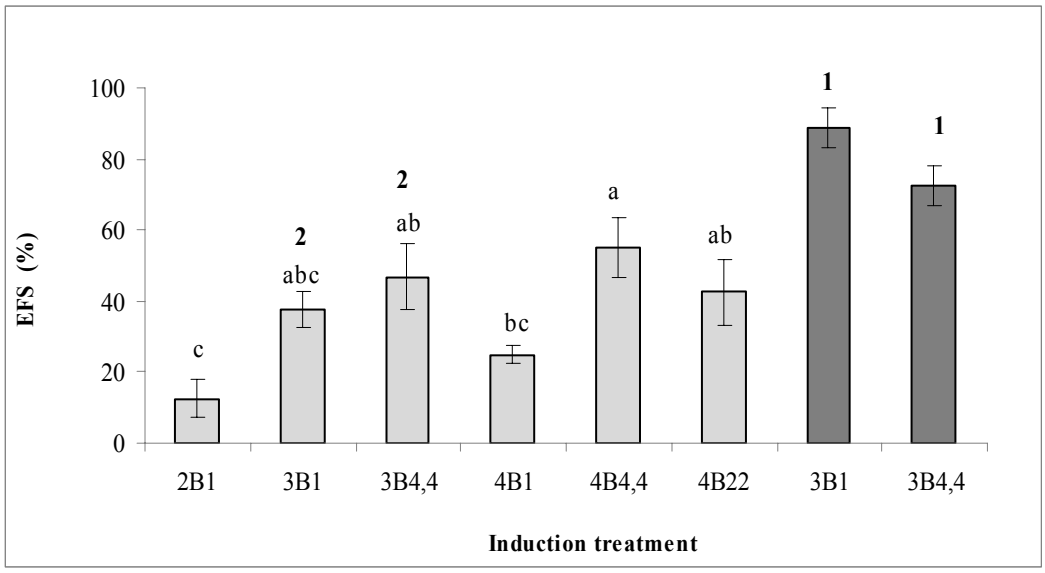

Fig. 2 Embryos forming shoots [EFS (%)] for non-germinated (NG, light grey) and germinated (G, dark grey) *Pinus radiata* somatic embryos cultured on 1/2LP supplemented with 1 µM BA for 2 weeks (2BA1), with 1 or 4.4 µM BA for 3 weeks (3BA1, 3BA4.4) and with 1, 4.4 or 22 µM BA for 4 weeks (4BA1, 4BA4.4, 4BA22) (M±S.E.). Different letters show significant differences among treatments in NG embryos; and different numbers show significant differences between NG and G embryos for treatments with 1 or 4.4 µM BA for 3 weeks by Duncan's post hoc test ($p \leq 0.05$).

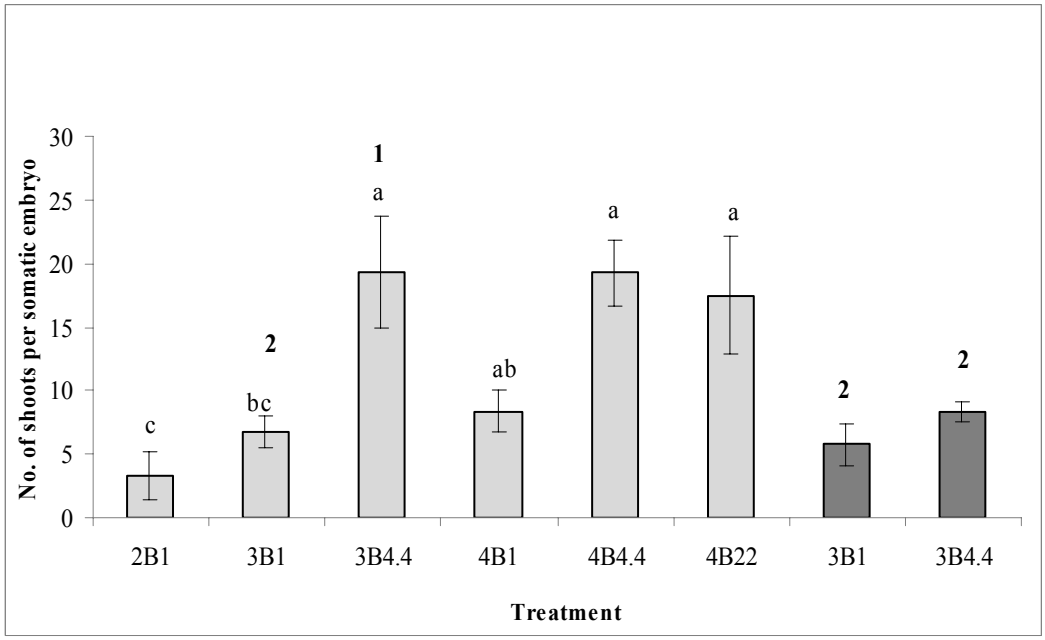

Fig. 3 Number of shoots per embryo in non-germinated (NG, light grey) and germinated (G, dark grey) *Pinus radiata* somatic embryos cultured on 1/2LP supplemented with 1 µM BA for 2 weeks (2BA1), with 1 or 4.4 µM BA for 3 weeks (3BA1, 3BA4.4) and with 1, 4.4 or 22 µM BA for 4 weeks (4BA1, 4BA4.4, 4BA22) (M±S.E.). Different letters show significant differences between different treatments in NG embryos; and different numbers show significant differences between NG and G embryos for treatments with 1 or 4.4 µM BA for 3 weeks by Duncan's post hoc test ($p \leq 0.05$).

When the SEC value was analysed, G embryos and NG embryos from 3BA4.4 treatment presented significantly higher SEC indices than NG embryos after treatment 3BA1 (Fig. 4). NG embryos cultured with 3BA4.4, 4BA4.4 and 4BA22 achieved the best SEC indices (9.1, 10.2 and 7.4, respectively). Moreover, NG embryos induced with 1 µM BA for 3 or 4 weeks led to significantly higher SEC values (2.5 and 2.1, respectively) than NG embryos treated with the same concentration of BA for 2 weeks (0.4) (Fig. 4).

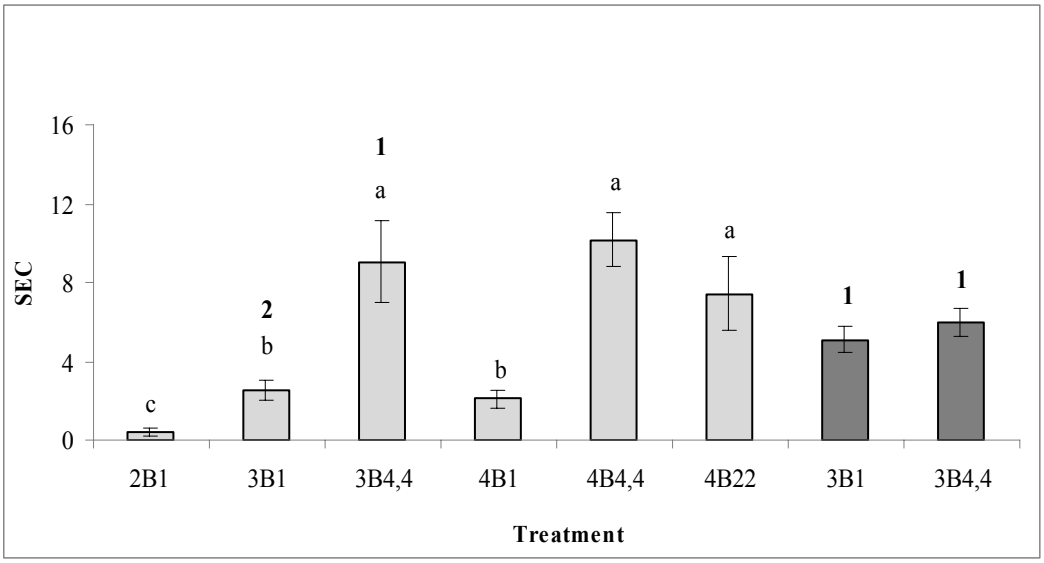

Fig. 4 Shoot elongation capacity (SEC) in non-germinated (NG, light grey) and germinated (G, dark grey) *Pinus radiata* somatic embryos cultured on 1/2LP supplemented with 1 µM BA for 2 weeks (2BA1), with 1 or 4.4 µM BA for 3 weeks (3BA1, 3BA4.4) and with 1, 4.4 or 22 µM BA for 4 weeks (4BA1, 4BA4.4, 4BA22) (M±S.E.). Different letters show significant differences between different treatment in NG embryos; and different numbers show significant differences between NG and G embryos for treatments with 1 or 4.4 µM BA for 3 weeks by Duncan's post hoc test ($p \leq 0.05$).

3.3. Root induction

There were significant differences for the percentage of shoots with roots among root induction treatments. The percentage of explants rooted with NAA or MIX under a 16 h photoperiod of 120 mmol m^{-2} s^{-1} was significantly higher than the percentage of rooted explants obtained when shoots were cultured with IBA under the same light conditions (Table 2). When explants were cultured in darkness for the first week of the root induction stage, the percentage of rooted explants was not significantly different for the different root induction treatments tested (IBA, MIX and NAA). Gathering the results for the two light regimes, the

percentage of explants rooted with NAA and MIX produced significantly higher percentages of rooted explants than IBA (Table 2).

When comparing the root induction treatment assayed separately for the different light regimes tested, shoots rooted in darkness with NAA or MIX gave significantly worse rooting percentages (25% and 20%, respectively) than those rooted under a 16 h photoperiod (60% with NAA and 50% with MIX). The percentage of explants rooted with IBA was the same under the two light regimes assayed (5%) (Table 2).

Table 2 *Pinus radiata* rooted somatic shoots (%) after different root induction treatments: on 1/4LP supplemented with 1.5 mg L^{-1} naphthaleneacetic acid (4NAA), 1.5 mg L^{-1} indole-3-butyric acid (4IBA), or 1 mg L^{-1} NAA with 0.5 mg L^{-1} IBA (4MIX). Shoots were obtained from somatic embryos after induction treatment 3BA4.4 (BA at 4.4 μM for 3 weeks) (M±S.E.). Different letters within a column and different numbers within a row show significant differences by Duncan´s post hoc test ($p \leq 0.05$) (n.s.: non significant).

Rooting treatment	Light	Darkness	Total
4IBA	5 ± 3.3 b n.s.	5 ± 3.3 n.s. n.s.	5 ± 3.3 b
4MIX	50 ± 10.2 a 1	20 ± 5.0 n.s. 2	33.3 ± 7.2 a
4NAA	60 ± 12.7 a 1	25 ± 7.9 n.s. 2	42.5 ± 9.2 a

4. DISCUSSION

The ability to produce morphologically and developmentally normal embryos and from undifferentiated somatic cells in culture, through the process of SE, resides uniquely within the plants (Zimmerman 1993). This unique developmental potential has been recognized both as an important pathway for the regeneration of plants and as a potential model for studying early regulatory and morphogenetic events in plant development (Zimmerman 1993). But, as we have pointed above, one of the major bottlenecks in conifer SE is the conversion of the ET into plants. It results, particularly in *Pinus* genus, in a limiting factor to broader application of SE in clonal propagation and genetic engineering (Klimaszewska et al. 2007).

In this study we have tried to overcome the low frequency of plantlet regeneration often encountered in pines by means of shoot organogenesis. Our results indicate that explant type, cytokinin induction period and BA concentration significantly affect the percentage of responding somatic embryos and the number of shoots per embryo obtained. However, the

success in the induction of organogenesis from somatic embryos resembled the results obtained when the same methodology was used in zygotic embryos (Chapter 1).

In *Pinus radiata* zygotic embryos, it has been proposed that the high bud-forming capacity of cotyledons is related to the undifferentiated state of cotyledonary cells at the time of culture (Yeung et al. 1981; Aitken-Christie et al. 1985). But in several pine species, it is a current practice to germinate whole embryos or cotyledons for a certain time, ranging from hours to days, before applying any induction treatment (Valdés et al. 2001; Villalobos-Amador et al. 2002; Hargreaves et al. 2005). In our experiments explants germinated for one week before the induction treatment showed the highest percentages of EFS (88.9% for treatment 3BA1 and 72.2% for treatment 3BA4.4), whereas NG embryos did not present percentages higher than 47% for the same treatments. On the contrary, in *Pinus ayacahuite*, Saborio et al. (1997) suggested that most cotyledonary cells of embryos kept in culture for six or more days might have lose ability to dedifferentiate and therefore were unable to respond to the bud induction conditions. Martinez Pulido et al. (1990) also found that explants of *Pinus canariensis* too young or too old (germinated for 1 day or 8 days) gave worse results than explants germinated for 3 days.

In our experiments, the presence of AC in the germination medium could have had a beneficial effect due to its property of adsorbing residual plant growth regulators (Von Aderkas et al. 2002). Auxins and cytokinins are the two main growth regulators in plants involved in the regulation of division and differentiation and have been proved to play an important role in the induction of somatic embryogenesis (Fehér et al. 2003); later, in SE process the development of somatic embryos is induced by application of abscisic acid to the medium (Stasolla and Yeung 2003). The germination step before shoot induction could have made the G embryos prone to an organogenic response, eliminating a growth regulator excess and preparing the explants for shoot induction.

In NG embryos, the percentage of organogenic embryos was higher when BA concentration was increased from 1 μM to 4.4 μM; a higher BA concentration did not gave better percentages; similar results were found in mature zygotic embryos of *Pinus halepensis* (Lambardi et al. 1993). In accordance with these authors, increasing the exposure time from 3 to 4 weeks had not a significant effect on the percentage of responding embryos. In the same line, Moncaleán et al. (2005) found in *P. pinea* that increasing the exposure time from 2 to 5 weeks did not affect the percentage of responding embryos. The highest EFS percentages

were obtained when G embryos were used as initial explants, independently of the BA concentration. Thus the source of material (G versus NG embryos) seemed to be the most critical factor; similar findings were reported by Martinez Pulido et al. (1990) in *P. canariensis* cotyledons germinated for 3 or 5 days, although these authors observed a decrease in the organogenic response when the explants were germinated for a longer period.

Hyperhydric shoots only appeared when induction treatments lasted 4 weeks, and in percentages ranging from 5 to 22%. In this sense, Capuana and Giannini (1995) observed in *P. pinea* that exposures longer than 3 weeks to BA produced shoots that showed callusing and hyperhydricity. In general terms, contamination percentages remained low.

The highest number of shoots per embryo was obtained in NG embryos induced with treatments 3BA4.4, 4BA4.4 and 4BA22 (19.3, 18.5 and 17.5, respectively). In previous experiments with *P. radiata* zygotic embryos cultured under the same conditions, we found treatment 3BA4.4 the most productive (data not shown). Biondi and Thorpe (1982) obtained similar results in germinated zygotic cotyledons of the same species, cultured for 3 weeks in medium supplemented with 5μM BA. When these NG embryos were cultured under a lower BA concentration or for a shorter period of time, the number of shoots per embryo was significantly lower; these results are in agreement with the observations made by Moncaleán et al. (2005) in stone pine cotyledons. In our experiments, the number of shoots per embryo from G embryos was not statistically different from the values obtained in NG embryos cultured with 1μM BA. Webb et al. (1988) also found that low BA concentration and germination for longer than 6 days, negatively affect the production of shoots in zygotic cotyledons of eastern white pine. In this context, Valdés et al. (2001) reported a drop in the number of buds per cotyledon in *Pinus pinea* when the explants were germinated before the induction treatment.

The SEC incorporates the EFS percentage and the number of shoots per embryo. The combination of these factors offers a realistic assessment of the efficacy of the culture conditions (Martínez Pulido et al. 1992). The lowest SEC values were found NG somatic embryos treated with 1μM BA. NG embryos induced with 4.4 μM BA for 3 and 4 weeks or BA at 22 μM for 4 weeks achieved the highest SEC values. In contrast, in previous experiments with *Pinus radiata* zygotic embryos (Chapter 1) the SEC values obtained in treatments 2BA1 and 3BA4.4 were similar to the SEC index obtained in NG embryos induced BA4,4 during 3 weeks.

When the initial type of explant was compared, the SEC value was similar in G embryos and NG embryos, except for NG embryos induced with 1 μM BA for 3 weeks. The SEC indices in G embryos were higher than the indices reported by Álvarez et al. (2009) in *P. pinaster* germinated cotyledons; the reason for better values in our experiments could be the abovementioned higher organogenic response in G explants when compared with NG explants. Similar findings have been found in some angiosperms species such as red horse chestnut (Zdravković-Korać et al. 2008).

Rooting percentage of shoots was significantly affected by the rooting treatment used. In accordance with our results in organogenesis from zygotic embryos, explants treated with NAA-L and MIX-L showed the higher rooting percentages (60% and 50% respectively) than explants treated with IBA (5%). In radiata pine *in vitro* organogenesis it is a common practice to use a mixture of IBA and NAA for rooting (Prehn et al. 2003; Hargreaves et al. 2005). NAA alone has also been reported to be effective in *in vitro* rooting of several pine species such as: *P. ayacahuite* (Saborio et al. 1997) and *P. pinaster* (Álvarez et al. 2009).

The light regime also influenced the process, in conifers a dark period at the beginning of the rooting stage is often required for root formation and generally a reduction of light intensity favours adventitious root development (Brassard et al. 1996; Alonso et al. 2006). Our results are according with other reports in *Pinus* genus where rooting of explants is higher under a 16 h photoperiod (Flygh et al. 1993; Tang and Guo 2001).

Further testing on the performance of the rooted explants in the greenhouse will determine if the best treatments for *in vitro* rooting lead to the best acclimatization rates or on the contrary, the explants rooted with IBA show a better *ex vitro* performance as observed in radiata pine zygotic embryos (data not shown). IBA is the auxin currently used for rooting of cuttings of *P. radiata* and has been used in *in vitro* rooting protocols of *P. canariensis* (Martínez Pulido et a. 1994) and *P. heldreichii* (Stojičić et al. 1999).

In the last few years several SE and organogenesis protocols have been developed in parallel instead of combining these *in vitro* techniques, in angiosperm species such as *Manihot esculenta* (Guohua 1998), *Gossypium* spp. (Khan et al. 2006), *Aesculus carnea* (Zdravković-Korać et al. 2008) and *Hippophae rhamnoides* (Sriskandarajah and Lundquist 2009). The goal of these studies was to study the potential of both techniques, through media and growth regulator adjustments, in the regeneration of these species.

A few protocols combining SE and organogenesis have been described in angiosperms in order to maximize plant production from genetically transformed ET (Kim et al. 2009) or to regenerate endangered species (Siva et al. 2009). In conifers there are no reports in this line, although this methodology could overcome some problems associated with SE such as the aforementioned seasonality of ET initiation or a low embryo production from the ET obtained. The latter problem is especially important in the case of genetically transformed ETs, where the plantlet regeneration is often low (Giri et al. 2004).

We have established an efficient plant regeneration system via a combined pathway of SE and organogenesis. Once the somatic embryos are obtained, up to 19 rootable shoots can be obtained from a single embryo, these shoots presented rooting percentages around 60%. Considering that we can obtain more than 150 embryos per 100 mg of ET (Montalbán et al. 2010), theoretically using the described method more than 1700 rooted shoots can be produced. Moreover, these shoots before rooting can be propagated and continuously used as a source for plant regeneration.

Furthermore, this approach facilitates the application of genetic manipulation procedures; and over time, numerous plants, representing clones, can be regenerated from a single shoot. Thus, *in vitro* micropropagation via induction of SE and organogenesis can be very useful for plant genetic resource management. To the best of our knowledge, this is the first attempt to report the induction of organogenesis in somatic embryos of *Pinus radiata* D. Don.

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CAPÍTULO 5

Endogenous cytokinin and auxin profiles during *in vitro* organogenesis from vegetative buds of radiata pine adult trees and their relationship with the organogenic response

Este capítulo se corresponde con el artículo: Endogenous cytokinin and auxin profiles during *in vitro* organogenesis from vegetative buds of radiata pine adult trees and their relationship with the organogenic response (En preparación).

Capítulo 5: Endogenous cytokinin and auxin profiles during *in vitro* organogenesis from vegetative buds of radiata pine adult trees and their relationship with the organogenic response

1. INTRODUCTION

Clonal forestry is widely discussed to cope with the expected increasing demand for wood during the next decades (Fenning and Gersheazon 2002). Vegetative propagation of trees is an effective way to capture genetic gain and produce large amounts of selected plant material. Cloning can be achieved by grafting, rooting of cuttings and *in vitro* techniques. All propagation methods have limitations, and conifers are generally more difficult to clone than most angiosperm woody trees (Von Aderkas and Bonga 2000). Conifers can be grafted at almost all ages, but grafting is not practical on a large scale. Moreover, rooting of cuttings is only possible in young trees (Bonga and Von Aderkas 1993). Organogenesis and somatic embryogenesis are difficult to achieve in adult tissues but the economic value of a tree can only be assessed after it reaches maturity (Greenwood 1995; Cortizo et al. 2009a).

Besides, maturation and ageing woody plants modify the physiology and morphology of the tree. In *Eucalyptus*, differences in the morphology of shoot apical meristem domes between juvenile, mature and *in vitro* material have been documented (Mankessi et al. 2010). In *Pinus radiata*, the transition from juvenile to mature phase is characterized by changes in meristem behaviour, reduced efficiency in rooting cuttings, decrease in the diameter growth rate of the rooted cuttings, onset of pollen and cone formation and changed bud and foliar morphology (Menzies et al. 2000). All these changes reduce the propagation potential of the tree (Von Aderkas and Bonga 2000; De Diego et al. 2008). The previously mentioned decrease in organogenic potential is usually reverted in breeding programs by grafting or other reinvigoration procedures to enable vegetative propagation of trees (Valdés et al. 2004a). Reinvigoration can be defined as a temporary increase in vigour that enables the recovery of some organogenic abilities (Valdés et al. 2003).

In order to overcome the abovementioned bottlenecks, it is necessary to develop new biotechnological tools (Davis and Becwar 2007). The application of these biotechnologies to breeding programs could raise the yield of plantation forests, assuring the future wood demand sustainably and finally protecting the natural forests (Walter 2004).

In *Pinus radiata*, whole plants have been regenerated from juvenile explants since the seventies (Reilly and Washer 1977; Aitken-Christie et al. 1988) and in the last decade protocols describing somatic embryogenesis in this species have been reported (Walter et al. 1998; Hargreaves et al. 2009). Although these techniques seem to be amenable for large scale production, they have to be combined with other techniques such as cryopreservation, because the plantlets produced may not bear the features of interest and need to be tested in the field (Park 2002). These problems could be solved with the implementation of an efficient system to clone adult trees, and thus eliminating the uncertainty prior to test in the field.

In this species one of the first reports on micropropagation from adult tissues was by Horgan (1987) who produced plants from shoot tips of 8-17 years old trees. In the same way, Prehn et al. (2003) cloned 3-7 years old trees using as initial explants apical meristems. In these protocols the reinvigoration of the tissue was achieved through BA application to the induction medium.

It is clear that the physical and chemical culture conditions, and especially the exogenously applied cytokinins (CKs), influence the organogenic process (Thorpe et al. 1991) as well as other aspects of plant growth, development and physiology such as cell division, organ formation and regeneration, apical dominance, vascular development, nutrient mobility, and senescence (Haberer and Kieber 2002; Hwang and Sakakibara 2006). Recently, in addition to aforementioned effects on plant development, CKs are been studied due to their ability to induce apoptosis and/or block cell cycling in a wide range of cancer cells, making them potential candidates as drugs for treating cancer (Voller et al. 2010).

CKs found in plants are adenine derivatives substituted at the N^6-position with either isoprenoid or an aromatic side chain (Mok and Mok 2001). While isoprenoid cytokinins are ubiquitous in plants, aromatic CKs (represented by N^6-benzyladenine (BA), and its hydroxylated derivatives, the Topolins) have only been identified, as yet, in a limited group of plant taxa (Strnad et al. 1997). Natural CK bases have the corresponding nucleosides,

nucleotides, and glycosides (Sakakibara 2006). Collectively, aromatic CKs are distinguishable from the isoprenoid CKs both chemically and in the spectrum of their biological activities.

CK metabolic pathways can be broadly classified into two types: the modification of the adenine moiety and the modification of the side chain. The simultaneous occurrence of CK bases with the corresponding nucleosides and nucleotides in plant tissues suggests that the metabolic flow from CK nucleotides to the active bases is probably not unidirectional but circular (Sakakibara 2006).

In 1983, Letham and Palni described CK bases and their riboses as active forms, and conversion of free to glucosylated forms was considered as storage or detoxification pathway. Glucosylation of CKs has been observed at the N^3, N^7, and N^9 position of the purine moiety as N-glucosides, and at the hydroxyl group of the side chain of several CKs as O-glucosides. O-glucosylation is reversible and deglucosylation is catalyzed by β-glucosidase (Brzobohaty et al. 1993). It has been suggested that the readily cleaved O-glucosides represent reversible inactive stable storage forms of CKs. On the other hand, N-glucoconjugates are not efficiently cleaved by β-glucosidase and they are considered to be irreversible deactivation products (Van Staden and Drewes 1992).

In summary, the active CKs pool is regulated during biosynthesis by uptake from extracellular sources, metabolic interconversions, inactivation, degradation, and signal transduction and transport (Mok and Mok 2001; Kakimoto 2003).

There are some studies describing the effect of the culture conditions in the endogenous CK levels in angiosperms (Auer et al. 1992; Feito et al. 1994; Moncaleán et al. 1999) and gymnosperms (Moncaleán et al. 2005; Cuesta et al. 2009). Recently, Zhang et al. (2010) studied the effect of the BA application to the culture medium in the reinvigoration of *P. radiata* buds *in vitro*, and in some endogenous CK levels in the buds.

As mentioned in Chapter 4, during the last years our research group has focused on the development of *in vitro* regeneration protocols for *P. pinaster* (De Diego et al. 2008), *P. pinea* (Cortizo et al. 2009a) and *P. sylvestris* (De Diego et al. 2010). In these protocols, zeatin (Z) and *meta*-topolin (mT) have been studied as an alternative to BA for micropropagation. Till date, there is no report on *P. radiata* organogenesis where the effect of these CKs has been studied.

The aim of this work was to determine the best culture conditions (testing different CK types and concentrations and induction periods) for the reinvigoration of *Pinus radiata* buds *in vitro*. Moreover, we are interested in studying the effect of these conditions in the endogenous levels of CKs and indole-3-acetic acid (IAA) at different stages of the organogenic process in order to find a physiological marker of the organogenic potential in *Pinus radiata*.

2. MATERIALS AND METHODS

2.1. Plant material

Five trees (7, 10, 67, 72 and 76) were selected from a seed orchard established by Neiker-Tecnalia in Amurrio-Spain (latitude: 43º03'00''N, longitude: 3º10'00''W, elevation: 200 m). These trees were 15 years old. Apical vegetative buds (3–5 cm long) were collected from mid-basal part of the trees, the first and the last week of January in 2009 (Fig. 1a); the dormant buds were wrapped in moist paper to prevent dehydration and stored in polyethylene bags at 4ºC for a maximum of a week.

Buds were sprayed with 70% (v/v) ethanol, and then rinsed with sterile distilled H_2O. Afterwards, the buds were submerged in 50% (v/v) commercial bleach (active chloride >5%) plus 2 drops of Tween 20® and agitated for 30 min. Finally, they were rinsed three times in sterile distilled H_2O in aseptic conditions.

Subsequently, bud scales were removed and the buds were cut transversely into 0.5–1.0 cm thick slices with a surgical scalpel blade. The slices were laid on culture medium.

2.2. Organogenic process

Buds slices were cultured on modified LP basal medium (Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988). Three CKs were individually assayed: BA, mT, and zeatin (Z) at two concentrations (25 and 50 μM) and for two different induction periods (6 or 8 weeks) (Fig. 1b).

The induction media were supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} Difco Agar® granulated. The pH was adjusted to 5.8 before autoclaving at 121ºC for 20 min. Five bud slices were cultured on each 90 x 20 mm Petri dish. Cultures were maintained at 21±1°C

under a 16 h photoperiod of 120 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France).

After induction period (6 or 8 weeks), explants were transferred to elongation medium (LPAC) for 4 weeks (Fig. 1c). This medium was the previously described LP medium but lacking plant growth regulators and supplemented with 0.2% (w/v) activated charcoal (AC) and 10 g L^{-1} Difco Agar® granulated. Then, explants were transferred again to the induction media in the same culture conditions for another 4 weeks (re-induction period). Finally, explants were subcultured again on LPAC for 4 weeks.

2.3. Quantitative analysis of cytokinins by liquid chromatography–single-quadrupole mass spectrometry

Samples were collected from trees 72 and 76 at the beginning of the experiment and after the first induction period of 8 weeks from each induction treatment. Another set of samples was collected from tree 76 after the second induction period of 4 weeks and at the end of the experiment. The plant material was deeply frozen in liquid nitrogen, ground with a mortar and pestle and lyophilised prior to extraction and purification.

The extraction, purification and quantitative analysis of CKs were performed in the Laboratory of Growth Regulators (Palacký University & Institute of Experimental Botany, Olomouc, Czech Republic) following the methodology described by Novák et al. (2003; 2008).

Samples of 50 mg dry weight (DW) were placed individually in 1 mL of Bieleski buffer (Bieleski 1964) (60% (v/v) methanol, 25% (v/v) $CHCl_3$, 10% (v/v) HCOOH) and re-extracted in 300 μl of 50% (v/v) methanol with 2% (v/v) HCOOH in 1.5 mL Eppendorf tubes. Stable isotope-labelled CK internal standards were added to each sample (1 pmol of each compound per sample) to check the recovery during purification and to validate the quantification. The following internal standards were used: [$^2H_5$]tZ, [$^2H_5$]tZR, [$^2H_5$]tZ9G, [$^2H_3$]DHZ, [$^2H_3$]DHZR, [$^2H_3$]DHZ9G, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]]iP9G, [$^2H_5$]tZOG, [$^2H_5$]tZROG, [$^2H_5$]tZRMP, [$^2H_3$]DHZRMP, [$^2H_6$]]iPRMP.

Plant material was extracted using a vibration mill at a frequency of 27 Hz for 3 min after adding 3 mm tungsten carbide beads to each tube to increase the extraction efficiency. The tube contents were sonicated for 3 min and then stirred for 30 min at 4ºC. After centrifugation

(3 min, 15000 rpm, 4ºC) the supernatants were transferred to glass tubes and stored at 4ºC. The pellets were re-extracted in the same way for 30 min at 4ºC and after centrifugation (3 min, 15000 rpm, 4ºC) both supernatants were combined and immediately purified.

The purification step, involved passing the samples through 100 mg SCX columns, activated with 2 mL of 100% methanol (high performance liquid chromatography, HPLC grade) and equilibrated with 2 mL of 50% (v/v) methanol containing 2% (v/v) HCOOH. In each sample, after applying the extract, the column was washed with 2 mL of Bieleski buffer followed by 2 mL of 100% methanol. Target compounds were subsequently eluted with 3 mL of 4 M NH_3 in 60% (v/v) methanol**.** After two ion-exchange chromatography (IEC) steps (SCX, DEAE-Sephadex combined with SPE C18-cartridges) the samples were split into two fractions. The first fraction included CK free bases, ribosides, 9-glucosides and O-glucosides, the second fraction contained ribonucleotides. Partially purified samples were evaporated to dryness, dissolved in 50 µl 70% (v/v) ethanol and 450 µl phosphate buffer (PBS) (50 mM NaH_2PO_4, 15 mM NaCl, pH 7.2).

The first fraction (CK free bases, ribosides, 9-glucosides and O-glucosides) was subsequently applied onto pre-immune and immunoaffinity columns.

Pre-immune columns were prepared by coupling rabbit IgG to Affi-Gel 10 (25 mg mL^{-1}) as a pre-cleaning step to eliminate compounds that non-specifically bound to immunoaffinity gel (IAG). IAG based on a generic monoclonal antibody raised against ortho-topolin riboside, as described by Novák et al. (2003), was used for the sample purification and CK isolation.

The pre-immune column was equilibrated with 10 mL PBS before sample loading. The sample (0.5 mL) was applied onto pre-immune column, and then the pre-immune column was washed with 0.5 mL H_2O.

The immunoaffinity column was equilibrated with 10 mL PBS before sample loading. The sample was repeatedly (five times) applied onto immunoaffinity column. Then the column was rinsed with 0.5 mL H_2O and this eluate (1.5 mL) contained O-glucoside derivatives not retained on the IA-columns. Then the column was rinsed with 10 mL PBS and 10 mL water and the bound CKs (CK free bases, ribosides and 9-glucosides) were eluted by 3 mL 100% methanol. O-glucoside derivatives were treated with β-glucosidase and immuno-purified again in order to obtain the O-glucoside (OG) fraction.

The second fraction (CK ribonucleotides) was treated with alkaline phosphatase and subsequently purified using pre-immune and immunoaffinity columns, as described above. According to this purification protocol, ribonucleotides and O-glucosides were analysed after enzyme treatment as appropriate ribosides and bases, respectively. Purified samples were evaporated to dryness in a Speed-Vac concentrator and analysed by ultra-performance liquid chromatography–electrospray tandem mass spectrometry (Novák et al. 2008).

2.4. Quantitative analysis of IAA by liquid chromatography–single-quadrupole mass spectrometry

Free indole-3-acetic acid (IAA) was determined in the samples described previously for the CK analyses. The extraction, purification and quantitative analysis of IAA were performed in the Laboratory of Growth Regulators (Palacký University & Institute of Experimental Botany, Olomouc).

The plant material was deeply frozen in liquid nitrogen, ground with a mortar and pestle and lyophilised, prior to the extraction and purification. Samples of 5 mg DW were extracted for 5 min with 1 mL of cold PBS containing 0.02% sodium diethyldithiocarbamate (w/v) and [$^2H_5$]IAA as internal standard.

After centrifugation (10 min, 15000 rpm, 4 ◦C) each sample was transferred into a tube, acidified with 1 M HCl to pH 2.7 and applied on a C18 column pre-washed with 2 mL of 100% methanol and equilibrated with 2 mL of 1% (v/v) HCOOH. The eluate was evaporated to dryness in vacuum. Final analysis was done by HPLC coupled to tandem MS/MS detection with the use of a triple-quadrupole mass spectrometer.

2.5. Data collection and statistical analyses

After one month on LPAC (Fig. 1d), the percentage of bud slices forming shoots (BFS), the number of shoots > 3mm per bud slice and the shoot elongation capacity (SEC) index were recorded. The SEC index was calculated as follows (Lambardi et al. 1993):

SEC= (average number of shoots > 3mm per bud slice) x (BFS, %)

Normality was evaluated by Shapiro–Wilk normality test (Shapiro and Wilk, 1965) and the equal variance of the data was analysed by Levene's test (Brown and Forsythe 1974). The analyses were performed using the SPSS© version 15.0 software package.

Analysis of variance (ANOVA) was carried out to determine differences between treatments for the BFS percentage, the number of shoots per bud slice and SEC index. Data for the number of shoots per bud slice and the SEC index were subjected to log (x) transformation. Multiple comparisons were made using Duncan´s post hoc test ($p \leq 0.05$).

Data for the different CK types and fractions, for the IAA and for the ratios were subjected to ANOVA to determine differences between treatments. Multiple comparisons were made using Scheffe´s post hoc test ($p \leq 0.05$).

3. RESULTS

3.1. Organogenic process

BFS did not show significant differences among induction periods (Table 1). When the induction periods (6 or 8 weeks) were analysed separately, considering all the induction treatments together, significant differences were found among genotypes for the BFS percentage. In both induction periods, genotype 7 and 76 showed the highest organogenic response (between 49.2% and 69.2 %) presenting significant differences with the other genotypes tested. Genotypes 67, 10 and 72 showed induction percentages below 23% (Table 1).

When all genotypes were analysed together, neither the CK type nor the concentration had a significant effect on the BFS (Tables 2). In both induction periods, BA at 50 µM induced the highest BFS percentage (52.5% and 57.5%). In the 6 weeks induction period the lowest BFS was observed in buds cultured with 50 µM Z, whereas in the 8 weeks induction period the lowest BFS recorded in explants cultured with 50 µM mT (Table 2).

When we analysed the results obtained in explants cultured in the presence of CK for 6 weeks (Table 3), mT induced in genotype 7 significantly higher BFS percentages than BA. Explants from this genotype treated with Z also showed higher BFS percentages than those induced with BA (Table 3). On the contrary, buds from genotype 67 cultured with 50 µM BA showed a significantly higher BFS percentage than the other treatments assayed. In this period, no significant differences were found among treatments in the other genotypes tested (Table 3).

Table 1 BFS (%) from *Pinus radiata* bud explants after *in vitro* culture on LP medium supplemented with cytokinins for 6 or 8 weeks (M±S.E.). Different letters within a column show significant differences among genotypes by Duncan's post hoc test ($p \leq 0.05$). In the last row differences among induction periods were analysed (n.s.: non significant).

BFS (%)		
Genotype	**Induction period**	
	6 weeks	**8 weeks**
7	69.2 ± 6.2 a	59.2 ± 7.6 a
10	17.5 ± 4.9 c	15.0 ± 5.0 b
67	22.5 ± 6.9 c	16.7 ± 4.9 b
72	5.8 ± 2.2 c	2.5 ± 1.4 b
76	49.2 ± 7.5 b	55.8 ± 6.9 a
Total	32.8 ± 3.3 n.s.	29.8 ± 3.2 n.s.

Table 2 BFS (%) from *Pinus radiata* bud explants after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM for 6 or 8 weeks (M±S.E.). Different letters within a column show significant differences among treatments by Duncan's post hoc test ($p \leq 0.05$) (n.s.: non significant).

BFS (%)					
Treatment	**Induction period**		**Induction period**		**CK type**
	6 weeks	**8 weeks**	**6 weeks**	**8 weeks**	
25 µM BA	36.2 ± 8.8 n.s.	30.0 ± 6.6 n.s.	37.0 ± 5.8 n.s	35.5 ± 5.6 n.s	**BA**
50 µM BA	52.5 ± 9.6 n.s.	57.5 ± 9.3 n.s.			
25 µM mT	41.2 ± 9.4 n.s.	38.7 ± 10.2 n.s.	31.5 ± 6.2 n.s	25.0 ± 5.2 n.s	**mT**
50 µM mT	35.0 ± 11.3 n.s.	22.5 ± 6.5 n.s.			
25 µM Z	48.7 ± 8.1 n.s.	33.7 ± 10.4 n.s.	30.0 ± 5.3 n.s	29.0 ± 6.1 n.s	**Z**
50 µM Z	23.7 ± 8.4 n.s.	37.5 ± 10.0 n.s.			

The results obtained in explants cultured in the presence of CKs for 8 weeks are shown in Table 4. Explants from genotype 76 cultured with BA at 50 µM presented the highest BFS percentage (90%), showing significant differences with the other treatments tested except for 50 µM Z. The other genotypes used in this experiment did not show significant differences among treatments for the BFS percentage (Table 4).

Table 3 BFS (%) from *Pinus radiata* bud explants after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM for 6 weeks (M±S.E.). Different letters

within a row show significant differences among treatments by Duncan's post hoc test ($p \leq 0.05$) (n.s.: non significant).

	BFS (%)					
Genotype	**Cytokinin treatment**					
	25 µM BA	**50 µM BA**	**25 µM mT**	**50 µM mT**	**25 µM Z**	**50 µM Z**
7	35.0 ± 17.1 c	50.0 ± 17.3 bc	90.0 ± 5.8 a	95.0 ± 5.0 a	70.0 ± 12.9 abc	75.0 ± 9.6 ab
10	15.0 ± 9.6 n.s.	15.0 ± 9.6 n.s.	15.0 ± 9.6 n.s.	5.0 ± 3.5 n.s.	50.0 ± 17.3 n.s.	5.0 ± 3.6 n.s.
67	25.0 ± 15.0 b	80.0 ± 14.1 a	5.0 ± 3.6 b	0 b	15.0 ± 10.5 b	10.0 ± 7.5 b
72	15.0 ± 9.6 n.s.	0 n.s.	5.0 ± 3.7 n.s.	5.0 ± 3.8 n.s.	5.0 ± 3.9 n.s.	5.0 ± 3.7 n.s.
76	70.0 ±19.1 n.s.	65.0 ± 22.2 n.s.	55.0 ± 9.6 n.s.	40.0 ± 24.5 n.s.	60.0 ± 8.2 n.s.	5.0 ± 3.8 n.s.

Table 4 BFS (%) from *Pinus radiata* bud explants from different genotypes after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM for 8 weeks (M±S.E.). Different letters within a row show significant differences among treatments by Duncan's post hoc test ($p \leq 0.05$) (n.s.: non significant).

	BFS (%)					
Genotype	**Cytokinin treatment**					
	25 µM BA	**50 µM BA**	**25 µM mT**	**50 µM mT**	**25 µM Z**	**50 µM Z**
7	35.0 ± 15.0 n.s.	75.0 ± 9.6 n.s.	85.0 ± 9.6 n.s.	30.0 ± 19.1 n.s.	70.0 ± 23.8 n.s.	60.0 ± 23.1 n.s.
10	5.0 ± 3.5 n.s.	40.0 ± 21.6 n.s.	5.0 ± 3.7 n.s.	15.0 ± 9.6 n.s.	20.0 ± 14.1 n.s.	5.0 ± 3.8 n.s.
67	35.0 ± 15.0 n.s.	25.0 ± 15.0 n.s.	15.0 ± 9.6 n.s.	0 n.s.	15.0 ± 10.7 n.s.	10.0 ± 8.1 n.s.
72	5.0 ± 3.6 n.s.	0 n.s.	0 n.s.	5.0 ± 3.8 n.s.	0 n.s.	5.0 ± 3.7 n.s.
76	45.0 ± 9.6 bc	90.0 ± 5.8 a	50.0 ± 23.8 bc	45.0 ± 5.0 bc	30.0 ± 20.8 c	75.0 ± 9.6 ab

When all CK treatments were gathered, no significant differences between induction periods were found for the number of shoots per bud (Table 5). In both induction periods analysed, genotype 7 produced the highest number of shoots per bud (6 and 6.4 shoots for induction periods of 6 and 8 weeks, respectively). When buds were cultured with CKs for 6 weeks, genotypes 10 and 72 presented a significantly lower number of shoots (1.7 and 1.4, shoots, respectively) than the other genotypes tested. The induction period corresponding to 8 weeks provoked in genotype 7 a significantly higher number of shoots than in genotypes 10, 67 and 72 (Table 5).

Table 5 Number of shoots per *Pinus radiata* bud in different genotypes after *in vitro* culture on LP medium supplemented with different cytokinins for 6 or 8 weeks (M±S.E.). Different letters within a column show significant differences among genotypes by Duncan's post hoc test ($p \leq 0.05$). In the last row differences among induction periods are analysed (n.s.: non significant).

Shoots per bud (No)		
Genotype	**Induction period**	
	6 weeks	**8 weeks**
7	6.0 ± 0.4 a	6.4 ± 0.6 a
10	1.7 ± 0.2 c	2.2 ± 0.4 bc
67	3.9 ± 0.5 b	2.6 ± 0.4 bc
72	1.4 ± 0.3 c	1.3 ± 0.3 c
76	3.2 ± 0.3 b	3.6 ± 0.3 ab
Total	4.1 ± 0.2 n.s.	4.4 ± 0.3 n.s.

Explants were induced for 6 weeks with CKs showed significant differences for the number of shoots per bud among CK types and concentrations (Table 6). Considering only CK type, culture with mT and Z provoked a higher number of shoots per bud than culture with BA. mT and Z (at 25 and 50 µM) produced significantly higher number of shoots than 25 µM BA. The highest number of shoots per bud was observed after treatments with 50 µM mT and 50 µM Z (5.3 and 5.4, respectively) and the lowest value for this parameter was obtained in explants treated with 25 µM BA (2.7 shoots per bud) (Table 6).

After an induction period of 8 weeks, neither the CK type nor the CK concentration presented significant differences for the number of shoots per bud. The lowest values were observed when mT was applied at 50 µM (2.4 shoots per bud) or BA at 25 µM (3.2 shoots per bud); values ranging from 4.7 to 5.5 shoots per bud were obtained with the other treatments used (Table 6).

The total SEC values corresponding to induction periods of 6 and 8 weeks did not differ statistically (Table 7). When the induction period lasted 6 weeks, the best result for the SEC index was obtained in genotype 7. This value was significantly higher than those obtained in other genotypes except for genotype 67. In genotypes 67 and 76 the values for the SEC index (below 3) were significantly higher than those obtained in genotypes 10 and 72 (below 1) (Table 7).

Table 6 Shoots per bud (No.) from *Pinus radiata* bud explants after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT or Z) at 25 or 50 µM for 6 or 8 weeks (M±S.E.). Different letters within a column show significant differences among genotypes by Duncan's post hoc test ($p \leq 0.05$) (n.s.: non significant).

No. of shoots per bud					
Treatment	**6 weeks**	**8 weeks**	**6 weeks**	**8 weeks**	**CK type**
25 µM BA	2.7 ± 0.4 b	3.2 ± 0.4 n.s.	3.3 ± 0.3 b	4.2 ± 0.5 n.s.	**BA**
50 µM BA	3.7 ± 0.4 ab	4.8 ± 0.7 n.s			
25 µM mT	4.6 ± 0.6 a	5.5 ± 0.8 n.s	4.9 ± 0.4 a	4.3 ± 0.6 n.s.	**mT**
50 µM mT	5.3 ± 0.6 a	2.4 ± 0.5 n.s			
25 µM Z	4.5 ± 0.5 a	4.7 ± 0.9 n.s	4.8 ± 0.5 a	4.7 ± 0.6 n.s.	**Z**
50 µM Z	5.4 ± 1.1 a	4.7 ± 0.8 n.s			

Table 7 SEC values in *Pinus radiata* buds after *in vitro* culture on LP medium supplemented with different cytokinins for 6 or 8 weeks (M±S.E.). Different letters within a column show significant differences among genotypes by Duncan's post hoc test ($p \leq 0.05$). In the last row differences among induction periods are analysed (n.s.: non significant).

SEC		
Genotype	**Induction period**	
	6 weeks	**8 weeks**
7	5.0 ± 0.4 a	5.5 ± 0.6 a
10	0.9 ± 0.1 c	1.5 ± 0.5 b
67	2.8 ± 0.4 ab	1.4 ± 0.3 b
72	0.4 ± 0.1 d	0.3 ± 0.1 c
76	2.4 ± 0.3 b	2.8 ± 0.3 ab
Total	3.2 ± 0.2 n.s.	3.5 ± 0.3 n.s.

When the induction period lasted 8 weeks, the best SEC indices were recorded in genotypes 7 and 76 (5.5 and 2.8, respectively) (Table 7). Genotypes 10 and 67 presented SEC indices significantly lower than genotype 7 and significantly higher than genotype 72. As observed in the 6 weeks induction period, genotype 72 presented the worst SEC index (0.3) (Table 7).

In the 6 weeks induction period, explants cultured with BA had significantly lower SEC indices than those cultured with mT (Table 8). When CK types and concentrations were considered separately, the highest SEC index was observed in explants cultured with 50 µM

mT (4.8) although no significant differences were observed with the other treatments assayed, except for 25 µM BA (1.9) (Table 8).

When explants were subjected to an induction period of 8 weeks, no significant differences were observed for the CK type (Table 8). In this period, BA at 25 µM produced a significantly lower SEC index than other treatments, except for 50 µM mT. The lowest concentration of mT induced the highest SEC index (4.9). On the contrary of the results obtained in the 6 weeks induction period, the lowest SEC index (1.2) was achieved after culture with the highest concentration of mT (Table 8).

Table 8 SEC values in *Pinus radiata* buds after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT or Z) at 25 or 50 µM for 6 or 8 weeks (M±S.E.). Different letters within a column show significant differences among genotypes by Duncan's post hoc test ($p \leq 0.05$) (n.s.: non significant).

SEC					
Treatment	**Induction period**		**Induction period**		**CK type**
	6 weeks	**8 weeks**	**6 weeks**	**8 weeks**	
25 µM BA	1.9 ± 0.3 b	1.7 ± 0.2 b	2.5 ± 0.2 b	3.1 ± 0.4 n.s.	**BA**
50 µM BA	3.0 ± 0.4 a	3.8 ± 0.5 a			
25 µM mT	3.5 ± 0.5 a	4.9 ± 0.8 a	4.1 ± 0.4 a	3.5 ± 0.6 n.s.	**mT**
50 µM mT	4.8 ± 0.6 a	1.2 ± 0.3 b			
25 µM Z	3.2 ± 0.5 a	4.4 ± 0.9 a	3.5 ± 0.5 ab	4.1 ± 0.6 n.s.	**Z**
50 µM Z	4.1 ± 1.1 a	3.8 ± 0.8 a			

As mentioned before, there were no significant differences among induction periods for the BFS percentage, the number of shoots per bud and the SEC index. On the contrary, significant differences were found among genotypes for the three parameters studied (Tables 1, 5 and 7). On the basis of these results, two genotypes cultured in presence of CK for 8 weeks were chosen for CK analysis. These genotypes were classified as highly organogenic (76) and non-organogenic (72) according to their BFS percentages (Table 1). The non-organogenic genotype (72) did not show SEC indices higher than 0.6. In contrast and as we can observe in Fig. 2, the results for the SEC indices in the organogenic genotype (76) ranged from 0.9 to 4.2. Buds from genotype 76 cultured with 50 µM BA achieved the highest SEC index (4.2), significantly lower values were obtained in those cultured with 25 µM BA (1.6) or 50 µM mT (0.9).

Taking into account the results obtained for the three parameters studied, we can consider 50 µM BA for 8 weeks as the best induction treatment.

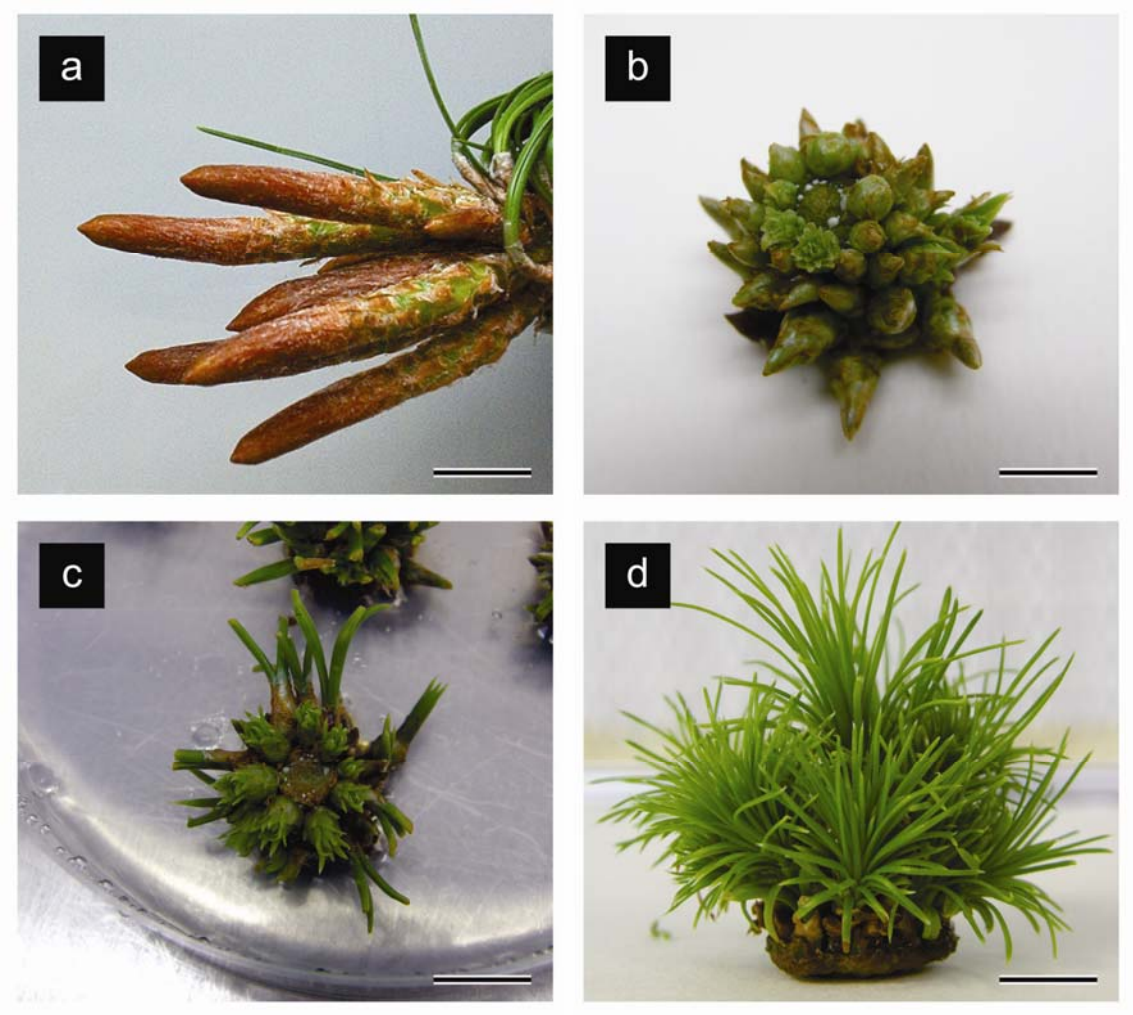

Fig. 1 Organogenesis in vegetative buds from *Pinus radiata* adult trees: (a) Buds from the field collected at the start of January (bar=12 mm); (b) bud slice after 8 weeks of culture on LP basal medium supplemented with Z at 50 µM (bar=8 mm); (c) bud slice cultured for 8 weeks on LP supplemented with mT at 25 µM transferred to LPAC for 4 weeks and then re-induced with the same CK treatment for 4 weeks (bar=12 mm); (d) Shoot organogenesis in a bud slice induced with Z at 50 µM for 6 weeks, after being re-induced with the same treatment for 4 weeks and then transferred to LPAC for 4 weeks (bar=10 mm).

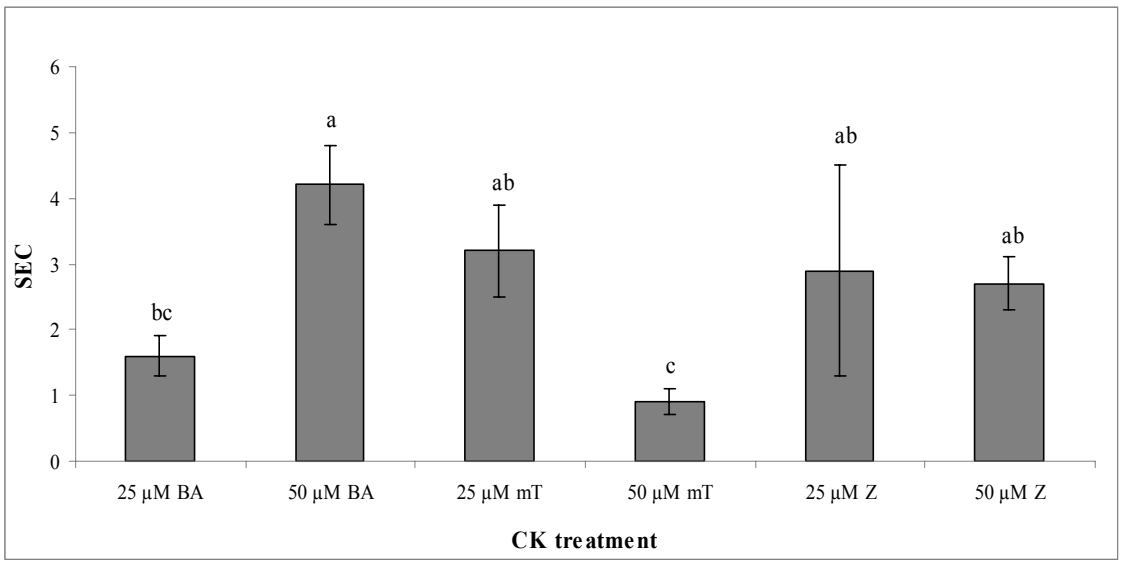

Fig. 2 SEC values in *P. radiata* buds from genotype 76 after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM for 8 weeks (M±S.E.). Different letters show significant differences among treatments by Duncan's post hoc test ($p \leq 0.05$).

3.2. Quantitative analysis of cytokinins

After the first induction period, buds cultured in presence of 50 µM Z showed higher values than those treated with 25 µM Z for all tZ-type CKs individually and for the total content (Table 9). These differences among concentrations were significant for tZ, tZROG and tZ9G. This trend was observed after re-induction in tZR, tZRMP, tZROG, tZ9G and the total content. After re-induction with 25 µM Z, tZ value was significantly higher than after re-induction with 50 µM Z. In this period tZOG values were similar independently of the Z concentration assayed (Table 9).

Buds from genotype 76 re-induced with mT at 50 µM presented the lowest tZ value. The lowest tZR and tZROG values were observed in genotype 72 after induction with 25 µM BA, whereas tZRMP and tZOG lowest values were recorded in the same genotype when it was incubated with 50 µM mT and 50 µM BA, respectively (Table 9). In buds collected from the field and in explants re-induced with 50 µM Z, the metabolite showing the highest values was tZRMP. At the end of the experiment in buds previously incubated with Z at 50 µM, the metabolite that presented the highest values was tZROG, whereas in the other samples tZRMP was again the majority metabolite (Table 9).

Table 9 Endogenous cytokinin content (pmol g^{-1} DW) of the tZ-type CKs in buds from *Pinus radiata* collected from the field (genotypes 72 and 76); in buds after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM (I-72, I-76); in buds after a re-induction period of 4 weeks (R-76); and in buds after 4 weeks on LP medium supplemented with 0.2% AC (C-76) (M±S.E.). Different letters within a column show significant differences among genotypes by Scheffe's post hoc test ($p \leq 0.05$). <*LOD*: Under limit of detection.

Code	tZ	tZR	tZRMP	tZOG	tZROG	tZ9G	Total
72	14.22±0,57 f	85.37±2.38 de	202.28±14.36 b	30.37±3.44 c	139.25±4.69 f	<*LOD*	471.48±24.39 b
76	22.96±0.60 f	67.30±0.48 de	195.61±10.91 b	27.57±2.18 c	84.03±1.40 f	<*LOD*	397.47±13.75 b
I-72-BA25	5.86±0,20 f	1.89±0.15 e	29.94±3.08 b	8.07±1.21 c	20.35±0.94 f	<*LOD*	66.12±2.78 b
I-76-BA25	26.64±2.57 f	3.80±0.23 e	38.30±2.97 b	9.64±0.50 c	41.17±0.36 f	<*LOD*	119.55±5.84 b
I-72-BA50	7.65±0.99 f	3.03±0.32 e	17.29±2.47 b	6.77±0.57 c	27.32±0.34 f	<*LOD*	62.05±3.17 b
I-76-BA50	24.63±3.78 f	32.12±0.77 de	49.10±5.32 b	12.42±1.87 c	51.81±7.53 f	<*LOD*	170.08±17.30 b
I-72-mT25	11.52±1.21 f	2.82±0,11 e	30.99±0.70 b	15.39±1.01 c	33.96±0.54 f	<*LOD*	94.69±1.51 b
I-76-mT25	13.61±0.86 f	5.26±0.35 e	25.91±1.83 b	11.77±0.43 c	44.63±0.85 f	<*LOD*	101.17±2.88 b
I-72-mT50	8.49±0.90 f	4.43±0,10 e	15.86±1.42 b	8.74±0.70 c	34.20±0.32 f	<*LOD*	71.71±1.32 b
I-76-mT50	17.26±0.61 f	5.28±0.91e	25.91±1.06 b	21.57±4.01 c	45.63±4.98 f	<*LOD*	115.65±7.70 b
I-72-Z25	935.26±21,13 e	261.74±5.14 cd	1594.18±35.30 b	1184.36±30.07 bc	5921.38±95.69 d	2.21±0.15 c	9899.13±105.56 b
I-76-Z25	320.48±22.57 f	221.44±26.62 de	1063.32±130.90 b	243.31±4.00c	1566.10±94.46 e	0.92±0.13 de	3415.58±210.73 b
I-72-Z50	1665.21±96,49 d	483.14±11.08 c	4699.27±894.39 b	2584.12±152.40 b	11129.31±230.62 b	4.32±0.45 b	20565.37±966.75 b
I-76-Z50	2575.52±103.96 c	**2543.93±116.23 a**	7913.75±167.75 b	**4587.76±733.94 a**	**13029.34±415.69 a**	**5.59±0.07 a**	30655.89±1038.30 b
R-76-BA25	23.35±0.86 f	5.22±0.64 e	66.66±12.74 b	16.96±1.33 c	34.32±2.07 f	<*LOD*	146.51±17.39 b
R-76-BA50	14.20±1.59 f	5.56±0.73 e	39.96±4.48 b	13.74±0.62 c	38.03±2.57 f	<*LOD*	111.49±4.56 b
R-76-mT25	26.52±0.73 f	4.95±0.41 e	46.22±1.85 b	10.98±0.84 c	46.77±1.57 f	<*LOD*	135.43±1.38 b
R-76-mT50	5.15±0.77 f	3.79±0.63 e	18.36±3.26 b	7.84±1.13 c	23.44±1.83 f	<*LOD*	58.59±3.73 b
R-76-Z25	**13098.52±186.63 a**	785.86±28.72 b	8910.26±685.50 b	**6020.67±325.55 a**	1713.41±13.37 e	2.06±0.19 cd	30655.89±1038.30 b
R-76-Z50	5060.69±55.05 b	**2452.58±36.81 a**	**156856.03±25001.92 a**	**5210,82±312.05 a**	7635.85±79.14 c	**6.68±0.06 a**	**177222.65±24693.44 a**
C-76-BA25	30.40±4.39 f	9.34±0.38 e	173.09±7.14 b	40.48±1.65 c	55.18±2.52 f	<*LOD*	308.49±4.16 b
C-76-BA50	27.75±4.31 f	7.04±1.22 e	216.10±26.39 b	19.24±2.43 c	36.78±5.37 f	<*LOD*	306.91±25.53 b
C-76-mT25	11.59±1,65 f	3.57±0.22 e	111.62±11.45 b	11.18±0.52 c	20.98±0.76 f	<*LOD*	158.94±9.77 b
C-76-mT50	19.15±0,25 f	36.33±0.13 de	308.88±21.43 b	69.07±1.86 c	143.21±1.81 f	<*LOD*	576.64±22.32 b
C-76-Z25	25.80±0.86 f	23.62±0.62 e	354.17±52.72 b	112.62±2.05 c	329.24±7.46 f	0.22±0,20 e	845.66±59.59 b
C-76-Z50	59.39±1.59 f	45.37±0.91 de	274.53±16.23 b	167.79±5.82c	626.56±10.91 f	0.23±0,01 e	1173.86±30.12 b

In the two genotypes analysed, the highest tZROG values were observed in explants incubated with Z at 50 μM after the first induction period. In the case of tZ9G, this metabolite only appeared in samples that have been treated with Z and at low concentrations (below 7 pmol g^{-1} DW); the highest tZ9G values were found in genotype 76 after induction and re-induction periods with 50 μM Z (Table 9).

Treatments with BA or mT did not induce any significant change in the endogenous content of any metabolite of the tZ-type CKs when compared with the endogenous content of these CKs in buds from the field (Tables 9).

Before the application of any induction treatment, the endogenous values of tZ-type CKs were at least 2.7-fold higher than cZ-type CKs (Tables 9 and 10). After the first induction period, the endogenous content of cZ, cZR, cZRMP and cZROG was higher in buds cultured with 50 μM Z than in buds cultured with 25 μM Z in each genotype analysed (Table 10). Buds from genotype 76 collected in the field showed a significantly higher cZOG content than those from genotype 72. This cZOG value in genotype 76 was also significantly higher than the values obtained after CK treatments. As it was observed for the endogenous content of tZ, re-induction with Z at 25 μM provoked the highest endogenous cZ value. Re-induction with 50 μM Z caused the highest total content of cZ-type CKs. This treatment also induced the highest cZR and cZRMP values. These differences were significant for cZRMP and the total content. Buds from genotype 76 after the first induction period with 50 μM BA also showed a high cZR value. This value was significantly higher than that obtained in genotype 72 after the same treatment (Table 10).

The lowest value for cZ was observed in genotype 72 in buds from the field and after the first induction period with 50 μM BA. In the same genotype, the lowest cZR and cZRMP values were recorded after the first induction period with 25 μM BA and 50 μM BA, respectively. The endogenous cZOG content was similar in all samples except in buds from genotype 76 before any treatment with CK, where it was significantly higher. On the other hand, the highest endogenous cZROG was observed after the first induction period in explants incubated with Z at 50 μM. The lowest cZOG was recorded in genotype 72 after the first induction period with 50 μM Z, and in genotype 76 re-induced with 25 μM BA the lowest cZROG level was found. The endogenous content of cZ9G was below the detection limit in all samples (Table 10).

Table 10 Endogenous cytokinin content (pmol g^{-1} DW) of the cZ-type CKs in buds from *Pinus radiata* collected from the field (genotypes 72 and 76); in buds after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM (I-72, I-76); in buds after a re-induction period of 4 weeks (R-76); and in buds after 4 weeks on LP medium supplemented with 0.2% AC (C-76) (M±S.E.). Different letters within a column show significant differences among genotypes by Scheffe's post hoc test ($p \leq 0.05$). <*LOD*: Under limit of detection.

Code	cZ	cZR	cZRMP	cZOG	cZROG	cZ9G	Total
72	1.30±0.02 d	9.36±1.04 ef	15.62±2.25 e	5.78±0.64 b	18.17±0.86 e	<*LOD*	50.24±2.76 ij
76	3.01±0.26 d	8.82±0.80 f	40.05±8.45 de	**10.18±0.78 a**	29.14±1.11 de	<*LOD*	91.21±6.59 ghij
I-72-BA25	1.65±0.08 d	5.50±0.67 f	28.77±1.21 de	4.16±0.63 b	17.04±2.91 e	<*LOD*	57.13±3.38 ij
I-76-BA25	6.51±0.52 cd	57.40±2.84 bcd	32.66±0.98 de	4.07±0,47 b	17.02±0.13 e	<*LOD*	117.66±3.15 defghij
I-72-BA50	1.35±0.19 d	13.88±2.07 ef	10.77±2.43 de	3.10±0.52 b	14.80±1.94 e	<*LOD*	43.90±3.60 j
I-76-BA50	5.39±0.88 cd	**87.26±15.77 ab**	26.09±3.24 e	3.64±0.63 b	19.59±1.85 e	<*LOD*	141.96±14.69 defgh
I-72-mT25	2.33±0.12 d	31.66±0.69 def	31.50±2.34 de	4.29±0.22 b	43.86±2.07 cde	<*LOD*	113.63±0.41 defghij
I-76-mT25	3.36±0.41 d	40.19±0.82 cdef	26.16±2.62 de	3.74±0.17 b	47.10±3.00 cde	<*LOD*	120.55±4.73 defghij
I-72-mT50	1.65±0.13 d	27.75±0.64 def	49.60±8.03 cde	2.72±016 b	51.42±1.93 cde	<*LOD*	133.14±6.19 defghi
I-76-mT50	5.64±0.26 cd	44.92±2.17 cde	33.79±5.31 de	2.77±0.46 b	57.85±9.19 bcd	<*LOD*	144.96±13.34 defgh
I-72-Z25	14.44±1.63 cd	45.08±3.75 cde	29.49±1.14 de	3.13±0.53 b	90.10±2.01 b	<*LOD*	182.24±6.75 de
I-76-Z25	6.06±1.04 cd	41.28±2.07 cdef	30.19±5.24 de	3.52±0.38 b	59.33±4.77 bcd	<*LOD*	140.38±2.41 defgh
I-72-Z50	19.03±0.48 bc	52.62±5.95 bcd	60.44±9.20 bcde	2.47±0.36 b	**138.56±3.96 a**	<*LOD*	273.13±10.37 bc
I-76-Z50	32.47±4.63 b	**74.69±6.39 abc**	48.01±5.48 cde	3.00±0.43 b	**160.51±14.74 a**	<*LOD*	318.68±23.93 b
R-76-BA25	2.62±0.35 d	11.02±0.65 ef	33.43±5.00 de	3.68±0.53 b	14.01±0.21 e	<*LOD*	64.76±5.93 hij
R-76-BA50	3.00±0.22 d	10.20±0.31 ef	36.74±3.97 de	3.70±0.20 b	16.78±0.54 e	<*LOD*	70.41±3.95 ghij
R-76-mT25	3.71±0.12 d	37.48±1.70 def	102.11±10.88 bc	3.29±0.30 b	30.87±1.86 de	<*LOD*	177.45±13.47 def
R-76-mT50	2.41±0.19 d	38.54±0.48 def	106.46±9.17 b	3.36±0.42 b	44.06±3.74 cde	<*LOD*	194.82±11.39 cd
R-76-Z25	**79.30±5.83 a**	32.98±2.28 def	18.89±2.76 de	4.50±0.28 b	17.30±0.87 e	<*LOD*	152.96±5.22 defg
R-76-Z50	29.58±1.65 b	**96.18±3.23 a**	**300.92±8.20 a**	3.09±0.56 b	78.64±4.06 bc	<*LOD*	**508.41±10.78 a**
C-76-BA25	1.67±0.07 d	7.59±0.62 f	47.62±3.85 cde	4.03±0.23 b	15.85±2.18 e	<*LOD*	76.75±6.69 ghij
C-76-BA50	3.18±0.20 d	8.75±0.58 f	56.93±2.19 bcde	4.93±0.56 b	16.81±1.22 e	<*LOD*	90.60±2.94 ghij
C-76-mT25	4.71±0.13 cd	15.65±1.81 ef	71.79±3.75 bcd	4.37±0.50 b	25.76±1.21 de	<*LOD*	122.29±4.50 defghij
C-76-mT50	2.78±0.20 d	10.12±0.29 ef	73.48±13.95 bcd	3.76±0.37 b	15.03±2.16 e	<*LOD*	105.17±13.22 efghij
C-76-Z25	2.87±0.39 d	10.78±1.22 ef	40.54±2.99 de	3.44±0.38 b	19.08±1.25 e	<*LOD*	76.71±3.49 ghij
C-76-Z50	2.86±0.30 d	11.41±1.09 ef	50.12±5.15 cde	4.41±0.23 b	27.35±1.62 de	<*LOD*	96.15±7.61 fghij

The results for the endogenous DHZ-type CKs are displayed in Table 11. Explants after induction and re-induction with Z showed higher endogenous contents of each DHZ-type CKs than explants treated with BA or mT. These differences were significant for DHZ and DHZR values specifically.

After the first induction period buds from genotype 72 cultured with 25 μM Z presented significantly higher endogenous DHZ, DHZR, DHZOG, DHZROG and total DHZ content than explants from genotype 76 incubated with the same CK treatment. In this period, DHZR and DHZ9G values were significantly higher in genotype 76 than in genotype 72 after culture with 50 μM Z (Table 11). The highest DHZOG and DHZROG values were observed in genotype 72 after the first induction period with 25 μM Z and 50 μM Z, respectively.

Re-induction with Z at 50 μM provoked endogenous peak values of DHZ, DHZR, DHZRMP and the total content. These values were significantly higher than those obtained in the rest of samples analysed. Except for DHZOG, values of all DHZ-type CKs individually and of the total content were higher after induction and re-induction with 50 μM Z than after induction and re-induction with 25 μM Z. At the end of the experiment, after culture in the presence of AC, the buds previously incubated with Z showed DHZOG and DHZROG values at least 3 times higher than the buds previously cultured with BA or mT, although no significant differences were observed among treatments (Table 11).

As it was observed in tZ-type CKs, endogenous levels of DHZ9G were only detected in explants incubated with Z and at low concentrations (below 5 pmol g^{-1} DW). The highest value for this metabolite was achieved in genotype 76 after the first induction period with 50 μM Z (Table 11). The lowest DHZ, DHZR and DHZROG values were observed in genotype 72 after induction with 25 μM BA. In the same genotype, after incubation with 25 μM mT, DHZRMP lowest value was recorded. After re-inducing buds from genotype 76 with mT at 50 μM the lowest DHZOG was observed (Table 11).

Table 11 Endogenous cytokinin content (pmol g^{-1} DW) of the DHZ-type CKs in buds from *Pinus radiata* collected from the field (genotypes 72 and 76); in buds after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM (I-72, I-76); in buds after a re-induction period of 4 weeks (R-76); and in buds after 4 weeks on LP medium supplemented with 0.2% AC (C-76) (M±S.E.). Different letters within a column show significant differences among genotypes by Scheffe's post hoc test ($p \leq 0.05$). *<LOD*: Under limit of detection.

Code	DHZ	DHZR	DHZRMP	DHZOG	DHZROG	DHZ9G	Total
72	9.43±0.74 e	11.70±0.20 f	25.90±1.99 d	50.22±9.70 d	109.55±1.21 e	<LOD	206.79±11.30 f
76	9.00±0.37 e	10.53±0.41 f	41.64±1.38 d	51.50±9.80 d	45.26±2.30 e	<LOD	157.93±7.40 f
I-72-BA25	3.50±0.19 e	2.28±0.15 f	6.69±1.00 d	32.95±5.48 d	21.23±3.42 e	<LOD	66.64±8.12 f
I-76-BA25	20.26±0.06 e	16.92±0.71 f	10.92±0.35 d	54.11±5.19 d	73.08±4.43 e	*<LOD*	175.28±8.92 f
I-72-BA50	11.30±1.23 e	9.66±1.31 f	11.63±1.65 d	60.18±8.64 d	33.18±3.00 e	<LOD	125.94±10.16 f
I-76-BA50	30.72±2.08 e	71.88±1.45 f	26.10±3.69 d	56.38±1.15 d	73.73±9.15 e	*<LOD*	258.82±10.82 f
I-72-mT25	7.83±0.13 e	5.04±0.41 f	5.87±0.60 d	41.71±1.27 d	47.27±1.44 e	<LOD	107.72±1.57 f
I-76-mT25	15.69±0.18 e	16.05±0.38 f	9.87±0.39 d	85.45±3.91 d	144.10±6.43 e	*<LOD*	271.19±9.79 f
I-72-mT50	23.33±0.87 e	19.37±0.45 f	22.16±0.08 d	76.35±7.25 d	90.94±2.94 e	<LOD	232.15±9.41 f
I-76-mT50	17.98±1.92 e	21.45±0.37 f	16.33±2.00 d	89.76±9.61 d	184.95±26.97 e	*<LOD*	330.47±39.59 f
I-72-Z25	1559.65±11.01 c	1203.20±17.65 d	1584.83±71.58 bcd	**3139.11±252.58 a**	7137.78±211.06 b	1.42±0.07 de	14625.98±358.90 c
I-76-Z25	441.21±18.53 d	466.99±63.01 e	644.26±107.21 cd	735.50±98.50 d	1660.52±135.89 c	0.85±0.06 ef	3949.33±403.85 de
I-72-Z50	2037.95±10.51 b	1622.91±48.33 c	3777.92±324.08 b	**2654.19±176.60 ab**	**10970.22±315.49 a**	1.94±0.22 cd	21065.13±788.14 b
I-76-Z50	2125.00±39.68 b	3234.38±61.41 b	3096.11±151.16 bc	2292.23±295.01 bc	**10800.47±292.51 a**	**4.42±0.07 a**	21552.60±382.24 b
R-76-B25	11.30±1.49 e	14.77±2.49 f	14.40±3.96 d	34.61±2.10 d	29.77±2.23 e	*<LOD*	104.84±11.60 f
R-76-B50	8.27±1.38 e	10.40±1.63 f	8.47±1.32 d	37.73±1.36 d	38.22±2.67 e	*<LOD*	103.09±4.13 f
R-76-mT25	14.32±0.24 e	14.79±0,10 f	29.28±4.05 d	61.99±3.44 d	91.03±1.43 e	*<LOD*	211.40±2.48 f
R-76-mT50	9.07±0.61 e	10.36±0.76 f	16.66±2.65 d	21.39±3.46 d	38.57±1.41 e	*<LOD*	96.05±2.05 f
R-76-Z25	2183.85±57.57 b	1435.93±36.76 cd	1273.59±31.33 bcd	838.77±25.98 d	1555.22±29.10 cd	2.54±0.08 c	7289.91±167.35 d
R-76-Z50	**2747.20±48.44 a**	**4618.34±74.06 a**	**23287.21±1505.96 a**	1692.11±92.36 c	6542.00±141.62 b	3.55±0.12 b	**38890.42±1518.80 a**
C-76-BA25	5.33±0.27 e	5.44±0.14 f	18.12±1.40 d	38.07±1.59 d	28.43±1.55 e	*<LOD*	95.39±1.44 f
C-76-BA50	8.08±1.14 e	8.81±1.24 f	23.01±3.83 d	43.00±1.36 d	28.38±1.61 e	*<LOD*	111.28±7.03 f
C-76-mT25	5.63±0.22 e	8.32±0.32 f	12.02±1.39 d	34.01±5.41 d	35.91±1.89 e	*<LOD*	95.88±5.02 f
C-76-mT50	28.89±2.11 e	33.46±1.16 f	22.22±3.25 d	87.48±3.56 d	94.61±1.55 e	*<LOD*	266.66±4.79 f
C-76-Z25	26.60±1.95 e	34.96±1.54 f	22.19±2.87 d	459.43±8.50 d	290.14±5.23 e	0.41±0.01 f	833.74±12.84 ef
C-76-Z50	57.07±0.19 e	54.71±0.76 f	24.69±0.73 d	763.66±38.34 d	637.84±31.59 de	1,17±0.07 e	1539.14±67.33 ef

Buds from genotype 72 collected from the field showed significantly higher endogenous iPR and iP9G values than those from genotype 76 before any CK treatment (Table 12). After the first induction period, in genotype 76 BA and mT provoked higher iP values than Z after the first induction period; whereas in genotype 72 the highest endogenous iP content was observed in buds cultured with Z (Table 12). In the case of induction with Z, these differences among genotypes were significant. The values for iP metabolite were always below 15 pmol g^{-1} DW.

Table 12 Endogenous cytokinin content (pmol g^{-1} DW) of the iP-type in buds from *Pinus radiata* collected from the field (genotypes 72 and 76); in buds after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM (I-72, I76); in buds after a re-induction period of 4 weeks (R-76); and in buds after 4 weeks on LP medium supplemented with 0.2% AC (C-76) (M±S.E.). Different letters within a column show significant differences among genotypes by Scheffe's post hoc test ($p \leq 0.05$). <*LOD*: Under limit of detection.

Code	iP	iPR	iPRMP	iP9G	Total
72	5.74±1.02 bcdef	**7.99±1.41 a**	83.66±7.72 b	0.86±0.02 b	98.24±8.93 b
76	3.76±0.09 cdef	2.63±0.24 cd	98.91±4.49 b	0.21±0.05 d	105.52±4.29 b
I-72-BA25	2.39±0.32 ef	0.62±0.07 d	32.98±3.68 b	**1.08±0.03 a**	37.06±4.03 b
I-76-BA25	**14.75±0.27 a**	**4.94±0.03 abc**	26.34±4.57 b	0.12±0.02 d	46.16±4.67 b
I-72-BA50	7.14±1.17 bcde	2.45±0.39 cd	9.92±1.76 b	0.72±0.05 bc	20.24±2.76 b
I-76-BA50	**10.04±1.67 ab**	**6.27±0.34 ab**	12.64±1.98 b	0.12±0.01 d	29.06±3.62 b
I-72-mT25	6.54±1.04 bcdef	2.65±0.40 cd	9.54±0.64 b	0.88±0.02 b	19.60±1.13 b
I-76-mT25	**10.20±0.54 ab**	3.82±0.22 bcd	9.16±0.81 b	0.12±0.01 d	23.29±0.62 b
I-72-mT50	6.09±1.02 bcdef	2.16±0.21 cd	9.18±0.19 b	0.69±0.03 bc	18.12±1.08 b
I-76-mT50	5.00±0.79 bcdef	2.48±0.36 cd	9.16±0.81 b	0.10±0.01 d	16.75±1.93 b
I-72-Z25	**9.16±0.53 abcd**	3.56±0.15 bcd	29.27±2.56 b	0.53±0.02 c	42.52±1.91 b
I-76-Z25	1.77±0.06 ef	1.43±0.23 d	9.21±1.29 b	0.09±0.01 d	12.50±1.09 b
I-72-Z50	**9.65±0.83 abc**	3.09±0.43 bcd	26.87±3.85 b	0.58±0.02 c	40.19±4.00 b
I-76-Z50	2.75±0.09 ef	2.24±0.23 cd	24.49±1.94 b	0.11±0.01 d	29.59±2.18 b
R-76-BA25	2.02±0.13 ef	2.02±0.27 cd	37.94±5.33 b	0.14±0.00 d	42.11±4.97 b
R-76-BA50	3.27±0.16 def	1.58±0.19 d	20.80±2.23 b	0.11±0.01 d	25.76±2.59 b
R-76-mT25	1.95±0.28 ef	1.37±0.12 d	33.01±1.97 b	0.08±0.01 d	36.41±2.13 b
R-76-mT50	0.74±0.11 f	1.40±0.04 d	14.90±2.52 b	0.11±0.02 d	17.15±2.37 b
R-76-Z25	2.77±0.32 ef	2.03±0.06 cd	17.01±2.52 b	0.04±0.01 d	21.85±2.89 b
R-76-Z50	1.79±0.15 ef	2.58±0.11 cd	**509.47±52.61 a**	0.05±0.01 d	**513.89±52.81 a**
C-76-BA25	0.66±0.12 f	1.35±0.07 d	70.56±3.90 b	0.13±0.02 d	72.70±4.02 b
C-76-BA50	1.81±0.23 ef	1.62±0.07 d	81.62±4.39 b	0.09±0.01 d	85.14±4.36 b
C-76-mT25	5.50±0.29 bcdef	1.62±0,11 d	72.97±5.22 b	0.10±0.01 d	80.19±5.50 b
C-76-mT50	2.96±0.47 ef	1.75±0.10 cd	97.27±13.45 b	0.16±0.01 d	102.14±13.96 b
C-76-Z25	3.05±0.17 ef	1.94±0.09 cd	72.78±11.95 b	0.08±0.01 d	77.86±11.81 b
C-76-Z50	3.96±0.63 cdef	1.92±0.20 cd	70.83±10.20 b	0.09±0.00 d	76.80±9.47 b

The endogenous content of iPR in the samples analysed was always below 8 pmol g^{-1} DW. The highest iPR content was obtained in genotype 72 in buds from the field, and in buds from

genotype 76 after the first induction period with BA. The lowest iPR value was recorded after this period in explants from genotype 72 incubated with BA at 25 µM. At the end of the experiment the endogenous iPR values were below 2 pmol g^{-1} DW (Table 12).

The endogenous iPRMP values recorded in buds from the field were higher than those found in buds after the first induction period although no significant differences were found. As observed for tZ-group, cZ-group and DHZ-group, buds after re-induction with 50 µM Z showed the highest endogenous iPRMP content. Except in I-76-mT50, in each sample analysed iPRMP showed the highest value when compared with other iP-type metabolites (Table 12). At the end of the experiment, after culture in the presence of AC, explants from different treatments showed iPRMP values similar to those observed in buds from the field (between 70 and 97 pmol g^{-1} DW). Buds from genotype 76 after induction with mT at 25 and 50 µM displayed the lowest iPRMP values (Table 12).

In all samples analysed, iP9G was the metabolite presenting the lowest endogenous concentrations (below 1.1 pmol g^{-1} DW), but on the contrary of the observations made in tZ-group, cZ-group and DHZ-group this metabolite appeared in all samples analysed. The total content of iP-type CKs followed the same pattern described for the ribonucleotide fraction (Table 12).

In Table 13, the results of the analyses for the BA-type CKs are presented. No detectable levels of BA-type CKs were observed in buds from the field. Endogenous levels of BA-type CKs were only detected in buds cultured with BA. At the different culture stages analysed, considering genotypes 72 and 76 separately, explants induced with 50 μM BA showed higher endogenous levels of all BA metabolites and their total content than explants cultured with 25 μM BA. At the end of the experiment, buds previously induced with 50 μM BA presented an endogenous BA content 76-fold higher than buds previously incubated with 25 μM BA (Table 13).

BA, BAR and BARMP and the total BA content achieved their highest values after the first induction period with BA at 50 μM. After induction and re-induction periods, considering the BA-type metabolites in each sample analysed, BA presented concentrations 9-fold higher than BA9G and BARMP fraction and concentrations 100-fold higher than BAR fraction (Table 13).

As observed for the BA-type CKs, neither buds from the field nor those cultured with Z showed detectable levels of any Topolin (oT, mT, and pT) (Tables 14, 15 and 16). Endogenous levels of oT, oTR, and oTOG were only detected in buds cultured with BA. The highest values for these metabolites were recorded in explants from genotype 76 after the first induction period with 50 μM BA. These oT and oTR values were significantly higher than those obtained in the other samples. In each period, BA at 50 μM provoked higher endogenous levels of all oT-type CKs and their total content than BA at 25 μM (Table 14).

In each sample analysed, the endogenous levels of oT were at least 18-fold higher than the endogenous levels of oTR and oTOG after induction and re-induction periods. At the end of the experiment, oT was the only metabolite of the oT-type CKs found in buds. The levels at this culture stage were at least 10 times lower than those observed after induction and re-induction. No detectable levels of oTRMP, oTROG or oT9G were found in any of the culture stages analysed (Table 14).

Table 13 Endogenous cytokinin content (pmol g^{-1} DW) of the BA-type in buds from *Pinus radiata* collected from the field (genotypes 72 and 76); in buds after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM (I-72, I-76); in buds after a re-induction period of 4 weeks (R-76); and in buds after 4 weeks on LP medium supplemented with 0.2% AC (C-76) (M±S.E.). Different letters within a column show significant differences among genotypes by Scheffe's post hoc test ($p \leq 0.05$). <*LOD*: Under limit of detection.

Code	BA	BAR	BARMP	BA9G	Total
72	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
76	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-72-BA25	**33907.44±3420.83 abc**	58.38±3.16 d	1439.09±135.98 cd	976.40±40.46 b	36381.30±3587.72 bc
I-76-BA25	21515.54±1516.57 bcd	75.21±3.05 cd	724.80±104.38 cd	392.25±14.91 c	22707.81±1461.42 cd
I-72-BA50	**52463.93±3928.61 ab**	136.94±13.85 b	**5754.52±296.05 a**	**2023.81±121.18 a**	**60379.19±3752.97 ab**
I-76-BA50	**63680.68±11327.18 a**	**440.97±13.50 a**	3235.82±525.45 b	886.80±31.11 b	**68244.27±11089.48 a**
I-72-mT25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-76-mT25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-72-mT50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-76-mT50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-72-Z25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-76-Z25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-72-Z50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-76-Z50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
R-76-BA25	**36676.40±5774.46 abc**	44.76±1.16 de	650.94±99.92 cd	83.51±0.83 d	37455.61±5675.12 bc
R-76-BA50	**49786.97±4409.08 ab**	105.31±1.68 bc	1572.94±182.25 c	221.32±5.80 cd	**51686.53±4482.88 ab**
R-76-mT25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
R-76-mT50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
R-76-Z25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
R-76-Z50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
C-76-BA25	106.20±15.84 d	4.46±0.31 e	124.00±82.05 d	15.73±0.16 d	250.38±84.72 d
C-76-BA50	8126.68±201.73 cd	71.04±0.84 cd	222.94±12.43 cd	68.01±2.06 d	8488.66±196.61 d
C-76-mT25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
C-76-mT50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
C-76-Z25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
C-76-Z50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*

Endogenous levels of mT, mTR, mTOG and mTROG were detected in explants cultured in the presence of BA or mT, whereas mTRMP and mT9G were only detected in buds incubated with mT (Table 15). Explants cultured with mT after induction and re-induction periods showed endogenous levels of mT, mTR, mTOG, and mTROG higher than those cultured with BA. These differences were significant for the O-glucosides (mTOG and mTROG) (Table 15). The highest endogenous levels of mT were found in buds after the re-induction period with mT, whereas the highest mTR and mTROG endogenous levels were observed in explants incubated with mT after the first induction period (Table 15).

Considering genotypes 72 and 76 separately, after the first induction period 50 µM BA and 50 µM mT provoked higher endogenous levels of mT, mTR, mTOG, mTROG and the total content than 25 µM BA and 25 µM mT, respectively. After re-induction with mT the same trend was observed in all mT type CKs except the bases fraction, whereas re-induction with BA only provoked this effect in endogenous values of mT and the total content (Table 15).

As observed in the oT-type CKS, endogenous levels of pT-type CKs were only detected in buds cultured with BA and these values were always below 68 pmol g^{-1} DW. BA at 50 µM caused higher endogenous levels of pT, pTR, pTOG and the total content than buds induced at 25 µM BA, at different culture stages. The highest values for the pT and pTR were observed in buds from genotype 76 after the first induction period with BA at 50 µM. In this genotype, buds incubated with 50 µM BA did not show significant differences among induction and re-induction periods in endogenous pTRMP, pTOG, pTROG and the total content (Table 16).

It is remarkable that BA provoke the appearance oT-type, mT-type and pT-type CKs, whereas mT only provoke the synthesis of mT-type CKs (Tables 14, 15 and 16). After induction and re-induction with BA the Topolin that appear at higher concentrations was oT, particularly the bases fraction. At the end of the experiment, in buds previously incubated with BA at 50 µM, the metabolite showing the highest concentration was pTROG (Table 16).

Table 14 Endogenous cytokinin content (pmol g^{-1} DW) of the oT-type in buds from *Pinus radiata* collected from the field (genotypes 72 and 76); in buds after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 μM (I-72, I76); in buds after a re-induction period of 4 weeks (R-76); and in buds after 4 weeks on LP medium supplemented with 0.2% AC (C-76) (M±S.E.). Different letters within a column show significant differences among genotypes by Scheffe's post hoc test ($p \leq 0.05$). <*LOD*: Under limit of detection.

Code	oT	oTR	oTRMP	oTOG	oTROG	oT9G	Total
72	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
76	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-72-BA25	115.09±6.76 cd	0.81±0.14 c	<*LOD*	3.75±0.57 b	<*LOD*	<*LOD*	119.65±7.36 cde
I-76-BA25	205.44±6.25 bc	3.71±0.18 b	<*LOD*	7.41±1.34 b	<*LOD*	<*LOD*	216.56±6.37 c
I-72-BA50	341.83±52.89 b	3.82±0.67 b	<*LOD*	**12.03±0.57 ab**	<*LOD*	<*LOD*	368.12±53.96 b
I-76-BA50	**543.64±31.17 a**	**11.71±0.57 a**	<*LOD*	**18.90±3.21 a**	<*LOD*	<*LOD*	**589.61±29.13 a**
I-72-mT25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-76-mT25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-72-mT50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-76-mT50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-72-Z25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-76-Z25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-72-Z50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
I-76-Z50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
R-76-BA25	90.46±10.88 cd	0.67±0.07 c	<*LOD*	4.98±0.86 b	<*LOD*	<*LOD*	96.11±11.07 def
R-76-BA50	156.44±12.50 c	1.23±0.10 c	<*LOD*	8.57±0.78 b	<*LOD*	<*LOD*	166.24±12.57 cd
R-76-mT25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
R-76-mT50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
R-76-Z25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
R-76-Z50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
C-76-BA25	1.31±0.05 d	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	1.31±0.05 f
C-76-BA50	8.30±0.40 d	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	8.30±0.40 ef
C-76-mT25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
C-76-mT50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
C-76-Z25	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*
C-76-Z50	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*	<*LOD*

The relative content of the different CK fractions in *P. radiata* buds at different culture stages is presented in Fig. 3. At the start of the experiment, the buds from the field presented similar relative contents of all metabolites (bases, riboses, ribonucleotides, O-glucosides, and N-glucosides) in genotypes 72 and 76, although genotype 76 had a slightly higher relative content of the ribonucleotide fraction and a slightly lower relative content of the O-glucoside fraction than genotype 72.

After the first induction period, buds cultured with BA presented the highest relative content of bases (between 86.4% and 92.7%). Explants cultured with 50 μM mT showed higher values than those cultured with 25 μM mT, this was particularly patent in genotype 72. Buds incubated with Z showed bases values ranging from 8.9% to 10.2% (Fig. 3). The two genotypes studied showed a similar pattern in the relative percentages of the different CK fractions. Explants from genotype 72 incubated with 50 μM BA or 25 μM mT had a slightly lower bases content than the buds from genotype 76 subjected to the same treatments, and the contrary occurred for buds from genotype 72 incubated with 50 μM mT.

O-glucoside fraction was predominant in buds cultured with mT or Z after the first induction period, with relative percentages ranging from 59.1% to 80.8% for mT, and from 56.8% to 70.6% for Z (Fig. 3). Higher O-glucoside relative percentages were found in explants incubated with 25 μM mT than in those cultured with 50 μM mT (Fig. 3).

The relative percentage of riboses together with the relative percentage of ribonucleotides was higher in explants treated with Z than in those treated with mT. After the first induction period, the relative content of the ribonucleotide fraction in buds treated with mT was similar to that observed in those treated with BA (Fig. 3).

The highest relative percentage of endogenous bases was achieved in buds cultured with BA after the re-induction period (between 95.5% and 96.7%). In those re-induced with mT or 25 μM Z, the relative content of the bases fraction ranged from 40.4% to 56.2%, whereas Z at 50 μM provoked values below 4% for this fraction. After the re-induction period, the relative percentage of the ribonucleotide fraction in buds cultured with BA was below 4% and the relative percentages of the other fractions analysed were below 1% (Fig. 3).

Table 15 Endogenous cytokinin content (pmol g^{-1} DW) of the mT-type in buds from *Pinus radiata* collected from the field (genotypes 72 and 76); in buds after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM (I-72, I76); in buds after a re-induction period of 4 weeks (R-76); and in buds after 4 weeks on LP medium supplemented with 0.2% AC (C-76) (M±S.E.). Different letters within a column show significant differences among genotypes by Scheffe's post hoc test ($p \leq 0.05$). *<LOD*: Under limit of detection.

Code	mT	mTR	mTRMP	mTOG	mTROG	mT9G	Total
72	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
76	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-72-BA25	37.83±0.87 e	0.98±0.07 f	*<LOD*	7.07±0.85 d	29.19±3.46 d	*<LOD*	75.07±5.08 f
I-76-BA25	25.01±2.61 e	2.94±0.24 f	*<LOD*	5.06±0.83 d	47.69±5.79 d	*<LOD*	80.69±9.45 f
I-72-BA50	65.37±3.69 e	2.26±0.35 f	*<LOD*	15.99±2.92 d	56.76±0.67 d	*<LOD*	140.38±6.25 f
I-76-BA50	75.82±1.82 e	4.93±0.11 f	*<LOD*	13.85±1.57 d	62.29±6.25 d	*<LOD*	156.88±9.59 f
I-72-mT25	2330.97±50.74 ef	751.97±21.67 d	352.13±39.55 c	**5979.43±667.96 ab**	9065.47±404.50 bc	45.31±1.82 b	18525.29±1088.65 d
I-76-mT25	2921.35±98.08 de	1055.11±51.81 c	1227.75±27.41 bc	3431.47±190.52 bc	8960.76±405.76 bc	22.21±1.43 cd	17618.66±713.58 d
I-72-mT50	8634.72±319.05 c	1596.29±73.95 b	**2870.83±163.16 ab**	**7457.73±214.27 a**	**11772.92±252.65 ab**	**179.80±8.25 a**	32512.29±643.22 bc
I-76-mT50	5575.55±210.05 d	**1846.04±62.64 a**	**2488.38±98.01 ab**	3539.99±239.05 bc	**14945.19±2408.38 a**	44.39±1.30 b	28439.53±2668.24 c
I-72-Z25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-76-Z25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-72-Z50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-76-Z50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
R-76-BA25	50.22±2.10 e	2.30±0.31 e	*<LOD*	13.62±2.33 d	39.80±5.67 d	*<LOD*	105.94±6.90 f
R-76-BA50	71.87±12.79 e	1.46±0.09 f	*<LOD*	13.09±2.06 d	34.51±5.01 d	*<LOD*	120.91±17.38 f
R-76-mT25	**21726.59±975.50 a**	464.38±4.95 e	**2998.56±161.51 a**	**6973.01±982.76 a**	6026.95±138.02 c	12.33±0.35 cd	**38201.83±99.17 ab**
R-76-mT50	18277.37±877.96 b	1223.90±32.58 c	**4179.74±729.34 a**	**8092.67±636.51 a**	9583.93±1009.23 bc	26.99±1.32 bc	**41384.60±1795.90 a**
R-76-Z25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
R-76-Z50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
C-76-BA25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
C-76-BA50	10.65±1.44 e	0.86±0.13 f	*<LOD*	4.20±0.59 d	23.28±3.40 d	*<LOD*	38.99±4.49 f
C-76-mT25	806.06±36.83 ef	622.36±18.55 de	557.59±37.88 c	2894.86±77.58 c	6067.19±25.82 c	3.44±0.21 d	10951.50±146.17 e
C-76-mT50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
C-76-Z25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
C-76-Z50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*

Explants re-induced with 25 µM and 50 µM mT showed similar values for the ribonucletide (8.3% and 10.4%, respectively) and O-glucoside fractions (34.2% and 42.7%, respectively). The relative percentage of the N-glucoside fraction was negligible (Fig. 3). The relative percentage of the endogenous ribonucleotide and O-glucoside fractions was the same (27%) when explants were re-induced with 25 µM Z, whereas after re-induction with 50 µM Z the ribonucleotides represented the 83.3% of the total CK content. The relative percentages of riboses ranged between 1% and 3% in explants cultured with mT and between 3% and 6% in buds induced with Z (Fig. 3).

At the end of the experiment, the relative percentage of bases found in buds previously cultured with 25 µM BA decreased to 17.3%. On the contrary, explants induced with 50 µM BA maintained a high relative percentage of bases (89%). Buds from treatments with mT and Z presented relative contents of bases below 8%; in these samples and in explants previously treated with 25 µM BA, the relative percentage of riboses ranged from 3.9% to 7.8%. Explants previously induced with 25 µM BA and 50 µM mT showed the highest relative percentage of ribonucleotides (53% and 47.8%, respectively). In explants previously cultured with 25 µM mT or Z, the O-glucosides were the most important fraction in terms of endogenous relative percentage. N-glucosides were below 2% in all samples (Fig. 3).

Table 16 Endogenous cytokinin content (pmol g^{-1} DW) of the pT-type in buds from *Pinus radiata* collected from the field (genotypes 72 and 76); in buds after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 µM (I-72, I76); in buds after a re-induction period of 4 weeks (R-76); and in buds after 4 weeks on LP medium supplemented with 0.2% AC (C-76) (M±S.E.). Different letters within a column show significant differences among genotypes by Scheffe's post hoc test ($p \leq 0.05$). *<LOD*: Under limit of detection.

Code	pT	pTR	pTRMP	pTOG	pTROG	Total
72	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
76	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-72-BA25	5.80±0.39 de	7.32±0.73 bc	**16.58±2.27 ab**	**22.07±3.28 abc**	41.86±7.65 bc	93.62±10.00 bc
I-76-BA25	5.06±0.42 de	11.62±0.01 b	13.08±1.98 b	15.43±1.86 cd	32.45±1.20 cd	77.64±3.95 bc
I-72-BA50	19.14±2.50 b	12.07±1.69 b	10.98±0.67 b	**24.77±2.32 abc**	37.69±1.53 bcd	104.65±4.28 b
I-76-BA50	**26.20±1.85 a**	**27.15±1.25 a**	**26.14±3.62 ab**	**34.74±0.71 a**	**52.01±3.47 ab**	166.23±6.62 a
I-72-mT25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-76-mT25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-72-mT50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-76-mT50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-72-Z25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-76-Z25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-72-Z50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
I-76-Z50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
R-76-BA25	8.75±0.51 cd	7.32±0.08 bc	**22.65±2.96 ab**	20.33±2.94 bc	41.22±0.16 bc	100.27±6.51 b
R-76-BA50	12.84±0.57 bc	10.60±0.33 b	**29.17±3.66 a**	**29.50±2.15 ab**	**67.34±1.10 a**	149.45±2.72 a
R-76-mT25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
R-76-mT50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
R-76-Z25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
R-76-Z50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
C-76-BA25	0.47±0.03 e	1.86±0.10 d	**14.36±2.61 ab**	3.44±0.23 d	19.52±0.51 d	39.65±3.22 d
C-76-BA50	1.30±0.06 e	2.99±0.32 cd	**17.45±1.71 ab**	12.70±2.23 cd	32.94±0.65 cd	67.39±3.75 cd
C-76-mT25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
C-76-mT50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
C-76-Z25	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*
C-76-Z50	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*	*<LOD*

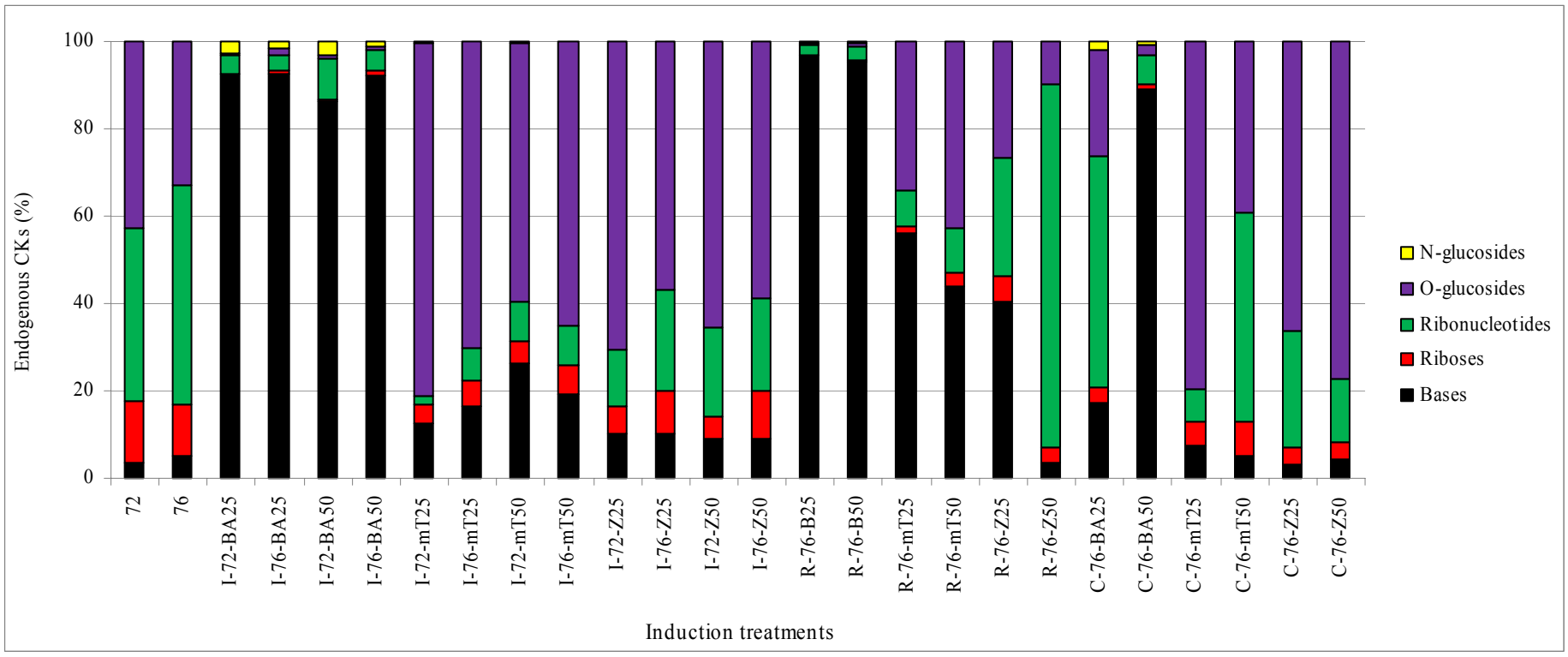

Fig. 3 Relative percentages of endogenous bases, ribosides, ribonucleotides, O-glucosides and N-glucosides in buds from *Pinus radiata* collected from the field (genotypes 72 and 76); in buds after *in vitro* culture on LP medium supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 μM (I-72 and I-76); in buds after a re-induction period of 4 weeks (R-76); and in buds after 4 weeks on LP medium supplemented with 0.2% AC (C-76).

3.3. Quantitative analysis of IAA

Buds collected from the field had a significantly higher endogenous IAA content than explants at any *in vitro* culture stage (279 and 282 pmol g^{-1} DW in genotypes 72 and 76, respectively). After the first induction period the endogenous content of all samples decreased to values between 31 and 100 pmol g^{-1} DW. The lowest endogenous levels of IAA in all the samples analysed (values above 28 pmol g^{-1} DW) were found in buds after the re-induction period. At the end of the experiment IAA values ranged from 28 to 49 pmol g^{-1} DW (Table 17).

Table 17 Endogenous IAA content (pmol g^{-1} DW), bases/IAA ratio, riboses/IAA ratio and ribonucleotides/IAA ratio in buds from *Pinus radiata* collected from the field (genotypes 72 and 76); in buds after *in vitro* culture on LP medium, supplemented with different cytokinin types (BA, mT, or Z) at 25 or 50 μM (I-72, I-76); in buds after a re-induction period of 4 weeks (R-76); and in buds after 4 weeks on LP medium supplemented with 0.2% AC (C-76) (M±S.E.). Different letters within a column show significant differences among genotypes by Scheffe's post hoc test ($p \leq 0.05$).

Code	***IAA***	***CK Bases/IAA***	***CK Riboses/IAA***	***CK Ribonucleotides/IAA***
72	**278.73±10.88 a**	0.11 d	0.41 e	1.18 b
76	**282.16±8.51 a**	0.14 d	0.32 e	1.34 b
I-72-B25	45.94±12.02 bc	838.92 bcd	1.86 e	37.78 b
I-76-B25	31.36±1.55 bc	695.14 cd	5.66 e	27.14 b
I-72-B50	41.72±3.12 bc	**1291.87 abc**	4.41 e	139.88 b
I-76-B50	51.57±3.09 bc	**1269.63 abc**	13.34 de	66.51 b
I-72-mT25	50.96±2.22 bc	46.39 d	15.60 de	8.51 b
I-76-mT25	100.07±28.81 b	34.42 d	13.02 de	14.86 b
I-72-mT50	40.29±0.96 bc	215.90 d	41.08 de	73.58 b
I-76-mT50	51.22±0.87 bc	109.67 d	37.54 de	50.21 b
I-72-Z25	47.79±1.83 bc	52.90 d	31.77 de	67.83 b
I-76-Z25	42.67±0.77 bc	18.01 d	17.07 de	40.83 b
I-72-Z50	41.94±1.70 bc	89.34 d	51.66 d	202.69 b
I-76-Z50	49.88±3.02 bc	95.95 d	118.42 c	223.51 b
R-76-B25	20.83±0.34 c	**1779.87 ab**	4.23 e	39.48 b
R-76-B50	27.70±1.93 c	**1802.72 a**	5.34 e	61.75 b
R-76-mT25	23.49±0.95 c	**928.04 abcd**	22.34 de	137.13 b
R-76-mT50	22.67±2.59 c	830.13 bcd	58.23 d	193.10 b
R-76-Z25	11.67±1.49 c	**1354.43 abc**	197.95 b	909.93 b
R-76-Z50	26.58±1.16 c	295.75 d	**270.73 a**	**6755.03 a**
C-76-B25	36.85±1.69 bc	4.00 d	0.82 e	12.41 b
C-76-B50	28.46±1.77 bc	289.31 d	3.59 e	22.03 b
C-76-mT25	34.15±1.13 bc	24.52 d	19.15 de	24.34 b
C-76-mT50	38.77±0.91 bc	1.39 d	2.11 e	12.97 b
C-76-Z25	33.62±0.36 bc	1.74 d	2.12 e	14.56 b
C-76-Z50	49.02±1.61 bc	2.52 d	2.32 e	8.58 b

Before any CK treatment, the lowest CK bases/IAA ratio was recorded in both genotypes ratio (below 0.2). After the first induction period, the CK bases/IAA ratio in buds induced with the different CK assayed (BA, mT or Z) at 25 μM was lower than the CK bases/IAA ratio in explants cultured with 50 μM of the same CKs. This trend was not observed after the re-induction period (Table 17).

The highest CK bases/IAA ratio (1802.72) was observed in buds re-induced with 50 μM BA; this ratio did not differ statistically from those achieved with the same treatment after the first induction period in both genotypes. Explants re-induced with 50 μM Z or 50 μM mT presented a CK bases/IAA ratio significantly lower than the ratio achieved after re-induction with 50 μM BA. At the end of the experiment, only the buds previously induced with 50 μM BA (289) or 25 μM mT (24) presented ratios higher than 4 (Table 17).

The lowest CK riboses/IAA ratios were observed in buds from the field (below 0.5) at the start of the experiment (Table 17). After the first induction period, buds from genotype 76 cultured with 50 μM Z presented a significantly higher CK riboses/IAA ratio than explants induced with 25 μM Z, and both concentrations of BA or mT. After induction and re-induction periods, considering genotypes 72 and 76 separately, the endogenous riboses/IAA ratio was higher in explants induced with each CK at 50 μM than in those induced with 25 μM of the same CK. The highest value for the CK riboses/IAA ratio was observed after re-induction with 50 μM Z, this ratio was significantly higher than the ratios obtained in the other samples analysed. The CK riboses/IAA ratios recorded at the end of the experiment did not differ statistically from those observed in buds from the field.

After the first induction period, Z at 50 μM provoked higher CK ribonucleotides/IAA ratios than BA or mT in both genotypes tested. As observed for the CK riboses/IAA ratio, significantly higher CK ribonucleotides/IAA ratio (6755.03) was achieved in buds re-induced with 50 μM Z than in the other samples analysed, being at least 7.4 times higher. In the re-induction period, explants cultured with 50 μM of each CK tested showed higher endogenous ratios than those incubated with 25 μM of the same CK (Table 17).

At the end of the experiment, after culture on LPAC, the values for the CK ribonucleotides/IAA ratio were similar in buds from different treatments (below 25). As it was observed in the other ratios considered, the lowest endogenous CK ribonucleotides/IAA ratios were observed in buds from the field (Table 17).

The high CK riboses/IAA and CK ribonucleotides/IAA ratios recorded after re-induction with Z at 50 μM were not caused by a low IAA content in these samples (Table 17), but for the high riboses and ribonucleotides levels of the isoprenoid type CKs (particularly tZR, tZRMP, DHZR and DHZRMP) achieved in explants re-induced with this treatment (Tables 9 and 11).

4. DISCUSSION

Maturational changes are particularly persistent and difficult to reverse in conifers. *In vitro* culture can be used to reinvigorate mature selected trees in order to enhance their rooting ability for a true-to-type cloning (Von Aderkas and Bonga 2000). In this sense *in vitro* axillary budding offers considerable potential for obtaining plants from superior mature trees (Cortizo et al. 2009a). However, there are not many studies focused on *in vitro* organogenesis from mature pine trees (Abdullah et al. 1987; Parasharami et al. 2003; De Diego et al. 2008; De Diego et al. 2010). As pointed in Chapter 4, *in vitro* vegetative propagation from physiologically mature tissue tends to be quite difficult as the reinvigoration or appearance of some juvenile characteristics is transient and can result in changes in the attributes of the resulting plants (Von Aderkas and Bonga 2000). Regarding to this, in woody plants the rooting capacity has been reported to vary noticeable among time replicates of the same rooting protocols (Dumas and Monteuuis 1995; Monteuuis 2004). In this study, we have obtained shoots with juvenile characteristics from different genotypes of *P. radiata* adult trees mature with the different CKs assayed (BA, mT and Z).

Zhang et al. 2010 showed that BA at 22 μM could reverse the maturation status of mature buds in tissue culture. They described anatomical changes such as the development of primary needles from the newly activated fascicle apical dome; moreover the fascicle meristems produced apical domes that were similar to the apical meristem structure of a seedling. However, neither different CKs or concentrations nor different initial induction periods were tested in this study.

In our experiments the different initial induction periods assayed (6 or 8 weeks) did not influence significantly the BFS percentage. Although all genotypes produced shoots showing juvenile characteristics, two genotypes (7 and 76) were highly organogenic with BFS percentages ranging from 49 % to 69 %, another two were moderately organogenic (10 and 67), and another one (72) showed a very low organogenic response (under 6% of BFS). The

genetic background of a tree has great influence on cloning efficiency, and it is generally accepted that all genotypes or families within a species are not equivalent in their propagation capacity (Davis and Becwar 2007). In this sense, De Diego et al (2008) tested the organogenic response of 20 adult maritime pine trees and also observed differences among genotypes.

When the genotypes were analysed together, although no significant differences were found among treatments for the organogenic response in the two initial induction periods assayed, the highest BFS percentages were obtained after application of 50 μM BA to the induction medium (between 52.5 and 57.5%). In this sense, in organogenesis from *P. pinaster and P. sylvestris* adult tissues De Diego et al. (2008; 2010) did not found significant differences in terms of BFS among treatments with BA and Z tested at 25 μM and 50 μM, and mT at 25 μM. In our experiments the lowest BFS percentages were observed after treatments with Z and mT at 50 μM (in the 6 and 8 weeks induction periods, respectively). Our results are in agreement with the studies by De Diego et al. (2008; 2010) in which the worst BFS percentages were obtained after treatments with 50 μM mT and 50 μM Z in *P. pinaster* and *P. sylvestris,* respectively.

The explants were subcultured on elongation medium with AC between induction periods and after the re-induction period in order to prevent the negative effects associated with an excess of CK in the explants or toxic compounds in the culture medium (Thomas 2008). In general, most of the explants did not present symptoms of hyperhydricity (data not shown), suggesting that the phytohormones and concentrations used did not reach toxic levels in the buds and that as mentioned above, the differences found in the *in vitro* performance of some genotypes could be due to their recalcitrance to micropropagation (Bonga et al. 2010).

The highest number of shoots per bud was found in genotypes 7 and 76, and the lowest in the less organogenic genotype (72); the SEC indices also followed this pattern. Gathering all the genotypes tested, in the 6 weeks induction period significantly lower SEC values were obtained after treatment with 25 μM BA. In the 8 weeks induction period treatments with 25 μM BA or 50 μM mT provoked significantly lower SEC indices. When the genotypes subjected to CK analysis (72 and 76) were considered separately for the SEC index, in genotype 72 (non-organogenic genotype) the differences among induction treatment were non-significant. In genotype 76 (organogenic genotype), the best SEC index was achieved after treatment with BA at 50 μM. In this sense, differences among genotypes in their *in vitro* performance have been widely documented in different conifer species (Bonga et al. 2010).

The results for the organogenesis parameters studied indicate that 50 µM BA is the most suitable treatment in terms of BFS percentage and SEC indices. Further studies on rooting and *ex vitro* performance of the regenerated shoots could elucidate possible differences among shoot induction treatments, as it has been tested in *in vitro* organogenesis from zygotic embryos (Chapter 1). To the best of our knowledge, this is the first study where the effect of different CKs and concentrations has been assayed for *in vitro* organogenesis from radiata pine adult trees.

We analysed endogenous concentrations of different CK types and fractions, in an attempt to relate them with the organogenic response observed in the buds. Some authors have pointed the importance of different CK fractions, auxin or ABA as markers of the physiological stage of an explant (Valdés et al. 2004a; 2004b; 2007; Kepinski 2006; Moncaleán et al. 2002). In radiata pine, Valdés et al. (2002) found a correlation between the maturity degree and loss of the morphogenic capacity and the decreasing iP-type/Z-type CKs ratio. In accordance with these authors, iP significantly increased in genotype 76 after the first induction with BA at 25 and 50 µM, and with mT at 25 µM; but no clear trend was observed in other samples analysed from this genotype or in genotype 72. Valdés et al. (2002) found increasing Z-type (Z and ZR) CKs throughout maturation; on the contrary, in our experiments tZ and tZR significantly increased after reinvigoration with 50 µM Z. When the buds were subjected to the other treatments tested, and at the end of the experiment in all samples, tZ and tZR values were similar to those observed in buds from the field. In buds induced and re-induced with Z, DHZ and DHZR levels were significantly higher, thus, zeatin reductase could have converted part of the Z applied to DHZ. Although the endogenous levels of cZ and cZR were low, they were significantly higher after induction and re-induction with 50 µM Z than in buds from the field.

Zhang et al. (2003) proposed nucleotide and glucoside fractions of the isoprenoid CKs as indicative markers of maturation status of buds in *P. radiata*; they found a higher endogenous content of these two metabolite groups in mature buds than in juvenile buds. According with these authors and on the contrary of the observation made by Bredmose et al. (2005) we observed that buds after induction and re-induction presented iPRMP lower than buds collected from the field (except for re-induction with 50 µM Z). In contrast, the ribonucleotide and O-glucoside fractions of tZ-type and DHZ-type CKs in explants cultured with Z were at least 5 times higher than in buds from the field. In this sense, Åstot et al. (2000) reported that CK biosynthesis occurs at the nucleotide level, being this reaction reversible; phosphoribosylation of CK base by adenine phosphoribosyltransferase reduces the biological

activity, playing an important role in the regulation of the level of active CKs (Moncaleán et al. 2005). In our experiments an external supply of 50 μM Z, could have favoured this phosphoribosylation as a mechanism of regulation, explaining the high levels of ribonucleotides found for the isoprenoid CKs analysed (tZRMP, cZRMP, DHZRMP and iPRMP) after re-induction period.

The analyses described in our study were able to discriminate between iP-type CKs and BA-type CKs; while Zhang et al. (2010) were not able to separate these two CK types due to cross-reaction with the antibody employed and considered the iP content negligible. With our analytical method (Novák et al. 2003; 2008) we were able to discriminate these two CK groups and found BA metabolites and iP metabolites above the detectable limit.

The methodology developed by Novák et al. (2003; 2008) provided sufficiently sensitive and selective analytical tools for determination of several endogenous CKs, even when they were present in minute amounts, from small amounts of tissue (50 mg DW). This methodology combined convenient, high-throughput purification steps, rapid but reliable separations and highly sensitive detection and quantitative analysis by electrospray tandem mass spectrometry.

The highest levels of the N-glucoside fraction were found in the aromatic CKs analysed, particularly BA9G; due to the fact that very high levels of endogenous BA were observed in BA treated buds, this N-glucosylation could have been a mechanism of detoxification (Feito et al. 1995; Sakakibara 2006).

It is remarkable that aromatic CKs and their derivatives were only found in buds treated with BA or mT. BA-type compounds were recognized as naturally occurring based on their isolation from poplar (Horgan et al. 1975), anise (Ernst et al. 1983), or tomato (Nandi et al. 1989). Jones et al. (1996) reported the occurrence of BA, mT and oT in various tissues of oil palm and Strnad et al. (1997) isolated mT from poplar leaves; but in radiata pine endogenous BA has only been found after supplying BA to the culture medium.

Zhang et al. (2010) reported an increase in the endogenous levels of BA, after CK withdrawal from the culture medium. On the contrary, after CK removal from the culture medium, we observed a decrease in the endogenous content of all BA-type metabolites analysed. These authors also found relatively low levels of BARMP and associate it with rejuvenation of the tissue whereas in our samples this metabolite was the most abundant after the bases,

particularly after induction and re-induction. The abovementioned decrease in the concentration of all BA metabolites, at the end of the experiment (after culture in the presence of AC), could have favoured the elongation of the shoots induced (Thomas 2008).

As pointed above, hydroxylated forms of BA also occur naturally (Strnad 1997), being mT much more active than pT and oT (Mok and Mok 2001). Interestingly, endogenous oT and pT-type CKs were only found in buds treated with BA; the endogenous content of oT was higher than the content of mT or pT in these explants after induction and re-induction periods. Kamínek et al. (1987) proposed that region-specific hydroxylation might regulate the activity of the aromatic CKs. The hydroxylation of the benzyl ring of BA in the *meta*-position increases CK activity; and in the *ortho* and the *para*-position decreases CK activity (Strnad 1997; Holub et al. 1998). At the end of our experiments mT-type CKs were high in explants from genotype 76 previously treated with 25 μM mT, but surprisingly this coincided with the lowest SEC in this genotype.

Endogenous mT-type CKs were found at high levels in buds induced with mT and at low levels in buds induced with BA. These results suggest the existence of a unidirectional enzymatic pathway to convert BA in Topolins and its metabolites. Although BA uptake and metabolism has been already described in *P. pinea* (Moncaleán et al. 2005; Cortizo et al. 2009b; Cuesta et al. 2009) and *P. radiata* (Zhang et al. 2010), this is the first study in radiata pine where the hydroxylation of BA to form oT, mT and pT-type CKs is reported.

We have studied for the first time the effect of different CKs (mT and Z) apart from BA, on organogenesis and on endogenous CK profiles of buds from radiata pine. Several authors have suggested that exogenously applied CKs instead of acting through endogenous CKs, act *per se* (Feito et al. 1995; Centeno et al. 1996; Moncaleán et al. 1999) and our results with the different CKs assayed support this hypothesis. In this sense, when we applied BA or mT to the induction medium, we did not observe any significant increase or decrease in the endogenous isoprenoid CK content of the treated explants; on the contrary of the reports in *P. pinea* by Moncaleán et al. (2005) or *P. radiata* (Zhang et al. 2010).

Further, we also report for the first time in *Pinus* the existence of O-glucosides (tZOG, tZROG, cZOG, cZROG, DHZOG, DHZROG) in explants from the field, before any CK treatment. O-glucosides represented around 35% of the total in buds from the field; after the first induction period the relative percentage of this fraction increased and reached values

around 70%. O-glucosides may have had a key role in regulating the endogenous levels of CKs, as they are stable and reversible storage forms. The natural occurrence of O-glucosides has been reported in bryophites (Von Schwartzenberg et al. 2007), angiosperms (Werner et al. 2003) and gymnosperms (Kong et al. 2009; Rasmussen et al. 2009). In the same way, our results have demonstrated the existence of the mechanism for side chain O-glucosilation *in vivo* and *in vitro* in *P. radiata* in contrast to the reports by Zhang et al. (2001; 2003; 2010) where they concluded that conifers and particularly *P. radiata* lacked this mechanism. These differences in the results obtained could have been due to differences in the analytical methods, as in our experiments O-glucoside derivatives were treated with β-glucosidase and immuno-purified again, giving the O-glucoside (OG) fraction.

Apart from the different CK types and fractions mentioned as potential markers of the physiological state of a plant or a certain tissue, Skoog and Miller (1957) proposed the balance between auxin and CK as determinant of the morphogenic competence of an explant. We measured the endogenous content of free IAA, and found that the levels of this auxin in buds from the field were significantly higher than in buds treated with different CKs. Materán et al. (2009) observed in this way that the endogenous content of IAA in adult buds from *P. radiata* was higher than in juvenile buds and micrografted explants. In our experiments, the lowest levels of IAA (10-fold lower than in buds from the field) were observed in buds after the re-induction period. On the contrary, Valdés et al (2004a) reported a decline in the IAA content in parallel with organ maturation in radiata pine. The same authors did not observe any variation in the IAA content in Stone pine buds throughout maturation of the trees tested (Valdés et al. 2004b). No differences among genotypes were found for the IAA content, thus we cannot attribute the differences in their organogenic performance to the endogenous content of this auxin in the explants.

Holub et al. (1998) proposed the activity sequence from the most active CK to the least active CK as follows: mT = Z > BA > oT. But in our assays, at the end of the experiment, the buds previously induced with 50 μM BA presented the highest bases/IAA ratio, coinciding with the highest BFS percentage and SEC values for these. At this culture stage buds from other treatments presented a relative content of bases below 20% whereas the relative endogenous content of bases in buds that have been treated with BA at 50 μM was 90%. Our results are in agreement with the study of Van der Krieken et al. (1988) where they pointed that when BA availability increases in the medium, relatively more BA remains in the unconjugated form.

Analysing our results, we can relate the best organogenesis results to a high CK bases and CK bases/IAA ratio.

The lowest bases/IAA was observed in buds from the field. After the first induction period buds from genotype 72 and 76 presented similar values but then, most of the buds from genotype 72 did not respond to the induction treatments and died. One of the properties of the AC present in elongation medium is to adsorb a CK excess (Pan and Van Staden 1998) but the residual CK accumulated in the explants might had not been rapidly adsorbed by the AC. As we have pointed above, different genotypes respond differently to external stimuli and the accumulation of high cytokinin levels inside the explants could have had a toxic effect in genotype 72, while promoting organogenesis in others as genotype 76.

We have analysed 43 CKs and IAA analysis offering the broadest spectrum till date in *P. radiata* buds before and during the reinvigoration process, but more information about enzymes involved in CK metabolism and CK receptors is needed to fully explain the events occurring in morphogenesis and why the same phytohormone dose has different effect in different genotypes.

In this study we have pointed the importance of ribonucleotide and O-glucosides fractions as storage forms with regulatory functions. We have also concluded that the relative CK bases content and the CK bases/IAA could be used as markers in *Pinus radiata* explants.

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CAPÍTULO 6

Aiming for somatic embryogenesis from *Pinus radiata* adult trees

Este capítulo se corresponde con la publicación: Recent observations regarding *Pinus radiata* somatic embryogenesis using juvenile and mature material (*2010 IUFRO Somatic Embryogenesis and Forest Trees Conference, Proceedings*, aceptado).

Capítulo 6: Aiming for somatic embryogenesis from *Pinus radiata* adult trees

1. INTRODUCTION

Clonal propagation of high-value forest trees through somatic embryogenesis has the potential to rapidly capture the benefits of breeding or genetic improvement programs and to increase the uniformity and quality of the nursery plant stock (Find et al. 1993).

Somatic embryogenesis (SE) in *Pinus* genus is usually initiated from immature seeds; either from immature embryos left in the excised megagametophytes (Yildirim et al. 2006) or from excised immature zygotic embryos (Hargreaves et al. 2009). There have been attempts to initiate SE from mature seeds in *Pinus strobus* (Garin et al. 1998) and *P. taeda* (Tang et al. 2001). However, the initiation frequencies obtained have been low to be considered for a commencial scale. When using mature or immature seeds, the embryogenic tissues (ET) obtained must be cryopreserved while field testing takes place to identify superior genotypes (Park et al. 1998; Nehra et al. 2005). In this sense, if elite mature trees could be cloned 'true-to-type' on a large scale by SE, superior characteristics could be captured immediately without having to depend on the clonal testing that is required when zygotic embryos are used as explants (Bonga 2004). Recently plant regeneration through SE from mature trees has been reported in *Olea europea* (Rugini and Caricato 1995) or *Quercus robur* (Toribio et al. 2004). In *Pinus* genus, SE has been achieved from mature tissues of *P. kesiya* (Malabadi et al. 2004), *P. patula* (Malabadi and Van Staden 2005) and *P. roxburghii* (Malabadi and Nataraja 2006). In *P. sylvestris* ET has been obtained but nowadays its conversion into plants still represents a challenge (Aronen 2010).

In conifers, the reduction in morphogenesis due to the phase change can be reverted *in vitro* with reinvigoration procedures such as culture in presence of cytokinins (Chapter 5; De Diego et al. 2008). To this respect, the term "reinvigoration" can be defined as a temporary increase in vigour that enables the recovery of some organogenic abilities (Valdés et al. 2003). This transient effect has been demonstrated through the analysis of the DNA methylation rate in *Acacia mangium* (Monteuuis et al. 2009) and in *P. radiata* (Fraga et al. 2002) in relation to their time in *in vitro* culture.

Our research team has developed protocols for organogenesis from tissues of adult *Pinus radiata* (Chapter 5), *P. pinaster* (De Diego et al. 2008) and *P. sylvestris* (De Diego et al. 2010) trees. However conifer SE has generally been far more productive than propagation by organogenesis. Furthermore, SE has the advantage that once the somatic embryos are obtained both root and shoot develop simultaneously, which simplifies the process of plant regeneration (Conde et al. 2004). Therefore, trying to obtain propagation of mature conifer trees by SE is worthwhile (Bonga 2004).

When trying to micropropagate adult trees there are some zones within trees that are more morphogenetically competent than other parts of the tree (Bonga et al. 2010) such as microcuttings in *Cedrus atlantica* and *C. libani* (Renau-Morata et al. 2005), vegetative buds in *Pinus patula* (Malabadi and Van Staden 2005) and *P. pinaster* (De Diego et al. 2010) or basal sprouts in *Sequoia sempervirens* (Boulay 1987). Also, SE-derived plantlets (emblings) are more responsive to *in vitro* propagation than those obtained from seed (Bonga et al. 2010). In this sense, SE has also been achieved from shoot buds of 10 years old *Picea glauca* somatic trees (Klimaszewska et al. 2010). In some studies in angiosperm species, *in vitro* shoot cultures have been used as a source of explants (Conde et al. 2004; San-José et al. 2010). These cultures enable better control of the growing conditions of stock material, avoid difficulties associated with possible differences in the physiological state of the explants, and guarantee a supply of an unlimited number of explants independently of the season.

ET initiation is influenced by the genotype of the plant, the developmental stage of the initial explant and the composition of the induction media (Radojevic et al. 1999). In this sense, factors such as carbon sources (Salajová and Salaj 2005), nitrogen sources, mineral elements (Hargreaves et al. 2009), gelling agents (Pullman and Johnson 2002), plant growth regulators (Klimaszewska et al. 2001; Lardet et al. 2009) and pH (Tautorus et al. 1991) have been identified as critical factors in the first stage of SE proccess.

At present, there is no report on SE from adult radiata pine. Taking into account the aforementioned factors, the purpose of this research was to induce SE and to study the effect on ET initiation of explant, basal media, plant growth regulators and aminoacids.

2. MATERIALS AND METHODS

2.1. Experiment 1

In 2008, seven trees were selected from a seed orchard established by Neiker-Tecnalia in Deba-Spain (latitude: 43º16'59''N, longitude: 2º17'59''W, elevation: 50 m). These trees were 19 years old at the moment of collection. Apical shoot buds (3–5 cm long) were taken from mid-basal part of the trees. The buds were collected fortnightly from the 18th of February (Fig. 1a) to the 29th of April (Fig. 1b), wrapped in moist paper to prevent dehydration and stored in polyethylene bags at 4ºC for a maximum of a week.

Buds were sprayed with 70% (v/v) ethanol, and then rinsed with sterile distilled H_2O. Afterwards, the buds were submerged in 50% (v/v) commercial bleach (active chloride >5%) plus 2 drops of Tween 20® and agitated for 10 min. Finally, they were rinsed three times in sterile distilled H_2O in aseptic conditions. When possible bud scales were removed, explants were cut transversely into 1–1.5 mm thick slices with a surgical scalpel blade and were laid on the culture medium (Fig. 1c).

In the first and the second collection dates (18th of February and 3th of March) buds slices were cultured on two initiation media. The first medium was embryo development medium (EDM) (Walter et al. 1998) with 30 g L^{-1}sucrose, 1 g L^{-1} inositol and a combination of 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 µM BA. Before autoclaving, the pH of the medium was adjusted to 5.7 and then 3 g L^{-1} Gelrite® were added. Medium was autoclaved at 121ºC for 20 min. After autoclaving, filter-sterilized solutions (pH=5.7) of 550 mg L^{-1} L-glutamine, 525 mg L^{-1} asparagine, 175 mg L^{-1} arginine, 19.75 mg L^{-1} L-citrulline, 19 mg L^{-1} L-ornithine, 13.75 mg L^{-1} L-lysine, 10 mg L^{-1} L-alanine and 8.75 mg L^{-1} L-proline were added to the cooled medium.

The second medium was full-strength DCR basal medium (DCRI, Gupta and Durzan 1985 modified by Malabadi and Van Staden 2005) containing 0.2 g L^{-1} polyvinylpyrrolidone-40 (PVP-40), 3.24 % (w/v) maltose, 1 g L^{-1} inositol and supplemented with 20 µM 2,4-D, 25 µM 1-naphthaleneacetic acid (NAA) and 9 µM BA. Before autoclaving, the pH of the medium was adjusted to 5.7 and then 1.5 g L^{-1} Phytagel was added. After autoclaving, filter-sterilized solutions (pH= 5.7) of 1 g L^{-1} casein hydrolysate and 1 g L^{-1} L-glutamine were added to the cooled medium prior to dispensing into gamma-irradiated Petri dishes (90 x 20 mm).

From the third to the sixth collection date (from the 17th of March to the 29th of April) half of the collected buds were cultured on DCRI, and the other half were subjected to a cold pre-treatment. This pre-treatment consisted of culturing the buds at 4ºC on full-strength DCR basal medium containing 0.2 g L^{-1} PVP-40, 3.24 % (w/v) maltose, 0.3% (w/v) activated charcoal (AC), and 1.5 g L^{-1} Phytagel; after 3 days, these buds were subcultured on DCRI.

At each collection date, five bud slices per Petri dish were cultured; seven Petri dishes per treatment were laid out randomly on the shelves of the growth chamber. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks. Then the bud slices and/or the proliferating tissues were transferred to maintenance medium. Maintenance medium for explants cultured on EDM was the same used for initiation; and maintenance medium for explants cultured on DCR (DCRM) had the same basal composition but contained 4.32 % (w/v) maltose, 1 g L^{-1} inositol and was supplemented with 2 µM 2,4-D, 2.5 µM NAA and 1 µM BA. The amino acid mixture was the same as for initiation. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks on maintenance medium.

2.2. Experiment 2

Ten trees over 20 years old were selected from a seed orchard established by Neiker-Tecnalia in Amurrio-Spain (latitude: 43º03'00''N, longitude: 3º01'00''W, elevation: 50 m). Apical shoot buds (3–5 cm long) were taken from mid-basal part of the trees (Fig. 2a). The buds were collected fortnightly from the December of 2009 to January of 2010. The buds were stored and disinfected as described in experiment 1.

Buds were cut transversely into 1-1.5 mm thick slices and 6-7 mm slices. These slices of two different thicknesses were cultured on EDM and on modified LP basal medium (LPI, Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988). This LPI medium was supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} inositol, 20 µM 2,4-D, 25 µM NAA and 9 µM BA; before autoclaving the pH of the medium was adjusted to 5.8 and 3 g L^{-1} Gelrite® were added. After autoclaving the same amino acid mixture used in EDM medium was added. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks.

Another set of buds cut into 6-7 mm slices was cultured on LP basal medium to induce organogenesis (LPO). LPO medium was supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} inositol and 22 µM BA; before autoclaving, the pH of the medium was adjusted to 5.8 and 8 g L^{-1} Difco Agar® were added. Cultures were maintained at 21±1°C under a 16 h photoperiod of

120 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France) for 8 weeks.

The bud slices cultured on EDM were transferred to the same medium. The slices cultured on LPI were subcultured either on LPI or on EDM. The buds cultured on LPO were subcultured to LPI.

2.3. Experiment 3

In 2010, *in vitro* adventitious buds were obtained from vegetative buds of ten trees over 20 years old from the same seed orchard mentioned in experiment 2 (Fig. 3a). These explants were obtained by culturing the buds from the field on LPO for 4 weeks to induced adventitious organogenesis and then transferring the explants to LP basal media lacking growth regulators and supplemented with 0.2% (w/v) AC (LPAC). The explants were transferred from one medium to another every month. After one year, *in vitro* buds were cut into halves, quarters or slices (Fig. 3b) and cultured on EDM or on EDM supplemented with 20 μM 2,4-D, 25 μM NAA and 9 μM BA (EDM2) (Fig. 3). After 4 to 8 weeks, when proliferation of the tissue was observed, the explants were transferred to maintenance medium. The maintenance media were those used for initiation. Cultures were maintained in the dark at 21±1ºC.

Small pieces of proliferating tissue were stained with acetocarmine (2% w/v) directly on glass slides for 4 min. Then, samples were rinsed with distilled sterile water and samples were mounted with a cover slide. Cover slides were sealed with commercial nail varnish to allow preservation of the tissue in the medium term (Gupta and Durzan, 1987). Samples were observed with an inverted microscope (LEICA DM4500) using a 40-fold magnification.

3. RESULTS

3.1. Experiment 1

In the first and second collection dates and in all the genotypes assayed, the bud slices cultured on EDM developed tissue in the peripheral part of the buds (Fig. 1d). These cell proliferations were formed by white-traslucent tissue formed by elongated cells (embryogenic-like cells, Fig. 1e) and a lower fraction of rounded cells (non-embryogenic like

cells). The buds cultured on DCRI showed the same growth of tissue but it grew less and more slowly than on buds cultured on EDM. When transferred to maintenance medium, the tissues continue proliferating at rapidly but the percentage of rounded cells increased and the tissue showed a yellow-brownish colour (Fig. 1f).

In all genotypes used from the third to the sixth collection dates, the growing tissues on bud slices cultured on DCRI showed the same trend mentioned above for the initiation and maintenance. The cold pre-treatment did not have a beneficial effect on the bud slices and half of these slices did not develop any tissue and necrosed. The other buds when transferred to DCRI formed tissue in the axillary zone of the buds, in the needle insertion zone. Although this tissue was white-traslucent and embryogenic-like cells could be seen (Fig. 1e), when proliferated in DCRM it tended to have a yellowish colour and after one month rounded cells started to appear (Fig. 1f).

3.2. Experiment 2

The bud slices cut transversely into 1-1.5 mm thick slices necrosed rapidly and did not produced any tissue (Fig. 2b). When the initial explants were buds cut into 6-7 mm slices and cultured on EDM, a developing white-traslucent tissue was observed with embryogenic-like cells (Fig. 2e); this tissue was subcultured to the same medium and became yellowish with a higher proportion of rounded cells.

On explants cultured and subcultured on LPI the tissue grew more slowly than on those subcultured on EDM or cultured from the beginning of the experiment on EDM. But after 4 to 8 weeks, the tissue growing on LPI showed the same macro and micro-morphological features as the ones growing on EDM (Fig. 2f).

When cultured on LP-O most buds showed a development in the brachyblast meristems; when transferred to LPI, 50% of them necrosed; the others developed globular structures (Fig. 2c) that finally burst showing the appearance of the tissue type previously observed on the other culture media assayed (Fig. 2d).

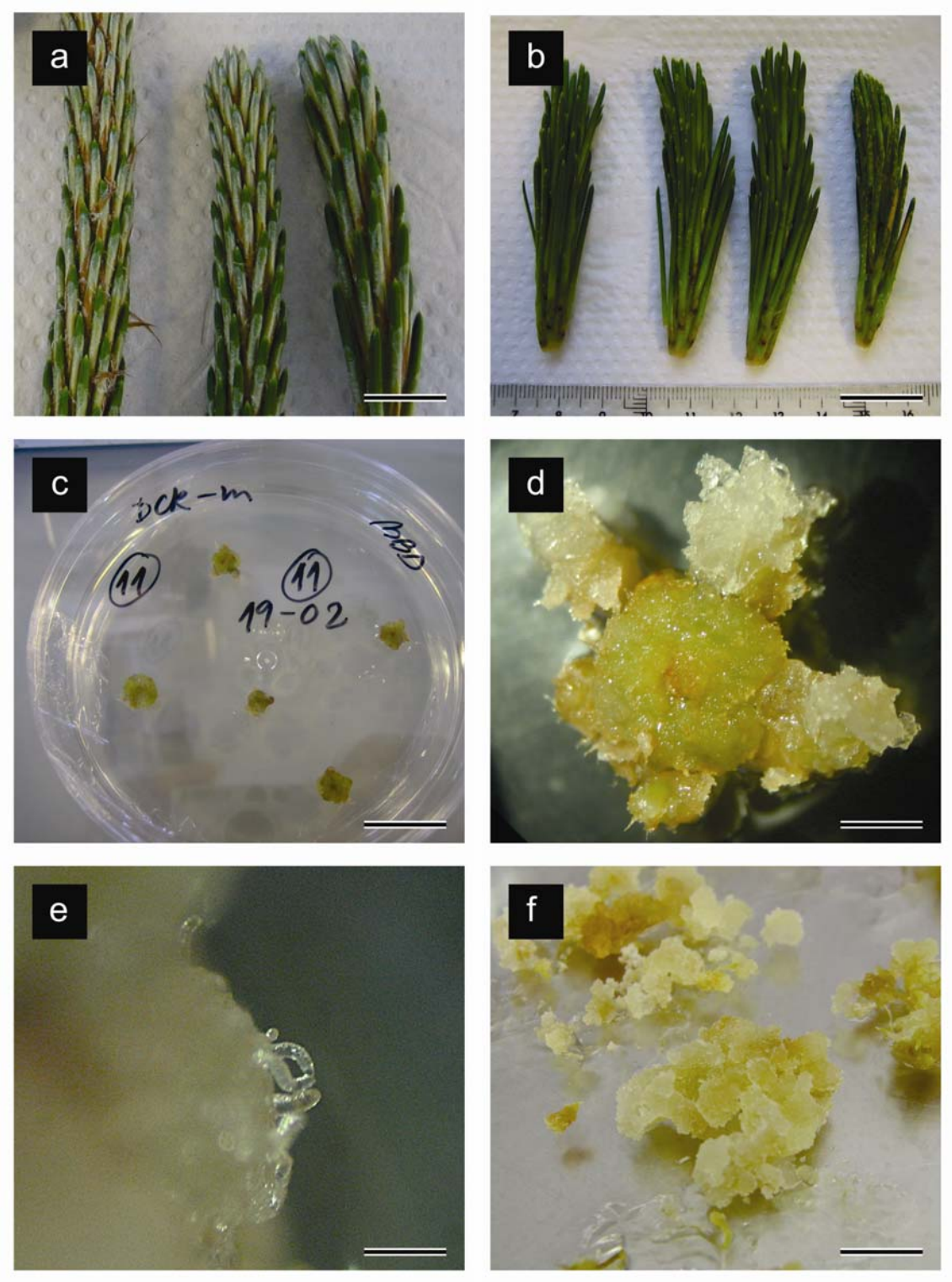

Fig. 1 Experiment 1: (a) shoot buds from the field collected at the end of February (bar=12 mm); (b) shoot buds from the field collected at the start of April (bar=19 mm); (c) bud slices collected on mid-February and cultured on DCRI medium (bar=14 mm); (d) bud slice cultured on DCRI for 3 weeks (bar=4 mm); (e) elongated cells in the proliferating tissue from the bud slices (bar=1 mm); (f) tissue proliferating on DCRM medium (bar=9 mm).

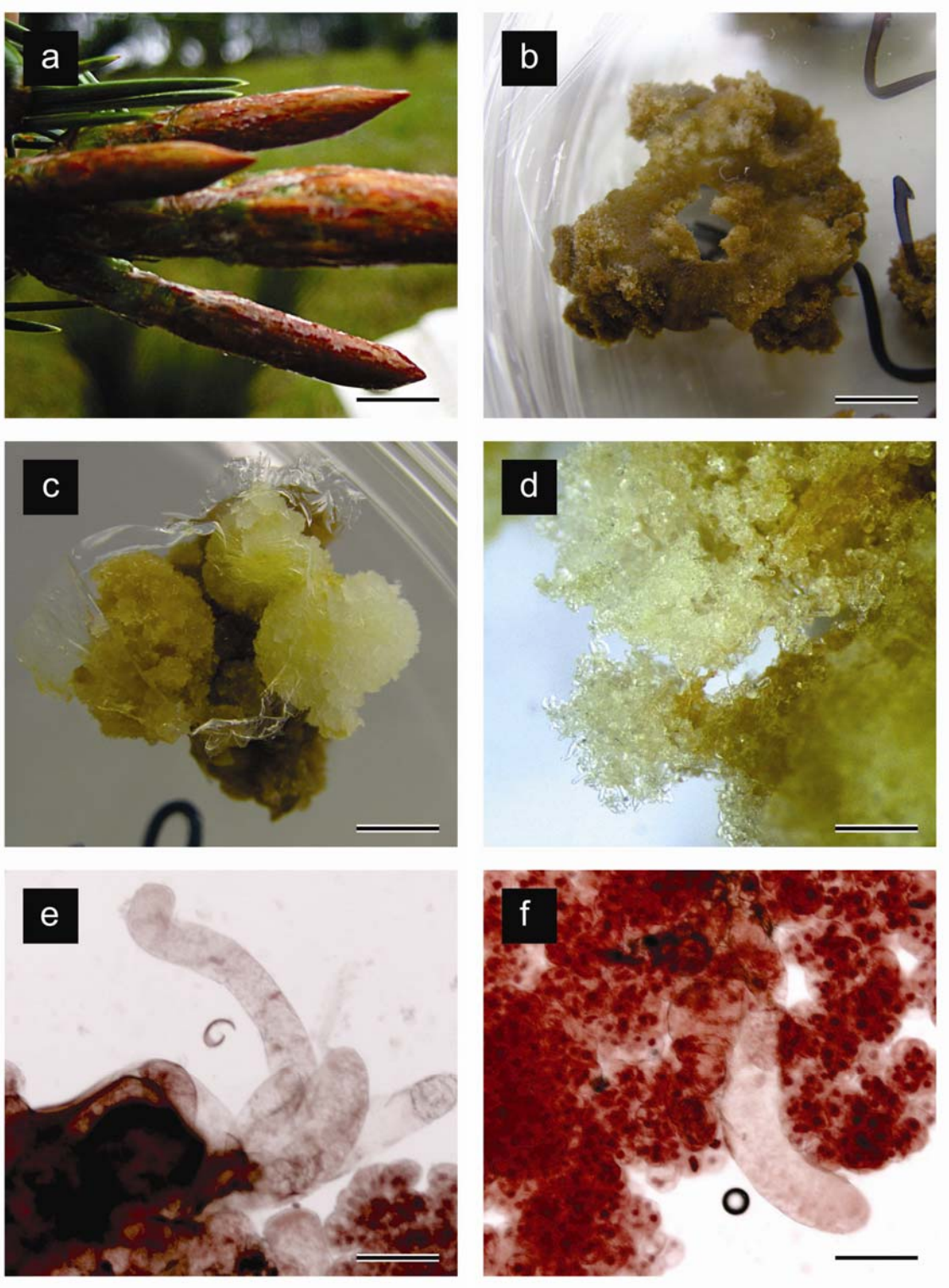

Fig. 2 Experiment 2: (a) shoot buds from the field collected at the end of December (bar=17 mm); (b) bud slice (1.5 cm thick) cultured on EDM (bar=4 mm); (c) tissue growing on 7 mm bud slices cultured on LPI medium (bar=4 mm); (d) elongated and rounded cells in the proliferating tissue (bar=3 mm); (e) micro-morphology of elongated cells in the proliferating tissue (bar=0.15 mm); (f) micro-morphology of elongated and rounded cells in the proliferating tissue (bar=0.2 mm).

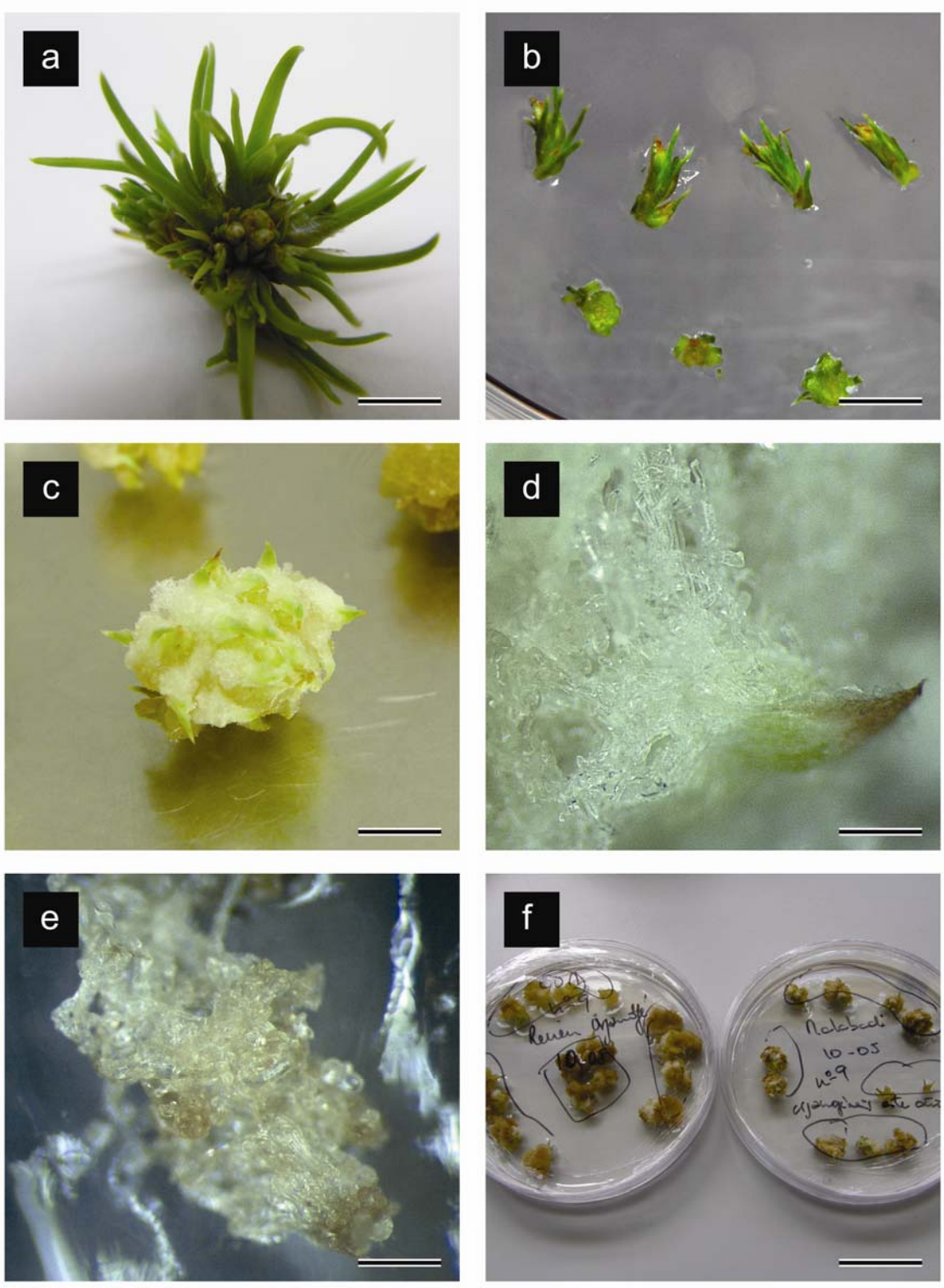

Fig. 3 Experiment 3: (a) *in vitro* adventitious buds (bar=7 mm); (b) *in vitro* buds cut into quarters, halves and sections (bar=5 mm); (c) *in vitro* bud after culture on EDM for 4 weeks (bar=6 mm); (d) elongated cells in proliferating tissue from *in vitro* buds cultured on EDM for 4 weeks (bar=0.8 mm); (e) elongated cells arranged into embryo-like structures from *in vitro* buds cultured on EDM for 6 weeks (bar=0.7 mm); (f) tissue growing on EDM on the left, and tissue growing on EDM2 on the right (bar=30 mm).

3.3. Experiment 3

Tissue proliferation was observed in the three types of explants tested (halves, quarters and slices) and this growth was higher in buds cut into quarters (Fig. 3c). In the explants cultured on EDM the tissue grew at a higher rate than in the explants cultured on EDM2 (Fig 3d). As observed in previous experiments, when transferred to maintenance medium the tissue became yellowish (Fig. 3f) and the cells showed rounded morphology in a higher proportion. But initiatlly, the proliferating tissue was white-translucent and embryogenic-like cells could be observed, showing promising macro-morphological features (Figs. 3d and 3e).

4. DISCUSSION

In this sense, there are only a few reports in conifers and in particular in *Pinus* genus where SE is described using mature tissues (Malabadi et al. 2004; Malabadi and Van Staden 2005; Malabadi and Nataraja 2006). SE in *P. radiata* is still mostly achieved from immature seeds, and there are no reports where SE has been obtained from explants over one year old (Smith 1997).

Cold pretreatment has been used to promote somatic embryogenesis in several angiosperm species (Krul 1993; Tomaszewski et al. 1994; Janeiro et al. 1995; Luo et al. 2003). In our first experiment and following the methodology described in *P. patula* (Malabadi and Van Standen 2005), buds slices were subjected to a cold pretreatment during 4 days. In contrast with the results obtained in *Larix decidua* and *L.* x *eurolepis* (Bonga 1996; 1997) and *P. patula* (Malabadi and Van Staden 2005), in radiata pine the cold treatment induced embryogenic-like tissue formation but did not have a significant effect on the initiation frequency as was also observed in *P. sylvestris* or *Abies cephalonica* SE (Krajňaková et al. 2008) using immature seeds as initial explant (Häggman et al. 1999).

In experiment 1, different induction media (DCR and EDM) were also tested for SE initiation. These media diferred in their basal composition and in the aminoacid mixture added, DCR was supplemented with casein and glutamine and EDM with the amino acid mixture currently used in this medium. Although the tissue growing in both media had the same aspect, on DCR it grew more slowly. In previous experiments with immature seeds from radiata pine, we have observed a negative effect of casein in SE proliferation (Chapter 2) and maturation

(Montalbán et al. 2010). Different performances of ET depending on the aminoacid formulation of the induction media have been also reported in *Sorghum bicolor* (Elkonin et al. 1995).

Our results showed that cutting the buds into thin slices (1-1.5 mm) had a negative effect in contrast with the observations made in *P. kesiya* (Malabadi et al. 2004), *P. patula* (Malabadi and Van Staden 2005) and *P. roxburghii* (Malabadi and Nataraja 2006) where only thin apical domes gave optimum callusing. This could be due to the fact that *P. radiata* buds were big and slices thicker than 3 mm were needed to preserve the whole whorl of brachyblast primordia. On the contrary, thinner slices cut this brachyblast primordial and the explants died shortly after.

Time of harvest and shoot selection are particularly important factors because the cells of a tissue differ in their ability to respond *in vitro* at different developmental stages (Becwar et al. 1990). In experiment 1, we collected the buds from February to April and we observed a similar *in vitro* response in this period for all the tested genotypes. Our results are opposite to those obtained in *P. patula* (Malabadi and Van Staden, 2005) where they found a strict correlation between the physiological status of the explant and its embryogenic potential.

In the abovementioned study on *P. patula*, it has been reported that white-traslucent embryogenic tissue only appeared in the central part of the explant sections. In contrast, in our experiments using buds from the field as initial explants, the tissue always proliferated from the peripheral part of the bud slices (in the insertion zone of the needles). Bonga (1996) reported growth of the ET from needles of adult *Larix*. Our tissue had the same colour and appearance of that described in *P. patula* so the location of proliferating tissue might depend on the species.

When we used adventitious buds obtained *in vitro* as initial explants, the tissue grew on the basal part of the shoot primordia. This tissue had the same macro-morphological aspect observed in previous experiments (white-translucent tissue formed by embryogenic-like cells). On the contrary, in experiments with adult tissues from *Larix decidua* and *L.* x *eurolepis* (Bonga 1997; Bonga 2004), the tissue arising from the explants was friable, small-celled and olive-colored. This tissue eventually formed green nodules, many of which developed into structures that resembled mature embryos.

Regarding to this, the *in vitro* adventitious buds offer the possibility of not depending on the seasonality of buds from the field on the contrary of the observations made by Malabadi et al. (2004) in *P. kesiya* SE and De Diego et al. (2008) in *P. pinaster* organogenesis.

The buds obtained *in vitro*, when subjected to SE induction treatments seemed to developed tissue at a higher rate than buds from the field. Moreover, exogenously applied cytokinins during organogenesis may provoke the reinvigoration of adult buds. Reinvigoration is accompanied by the acquisition of juvenile characteristics (De Diego et al. 2010) such as a low DNA methylation rate observed in other woody plants (Monteuuis et al. 2009) or the attainment of some polyamines characteristic of juvenile trees. Reinvigoration of radiata pine was correlated with the attainment of some, but not all, polyamines characteristic of juvenile trees (Fraga et al. 2003). The acquisition of these juvenile characteristics can make possible to the development of ET from the newly formed adventitious buds. This approach, using juvenile explants developed *in vitro* from adult trees, has been successfully used in *Quercus robur* (San José et al. 2010) and *Ulmus minor* (Conde et al. 2004).

In *P. kesiya*, *P. patula* and *P. roxburghii*, when the bud slices were not given a cold pre-treatment, the calli produced were hard and non-embryogenic (Malabadi et al. 2004; Malabadi and Van Staden 2005; Malabadi and Nataraja 2006). In our experiments the tissue proliferated was not hard and it showed a similar consistency of the embryogenic tissue initiated from immature seeds. In this sense, Aronen (2010) noted the importance of identifying the ET in adult *P. sylvestris* cultures. Once this ET is identified, separating this tissue carefully and following its evolution is a key issue (Aronen 2010).

The embryogenic-like tissue established showed promising features, especially in the case of the tissue obtained from *in vitro* adventitious buds (experiment 3), further micro and macro-morphological observations will establish definitive statements about this tissue.

If the tissue initiated resulted to be embryogenic, the next step would implicate maintenance medium and culture conditions optimization. In our experiments, the initiated tissue changed its morphology, colour and behaviour when transferred from induction to maintenance medium, but we could not confirm if it changed its competence for SE.

In order to discern whether the tissue produced at early stages of SE induction experiments is embryogenic or not, some authors had proposed analysing the protein pattern of the tissues (Von Arnold et al. 2002; Šamaj et al. 2008), the endogenous content of different growth

regulators (Pintos et al. 2002; Jiménez and Thomas 2006) or the expression of genes known to be involved in SE initiation (Fehér et al. 2003; Fehér 2008). In this sense, Bonga et al. (2010) have reviewed different gene families involved in SE induction such as oxidative stress-related genes (Stasolla et al. 2004; Sharma et al. 2008), molecular markers that identify cells that are competent to initiate SE (SERK gene family) (Rose and Nolan 2006), genes expressed only in embryogenic lines (WUS related WOX genes) (Palovaara and Hakman 2008; Park et al. 2010), or a transcription factor which ectopic expression induces cell proliferation leading to SE in *Arabidopsis* (*Brassica napus* BABY BOOM) (Passarinho et al. 2008).

Our next challenge is to choose one of these SE indicators and follow its evolution through initiation and maintenance. Moreover, we are focusing in the correct isolation of ETs, the proliferation of these ETs and their conversion into plantlets.

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CONCLUSIONES

Conclusiones

1- La inducción organogénica de embriones zigóticos de *Pinus radiata* se ve favorecida en términos de capacidad de elongación de tallo (SEC) al cultivarlos en un medio de cultivo con 1 μM de benciladenina o zeatina durante 2 o 3 semanas, respectivamente. El ácido naftalenacético aplicado al medio 1/2LP provoca una respuesta rizogénica *in vitro* significativamente mayor que las otras auxinas evaluadas. La supervivencia y el enraizamiento *ex vitro* son significativamente superiores cuando los explantos se cultivan en un medio 1/4LP en presencia de ácido indolbutírico.

2- Las distintas concentraciones y reguladores de crecimiento aplicados a los embriones zigóticos durante la fase de inducción organogénica no tienen un efecto significativo en el enraizamiento y la aclimatación de las microplantas producidas.

3- Existe una correlación positiva entre la máxima iniciación y proliferación de tejido embriogénico y un estadío de desarrollo entre 2 y 4 del embrión zigótico de *P. radiata*.

4- Las condiciones más adecuadas para la maduración de tejido embriogénico son 100 mg de peso fresco, cultivado en medio EDM con 60 μM de ácido abscísico, 60 g L^{-1} de sacarosa y 9 g L^{-1} agar. En estas condiciones, se obtiene el mayor número de embriones somáticos por gramo de peso fresco descrito hasta la fecha en el género *Pinus*. La germinación de embriones en medio 1/2LP con 2 g L^{-1} carbón activo fue del 95%.

5- La mayor respuesta caulogénica de embriones somáticos de *P. radiata* se obtiene tras su germinación y posterior cultivo en presencia de benciladenina (1 o 4.4 μM) durante 3 semanas.

6- La inducción caulogénica en yemas vegetativas de individuos adultos de *P. radiata* fue superior cuando los explantos se cultivaron en presencia de 50 μM de benciladenina durante 8 semanas.

7- Un mayor contenido relativo de bases y mayores valores en el ratio bases/IAA se relacionan con una mayor respuesta caulogénica.

8- Se ha detectado por primera vez en el género *Pinus* la existencia de derivados O-glucósilados de distintas citoquininas antes y durante la inducción caulogénica en yemas vegetativas de individuos adultos.

9- Las yemas procedentes de individuos adultos revigorizadas *in vitro* parecen ser los explantos iniciales más adecuados para la iniciación de tejido embriogénico.