Development of biotechnological tools for the *in vitro* propagation of elite trees for the Basque Country forest

Alejandra Rojas Vargas

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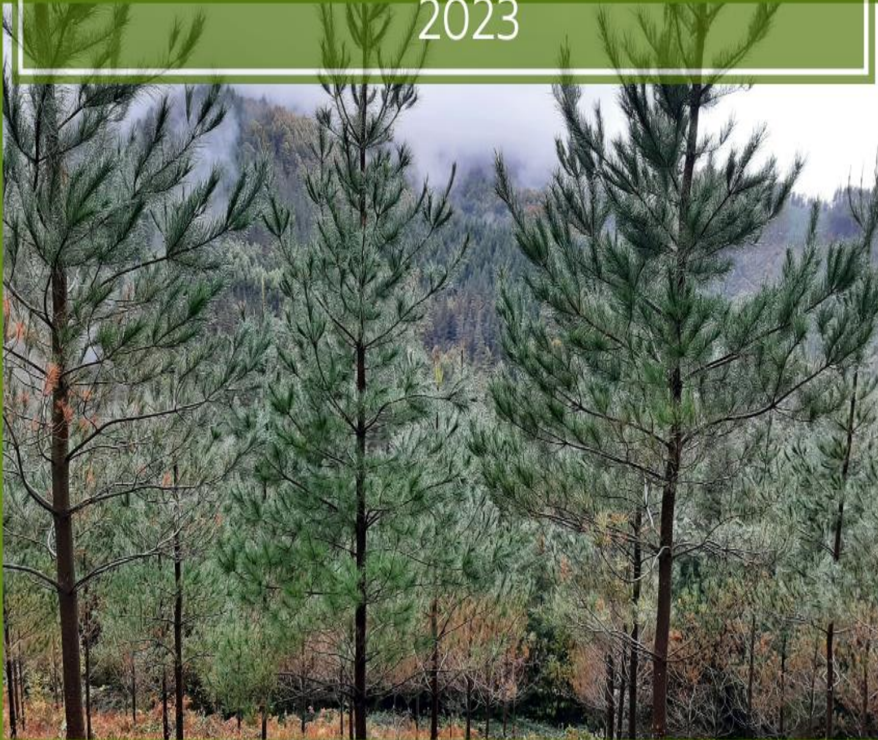

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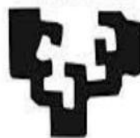

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Development of biotechnological tools for the *in vitro* propagation of elite trees for the Basque Country forest

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A mis padres: Victor e Isabel

A mis hermanos : Cindy, Melissa y Felipe

A Julio e Ignacio

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	analysis of variance
AC	activated charcoal
AS	apical explants < 1.0 cm
AT	apical explants > 1.5 cm
BA	6-benzyladenine
BS	basal explants < 1.0 cm
BT	basal explants > 1.5 cm
CK	cytokinin
DCR	DCR medium (Gupta and Durzan, 1985)
df	degrees of freedom
EFS	explants forming shoots
EM	elongation medium
FL	fluorescent light
HLP	half LP macronutrients medium
HPLC	high-performance liquid chromatography
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IM	induction medium
KIN	kinetin
LEDs	light emitting diodes
LLR	length of the longest root
LP	LP medium (Quoirin and Lepoivre, 1977, modified by Aitken-Christie et al. 1988)
m-T	meta-topolin
MS	MS medium (Murashige and Skoog, 1962)
NAA	1-naphthaleneacetic acid
NR/E	number of roots per explant
ns	non significant
NS/E	number of shoots per explant
PGRs	plant growth regulators
PSR	percentage of shoots elongated enough to be rooted
PVP	polyvinylpyrrolidone
QL	QL medium (Quoirin and Lepoivre, 1977)
REM	root expression medium
RI	root induction,
RIM	root induction medium
SE	somatic embryogenesis
SE	standard error
SEM	scanning electron microscopy
TDZ	thidiazuron

ABSTRACT

ABSTRACT

Located in the North of Spain, Basque Country has edaphoclimatic conditions that have allowed the establishment and development of an important forestry sector at European level. In this sense, 54.8% of the territory is dedicated to natural and cultivated forests. The most cultivated species in the Basque territory is *Pinus radiata*, whose first report of forest plantation establishment dates back to 1897. From that year to the present, its cultivation has increased in the Basque Country, reporting for the year 2022 the largest total area (25.9 %) and the highest forest productivity (80-85% of the annual cuts).

Pinus radiata, has a natural distribution from the central coast of California to Mexico. The versatility and quality of the wood, together with the rapid growth of the species, has allowed its plantations to extended to countries such as Australia, Chile, New Zealand, South Africa and Spain. However, in recent years *P. radiata* in the Basque Country has been affected by several diseases caused mainly by fungi: Pine canker caused by *Fusarium circinatum*, Dothistroma needle blight caused by *Dothistroma septosporum* and *Dothistroma pini*, and brown spot needle blight caused by *Lecanosticta acicula*. This last disease has affected 3,300 ha in Gipuzkoa, Basque Country. In addition, *P. radiata* plantations in the Basque Country have decreased from 123,921 ha (2016) to 102,488 ha (2022), a decrease that coincides with a historical outbreak of the needle blight during the years 2018-2019. For all of the abovementioned reasons, and together with the increase in the world population that causes a strong demand for timber and non-timber products, the forestry sector and HAZI (www.hazi.eus) proposed a list of candidate species to be used, compatible with the demand of the forestry sector, as well as socially and culturally accepted.

Using the list of possible alternative species, the following were selected for this study*: Cryptomeria japonica* (Thunb. ex L.f.) D. Don, *Pinus ponderosa* (P. Lawson and C. Lawson), *Sequoia semper*virens (D.Don). Endl and apparently tolerant genotypes of *P. radiata*. However, these species display desirable characteristics for clonal propagation at maturity, when traditional vegetative propagation and rooting are unfeasible. Therefore, the development of alternative methods of propagation, such as *in vitro* propagation, for these species is necessary. *In vitro* propagation through tissue culture could be performed mainly by organogenesis or somatic embryogenesis; those techniques combined with traditional ones could be efficient for the vegetative propagation of the abovementioned alternative species. In addition, these methods allow to improve the quality, yield and health of commercially valuable forest species. In this context, organogenesis in species of the Pinaceae family is generally restricted to zygotic embryos, part of them or young seedlings as initial source of explant, and attempts to clone selected adult individuals have been feasible but have faced several difficulties. In this regard, in our laboratory at Neiker, several *in vitro* propagation protocols have been successfully developed for *Pinus pinaster* Ait, *Pinus sylvestris* L., *Pinus pinea* L., *Pinus radiata* or *Pinus halepensis* among others.

Taking into account all this information, the general hypothesis of this work is that the modification of the physico-chemical environment in the micropropagation process can influence the regeneration efficiency of elite trees of forest species such as *C. japonica*, *P. ponderosa* and *S. sempervirens* to be considered as an alternative to *P. radiata* in the Basque Country. The second hypothesis to be corroborated is that adult *P. radiata* trees with apparent tolerance to pine needle blight can be used as a source of material for *in vitro* propagation.

In this context, the objective of the first chapter of the thesis was to analyze the influence of collection dates and different concentrations of 6-benzyladenine (BA) in the shoot induction phase, as well as the effect of different types of light on growth success of rooting. Also, the effect of physico-chemical factors in the content of amino acids and carbohydrates in the shoots developed *in vitro* was studied. Although reinvigorated shoots were obtained in both BA concentrations (22 or 44 μM), the highest BA concentration showed the best results in terms of shoot induction and number of shoots per explant when the explants were collected in the first week of February. In addition, in shoots developed *in vitro* a significantly higher differences were found in the different phases of the rooting process in the levels of fructose, glucose and sucrose and in the threonine and tyrosine contents.

The objective in the second chapter was to optimize the micropropagation method of *C. japonica* using adult trees as a source of plant material. In a first experiment, the effect of different types of initial explants and three basal culture media on shoot induction was studied. In addition, two sucrose concentrations and two light treatments (LED versus fluorescent lights) were tested for rooting stage. In a second experiment, the effect of different growth regulators (6-benzylaminopurine, metatopolin and thidiazuron) on shoot induction and subsequent phases of the organogenesis process were analysed. As main results, the highest number of shoots was obtained when using basal explants longer than 1.5 cm of length grown in the QL culture medium (Quoirin and Lepoivre, 1977). The shoots produced a higher number of roots when grown under red LED lights. Finally, root induction was significantly higher in shoots previously induced with meta-topolin.

The third chapter was focused on the analysis of the influence of different types of explants, basal culture media, cytokinins, auxins and light treatments on the *in vitro* propagation success in *P. ponderosa*. In this sense, whole zygotic embryos cultured

in 44 μM BA performed better in terms of explant survival. For shoot organogenesis, whole zygotic embryos and half LP (LP medium, Quoirin and Lepoivre, 1977, modified by Aitken-Christie et al., 1988) macronutrients were selected. Regarding the light treatments applied, a significantly higher percentage of rootable shoots was obtained under blue LED light. However, the percentage of acclimatization was higher in shoots previously cultivated under fluorescent light. Furthermore, anatomical studies using optical and scanning microscopy showed that light treatments promoted differences in anatomical aspects in *in vitro* shoots; needles of plantlets exposed to red and blue LEDs showed less stomata compared with needles from plantlets exposed to fluorescent light.

The objective in the fourth chapter was to optimize the micropropagation of adult elite trees of *S. sempervirens*. To accomplish this objective, the effect of different cytokinins (6-benzylaminopurine, meta-Topolin and Kinetin), and four types of explants in shoot induction were studied. At rooting stage, the effect of two types of auxins was evaluated: 1-naphthalene acetic acid, indole-3-butyric acid and a mixture of both. The best induction rate was obtained when apical explants longer than 1.5 cm were induced with 4.4 μM BA. Regarding the rooting, shoots previously induced in 4.4 μM BA and rooted in a culture medium with 50 μM 1-naphthalene acetic acid showed the best result. However, acclimatization was better when explants had been cultivated with Kinetin and rooted in a culture medium with indole-3-butyric acid.

Our study demonstrated that *in vitro* culture method can be used as an alternative for the propagation of elite individuals of *C. japonica*, *P. ponderosa*, *P. radiata* and *S. sempervirens*. Furthermore, the success of *in vitro* propagation in these species is influenced by genetic factors, collection dates, and physicochemical conditions of the culture environment.

RESUMEN

RESUMEN

Situado en el norte de España, el País Vasco cuenta con unas condiciones edafoclimáticas que han permitido el establecimiento y desarrollo de un importante sector forestal. En este sentido, el 54,8% del territorio está dedicado a bosques naturales y cultivados. La especie más cultivada en el territorio Vasco es *Pinus radiata,* siendo el primer reporte de su establecimiento del año 1897. Desde ese año a la actualidad su cultivo se ha incrementado en Euskadi, reportando para el año 2022 la mayor superficie total (25.9%) y la mayor productividad forestal (80-85% de las cortas anuales).

Pinus radiata, tiene una distribución natural desde la costa central de California hasta México. La versatilidad y calidad de la madera unido al rápido crecimiento de la especie han permitido que sus plantaciones se extiendan en países como Australia, Chile, Nueva Zelanda, Sudáfrica y España. Sin embargo, en los últimos años, el *P. radiata* en el País Vasco se ha visto afectado por varias enfermedades causadas principalmente por hongos: Chancro resino del pino, causado por *Fusarium circinatum*, banda causado por *Dothistroma septosporum* and *Dothistroma pini* y la banda marrón causado por *Lecanosticta acicula*. Esta última enfermedad ha ocasionado la pérdida de 3.300 ha en Gipuzkoa, País Vasco. Además, las plantaciones de *P. radiata* en Euskadi han disminuido de 123.921 ha (2016) a 102.488 ha (2022); disminución que coincide con un brote histórico de la enfermedad (bandas) durante los años 2018-2019. Todo esto unido al aumento de la población a nivel mundial provoca una fuerte demanda de productos maderables y no maderables. Por ello, el sector forestal vasco junto con HAZI (www.hazi.eus) han creado una lista de especies alternativas para ser utilizadas en sustitución del *P. radiata*, compatibles con la demanda del sector forestal así como social y culturalmente aceptadas.

En base a la lista de especies candidatas antes mencionada, se seleccionaron en este estudio: *Cryptomeria japonica* (Thunb. Ex L.f.) D. Don, *Pinus ponderosa* (P. Lawson and C. Lawson), *Sequoia sempervirens* (D. Don). Endl y genotipos aparentemente tolerantes de *P. radiata*. Sin embargo, esas especies muestran las características deseables para la propagación clonal cuando se ha producido el cambio de fase y por lo tanto es inviable la posibilidad de ser propagadas por propagación vegetativa tradicional.

Por lo tanto, es necesario el desarrollo de métodos alternativos como la propagación *in vitro*. La propagación *in vitro* a través del cultivo de tejidos se realiza principalmente por organogénesis o embriogénesis somática. Estos métodos permiten mejorar la calidad, el rendimiento y la sanidad de especies forestales comercialmente valiosas. En este contexto, la organogénesis en especies de la familia *Pinaceae* generalmente se restringe a embriones cigóticos, parte de ellos o plántulas jóvenes como fuente inicial de explante, y los intentos de clonar individuos adultos seleccionados han sido factibles pero han enfrentado varias dificultades. En ese sentido, en nuestro laboratorio en Neiker se han desarrollado de manera exitosa varios protocolos de propagación *in vitro* en *Pinus pinaster* Ait, *Pinus sylvestris* L., *Pinus pinea* L., *Pinus radiata* y *Pinus halepensis* entre otros.

En base a la información antes mencionada, la hipótesis general de este trabajo es que la modificación del entorno físico-químico en el proceso de micropropagación puede influir en la eficiencia de regeneración de plántulas élite de especies forestales como *C. japonica*, *P. ponderosa* y *S. sempervirens* para ser consideradas como alternativa a *P. radiata* en el País Vasco. La segunda hipótesis a ser corroborada es que los árboles adultos de *P. radiata* con aparente tolerancia a la enfermedad de las bandas pueden usarse como fuente de material para la propagación *in vitro*.

En ese contexto, el objetivo del primer capítulo de la tesis fue analizar la influencia de las fechas de recolección y diferentes concentraciones de 6-benciladenina (BA) en la fase de inducción de brotes, así como el efecto de diferentes tipos de luz sobre el éxito de la inducción de raíces. A su vez, se estudió el efecto de los factores fisicoquímicos antes mencionados sobre el contenido de aminoácidos y carbohidratos en los brotes desarrollados *in vitro*. Aunque se obtuvieron brotes revigorizados en ambas concentraciones de BA (22 ó 44 μM), la mayor concentración de BA mostró los mejores resultados en términos de inducción y número de brotes por explanto cuando estos se colectaron en la primera semana de febrero. Además, se encontraron diferencias significativas en las diferentes fases del proceso de enraizamiento en las cantidades de fructosa, glucosa y sacarosa y en el contenido de treonina y tirosina de los brotes desarrollados *in vitro*.

El objetivo en el segundo capítulo fue optimizar el método de micropropagación de *C. japonica* mediante el uso de árboles adultos como fuente de material vegetal. En un primer experimento, se estudió el efecto de diferentes tipos de explantos iniciales y tres medios de cultivo basales en la inducción de brotes. Además, se probaron dos concentraciones de sacarosa y dos tratamientos de luz (LED versus luces fluorescentes) para la mejora del enraizamiento. En un segundo experimento, se analizaron los efectos de diferentes reguladores del crecimiento (6-bencilaminopurina, metatopolina y tidiazuron) en la inducción de brotes y las fases posteriores del proceso de organogénesis. Como principales resultados, el mayor número de brotes se obtuvo cuando se utilizó explantos basales de una longitud mayor de 1,5 cm cultivados en el medio QL (Quoirin y Lepoivre, 1977). Los brotes obtenidos produjeron un mayor número de raíces cuando se cultivaron bajo luces LED rojas. Finalmente, la inducción de raíces fue significativamente mayor en los brotes previamente inducidos con meta-topolina.

El objetivo del tercer capítulo fue analizar la influencia de diferentes tipos de explantos, medios de cultivo basales, citoquininas, auxinas y tratamientos de luz sobre el éxito de la propagación *in vitro* en *P. ponderosa*. En ese sentido, embriones cigóticos completos y cultivados en 44 µM BA mostraron los mejores resultados en términos de supervivencia de los explantos. Para la organogénesis de brotes, se seleccionaron los embriones cigóticos completos y el medio de cultivo LP (Quoirin y Lepoivre, 1977, modificado por Aitken-Christie et al., 1988) reducido a la mitad de las sales. En cuanto a los tratamiento de luz aplicados, se observó un porcentaje significativamente mayor de brotes lo suficientemente alargados como para enraizarse cuando estos crecieron bajo luz LED azul. Sin embargo, el porcentaje de aclimatación fue mayor en los brotes previamente cultivados bajo luz fluorescente. Por otra parte, los estudios anatómicos mediante microscopía óptica y barrido mostraron que los tratamientos con luz promovieron diferencias en aspectos anatómicos en brotes *in vitro*; acículas de plántulas expuestas a LED rojos y azules mostraron menos estomas en comparación con acículas de plántulas expuestas a luz fluorescente.

El objetivo en el cuarto capítulo fue optimizar la micropropagación de árboles élite adultos de *S. sempervirens*. Para lograr este objetivo se estudio el efecto de diferentes tipos de citoquininas (6-bencilaminopurina, meta-Topolin y Kinetin), y cuatro tipos de explantos en etapa de inducción de brotes. Además, se evaluó el efecto de dos tipos de auxinas: ácido 1-naftalenacético, ácido indol-3-butírico y una mezcla de ambos. La mejor tasa de inducción de brotes se obtuvo cuando se utilizaron explantos apicales con longitud superior a 1,5 cm inducidos con 4,4 µM BA. El mejor resultado en términos de inducción de raíces se obtuvo cuando los brotes previamente inducidos en 4.4 µM BA se cultivaron en un medio de cultivo suplementado con 50 µM de ácido 1-naftalenacético. Sin embargo, la aclimatación

fue mejor cuando los explantos habían sido cultivados con Kinetina y enraizados en un medio de cultivo con ácido indol-3-butírico.

Nuestro estudio demostró que el cultivo de *in vitro* puede ser utilizado como un método alternativo de propagación de invididuos élites de *C. japonica*, *P. ponderosa*, *P. radiata* and *S. sempervirens*. Además, el éxito de la propagación *in vitro* en estas especies está influenciada por factores genéticos, fechas de colecta y condiciones físico-químicas del ambiente de cultivo.

CHAPTER 1

INTRODUCTION

INTRODUCTION

1. Cultivated forests in the Basque Country: current scenario.

The Basque Country have an extension of 7.226 km^2 and it is located in the North of Spain (42º28'-43º27' N; 1º44'W-3º27'W). Historically, the edaphoclimatic conditions of the Basque Country have lead to an important silvicultural development (Ibarrondo, 2008). In this context, the 54.8% (396. 191 ha) of the territory is dedicated to natural and cultivated forests, being an important surface in the European region (HAZI, 2022). Therefore, in the Basque Country, the forestry industry plays an important role at the economical, social and cultural level.

According with the last forest map of the Basque Country (2022), the wooded forest area is composed mainly of the following species: *Pinus radiata* (102.488 ha), *Fagus sylvatica* (55.291 ha), Atlantic mixed forest (38.138 ha), *Quercus faginea* (26.892 ha), *Quercus ilex* (26.757 ha), *Eucalyptus* spp (25.316 ha), *Quercus robur* together *Quercus petraea* (17.488 ha), *Pinus sylvestris* (17.493 ha), *Pinus nigra* (13.857 ha) and *Quercus pyrenaica* (13.258 ha) (Hazi, 2022).

In Spain, the largest plantations of *Pinus radiata* are in the Basque Country (HAZI, 2022). The first plantation of *P. radiata* was established in 1897 (Puerta et al., 2004). From then until nowdays, its cultivation has increased in the Basque Country, showing in 2022 the largest total wooded area (25.9%) and highest forest productivity (80-85% of the annual cuts) (HAZI, 2022).

2. *Pinus radiata* characteristics

Pinus radiata D. Don, commonly known as radiata pine or Monterey Pine, is naturally distributed from the Central Coast of California to Mexico. Due to the versatility, quality of the timber and rapid growth of the species, its plantations have been extended along Australia, Chile, New Zealand and South Africa (Martínez-Álvarez et al., 2014; Escandón et al., 2017; Farjon, 2018; Castander-Olarieta et al., 2019).

Radiata pine is an evergreen tree (Figure 1A), 30 m tall, trunk to 0.9 m diameter, leaves (needles) in fascicles of 2 or 3 needles (8- 20 cm long) with dark green color (Figure1B). The cones (7-15 cm long) have ellipsoid-cylindric shape and need two years for maturing, the seeds are characterized by two parts, the body (0.6 cm long) of dark brown color and the wing (2–3 cm long) (Bussmann et al., 2020).

Figure 1. **(A)** *Pinus radiata* D. Don tree in a seed orchard established by Neiker-BRTA in Arcaute, Álava, Spain and **(B**) apical buds and leaves (needles).

Radiate pine natural populations grow in climatic conditions characterized by monthly temperatures means ranging from 9 °C to 11 °C in winter, 16 °C to 18 °C in summer and annual precipitations ranging from 380 to 890 mm, with dry summers and snowless winters (Mead, 2013).

3. Current health situation of cultivated forest in the Basque Country

During the last years, *Pinus radiata* in the Basque Country has been affected by several diseases caused mainly by fungi. This situation has provoked a deep reduction of the survival, vigor and yield of cultivated forests (Elvira-Recuenco et al., 2020). Among the fungi that have caused the most damage to Basque forest systems are the following:

a.-Pine pitch canker (PPC), caused by *Fusarium circinatum* (Nirenberg and O'Donnell), provoked an affectation of 353.65 ha of radiata pine plantations during the years 2006-2011 in Gipuzkoa, Basque Country (Cantero, 2019).

b.-Dothistroma needle blight (DNB), caused by *Dothistroma septosporum* (Dorog.) Morelet and *Dothistroma pini* Hulbary.

c.-Brown spot needle blight caused by *Lecanosticta acicula* (Thümen) Sydow. According to Cantero (2019), during 2011-2012 an affectation of 3.300 ha of radiata pine plantations caused by this disease was reported in Gipuzkoa.

d.-*Mycosphaerella dearnessii* teleomorph of *L. acicula* also have been reported in *P. radiata* (Elvira-Recuenco et al., 2020; Mesanza et al., 2021a, 2021b).

In the last years (2018-2019), plantations of radiata pine in the North of Spain were affected by a historical outbreak of needle blight disease. In Basque forest systems this disease mainly caused by *D. septosporum* and *L. acicula* (Mesanza et al., 2021c), causing an affectation of 53.227 ha in the above-mentioned period (Cantero, 2019).

In the Basque country, the presence of *L. acicula* dates back to 1942, but for unknown scientific reasons, the disease in the last years has been a very rapid expansion. In

this context, the plantations of radiata pine in the Basque Country have decreased from 123.921 ha (2016) to 102.488 ha (2022), a decrease that coincides with the historical outbreak of this type of needle blight disease (HAZI, 2022). Also, several reports have mentioned problems in the commercial viability of these plantations. In this sense, it has been described that climate change is the responsible of the emergence of needle blight disease (Jankovský et al., 2009; Allen et al., 2010; Woods et al., 2016; Castander-Olarieta et al., 2019).

The increase of population worldwide provokes a strong demand of wood and non-wood products (Farjon, 2018; Bueno et al., 2021). For this reason, it is urgent to find alternative species that could be used in the Basque forest plantations and that are compatible with the demands of the industrial sector as well as being socially and culturally accepted.

At this point, a great effort has been made to find out which species could be planted in the areas where the radiata pine is being eliminated, which are resistant to the diseases mentioned and grow rapidly in our edaphoclimatic conditions. In recent years, forestry sector has analysed different possibilities and created a list of candidate species to be used.

In this regard HAZI (www.hazi.eus) proposed a list of alternative species (Cantero, 2019): *Larix leptolepis*, *L. decidua*, *Pseudotsuga menziesii*, *Cryptomeria japonica*, *Sequoiadendron giganteum*, *Sequoia sempervirens*, *Picea abies*, *P omorika*, *P. pungens*, *P. sitchensis*, *P. shrenkiana.*

4. Possible alternative species to radiata pine for the Basque Country

From the list of possible alternative species, three of them were chosen to work with: *Cryptomeria japonica* (Thunb. ex L.f.) D. Don, *Pinus ponderosa* (P. Lawson and C. Lawson) and *Sequoia sempervirens* (D. Don). Endl. Furthermore, *Pinus radiata* apparently tolerant genotypes were used for *in vitro* propagation experiments.

4.1. *Cryptomeria japonica* (Thunb. ex L.f.) D. Don

Cyptomeria japonica (Thunb. ex L.f.) D. Don trees (Figure 2), is monoecious conifer, belonging to the Cupressaceae family (20 genera with 125 species) and Taxodioideae subfamily (Onuma et al., 2023). This species also known as Japanese cedar or Sugi occurs naturally throughout East Asia (Taniguchi et al., 2020). According with Tsumura (2023) *C. japonica* forest area is naturally distributed and adapted to different ecosystems where the climatic conditions are characterized by an annual precipitation over 1500 mm and local temperatures ranging from 8.4 °C to 13.9 °C with cold winters. Sugi is an evergreen tree can reach up to 50 m tall , it shows slender leaves, awl-shaped, bluntly pointed; male cones with axillary and lateral female globular cones with wedgeshaped scales (Farjon, 1999).

Figure 2. *Cryptomeria japonica* (Thunb. ex L.f.) D. Don adult tree in Arkaute, Álava, Spain.

In Japan, Sugi is an important forestry species, it constitutes around 44% of forest plantations, its timber is used for wood construction or for obtaining biomass (Taniguchi et al., 2020; Onuma et al., 2023; Tsumura, 2023). In this sense, Japanese Cedar tree has been introduced in different countries as an alternative species due to its valuable timber and fast-growing (Page, 1990; Shimizu and Maiochi, 2007; Koguta et al., 2017).

In the Paraná region of Brazil, it has been introduced for the last 40 years, and in other countries such Russia, England or Indonesia was introduced in the nineteeth century (Farjon, 1999; Shimizu and Maiochi, 2007; Koguta et al., 2017). In the Basque Country, *C. japonica* plantations occupied an area of 3.372 ha in 2022 (HAZI, 2022)

4.2. *Pinus ponderosa* (P. Lawson and C. Lawson)

Pinus ponderosa (P. Lawson and C. Lawson) belongs to the Pinaceae family, where the genus *Pinus* occupies the first place among the all conifers in economic importance (Farjon, 2018; Charpin et al., 2022; Neale and Wheeler, 2019).

P. ponderosa (Figure 3) also known as ponderosa pine or western yellow pine is native to the United States, Southern Canada, and Northern Mexico (Ellis and Bilderback, 1991; Raish et al., 1997; Fitzgerald, 2005). This species is the largest of the western pine species and is a major lumber tree in Western North America (Ellis and Bilderback, 1991; Raish et al., 1997; Fitzgerald, 2005).

Figure 3. *Pinus ponderosa* (P. Lawson and C. Lawson) in a seed orchard established by Instituto Nacional de Tecnología Agropecuaria in Trevelin, Argentina.

Ponderosa pine is characterized for its protective outer corky bark and long needles (in fascicles of two or three needles) with high moisture content surrounding the terminal buds (Hall, 1980; Miller, 2000; Fitzegarld, 2005). Due to these adaptations

that promote its survival, is considered one the most fires-resistant conifers in the West of the United States (Hall, 1980; Miller, 2000; Fitzegarld, 2005).

P. ponderosa forests are important in ecology terms, since they are wildlife habitats for animals and plants, growing in sites characterized by elevations ranging from sea level to 3. 281 m, predominantly dry and moist. The seedling survival is affected by water availability and soil conditions; therefore, in the current climate change scenario, the development of new propagation technologies in the species is justified. They are also part of recreational, aesthetic and cultural values; the species itself constitute a great economic asset due to the value of its timber (Ellis and Bilderback, 1991; Raish et al., 1997; Zhang et al., 2022;). In this respect, ponderosa pine cultivated forests have been established in California with lumber purposes on private lands, while in countries as Chile and Argentina has been introduced as a promising species to optimize and diversify the wood industry (Zhang et al., 2019; Espinoza et al., 2021; Solans et al., 2021).

4.3. *Sequoia sempervirens* (D. Don). Endl

Sequoia sempervirens (D. Don). Endl trees belongs to the family Taxodiaceae (nine genera with 16 species), it shows an extreme size (400 Mg biomass) and can live up 2.500 years (Figure 4) (Burns et al., 2018; Sillett et al., 2020). *S. sempervirens* is commonly known as coastal redwood and naturally occurs in the west coast of North America, it is also cultivated in countries such as Romania as ornamental in parks and botanical gardens (Stoiculescu et al., 2009). Burns et al., 2018, mentioned that *Sequoia* has ~5% of primary forests remaining located in the native distribution of the genera, 740 km from Southwestern Oregon to central California, in sites with < 1000 m elevation and north-south latitudinal gradient (Sillett et al., 2022).

Figure 4. *Sequoia sempervirens* (D. Don.) Endl adult tree in Vitoria-Gasteiz, Álava, Spain.

Redwood is a shade-tolerant evergreen tree with fast growth rates that can reach up to 116 m in height. It has linear leaves (0.3- 1.0 cm long), entire edge of the leave with 6–12 leaves arranged per centimeter along the axes and elliptical female cones (1.1- 1.7 cm long) (Ma et al., 2005; Sillett et al., 2020; Watt et al., 2021).

Coastal redwood is an important species, due to the economic value of its wood (Laslo et al., 2015; Sillett et al., 2020). Several countries such as France, Chile, New Zealand and Germany have established *S. sempervirens* plantations with timber production purpose (Rapley, 2018; Arnaud et al., 1993; Ibañez et al., 2009; Breidenbach et al., 2020). In addition, in Spain and specifically in the Basque Country, it occupies an area of 1.586 ha (HAZI, 2022).

5. Biotechnology: alternative for the forestry sector

In the last years the climate change, the increase of population and their demand over forestry products leads to find elite trees with higher productivity to be propagated for afforestation purposes.

Because of the often-poor correlation between juvenile and mature traits in conifers, proper testing in breeding programs generally requires at least about one-third to one-half the rotation age (Weng et al., 2008). This, coupled with the fact that conifer stem cuttings suitable for rooting are generally available only from juvenile plants has led to limitations in traditional breeding techniques. Conifer trees usually develop the characteristics to determine their suitability for clonal planting after phase-change (during maturity), a developmental moment when rooting of cuttings becomes unfeasible (Bonga, 2015). Moreover, the intrinsic heterozygosity found in conifers makes it difficult to fix desirable alleles by conventional techniques (Williams and Savolainen, 1996.).

Consequently, traditional breeding techniques should be combined with new biotechnological tools (forest biotechnology) such as *in vitro* tissue to effectively improve the performance, quality and health of commercially valued forest species (Giri et al., 2004).

The generation of new plants *in vitro* can be achieved by three major pathways of micropropagation: 1) enhance of axillary bud break, 2) organogenesis and 3) somatic embryogenesis (SE) (Loberant and Altman, 2010). In this way, the first pathway uses bud meristems exist in the shoot tip and leaves and requires elongation and grow stage of the shoot. The axillary bud break it considered the simplest technique of micropropagation, but the regenerated plants present the highest rates of genetic stability (Loberant and Altman, 2010; De Oliveira et al., 2012). In regard to organogenesis it involves the production of adventitious buds

(shoots or roots) directly (without callus formation) or indirectly via callus, where the regenerated plant are finally rooted (Phillips, 2004; Loberant and Altman, 2010; Loyola- Vargas and Ochoa-Alejo, 2012). Finally, SE defined as embryos regeneration from cells or tissues directly (without callus formation) or indirectly on explants (George, 2008). The indirect organogenesis and the somatic embryogenesis involve the induction from the unspecialized, unorganized and dedifferentiated cells of callus tissues (George, 2008).

Organogenesis has successfully employed to propagate a wide variety of angiosperm species with high economic importance, such as *Eucalyptus* and *Populus*, and has also show great potential with certain conifer species, including *P. radiata* (Aitken-Christie et al., 1988; Montalbán et al., 2011). However, in the *Pinaceae* family this technique is restricted to zygotic embryos, parts of them, or young seedlings as explant sources, and attempts to clone selected adult trees, albeit feasible, have not shown the desired results. In our laboratory, organogenesis from vegetative buds of adult trees from various *Pinus* spp. was carried out (De Diego et al., 2008, 2010; Cortizo et al., 2009; Montalbán et al., 2013).

Micropropagation also known as *in vitro* culture is a procedure involving the manipulation of small plants parts (explants), tissues, organs or cells isolated from a mother plant that produces or regenerates plantlets under aseptic conditions on culture media and controlled environment (Loyola- Vargas and Ochoa-Alejo, 2012; George, 2008). This procedure is based on the totipotency of plants cells which is defined as the ability of single plant cell and its genetic program to regenerate a complete and functional plant in response to hormone conditions (Haberlandt, 1902).

In general, the organogenesis needs four stages for the success of the process: 1) establishment of the culture under *in vitro* conditions, 2) shoot induction or multiplication, 3) rooting and 4) acclimatization to *ex vitro* conditions (George and Debergh, 2008; Dobránszki and Da Silva, 2010). The first stage star with the selection of the explants from the mother plant, then surface sterilization of the explant and finally the culture on the artificial media (George, 2008; Loberant and Altman, 2010; Loyola- Vargas and Ochoa-Alejo, 2012).

After several subcultures *in vitro* plants are rooted, this stage involves media and environmental changes as the addition or increase of auxins, reduced sugar levels, or modifications in light quality (Loberant and Altman, 2010). Finally, they are transferred from *in vitro* to *ex vitro* control conditions to be acclimatized (George and Debergh, 2008; Loberant and Altman, 2010).

The micropropagation technique compared to conventional propagation enables propagate independent on the season, high coefficient of multiplication in short time, aseptic cultures or disease-free cultures, fast propagation, and is valuable technique when exist rare genotypes, sterile seeds or for the multiplication of species with economic or ecological importance (Valverde-Cerdas et al., 2008.; Tsubomura and Taniguchi, 2008; Dobránszki and Da Silva, 2010; Sahu and Sahu, 2013). Forthermore Bonga (2015), explain that conventional propagation will always be cheaper compared to biotechnological techniques. However, this cost is canceled when, through micropropagation, plants with a gain in genetic composition such as disease resistance and stem quality are obtained (Bonga, 2015).

In this context, Neiker's tissue culture laboratory has developed several successful protocols for adult pine species: *Pinus pinaster* Ait. (De Diego et al., 2008), *Pinus sylvestris* L. (De Diego et al., 2010), *Pinus pinea* L. (Cortizo et al., 2009), *Pinus radiata* (Montalbán et al., 2013) and *Pinus halepensis* (Pereira et al., 2021).

6. Factors influencing the success of the micropropagation process

Plant tissue culture was developed more than half a century ago, but nowdays scientists use these biotechnological techniques to propagate plants, as well as to study physiological, morphological, biochemical and molecular aspects related to plant development, as these aspects are implicated in tissue culture success (Neelakandan and Wang, 2012).

It is generally known that the genotype, the source and explant type and the physico-chemical environment affect the success of *in vitro* morphogenic processes (Phillips and Garda, 2019). In this context, several works have demonstrated that the genotype has a significant role in the reliability and repeatability of a micropropagation protocol, since a different organogenic response can be obtained depending on the genotype (Coleman and Ernst, 1989; Meneguzzi et al., 2019). For example, in previous studies with *P. radiata*, *P. taeda* and *P. halepensis* a different organogenic response depending on the genotype was reported (Montalbán et al., 2013; Cézar et al., 2015; Pereira et al., 2021).

The size of the explants may play an important role in *in vitro* propagation (Da Silva et al., 2016; Bonga, 2017). Several studies have been demonstrated that the explant size can affect axillary bud multiplication and due to larger explants having more endogenous hormones and nutrients to maintain the culture (Renau-Morata et al., 2005; George and Debergh, 2008; Desai et al., 2015). Furthermore, bigger explants from parts of shoot apex or stems with lateral buds show advantages over smaller explants for the *in vitro* proliferation (George and Debergh, 2008). For example, an *in vitro* protocol was obtained in *S. sempevirens* using explants bigger than 1.5 cm in length (Clapa et al., 2010; Meneguzzi et al., 2020). Additionally, the culture establishment can be affected by the explant size, it is probable that smaller size and lower exposure of the explant’s surface favoured their sterilization protocol (George

and Debergh, 2008; Sathyagowri and Seran, 2011). For example, the best survival response was observed in *Cedar libani* where shoot apices (<1.0 cm) were used (Renau-Morata et al., 2005). Similar result were reported in *Taxus mairei* using small stems (<1.0 cm), where the highest survival response was obtained (Chang et al., 2001).

Physical factors such as light quality and intensity, temperature, humidity, gaseous environment are manipulated in different ways to induce and optimize organogenesis or SE (Phillips, 2004; Kumar and Reddy, 2011). In this context, light quantity and spectral quality is one of the most important factors controlling plant growth and development; and have effects on plants photosynthesis and photomorphogenesis (Fang and Jao, 2000; Kumar and Reddy, 2011; Alallaq et al., 2020). According with George and Davies (2008) the *in vitro* plant material can be affected during its growth and morphogenesis due to changes in the photo flux, the day length also called as photoperiod and the wavelength.

Generally, in most of plant tissue culture laboratories the use of tubular fluorescent light with light intensity between 25 and 150 μmol m^{-2} s^{-1} for 16 h photoperiod (8 h dark) per day is commonly used, depending on the species conditions (Larraburu et al., 2018; Phillips and Garda, 2019). However, fluorescent lights increase costs due to high power consumption and limited efficiency, and culture rooms need to remove the heat emitted by using air conditioners (Kulus and Woźny, 2020). In the last years, light emitting diodes (red, blue, white and far-red LEDs) are recommended due their low power consumption, smaller size, durability, negligible heat production and the possibility to fix specific wavelengths, giving them advantages over fluorescent light (Fang and Jao, 2000).

Several studies reported that LEDs showed positive effects in *in vitro* propagation, when used as an alternative to conventional lighting (Gupta and Jatothu, 2013;

Chen et al., 2020). For example, *in vitro* shoots of *Gerbera jamesonii* growing under red LEDs displayed the greatest elongation rates (Pawłowska et al., 2018). A similar tendency was reported by Chen et al., (2020), where the highest stem length was observed in *in vitro* potato plantlets under red LEDs. Additionally, *in vitro* shoot regeneration was promoted in *Curculigo orchioides* and *Zingiber officinale* cultures exposed to blue LEDs (Gupta and Sahoo, 2015; Gnasekaran et al., 2021). Furthermore, *in vitro* explants of *Cucumis metuliferus* exposed to white LEDs and a mixed circuit of blue and red LEDs obtained higher root induction rates (Lai et al., 2022). Also, *in vitro* shoots of *Cedrela odorata* growing under white LEDs showed a higher number of roots formed (Dos Santos et al., 2022).

Chemical factors including basal medium composition (for example, organic or inorganic nitrogen sources, pH, carbohydrates sources and concentrations) and plant growth regulators (types and concentrations), are also factors involved in the induction and optimization of micropropagation success through organogenesis or SE (Phillips, 2004). The basal medium are generally composed of macronutrient salts (N,P, K, Ca, Mg and S), micronutrient salts (Fe, B, Mn, Mo, Cu, Co and I), vitamins, plant growth regulators (auxins, cytokinins, giberelins, abscisic acid, etc.) and carbohydrates (Phillips and Garda, 2019). The basal medium supplies all the necessary mineral nutrients for the plant tissue growth and the nature of the basal medium composition and its modifications such as reducing macronutrients to half-strength, can affect the success of the micropropagation (Phillips and Garda, 2019). For example, in *P. pinea* differences in shoot proliferation were obtained when different culture media were tested (Sul and Korban, 2004). Likewise, several studies in *Pinus* species such as *P. halepensis*, P. *pinaster* and *P. ayacahuite* reported that salt concentrations in the basal medium influenced the *in vitro* morphogenesis response (Lambardi et al., 1993; Saborio et al., 1997; De Diego et al., 2011).

Carbohydrates in plants have an important role in the synthesis pathway of many compounds (Gibson, 2000; Calamar and De Klerk, 2002). Generally, sucrose is commonly used in micropropagation because it is cheap, relatively stable to autoclaving, readily assimilated by plants and it is the main sugar translocated in the phloem of several plants (Ragonezi et al., 2010; Kumar and Reddy, 2011). The carbohydrates are involved in the growth of adventitious roots in shoots and supply the energy necessary for root development (Corrêa et al., 2005). However, during the root expression stage sucrose at high concentrations can have a negative effect (Ragonezi et al., 2010). In this context, in *Metroxylon sagu* the highest rooting response was obtained the 3% (w/v) sucrose compared with the 4.5% and 6.0% (w/v) sucrose concentrations (Sumaryono et al., 2012). In addition, sucrose in concentrations ranging from 1% to 9% in apple rootstock MM 10 showed a direct effect on root induction response where highest rooting rates was obtained using sucrose concentrations ranging from 1% to 7% (Calamar and De Klerk, 2002).

The higher plants produce organic compounds called growth regulators, which are involved in their growth and development (Kumar and Reddy, 2011). As above mentioned, the basal medium can be supplemented with different plant growth regulators depending on the morphogenic response to be induced (Phillips and Garda, 2019). According with van Staden et al., (2008), the balance and interaction between the growth regulators endogenously synthesized, and the plant growth regulators added to the basal medium regulate the morphogenesis *in vitro*. Among all plant growth regulators, auxins and cytokinins are the most commonly used (Phillips and Garda, 2019).

The most common auxins used are α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), followed by 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-acetic acid (IAA, natural auxin). Auxins induce rooting and alone or in

combination with cytokinins promote SE induction (Phillips, 2004; Kumar and Reddy, 2011; Phillips and Garda, 2019). For example, in *P. pinaster* (Álvarez et al., 2009) and in *P. radiata* (Montalbán et al., 2011) the highest rooting rates were observed in root induction medium supplemented with NAA. Moreover, different auxin / cytokinin ratios have been successfully applied for SE induction in *Pinus* (Montalbán et al., 2016).

Regarding to cytokinins, they are derived from adenine (aminopurine) and BA is the most commonly used, followed by Kinetin (KIN), Thidiazuron (TDZ) and 2-isopentenyladenine (2-IP), among others. The involvement of cytokinins in the promotion of *in vitro* shoot formation is due to their effect in plant cell division stimulation; morphogenesis, organogenesis and senescence regulation (Bairu et al., 2007; Valverde-Cerdas et al., 2008; De Diego et al., 2011; Kumar and Reddy, 2011; Aremu et al., 2012). For example, in *P. halepensis* BA at 44 μM provided a satisfactory shoot induction response (Pereira et al., 2021). Furthermore, cotyledons cultured in the presence of 10 μM BA from *P. pinaster* showed a high organogenic response (Álvarez et al., 2009). In the last years, a new class of aromatic cytokinins called topolins have been used in plant tissue culture as an alternative to conventional cytokinins (Aremu et al., 2012). The structure of topolins differs from that of BA in the presence of a hydroxyl group on the benzyl ring; and they have been used in *in vitro* cultures since topolins are involved in the improvement of parameters such as proliferation rates, root induction and alleviation of physiological disorders (Aremu et al., 2012). For example, in *P. sylvestris* the highest multiplication rates was observed when *meta*-Topolin [6-(3-hydroxybenzylamino)purine, *m*-T] was used during the induction stage (De Diego et al., 2010). In addition, a higher shoot induction response was obtained in *P. pinaster* when *m*-T was used instead BA (De Diego et al., 2011).

The approaches related to environment modifications for the improvement of micropropagation procedures can be combined with micromorphological and metabolomic analysis to understand some of the mechanisms leading to their success. Together with the implementation of advanced micropropagation techniques, these strategies could contribute to achieve efficient propagation of elite trees of alternative species and meet the current necessities derived from current climate change.

Objectives

General Hypothesis and Objectives

This work wants to confirm the following hypothesis:

- The modification of the physical and chemical environment in the micropropagation process can influence the regeneration efficiency of elite plantlets of forestry species such as *Cryptomeria japonica, Pinus ponderosa, and Sequoia sempervirens* to be considered as alternative to *Pinus radiata* in the Basque Country.
- Adult *Pinus radiata* trees with apparent tolerance to needle blight can be used as a source of material for *in vitro* propagation.

To validate these hypothesis, this work has been divided into four different objectives*:*

1. To evaluate the effect of genotypes, collection dates and BA concentrations on the shoot induction stage; and investigate the influence of different light types on the success of root induction of *Pinus radiata* shoots coming from adult trees. To determine if the assayed environmental conditions modified the content of amino acids and carbohydrates during the rooting phase.
2. To study the effect of different types of explants, cytokinins and culture media in the micropropagation of *Cryptomeria japonica* adult trees. To evaluate the influence of different sucrose concentrations, ventilated culture containers and light treatments in the rooting stage.
3. To analyze the influence of different types of explants, cytokinins, culture media, and light treatments in the improvement of organogenesis in *Pinus ponderosa.* To determine through anatomical analyses if the resulting *in vitro* needles present anatomical differences after different light treatments.
4. To determine the influence of different explants and cytokinin types in the micropropagation process of *Sequoia sempervirens* elite adult trees. To study

the effect of different auxins on the *in vitro* rooting and subsequent *ex vitro* acclimatization stage.

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CHAPTER 2

Influence of Physico-Chemical Factors on the Efficiency and Metabolite Profile of Adult *Pinus radiata* D. Don Bud Organogenesis

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ABSTRACT

Genetic improvement programs for conifer forest species face the challenge of propagating elite individuals with superior characteristics in the present landscape of climate change; the problem is focused on the fact that when these individuals have shown the desirable traits, they have changed phase and therefore have lost the ability to be propagated by traditional methods. Based on our previous works on *Pinus* spp. regeneration of adult trees through organogenesis and trying to improve the protocol in *Pinus radiata*, our objective was to analyze the influence of collection dates and different 6-benzyladenine (BA) concentrations in the first phase of shoot induction, as well as the effect of different light types on the success of root induction. Moreover, we were interested in studying the effect of the abovementioned physico-chemical fac-tors on the amino acid and carbohydrate content in the shoots developed *in vitro*. Reinvigorated shoots were obtained in both BA concentrations (22 or 44 μM), although the highest BA concentration showed the best results in terms of shoot induction (explants forming shoots (46%) and number of shoots per explant (1.95 ± 0.52)) when using initial explants collected in the first week of February. The percentage of explants forming shoots (EFS) was genotype-dependent. Explants from genotype A induced with the highest BA concentration showed the highest EFS (91%). With respect to the light treatment applied, significant differences in root induction (20%) and in the number of roots per explant (4.62 ± 0.65) were observed in shoots cultured under white FL. Finally, significant differences in different phases of the rooting process were detected in the amounts of fructose, glucose and sucrose and in the content of threonine and tyrosine.

RESUMEN

Los programas de mejoramiento genético para especies forestales de coníferas enfrentan el desafío de propagar individuos élite con características superiores en el panorama actual de cambio climático; el problema se centra en que cuando estos individuos han mostrado los rasgos deseables, han cambiado de fase y por lo tanto han perdido la capacidad de ser propagados por métodos tradicionales. Basado en nuestros trabajos previos con árboles adultos en *Pinus* spp. sobre la regeneración mediante organogénesis y tratando de mejorar el protocolo en *Pinus radiata*, nuestro objetivo fue analizar la influencia de las fechas de colecta y diferentes concentraciones de 6-benciladenina (BA) en la primera fase de inducción de brotes, así como el efecto de diferentes tipos de luz en el éxito de la inducción de raíces. Además, nos interesó estudiar el efecto de los factores físico-químicos antes mencionados sobre el contenido de aminoácidos y carbohidratos en los brotes desarrollados *in vitro*. Se obtuvieron brotes revigorizados en ambas concentraciones de BA (22 o 44 μM), aunque la mayor concentración de BA mostró los mejores resultados en términos de inducción de brotes (explantos que forman brotes (46%) y número de brotes por explanto (1,95 ± 0,52)) al utilizar explantos iniciales colectados en la primera semana de febrero. El porcentaje de explantos que forman brotes fue dependiente del genotipo. Los explantos del genotipo A inducidos con la concentración más alta de BA mostraron la respuesta de inducción más alta (91 %). Con respecto al tratamiento de luz aplicado, se observaron diferencias significativas en la inducción de raíces (20%) y en el número de raíces por explante (4,62 ± 0,65) en los brotes cultivados bajo luz fluorescente blanco. Finalmente, se detectaron diferencias significativas en las distintas fases del proceso de enraizamiento en las cantidades de fructosa, glucosa y sacarosa y en el contenido de treonina y tirosina.

1 INTRODUCTION

Conifers are an important gymnosperm group, distributed worldwide, and they form extensive, circumboreal forests across North America and Eurasia (Neale and Wheeler, 2019). Conifers are particularly important since they supply over 50% of the world's timber harvest and present technical and economic advantages for wood-based industries. In forestry plantations, conifers are preferred over angiosperms as they produce a much faster economic yield with more predictable shapes and sizes of timber (Farjon, 2018).

Among all conifers, the family Pinaceae far outweighs others in economic importance and the genera *Pinus* ranks first. Radiata pine (*Pinus radiata* D. Don), native to the Central Coast of California and Mexico, is the most planted pine species, and its plantations have been extended throughout Australia, Chile, New Zealand, South Africa and Northern Spain (Escandón et al., 2017; Castander-Olarieta et al, 2019). However, in recent years, many countries have reported problems regarding the commercial viability of plantations due to effects provoked by fungus and other stresses derived from climate change (Castander-Olarieta et al, 2019; Allen et al., 2010). Brown spot needle blight caused by *Lecanosticta acicola* and Dothistroma needle blight caused by the fungi *Dothistroma septosporum* and *D. pini* are forest diseases that affect this species (Jankovský et al., 2009; Woods et al., 2016). For this reason, great efforts are being carried out to develop efficient methods to propagate selected trees with tolerance to several biotic and abiotic stresses. In this sense, *in vitro* methods provide tools that can be used for the clonal propagation of *Pinus* species, as well as for the creation of backup collections as an alternative to the in situ conservation of species (Reed et al., 2011; De Diego et al., 2008, 2010; Cortizo et al., 2009).

In *in vitro* culture, the physico-chemical environment is very important and some times determines the success of the morphogenic process. The type and concentration of plant growth regulators, vitamins, basal media, solidifying agents, pH, etc., are crucial to obtain a highly efficient *in vitro* regeneration procedure (Phillips, 2004; Kulus and Woźny, 2020). In this sense, cytokinins added to the culture medium directly affect organogenesis, having a direct response to the induction of axillary shoot buds due to their effect on the endogenous phytohormone balance (Phillips, 2004; de Almeida et al., 2012).

Light also affects plant morphogenesis, and it may not only induce plant development but also induce photo-inhibition when leaves are exposed to more light than they can utilize (Thorpe et al., 2008). Usually, fluorescent tubes (FL) are used for plant micropropagation in growth chambers, with irradiances between 25 and 150 mmol m^{-2} s^{-1} for a 16 h photo-period (Larraburu et al., 2018). However, this illumination method has some drawbacks, such as its short lifespan (10.000 h) and the fact that it produces heat, which leads to the need for an extensive cooling system and high maintenance costs (Thorpe et al., 2008; Jao and Fang, 2003). Light-emitting diodes (LEDs) are being used currently as an alternative light source for controlled environment horticulture technology (Riikonen et al., 2016). The high efficiency in the energy conversion of LEDs reduces the heat emissions and thereby saves energy. LEDs are also environmentally safer than FL tubes and are made of recyclable material (Astolfi et al.,2012).

When optimizing a tissue culture protocol, the source of explant, the explant type and its developmental stage are also key factors (Kumar and Reddy, 2011). In the specific case of cells from adult explants, they are less prone to initiating dedifferentiation and reprogramming processes than juvenile explant cells (Narváez et al., 2019). Moreover, the effect of the initial explant collection date on

the morphogenic and embryogenic success has been demonstrated (Montalbán et al., 2011a).

The development of a successful tissue culture protocol to achieve *in vitro* shoots from *Pinus* adult trees requires not only the optimization of adventitious shoot initiation and elongation but also the improvement of the rooting process. There are many chemical factors involved in the success of the process; the carbohydrate source has been described as one of them, involved in the growth of adventitious roots in shoots (Ragonezi et al., 2010). Traditionally, sucrose is commonly used in tissue culture because it is readily assimilated by plants, but, in some cases, high concentrations of this compound could have negative effects in the root elongation phase (Kumar and Reddy, 2011; Ragonezi et al., 2010).

In the case of *Pinus*, the Neiker-BRTA research group established successful protocols for adult pine organogenesis in *Pinus pinaster* Ait., *Pinus sylvestris* L., *Pinus pinea* L., *Pinus radiata* and *Pinus halepensis* Mill. (De Diego et al., 2008, 2010; Cortizo et al., 2009; Montalbán et al., 2011b; Pereira et al., 2021). These kinds of procedures showed low success and were sometimes extremely dependent on the genotype. For this reason, and trying to increase the yield of this *in vitro* organogenic tool, our main goal was to study the effects of different physico-chemical factors on the organogenesis process of *Pinus radiata* buds from adult trees. Moreover, we focused our study on the involvement of the assayed environmental conditions in the content of amino acids and carbohydrates during the rooting phase.

2 MATERIALS AND METHODS

2.1. Plant Material

Vegetative shoot buds (1–7 cm length) (Figure 1a) were collected every 15 days from 13 January to the last week of January 2020, and every 8 days from the first to

the fourth week of February 2020, comprising a total of 6 collection dates. Eight 13-year-old adult trees (A, B, C, D, E, F, G and J), located in Neiker, Arkaute (Spain; 42°51′08.5″ N, 2°37′37.1″ W), and one 9-year-old juvenile somatic tree (N) (Spain; 42°51′5.51″ N, 2°37′19.43″ W) were chosen to carry out the experiment. After cutting and collecting buds from the trees, they were wrapped in moist paper to prevent dehydration and stored in polyethylene bags at 4 °C for a maximum of 3 days. The vegetative buds were disinfected as follows: all explants were washed with commercial detergent, rinsed under running water for 10 min, immersed in ethanol 96% for 2 min and then washed three times with sterile distilled water. After this, explants were disinfected in 1 mL L^{-1} silver nanoparticle solution (Argovit®, Vector Vita LLC, Novosibirsk, Russia) (1.2% (w/w) of metallic silver stabilized with 18.8% (w/w) of polyvinylpyrrolidone (PVP) suspended in water (80% w/w)) for 15 min and then rinsed three times with sterile distilled water (Stephano-Hornedo et al., 2020). Finally, the bud scales were removed, and the explants were cut transversely with a surgical scalpel blade into 0.5–1.0-cm-thick slices (Figure 1b) under sterile conditions in a laminar-flow unit. The slices were directly used to study the effect of 6-benzyladenine (BA, Duchefa Biochemie, Haarlem, Netherlands) on the induction medium.

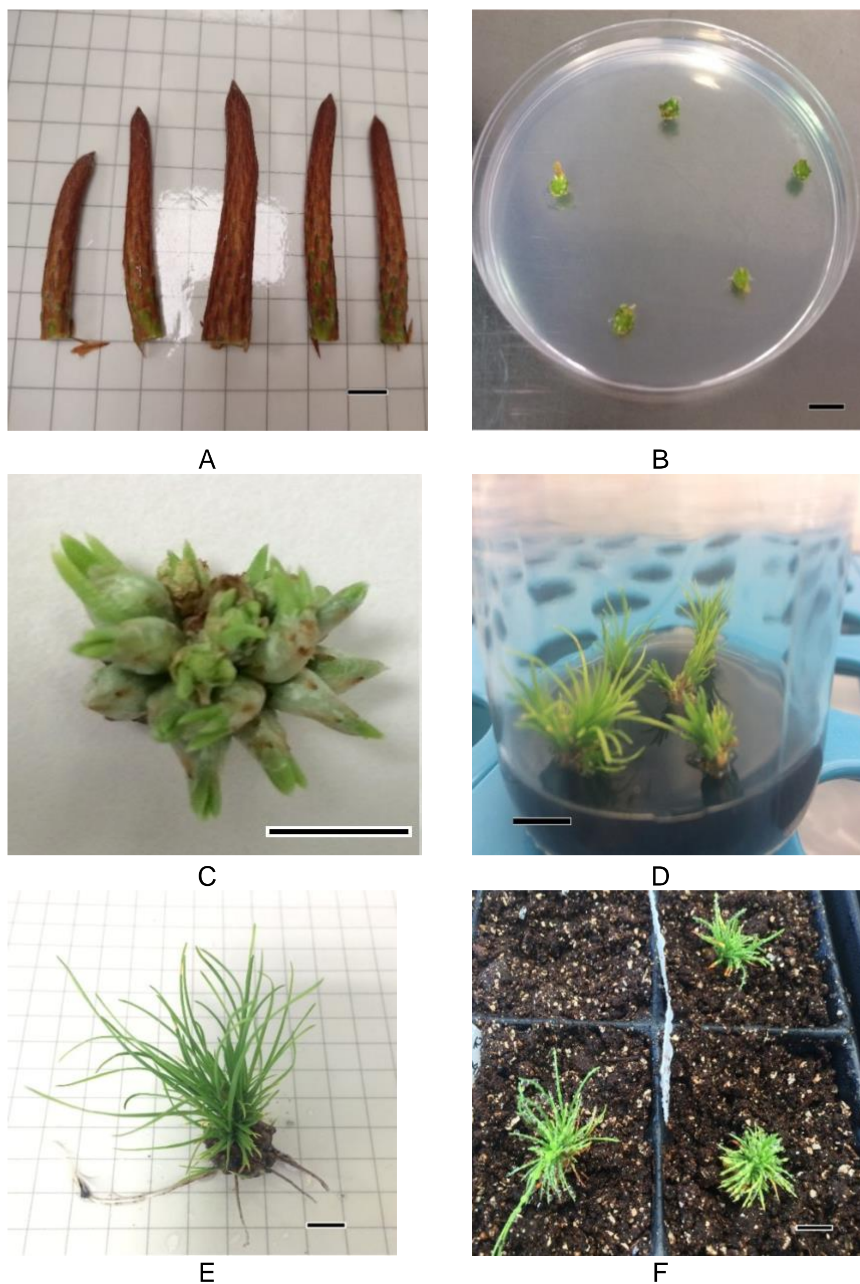


Figure 1. Organogenic process in apical shoot buds from *Pinus radiata* D. Don adult trees: (**a**) apical shoot buds, bar = 1 cm; (**b**) shoot bud slices in culture, bar = 1 cm; (**c**) shoot bud slice after induction with 22 μM 6-benzyladenine (BA), bar = 1 cm; (**d**) shoots in elongation medium, bar = 1 cm; (**e**) rooted shoots, bar = 1 cm; (**f**) acclimatized shoots, bar = 3 cm.

2.2 Organogenic Process

Four to five slices were cultivated vertically on Petri dishes (90 × 15 mm) containing 20 mL of bud induction medium (IM) (Figure 1b). IM consisted of Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), as modified by Aitken-Christie et al.,1988, supplemented with 3% (w/v) sucrose and solidified with 8 gL^{-1} Difco Agar®. The effect of two concentrations of BA was evaluated (22 and 44 μM). The pH of the medium was adjusted to 5.8 before autoclaving (121 °C, 20 min). Four to five Petri dishes per BA concentration and genotype were laid on the growth chamber at a temperature of 21 ± 1 °C, under a 16 h photoperiod, with 120 $\mu mol\ m^{-2}\ s^{-1}$ of light intensity provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France).

2.2.1. Shoot Growth and Elongation

As soon as bud induction was observed (between 7 and 9 weeks in IM) (Figure 1c), explants were transferred to Petri dishes with elongation medium (EM). EM consisted of hormone-free LP medium supplemented with 2 gL^{-1} activated charcoal and 3% (w/v) sucrose and solidified with 8.5 gL^{-1} Difco Agar®. The pH of the medium was adjusted to 5.8. After 35–50 days in culture (when elongating needle fascicles were evident), explants were subcultured into baby food jars (150 mL) containing 25 mL of EM (Figure 1d). The shoots were subcultivated every six weeks to the same medium. The light intensity, photoperiod and temperature of the growth chamber were the same as described above.

When shoots were 1.0–1.5 cm in length in the original bud slice, they were separated and cultivated individually in fresh EM (Figure 1d). The explants that showed new axillary shoot formation were cultivated into fresh EM, comprising a total of 6 subcultures, and the remaining explants showing secondary needles were

separated and cultivated again with IM treatment to promote axillary bud development (re-induction).

2.2.2 Root Induction and Acclimatization of Rooted Plants

After the elongation phase, shoots of at least 2.0–2.5 cm length were used for root induction. The explants were cultivated in Ecoboxes (Eco2box/green filter: a polypropylene vessel with a "breathing" hermetic cover, Duchefa®) containing 100 mL of root induction medium (RIM), which consisted of LP medium supplemented with a mixture of 5 μM 1-Naphthaleneacetic acid (NAA) and 10 μM indole-3-butyric acid (IBA), 8 gL^{-1} Difco Agar® and (A) 3% (w/v) sucrose or (B) 1.5% (w/v) sucrose. Eight shoots per genotype and BA concentration were cultivated for each sucrose concentration. The pH of the culture media was adjusted to 5.8 before autoclaving. The shoots were placed in dim light at 21 ± 1 °C for 8 days, followed by four weeks under a 16 h photoperiod. Two different light treatments were tested: (A) white light, 120 μmol m^{-2} s^{-1} of light intensity provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France); and (B) red light, 67 μmol m^{-2} s^{-1} of light intensity provided by adjustable LEDs (RB4K Grow LEDs, GureLED-Meetthings,Vitoria-Gasteiz, Spain).

After four weeks of culture in RIM, explants were cultured in Ecoboxes containing 100 mL of root expression medium (REM), which consisted of LP medium supplemented with 2 gL^{-1} activated charcoal, 3% (w/v) sucrose and 8.5 gL^{-1} Difco Agar®, for six weeks. Then, explants with visible roots were transferred to wet peat moss (Pindstrup, Aarhus, Denmark):vermiculite (8:2, v/v) and acclimatized in the greenhouse under controlled conditions at 21 ± 1 °C, decreasing the humidity progressively over one month from 95 to 80%.

2.3. Metabolite Extraction and Soluble Sugar and Amino Acid Quantification

Metabolite extractions from explants collected after four weeks of culture in RIM treatments and after six weeks cultured in REM medium were performed following the protocol described by Valledor et al., 2014. Five to eight explants were taken from each condition (previous BA treatment, light type and sucrose content in the medium); no particular selection of the material was made. Each explant was considered as a biological replicate. Explants were ground in liquid nitrogen and 100 mg of the resulting fine powder was transferred to 2 mL microcentrifuge tubes containing 800 μL cold metabolite extraction buffer (methanol:chloroform:water, 2.5:1:0.5, v:v:v). Then, tubes were centrifuged at 20, 000 g 6 min at 4 °C and the supernatants were transferred to new tubes containing 800 μL of phase separation mix (chloroform:water, 1:1, v:v). After centrifugation at 10,000 g for 5 min at room temperature, the upper aqueous phases containing polar metabolites were saved in new 1.5 mL tubes. Aliquots of 200 mL were then obtained from these tubes and completely dried on a Speedvac to remove the remaining methanol.

Finally, the samples were resuspended in 100 μL ultra-pure water and soluble sugars and amino acids were quantified by High-Performance Liquid Chromatography (HPLC) (Agilent 1260 Infinity II, Agilent Technologies, Santa Clara, CA, United States). Soluble sugars, including sugar alcohol (fructose, galactose glucose, mannitol and sucrose), were separated using a Hi-Plex Ca column (7.7 mm × 300 mm, 8 μm) and detected using a refractive index detector at a flow rate of 0.15 mL min^{-1} pure water at 80 °C for 30 min, as described by Castander-Olarieta et al., 2021. In the case of amino acids, a Poroshell 120 column (4.6 mm × 100 mm, 2.7 μm, Agilent Technologies, Santa Clara, CA, United States) was used, coupled to a fluorescence detector. The samples were injected into the column at a flow rate of 1.5 mL min^{-1} at 40 °C for 18 min with a discontinuous

gradient. Solvent A was a mixture of 10 mM Na_2HPO_4 (Scharlau Chemie, Barcelona, Spain) and 10 mM $Na_2B_4O_7$ (pH 8.2) (Scharlau Chemie, Barcelona, Spain) and solvent B was acetonitrile: methanol:water (45:45:10, v:v:v) (Scharlau Chemie, Barcelona, Spain, HPLC-grade). The gradient program was the following: min 0–13.40, solvent A 98% and solvent B 2%, min 13.40–13.50, solvent A 43% and solvent B 57%, min 13.50–15.80, solvent B 100%, and min 15.80–18, solvent A 98% and solvent B 2%. Amino acids were estimated after pre-column derivatization by mixing 1 µL sample with 2.5 µL borate buffer (Agilent Technologies, Santa Clara, CA, United States), 32 µL diluent (100 mL solvent A and 0.4 mL concentrated H_3PO_4) (PanReac AppliChem, Barcelona, Spain, pure pharma-grade) and 0.5 µL o-phthaldialdehyde (OPA) (Agilent Technologies, Santa Clara, CA, United States). In the specific case of amino acids hydroxyproline and proline, samples were mixed with 0.5 µL of 9-fluorenyl-methylchloroformate protecting group (FMOC) (Agilent Technologies, Santa Clara, CA, United States) instead of OPA. Detection was performed by analyzing fluorescence with excitation at 260 nm and emission at 450 nm for OPA derivatives and emission at 325 nm for FMOC derivatives.

In the case of amino acids, concentrations were determined from internal calibration curves constructed with the corresponding commercial standards (Agilent Technologies, Santa Clara, CA, United States). Carbohydrates such as fructose, galactose, mannitol and sucrose (Merck, Darmstadt, Germany) and glucose (Duchefa Biochemie, Haarlem, Netherlands) were determined from internal calibration curves constructed with the corresponding commercial standards. Results were conveniently adjusted considering the concentration step after methanol removal (2 times), and the results were expressed as µmol g FW^{-1}. In the case of certain amino acids, samples were re-injected after 1/32 dilution to

avoid signal saturation, and the resulting concentrations were conveniently adjusted.

2.5. Data Collection and Statistical Analysis

Two to four Petri dishes per genotype (nine genotypes) and four to five bud slices per Petri dish were cultured for each BA concentration and collection date. Contamination, survival and the percentage of explants forming shoots (EFS) (%) at each collection date were measured after two months of culture. When the axillary shoots were isolated and cultured individually in elongation medium, the EFS (%) and the mean number of shoots per explant (NS/E) were calculated with respect to the non-contaminated explants. A logistic regression model was used to analyze the collection date, genotype and BA concentration's effects on the EFS (%). Tukey's post hoc test (α = 0.05) was used for multiple comparisons. Data on the NS/E were analyzed by analysis of variance (ANOVA). When necessary, multiple comparisons were made using Duncan's post hoc test (α = 0.05).

The root induction percentage (RI) (%), the mean number of roots per explant (NR/E) and the length of the longest root (LLR) (cm) were recorded after six weeks of culture in REM medium. A completely randomized design was carried out using eight stems per BA and sucrose concentrations and light treatments.

To assess the effect of the cytokinin and sucrose concentration and light treatments' effects on the RI (%), a logistic regression was performed. Data on NR/E were x^2 transformed to meet homoscedasticity and were analyzed by ANOVA. Data on LLR (cm) were subjected to ANOVA. When necessary, multiple comparisons were made using Duncan's post hoc test (α = 0.05). The acclimatization percentage was calculated after four weeks under *ex vitro* conditions.

Data on free amino acid and carbohydrate content after four weeks of culture in RIM medium and after six weeks cultured in REM medium were analyzed. The confirmation of the homogeneity of variances and normality of the data was performed, and, when necessary, they were log(x) and $\sqrt{x}$ transformed. Data on free amino acids and carbohydrates were subjected to ANOVA, and, when necessary, multiple comparisons were made using Duncan's post hoc test (α = 0.05). The data were analyzed using R Core Team software® (version 4.2.1, Vienna, Austria) .

3. RESULTS

3.1. Organogenic Process

Genotype A showed the highest survival of the explants, independently of the collection date (72%). On the contrary, the lowest survival was observed in genotype J (43%). The highest survival values of the explants were observed on the first collection date (second week of January). In the same way, the highest contamination rates were recorded in samples collected in the third and fourth weeks of February (44 and 43%, respectively), and the lowest were found from January to the second week of February (between 17 and 34%).

The collection date had a significant effect on the EFS (%) (Figure 2, Supplementary Table S1). A significantly higher EFS (%) was obtained during the first week of February than during the third and fourth weeks of February (4%). Shoots induced in the second and third weeks of January and the second week of February displayed intermediate values (29 to 34%).

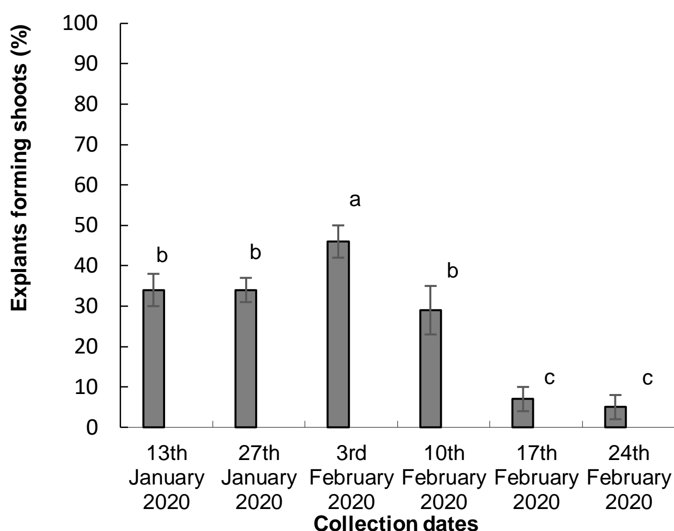


Figure 2. Explants forming shoots (%) shown in buds of *Pinus radiata* D. Don adult trees collected on different dates and cultured in Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al., 1988. Data are presented as mean values ± S.E. Different letters indicate significant differences by Tukey's post hoc test ($p < 0.05$).

Reinvigorated shoots were obtained after culture media were supplemented with both BA concentrations (22 and 44 μM). Regarding EFS (%), BA concentrations alone did not provoke any statistically significant differences in the EFS (%) (Supplementary Table S2), but the genotype and the interaction between genotype and BA concentration showed significant differences (Figure 3). A significantly higher EFS (%) was obtained in buds of genotype A induced with 44 μM BA than in the rest of the genotypes and BA concentrations tested. Buds of genotype N cultured with 44 μM BA showed a significantly lower EFS (%) than buds from genotypes A, F, G and J and genotypes E and J cultured at 22 and 44 μM BA, respectively (between 33 and 45%, Figure 3).

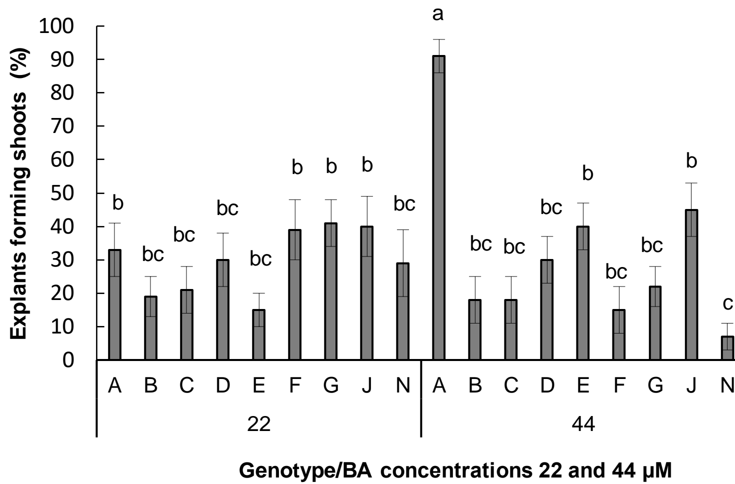


Figure 3. Explants forming shoots (%) shown in buds of different genotypes of *Pinus radiata* D. Don cultured in Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al., 1988, supplemented with 6-benzyladenine (BA at 22 and 44 μM). Data are presented as mean values ± S.E. Different letters indicate significant differences by Tukey's post hoc test ($p < 0.05$).

No statistically significant differences were found in NS/E when the effect of the BA concentration in the culture media during the induction phase was analyzed (Supplementary Table S3). The NS/E ranged from 1.66 ± 0.40 to 1.95 ± 0.52 in explants induced with 22 and 44 μM BA, respectively.

3.2. Rooting Induction and Acclimatization of Rooted Plants

The different BA concentrations (22 and 44 μM) used in the shoot induction phase did not have a statistically significant effect on RI (18% and 14%), on NR/E (4.50 and 3.56) or on LLR (1.83 cm and 1.60 cm), respectively (Supplementary Tables S4 and S5).

A significantly higher RI (%) was observed in shoots cultured under white FL when compared with shoots growing under red LEDs (Figure 4a). In the same way, NR/E

was significantly higher in shoots cultured under white FL than in those under red LEDs (Figure 4b). Light treatments did not provoke any statistically significant differences in the LLR. Shoots growing under red LEDs showed the longest length (2.22 cm) (Supplementary Tables S4 and S6).

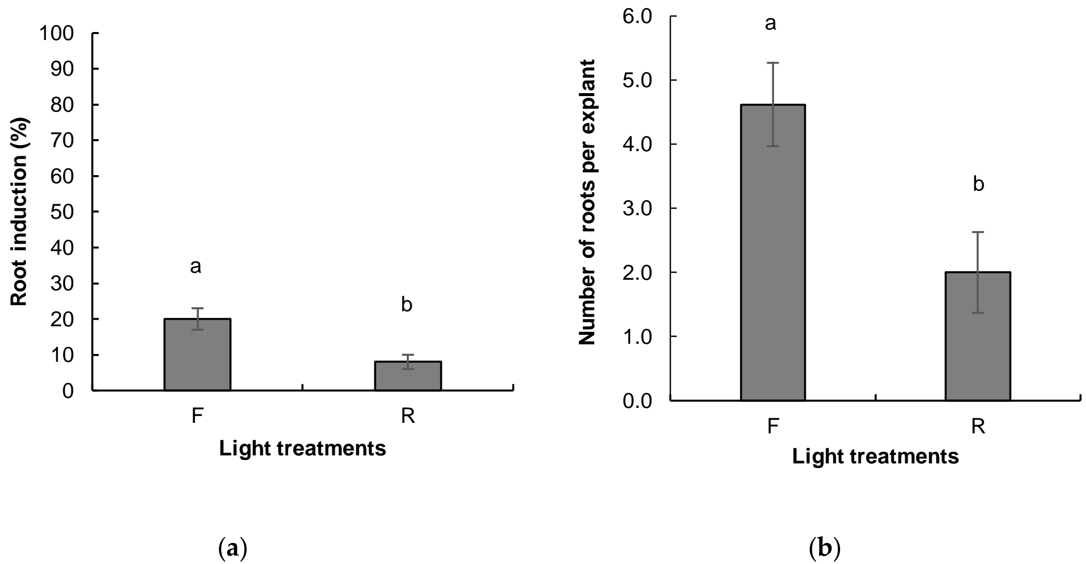


Figure 4. Effect of light treatments (white fluorescent (F) and red LEDs (R)) on root induction (%) (**a**) and number of roots per explant (**b**) of *Pinus radiata* D. Don shoots cultured in Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al., 1988. Data are presented as mean values ± S.E. Different letters indicate significant differences by Tukey's post hoc test (a) and by Duncan's post hoc test (b) ($p < 0.05$).

For the LLR, a significant effect of the interaction between BA and light treatment (Figure 5a) and between BA and sucrose concentration (Figure 5b) was observed. Explants induced with 44 μM BA and exposed to red LEDs displayed higher values compared with explants induced at the lowest BA concentration and exposed to the same light conditions (Figure 5a). On the contrary, explants cultured in the presence of 22 μM BA and exposed to white FL displayed significantly higher

values on LLR compared with explants induced at the highest BA concentration and exposed to the same light conditions (Figure 5a). Additionally, explants induced in the presence of 44 μM BA and cultured in medium supplemented with 1.5% sucrose during root induction showed significantly higher values than shoots induced at the same BA concentration followed by a medium supplemented with 3.0% sucrose (Figure 5b). When a culture medium containing 22 μM BA was used for shoot induction, regardless of the sucrose concentration evaluated, intermediate values of LLR were obtained (Figure 5b).

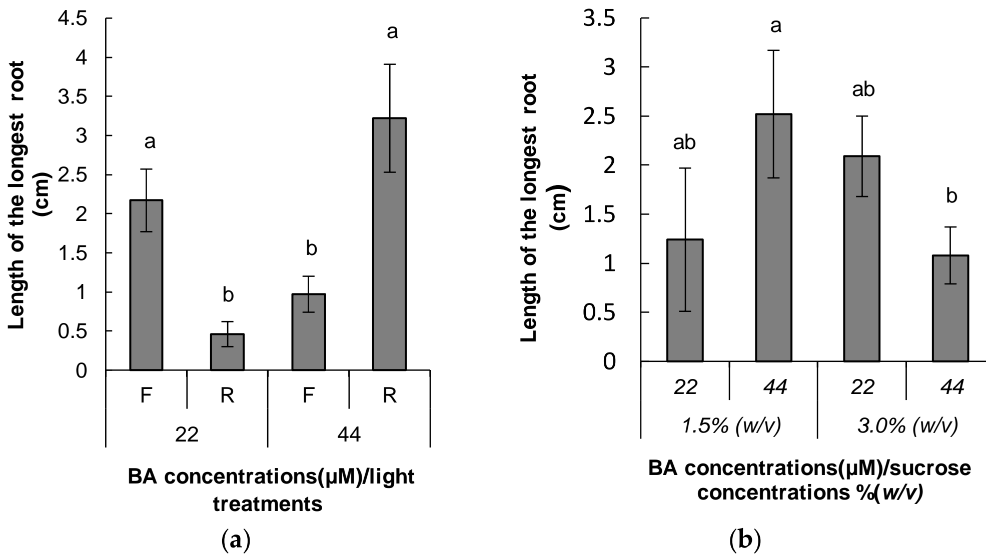


Figure 5. Effect of 6-benzyladenine (BA) concentration (22 and 44 μM), light treatment (white fluorescent (F) and red LEDs (R)) (**a**) and BA concentration (22 and 44 μM) and sucrose concentration (1.5% and 3.0% (w/v) (**b**) on length of the longest root (cm) of *Pinus radiat*a D. Don shoots cultured in Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al., 1988. Data are presented as mean values ± S.E. Different letters indicate significant differences by Duncan's post hoc test ($p < 0.05$).

Regarding the sucrose concentrations, shoots cultured in medium with 3% sucrose showed a significantly higher RI value (22%) than those cultured in 1.5% sucrose (9%). No statistically significant differences in NR/E (3.73 and 4.10) and LLR values

(2.01 and 1.55 cm) were observed in shoots cultured in the presence of 1.5 and 3% sucrose, respectively (Supplementary Tables S5 and S6).

Additionally, the interaction between BA concentration and light treatment and between BA and sucrose concentrations assayed throughout shoot induction did not show significant differences in RI (%) and NR/E (Supplementary Tables S4 and S5). In the same way, no statistically significant differences were observed in the interaction between light treatment and sucrose concentration evaluated during the shoot induction phase in RI (%), NR/E and LLR (Supplementary Table S6).

Shoots induced in the presence of 22 μM BA, rooted in medium supplemented with 3% sucrose and exposed to white FL from genotypes A, E and K developed *in vitro* roots (Figure 1e). In all genotypes, in the bases of shoots, a large and profuse callus was observed (Figure 1e). Acclimatized shoots were successfully obtained only from genotype K (33.33%) (Figure 1f).

3.3. Metabolite Analysis

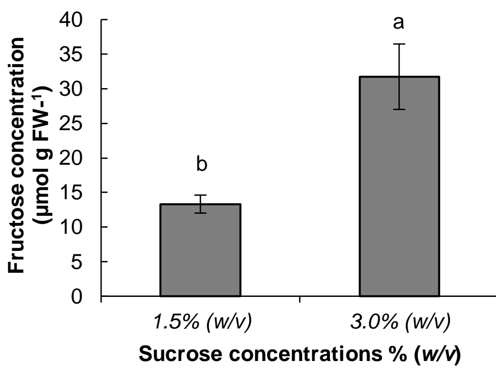
BA concentrations and light treatments, as well as the interaction between both variables, did not show significant differences for the concentration of carbohydrates in shoots analyzed after four weeks under RIM treatment (Supplementary Tables S7–S9). However, explants cultured in media with the highest BA concentration presented a lower sucrose concentration than explants cultured in media with 22 μM BA (Table 1). Additionally, the levels of all carbohydrates analyzed were lower in explants cultured under red LEDs, except for the galactose concentration (Table 1)

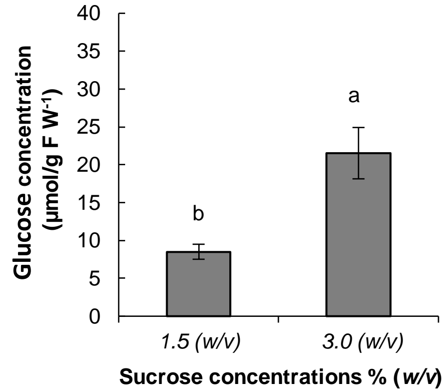
Table 1. Effect of 6-benzyladenine (BA) concentration (22 and 44 µM) and light treatment (white fluorescent or red LEDs) on carbohydrate content of *Pinus radiata* D. Don shoots cultured in rooting medium (Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al., 1988, supplemented with a mixture of 5 µM 1-Naphthaleneacetic acid (NAA) and 10 µM in-dole-3-butyric acid (IBA)).

Carbohydrates (µmol g FW^{-1})	**Retention Time (min)**	**BA Concentration (µM)**		**Light Treatment**	
		22	**44**	**White Fluorescer**	**Red LEDs**
Fructose	18.076	21.36 ± 6.28	22.71 ± 2.90	24.91 ± 5.21	19.20 ± 2.59
Galactose	16.096	4.82 ± 2.18	2.75 ± 0.28	2.96 ± 0.37	4.25 ± 1.82
Glucose	14.653	13.66 ± 4.37	15.50 ± 2.17	16.70 ± 3.57	12.66 ± 2.19
Mannitol	22.911	7.59 ± 0.45	7.04 ± 0.44	7.36 ± 0.36	7.15 ± 0.55
Sucrose	12.640	12.29 ± 3.82	5.67 ± 0.83	10.73 ± 3.30	5.70 ± 0.99

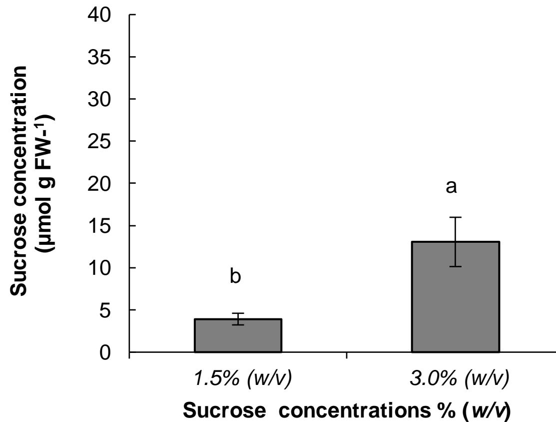
Data are presented as mean values ± S.E.

Regarding the effect of the sucrose concentration on the carbohydrate levels of shoots analyzed after root induction treatment, no significant differences were found in galactose and mannitol concentrations (Supplementary Tables S8 and S9). Galactose displayed values from 2.41 to 4.85 µmol g FW^{-1} and mannitol from 6.69 to 7.87 µmol g FW^{-1} in media supplemented with 1.5 and 3% sucrose, respectively. In the case of fructose, glucose and sucrose, significantly higher concentrations were obtained in shoots cultured in the presence of the highest sucrose concentration when compared with the results at the lowest sucrose concentration (Figure 6a–c). The interaction between BA and light treatment, the interaction between BA and sucrose concentration and the interaction between light treatment and sucrose concentration did not show statistically significant differences (Supplementary Tables S7–S9).





(**a**) (**b**)



(**c**)

Figure 6. Effect of sucrose concentration (1.5% and 3.0% (w/v)) on fructose (**a**), glucose (**b**) and sucrose (**c**) concentration of *Pinus radiata* D. Don shoots cultured in rooting medium (Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al.,1988, supplemented with a mixture of 5 µM 1-Naphthaleneacetic acid (NAA) and 10 µM indole-3-butyric acid (IBA)). Data are presented as mean values ± S.E. Different letters indicate significant differences by Dun-can's post hoc test ($p < 0.05$).

At the end of the rooting phase, no statistically significant differences were found for any of the carbohydrates analyzed in shoots during the root expression phase in relation to BA, light treatments, sucrose concentrations or the interactions among them (Supplementary Tables S10–S12). The highest glucose content (26.44

μmol g FW^{-1}) was found when shoots were cultured in the presence of the lowest BA concentration during the induction phase (Table 2). Additionally, shoots cultured under white FL exhibited an increase in fructose, glucose and sucrose levels compared with those cultured under red LEDs (Table 2). The fructose, glucose and sucrose levels decreased in shoots cultured during the induction phase in medium supplemented with the highest sucrose concentration. The galactose concentration in shoots after the rooting phase was the lowest of all the sugars analyzed, independently of the chemical characteristics of the culture media.

Table 2. Effect of 6-Benzyladenine (BA) (22 and 44 μM) and sucrose concentration (1.5 and 3.0% (w/v)) and light treatment (white fluorescent or red LEDs) on carbohydrate content of *Pinus radiata* D. Don shoots cultured in Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al., 1988, supplemented with 2 gL^{-1} activated charcoal after the rooting phase.

Carbohydrate	**Retention Tim**	**BA Concentration (μM**		**Light Treatment**		**Sucrose Concentration (*w/v***	
(μmol g FW^{-1}	**(min)**	**22**	**44**	**White Fluorescent**	**Red LEDs**	**1.5%**	**3.0%**
Fructose	18.076	25.74 ± 7.18	22.87 ± 1.9	25.98 ± 4.79	21.57 ± 2.3	27.17 ± 4.44	19.58 ± 1.95
Galactose	16.096	3.12 ± 0.59	3.87 ± 0.59	4.00 ± 0.47	3.19 ± 0.75	3.42 ± 0.66	3.86 ± 0.53
Glucose	14.653	26.44 ±15.0	14.41 ± 1.5	24.22 ± 9.88	12.46 ± 1.3	22.50 9.11	13.51±2.28
Mannitol	22.911	5.38 ± 0.75	5.85 ± 0.36	5.66 ± 0.56	5.71 ± 0.41	5.83 ± 0.56	5.49 ± 0.33
Sucrose	12.640	10.10 ± 4.2	5.29 ± 0.79	8.63 ± 2.88	5.15 ± 0.94	8.38 ± 2.71	5.12 ± 0.79

Data are presented as mean values ± S.E.

Amino acid concentrations in shoots after root induction were not significantly different in relation to BA, light treatments, sucrose concentrations or the interactions between them (Table 3 and Supplementary Tables S13–S15). Alanine, arginine, asparagine and glutamine levels were the highest in shoots after the rooting phase, regardless of the *in vitro* treatment applied during the induction phase (Table 3).

Table 3. Effect of 6-Benzyladenine (BA) (22 and 44 μM) and sucrose concentration (1.5 and 3.0% (w/v)) and light treatment (white fluorescent or red LEDs) on amino acid content (μmol g FW^{-1}) of *Pinus radiata* D. Don shoots cultured in rooting medium (Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al., 1988, supplemented with mixture of 5 μM 1-Naphthaleneacetic acid (NAA) and 10 μM indole-3-butyric acid (IBA)).

Free Amino Acids (μmol g FW^{-1})	Retention Time (min)	BA Concentration (μM)		Light Treatment		Sucrose Concentration (w/v)	
		22	44	White Fluoresce	Red LEDs	1.5%	3.0%
Alanine	5.323	19.14 ± 9.72	16.08 ±4.82	15.40 ± 6.71	19.43 ± 6.64	11.98 ± 4.54	22.54 ± 8.08
Arginine	5.035	29.98 ± 10.39	28.22 ±7.34	34.10 ± 8.77	22.8 ± 7.72	22.45 ± 6.74	35.34 ± 9.64
Asparagine	3.115	13.72 ± 8.27	24.78 ±8.18	16.09 ± 7.67	25.70 ± 9.39	30.53 ± 9.68	10.52 ± 6.05
Aspartic acid	1.204	0.57 ± 0.12	0.61 ± 0.07	0.68 ± 0.10	0.50 ± 0.05	0.60 ± 0.08	0.59 ± 0.10
Cysteine	7.140	3.47 ± 0.82	4.36 ± 0.55	4.61 ± 0.67	3.32 ± 0.60	3.95 ± 0.79	4.07 ± 0.53
Glutamic acid	1.849	9.31 ± 8.07	5.55 ± 2.64	11.78 ± 6.17	1.42 ± 0.10	3.60 ± 2.04	10.39 ± 6.54
Glutamine	3.827	52.78 ± 16.66	70.16 ±8.58	65.37 ± 12.38	61.26 ± 11.19	63.54 ± 12.57	63.41 ± 11.29
Glycine	4.231	0.11 ± 0.02	0.12 ± 0.01	0.13 ± 0.02	0.10 ± 0.01	0.10 ± 0.02	0.13 ± 0.01
Hydroxyproline	10.330	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Histidine	4.016	0.15 ± 0.04	0.18 ± 0.02	0.18 ± 0.03	0.16 ± 0.02	0.18 ± 0.03	0.17 ± 0.02
Isoleucine	9.079	0.08 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
Leucine	9.592	0.09 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
Lysine	10.018	0.14 ± 0.03	0.17 ± 0.02	0.16 ± 0.03	0.16 ± 0.01	0.17 ± 0.02	0.14 ± 0.02
Methionine	7.905	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
Phenylalanine	8.927	0.05 ± 0.01	0.06 ± 4.9^{-3}	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 4.3^{-3}
Proline	12.543	0.27 ± 0.07	0.30 ± 0.08	0.33 ± 0.09	0.23 ± 0.06	0.25 ± 0.07	0.31 ± 0.08
Serine	3.335	3.82 ± 3.16	1.01 ± 0.11	3.18 ± 2.24	0.83 ± 0.12	0.92 ± 0.14	3.27 ± 2.42
Threonine	4.386	0.12 ± 0.02	0.15 ± 0.01	0.14 ± 0.02	0.12 ± 0.01	0.13 ± 0.02	0.14 ± 0.02
Tryptophan	8.653	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 5.0^{-3}	0.05 ± 0.01	0.06 ± 4.6^{-3}
Tyrosine	6.334	0.06 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
Valine	7.760	0.29 ± 0.07	0.40 ± 0.04	0.38 ± 0.05	0.33 ± 0.04	0.32 ± 0.05	0.39 ± 0.05

Data are presented as mean values ± S.E.

Shoots cultured with the highest sucrose concentration showed higher levels of alanine, arginine and glutamic acid than those cultured at the lowest sucrose concentration (Table 3). In contrast, the asparagine content was higher in rooted shoots coming from explants cultured in induction media supplemented with 1.5% sucrose than in those cultured with 3.0% sucrose.

After six weeks of culture in REM medium, no significant differences were found for the amino acid concentrations in shoots coming from explants induced in the presence of different concentrations of BA, light treatment and the interaction between both variables (Supplementary Tables S16–S18). However, the concentrations of alanine, arginine, glutamine, glycine, and lysine exhibited a decrease when shoots had been cultured with the highest BA concentration (Table 4). *Pinus radiata* shoots contained concentrations below 1.00 μmol g FW^{-1} of aspartic acid, glycine, hydroxyproline, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine, regardless of the BA concentration used in the induction media (Table 4).

Table 4. Effect of 6-Benzyladenine (BA) (22 and 44 μM) and sucrose concentration (1.5 and 3.0% (w/v)) and light treatment (white fluorescent or red LEDs) on amino acid levels (μmol g FW−1) of *Pinus radiata* D. Don shoots cultured in Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al.,1988, supplemented with 2 gL^{-1} activated charcoal after the rooting phase.

Free Amino Acids (μmol g FW^{-1})	Retention Time (min)	BA Concentration (μM)		Light Treatment		Sucrose Concentration (w/v)	
		22	44	White Fluoresce	Red LEDs	1.5%	3.0%
Alanine	5.323	27.12 ± 9.12	15.29 ± 5.17	18.89 ± 7.55	19.96 ± 5.62	24.53 ± 6.87	12.74 ± 5.63
Arginine	5.035	67.60 ± 24.44	63.97 ± 15.2	79.68 ± 19.55	49.47 ± 16.80	68.70 ± 15.72	60.71 ± 23.06
Asparagine	3.115	0.76 ± 0.09	3.98 ± 3.30	0.73 ± 0.07	5.17 ± 4.50	4.56 ± 3.80	0.64 ± 0.06
Aspartic acid	1.204	0.81 ± 0.08	0.87 ± 0.08	0.82 ± 0.04	0.89 ± 0.12	0.90 ± 0.09	0.78 ± 0.06
Cystine	7.140	7.08 ± 0.74	7.39 ± 0.53	7.17 ± 0.60	7.41 ± 0.62	7.74 ± 0.66	6.69 ± 0.42
Glutamic acid	1.849	19.35 ± 10.99	27.51 ± 6.85	20.38 ± 8.18	29.34 ± 8.35	31.59 ± 9.19	15.67 ± 5.12
Glutamine	3.827	52.86 ± 12.07	39.56 ± 6.66	49.22 ± 10.51	38.70 ± 5.38	46.58 ± 5.06	41.08 ± 12.57
Glycine	4.231	0.20 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.19 ± 0.03	0.20 ± 0.03	0.17 ± 0.01
Hydroxyproline	10.330	0.05 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.09 ± 0.02	0.06 ± 0.0047
Histidine	4.016	0.24 ± 0.03	0.23 ± 0.02	0.23 ± 0.03	0.23 ± 0.03	0.26 ± 0.03	0.20 ± 0.02
Isoleucine	9.079	0.17 ± 0.01	0.17 ± 0.02	0.16 ± 0.01	0.18 ± 0.02	0.19 ± 0.02	0.15 ± 0.01
Leucine	9.592	0.22 ± 0.02	0.21 ± 0.02	0.20 ± 0.01	0.22 ± 0.03	0.22 ± 0.02	0.19 ± 0.01
Lysine	10.018	0.41 ± 0.16	0.25 ± 0.04	0.36 ± 0.11	0.25 ± 0.04	0.30 ± 0.04	0.32 ± 0.13
Methionine	7.905	0.02 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Phenylalanine	8.927	0.11 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
Proline	12.543	1.00 ± 0.18	1.02 ± 0.26	0.91 ± 0.17	1.12 ± 0.32	1.28 ± 0.28	0.67 ± 0.13
Serine	3.335	1.44 ± 0.14	1.41 ± 0.15	1.31 ± 0.11	1.53 ± 0.19	1.60 ± 0.16	1.19 ± 0.09
Threonine	4.386	0.23 ± 0.02	0.24 ± 0.02	0.24 ± 0.01	0.24 ± 0.03	0.27 ± 0.02 [a]	0.21 ± 0.01 [b]
Tryptophan	8.653	0.15 ± 0.02	0.12 ± 0.01	0.14 ± 0.01	0.12 ± 0.02	0.13 ± 0.01	0.13 ± 0.01
Tyrosine	6.334	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 4.0^{-3}	0.14 ± 0.01	0.15 ± 0.01 [a]	0.12 ± 0.01 [b]
Valine	7.760	0.63 ± 0.09	0.56 ± 0.06	0.56 ± 0.05	0.61 ± 0.09	0.63 ± 0.07	0.52 ± 0.06

Data are presented as mean values ± S.E. Different letters indicate significant differences by Dun-can's post hoc test ($p < 0.05$).

Arginine, glutamine and lysine concentrations were the highest in shoots growing under white FL. In contrast, concentrations of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, hydroxyproline, isoleucine, leucine, methionine, phenylalanine, proline, serine, tryptophan, tyrosine and valine increased in shoots exposed to red LEDs (Table 4).

Threonine and tyrosine concentrations in rooted shoots cultured in induction media with the lowest sucrose concentration were significantly higher than in shoots cultured in media with the highest sucrose concentration (Table 4). Although not significant, it is worth noting that alanine, arginine, asparagine,

aspartic acid, cysteine, glutamic acid, glutamine, glycine, hydroxyproline, histidine, isoleucine, leucine, proline, serine and valine levels at the end of the rooting phase were also lower in shoots cultured in medium with the highest sucrose concentration during root induction (Table 4).

Significantly higher values of hydroxyproline were found in shoots developed in a culture medium with 44 μM BA and 1.5% sucrose than in those from 44 μM BA and 3.0% sucrose. Additionally, the hydroxyproline levels were significantly higher in shoots cultured in a medium with 44 μM BA and 1.5% sucrose than in those from 22 μM BA and 1.5% sucrose (Figure 7). The lowest hydroxyproline level was obtained in shoots induced under the lowest BA concentration and cultured in medium supplemented with 1.5% sucrose during the root induction phase (Figure 7).

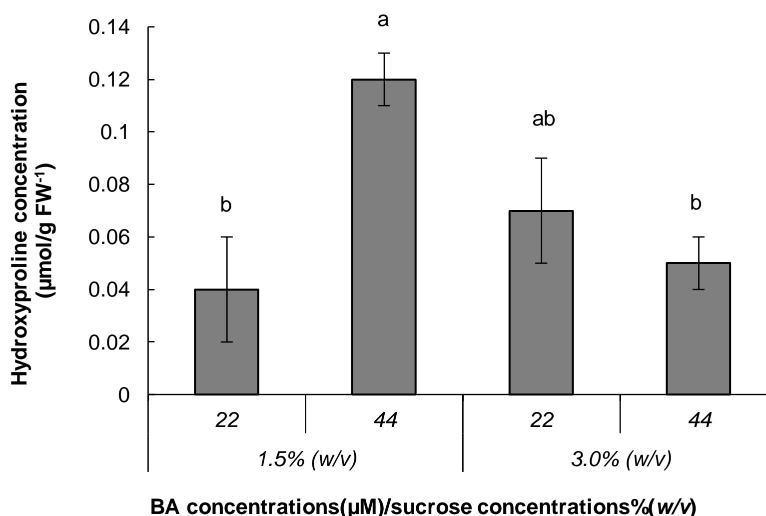
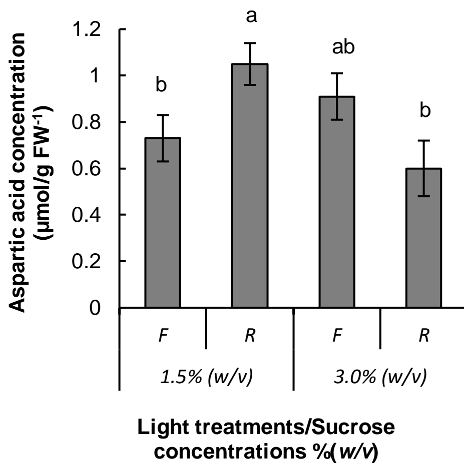
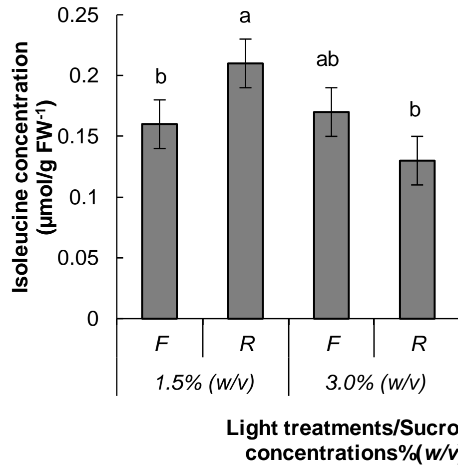


Figure 7. Effect of 6-Benzyladenine (BA) (22 and 44 μM) and sucrose concentration (1.5 and 3.0% (w/v)) on hydroxyproline amino acid level of *Pinus radiata* D. Don shoots cultured in Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al., 1988, supplemented with 2 gL^{-1} activated charcoal. Data are presented as mean values ± S.E. Different letters indicate significant differences by Duncan's post hoc test ($p < 0.05$).

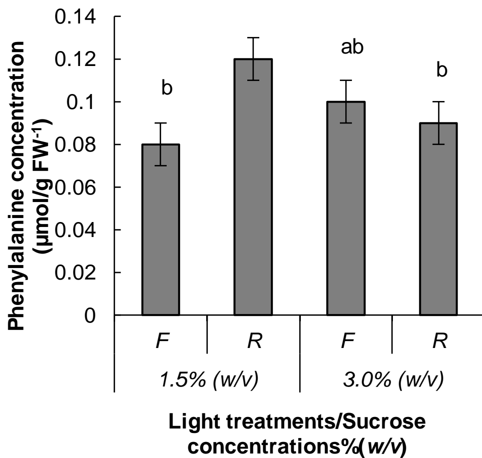
After the rooting expression phase, the interaction between light treatment and sucrose concentration showed statically significant differences for the aspartic acid, isoleucine, phenylalanine, threonine and tyrosine levels (Figures 8a–e). Significantly higher concentrations of the abovementioned amino acids were detected in shoots from medium with the lowest sucrose concentration and exposed to red LEDs (Figure 8a–e).



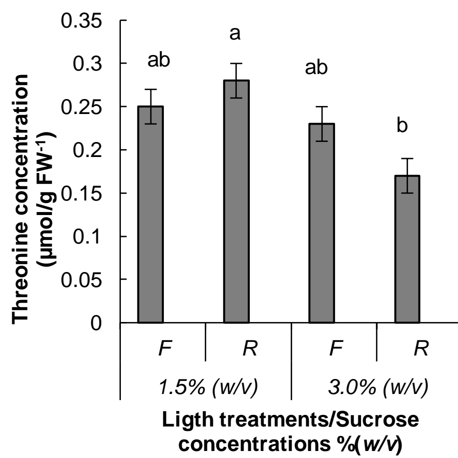
(a)



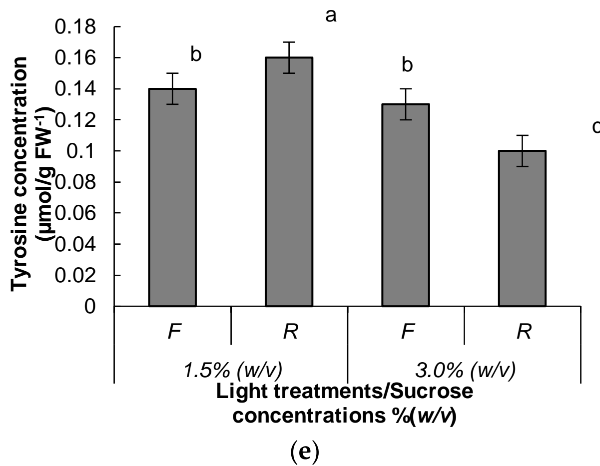
(b)



(c)



(d)



(**e**)

Figure 8. Effect of light treatment (white fluorescent (F) or red LEDs (R)) and sucrose concentration (1.5 and 3.0% (w/v)) on aspartic acid (**a**), isoleucine **(b**), phenylalanine (**c**), threonine (**d**) and tyrosine (**e**) amino acid levels of *Pinus radiata* D. Don shoots cultured in Quoirin and Lepoivre (LP) medium (Quoirin and Lepoivre, 1977), modified according to Aitken-Christie et al., 1988, supplemented with 2 gL^{-1} activated charcoal. Data are presented as mean values ± S.E. Different letters indicate significant differences by Duncan's post hoc test ($p < 0.05$).

Additionally, shoots coming from root induction at 3.0% sucrose and exposed to red LEDs presented the lowest aspartic acid, isoleucine, threonine and tyrosine levels (Figure 8a–e).

4. DISCUSSION

In this study, the genotype affected the survival of the explants independently of the collection date. This result could be partially explained since the genotype is an endogenous factor that has a significant role in the regenerative potential, overall repeatability and reliability of the tissue culture protocol (Coleman, and Ernst, 1989). As was reviewed in (Jiménez, 2005; Fehér, 2005), the response of explants to particular tissue culture conditions is highly dependent on the genetic and physiological determination. In this sense, *Pinus radiata*, *P. taeda* L. and *P. halepensis*

showed a differential organogenic response *in vitro* depending on the genotype in previous studies (Pereira et al., 2021; Montalbán et al., 2013; Cézar et al., 2015).

The highest survival and the lowest contamination rates were found during the first collection date. Several protocols mention that factors such as the genotype, explant source and plant growth regulators influence the *in vitro* regeneration of plants via organogenesis or somatic embryogenesis (Parzymies, 2021; Tung et al., 2021; Rojas-Vargas et al., 2021). In our case, the developmental stage of the explant can be related to the contamination rate. On the first collection date, explants consisted of apical shoot buds with closed scales; this source of explant could be better for the effective elimination of contamination. Analogous results were obtained in *Pseudotsuga menziesii* Mirb. Franco (Traore et al., 2005) organogenesis, where spring buds showed higher contamination rates than winter buds.

Silver nanoparticles have various applications in plant biotechnology, mainly to eradicate microbial contamination and promote *in vitro* development (Pastelín-Solano et al., 2020). In this sense, the use of a silver nanoparticle solution in our study favored the disinfection efficiency of the explants, in agreement with the results for *Fragaria × ananassa*, *Vanilla planifolia* Andrews and *Psidium friedrichsthalianum* (O. Berg) Nied, where the contamination rates of 23, 28 and 40%, respectively (Tung et al., 2021; Pastelín-Solano et al., 2020; Andújar et al., 2020), were reported. In contrast, in *P. halepensis*, a negative effect of the silver nanoparticle solution for the sterilization of apical shoot buds was found with a contamination rate of 89% (Pereira et al., 2021).

The timing of explant collection and the explant developmental stage influence the response to organogenesis or somatic embryogenesis (Andersone and Ievinsh, 2002; Corredoira et al., 2012). When the EFS was evaluated, apical buds collected

in the first week of February showed higher percentages than those collected during the third and fourth weeks of February. A similar pattern has been reported in other species, such as *Quercus alba* L. and *Allamanda cathartica* L., showing that the explant developmental stage or the season of collection of explants played an important role in the *in vitro* response (Corredoira et al., 2012; Khanam and Anis, 2018). During our study, a morphogenic peak from dormancy to bud initiation was observed during the January and February collection dates, wherein tissues became very active in terms of morphogenesis by increasing the shoot formation. These results are in accordance with those obtained in shoot explants of *Larix decidua* Mill. (Bonga, 2004, 2017), where two short morphogenic peaks were observed, the first before bud break and the second in late summer.

When we focused on the study of the effect of the BA concentration on EFS, no statistically significant differences were obtained. In plant tissue culture, BA is the most commonly used cytokinin (Phillips and Garda, 2019); in addition, BA, used alone or in combination with other cytokinins, has been shown to efficiently promote *in vitro* bud induction (De Diego et al., 2008; Valverde-Cerdas et al., 2008). In our experiments, BA at the concentrations tested (22 and 44 µM) provided a satisfactory organogenic response, and, although not significant, a slightly higher response in explants cultured in media supplemented with 44 µM BA was observed. An analogous result was obtained in *P. halepensis* using the same BA concentration (Pereira et al., 2021). Likewise, in *P. pinea*, a higher number of buds formed per explant was observed during the first 16 days of culture at the highest concentration of BA tested (44.4 µM) (Moncaleán et al., 2005). This behavior may be attributed to the maturity of the explant used, as, most likely, the aged buds are not as physiologically active as the young ones. For the above, higher concentrations of exogenously applied growth regulators may be needed in mature

explants to obtain similar morphogenic responses, as reported for *Tetraclinis articulata* (Vahl) Masters (Juan-Vicedo et al., 2022). In this regard, Wendling et al., 2014 mentioned that, through the continuous *in vitro* subculture of shoots in media supplemented with cytokinins, reinvigoration can be obtained. In our study, all genotypes evaluated were able to develop reinvigorated shoots in media with both BA concentrations tested. These results agreed with those observed in *P. pinea*, *P. pinaster*, *P. silvestris* and *P. halepensis*, where reinvigorated axillary shoots were obtained through organogenesis, using buds as initial explants (De Diego et al., 2008, 2010; Cortizo et al., 2009; Pereira et al., 2021).

In order to obtain a root induction response in the explants, the culture medium was supplemented with NAA combined with IBA, a mixture traditionally used to induce root differentiation in *Pinus* species (Álvarez et al., 2009; Cortizo et al., 2009; Montalbán et al., 2011b, 2013). However, in this work, the rooting percentage was low, as observed in *P. pinea* when NAA alone was applied (Cortizo et al., 2009). Montalbán et al. (Montalbán et al., 2011b), studying *P. radiata*, reported that IBA alone was more efficient in the adventitious root induction of shoots coming from explants of juvenile origin. In *Pinus elliottii* Engelm. var. elliottii, Nunes et al. (Nunes et al., 2018) observed that different concentrations and combinations of IBA (4.9 and 9.8 μM) and NAA (1.1, 2.1 and 2.7 μM) led to low root frequency using shoots developed from juvenile material. Likewise, in *P. halepensis*, long exposure to IBA was not effective in inducing roots in shoots of mature origin (Pereira et al., 2021). It is widely recognized that the most common problem encountered in the micropropagation of mature conifers is the adventitious root formation in shoots (Cortizo et al., 2009).

As reviewed by Bairu et al. (Bairu and Kane, 2011), the development of basal callus is one of the main physiological disorders that affects the rooting competence of

microplants; this basal callus could interfere with physiological processes by trapping essential growth constituents such as plant growth regulators and creating a physical barrier. In our work, in the bases of shoots, a large and profuse callus was observed, and this fact could have affected the development of adventitious roots. In contrast, in slash pine, when inducing adventitious roots using IBA combined with NAA, no callus formation was observed in shoots developed from juvenile explants (Nunes et al., 2018). Similarly, in *Pinus. roxburghii* Sarg., using shoots developed from juvenile explants, the root induction medium supplemented with NAA and solidified with 0.6% agar produced shoots with low or no callusing at the base of the shoots (Kalia et al., 2007). In *P. taeda* (De Oliveira et al., 2012; Cézar et al., 2015), a combination of auxin (2.68 µM NAA) with cytokinin (0.44 µM BA) was evaluated and showed root induction of 55.6 to 47.5%, respectively. More recently, a pulse with IBA 500 mg L^{-1} for 5 min was used as an *ex vitro* rooting preconditioning treatment in *Mitragyna parvif*olia (Roxb.) Korth., obtaining approximately 90% micropropagated shoots rooted *ex vitro* (Patel et al., 2020). Soumare et al. (Soumare et al., 2021) reported that the use of plant-growth-promoting microbes, specifically *Streptomyces griseorubens* and *Norcardiopsis alb*a, increased the rooting and root hair in maize. In this sense, these recent strategies, or physical factors such as the photoperiod, temperature and substrates for rooting, could be considered to improve *P. radiata* rooting.

Carbohydrates in plants have several essential functions: they are basic elements of macromolecules, constitute substrates for respiration, play an important role in the synthesis pathway of many compounds and are indispensable for many other processes related to plant development or gene expression (Gibson, 2000; Calamar and De Klerk, 2002). In our work, the sucrose treatment had a significant effect on the RI percentage, and the highest result was observed in 3% (w/v) sucrose. Similar

results were observed when comparing sucrose concentrations (1–9%) for the root induction of Apple Rootstock MM 10, concluding that sucrose had a direct effect on rooting (Calamar and De Klerk, 2002). Likewise, in banana (*Musa* sp) and *Metroxylon sa*gu Rottb., the highest values for rooting were found in a medium supplemented with 3% (w/v) sucrose (Ahmed et al., 2014; Sumaryono et al., 2012). In *Eucalyptus globulus* Labill. and *P. pinea*, glucose had a positive effect during root induction (Corrêa et al., 2005; Zavattieri et al., 2009). Additionally, in *E. globulus* (Corrêa et al., 2005), it was reported that the carbohydrates are an energetic requirement for root development and sucrose, which is commonly used in tissue culture because it is the main sugar translocated in the phloem of several plants (Ragonezi et al., 2010; Kumar and Reddy, 2011).

The light type and wavelength specificity have an influence on morphogenetic responses such as adventitious root formation (Ragonezi et al., 2010). In several studies, LED lights have been used as an alternative to conventional lighting sources for plant tissue culture (Gupta and Jatothu, 2013). Supporting this, the spectral properties of LEDs have been found to regulate the morphological, anatomical and physiological responses of *in vitro* plants (Gupta and Jatothu, 2013). In this work, it was stated that the effectiveness of red LEDs on rooting depends on the genotype and the concentration of growth regulators applied in the root induction medium (Gupta and Jatothu, 2013). LED treatments (red or blue) showed a higher response in the rooting of plantlets in *Tripterospermum japonicum* Maxim, *Gossypium hirsutum* L. and *Doritaenopsis* (Moon et al., 2006; Shin et al., 2008; Li et al.,2010). In *Anthurium*, root formation was progressively induced under a higher portion of red LEDs in a mixed circuit of blue and red (Budiarto, 2010). However, in this work, shoots grown under white FL showed a significantly higher RI and NR/E. A similar pattern has been reported in *Handroanthus ochraceus* (Cham.)

Mattos, *Achilea millefolium* L. and *Alocasia amazonica*, where higher values of root induction were obtained in plants growing under low FL irradiances (Jo et al., 2008; Alvarenga et al., 2015; Larraburu et al., 2018). In future experiments to improve the micropropagation procedure in radiata pine should evaluate a combination of different types of light. In this way, in *P. radiata* the used of blue LEDs improved the shoot height of somatic plants (Castander-Olarieta et al., 2023).

When studying the effect of the BA concentration and light treatment on LLR, explants induced with 44 μM BA and exposed to red LEDs showed the highest values. A similar pattern has been reported in *Vitis ficifolia* Bunge var. ganebu, where longer roots were obtained in plants growing under red LEDs (Poudel et al., 2008). In contrast, higher values of root length were obtained in plants of *H. ochraceus* exposed to low FL irradiances (Larraburu et al., 2018).

Metabolite analysis showed significant differences for several carbohydrate and amino acid concentrations in the different treatments tested. Carbohydrates in *in vitro* culture media have functions such as the maintenance of osmotic potential and to serve as a carbon source for developmental processes including root induction (Yaseen et al., 2013; Moradi et al., 2017). When studying the effect of the sucrose concentration supplemented in RIM, higher fructose, glucose and sucrose content was found in shoots growing in culture medium supplemented with the highest sucrose concentration. In *A. amazonica*, media supplemented with 3 or 6.0% sucrose showed higher glucose, fructose and sucrose content in *in vitro* leaves compared with *ex vitro* leaves (Jo et al., 2009). Moreover, Mingozzi et al. (Mingozzi et al., 2011), studying *Cydonia oblonga* Mill., concluded that the sucrose content in leaves was associated with its content in the culture media, and shoots cultured with 30 gL^{-1} sucrose showed higher content of sucrose, glucose and fructose. Additionally, (Thorpe et al., 2008) explained that *in vitro* plants release invertase

enzymes to the culture medium that act in the hydrolysis of sucrose, giving rise to glucose and fructose. In the present work, the levels of the carbohydrates were unaffected by lighting conditions. Contrasting our results, the content of sucrose, starch and soluble sugars was higher in plantlets of *Doritaenopsis* and *G. hirsutum* exposed to red plus blue and red LEDs, respectively (Shin et al., 2008; Li et al., 2010).

Amino acid metabolism plays an essential role in plant protein biosynthesis, represents a building block for other biosynthesis pathways and is essential during signaling processes (Hildebrandt et al., 2015). Apart from the abovementioned functions, amino acids may also be involved in the plant stress response through intracellular pH regulation and the detoxification of reactive oxygen species (Campalans et al., 1999; Nuccio et al., 1999). Tyrosine is the precursor of secondary metabolites such as betacyanin in *Alternanthera brasiliana* (L.) Kuntze and certain enzymes such as tyrosine decarboxylase, which is responsible for redirecting essential primary metabolites into secondary metabolic pathways (Silva et al., 2005; Zhang et al., 2011). In addition, in *P. radiata* embryonal masses initiated under stressful conditions, Castander et al. al (Castander-Olarieta et al., 2019) discussed that tyrosine could be involved in antioxidative processes and osmotic adjustment. Likewise, the tyrosine metabolism pathway serves as a starting point for the production of tyrosine-derived metabolites essential to plant survival, such as tocopherols, plastoquinone and ubiquinone (Xu et al., 2020). With regard to threonine, it is an essential amino acid, and it is a substrate for isoleucine synthesis (Joshi et al., 2010). Moreover, threonine metabolites have an important role in plant growth and development, cell division and responses to abiotic stresses (Joshi et al., 2010; Muthuramalingam et al., 2018. However, in our study, the shoots with higher content of these two amino acids showed worse *in vitro* rooting (those

coming from root induction medium with 1.5% (w/v) sucrose, at the end of the rooting phase).

Krasenski et al., Lugan et al. and Joshi et al. (Joshi et al., 2010; Lugan et al., 2010; Krasensky and Jonak, 2012) observed amino acid accumulation in plants exposed to abiotic stress, such as drought, extreme temperature, salinity and osmotic stress. In this sense, tobacco plants showed high levels of aromatic amino acids, and this fact was related to the improvement of salt stress tolerance (Oliva et al., 2021). In this study, our results suggest that low sucrose concentrations could be related to abiotic stress similar to that observed in nutrient deficiencies or carbohydrate starvation, such as the results observed in *Camellia sinensis* (L.) Kuntze leaves, where increased threonine levels were observed under phosphate starvation (KC, S et al., 2018). However, the amino acid pool differs and changes depending on the developmental and physiological stage of the plant (Hildebrandt et al., 2015) .

5. CONCLUSIONS

The regeneration of *P. radiata* from the vegetative shoot buds of adult trees via organogenesis was achieved, and it depended on environmental physico-chemical factors. The collection date and genotype had a strong effect on the efficiency of the process. Reinvigorated shoots from adult trees were obtained at both of the BA concentrations tested. The optimal result in terms of shoot induction was obtained at the highest BA concentration. Our results suggest that the use of white FL and a 3% sucrose concentration was better for root induction. The low RI percentage suggests that further research should be encouraged to improve this phase, which is still a bottleneck in the micropropagation of mature conifers.

Moreover, shoots showing better *in vitro* rooting had higher fructose, glucose and sucrose content, whereas those from the worse root treatment showed higher threonine and tyrosine levels.

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CHAPTER 3

Adult trees *Cryptomeria japonica* (Thunb. ex L.f.) D. Don Micropropagation: Factors Involved in the Success of the Process

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ABSTRACT

Cryptomeria japonica (Thunb. ex L.f.) D. Don is a commercial tree native to Japan and is one of the most important forest species in that country and the Azores (Portugal). Because of the quality of *C. japonica* timber, several genetic improvement programs have been performed. Recently, some studies focusing on *C. japonica* somatic embryogenesis have been carried out. However, in this species, this process uses immature seeds as initial explants, and for this reason, it is not possible to achieve the maximum genetic gain (100% genetic of the donor plant). Although some studies have been made applying organogenesis to this species, the success of the process in adult trees is low. For this reason, our main goal was to optimize the micropropagation method by using trees older than 30 years as a source of plant material. In this sense, in a first experiment, we studied the effect of different types of initial explants and three basal culture media on shoot induction; then, two sucrose concentrations and two light treatments (LEDs versus fluorescent lights) were tested for the improvement of rooting. In a second experiment, the effects of different plant growth regulators (6-benzylaminopurine, meta-topolin, and thidiazuron) on shoot induction and the subsequent phases of the organogenesis process were analyzed. The cultures produced the highest number of shoots when QL medium (Quoirin and Lepoivre, 1977) and long basal explants (>1.5 cm) were used; the shoots obtained produced a higher number of roots when they were grown under red LED lights. Moreover, root induction was significantly higher in shoots previously induced with meta-topolin.

RESUMEN

Cryptomeria japonica (Thunb. ex L.f.) D. Don es un árbol comercial originario de Japón y es una de las especies forestales más importantes de ese país y de las Azores (Portugal). Debido a la calidad de la madera de *C. japonica*, se han llevado a cabo varios programas de mejora genética. Recientemente, se han llevado a cabo algunos estudios centrados en la embriogénesis somática de *C. japonica*. Sin embargo, en esta especie, este proceso utiliza semillas inmaduras como explantos iniciales, por lo que no es posible lograr la máxima ganancia genética (100% genética de la planta donante). Aunque se han realizado algunos estudios aplicando organogénesis a esta especie, el éxito del proceso en árboles adultos es bajo. Por esta razón, nuestro principal objetivo fue optimizar el método de micropropagación utilizando árboles mayores de 30 años como fuente de material vegetal. En este sentido, en un primer experimento estudiamos el efecto de diferentes tipos de explantos iniciales y tres medios de cultivo basales sobre la inducción de brotes; luego, se probaron dos concentraciones de sacarosa y dos tratamientos de luz (LEDs versus luces fluorescentes) para mejorar el enraizamiento. En un segundo experimento, se analizaron los efectos de diferentes reguladores del crecimiento vegetal (6-bencilaminopurina, metatopolina y tidiazuron) sobre la inducción de brotes y las fases posteriores del proceso de organogénesis. El mayor número de brotes se obtuvo cuando los explantos basales largos (>1,5 cm); se desarrollaron el medio de cultivo QL (Quoirin y Lepoivre, 1977); los brotes obtenidos produjeron un mayor número de raíces cuando se cultivaron bajo luces LEDs rojas. Además, la inducción de raíces fue significativamente mayor en los brotes previamente inducidos con metatopolina.

1. INTRODUCTION

The Japanese cedar, *Cryptomeria japonica* (Thunb. ex L.f.) D. Don, subfamily Taxodioideae, family Cupressaceae, is a monoecious conifer distributed across East Asia (Kusumi et al., 2000; Onuma et al., 2023). *C. japonica* covers approximately 4.5 million ha, representing 44% of the total reforested area (Forestry Agency, 2020). This conifer is one of the most important timber tree species in Japan, and it is traditionally used for construction wood and for obtaining biomass (Taniguchi et al., 2020).

Tsubomura and Taniguchi (2008) mentioned that Japanese cedar is clonally propagated by cuttings, but this type of propagation requires many hours of manual labor, therefore it is difficult to establish a short-term propagation protocol. For the abovementioned reasons, biotechnological approaches for *C. japonica* clonal propagation, such as *in vitro* methods including somatic embryogenesis (SE) or shoot organogenesis, are valuable tools for the propagation of this conifer (Phillips, 2004). In this sense, SE is a recognized technique for the large-scale propagation of conifers (Park et al., 1998). However, SE is a complex, multistage process initiated from immature seeds, so it is not possible to reproduce the genotype of the donor plant. In contrast, micropropagation by nodal tissue culture is faster, and higher multiplication rates are possible (Tsubomura and Taniguchi, 2008; Sahu and Sahu, 2013). Micropropagation starting from nodal segments uses smaller explants than conventional techniques, and selection of juvenile explants is possible (Sahu and Sahu, 2013). With regard to explants coming from mature trees, they are less likely to dedifferentiate and reprogram (Narváez et al., 2019), but the use of explants from mature trees for conifer micropropagation has also been reported (Rojas-Vargas et al., 2021; Juan-Vicedo et al.,2022). However, because these trees are selected after

reaching maturity, there has been limited success reported in the vegetative propagation of mature conifers, and procedures should be improved.

Although the benefits of tissue culture for the propagation of forest trees have been recognized, the success of such methods is still highly dependent on the species, the explant quality, the age of the donor plant, the culture medium, plant growth regulators, and/or the interaction among all these different factors. As a result, morphogenesis determines the growth and development of plant tissues, and it is influenced by several physico-chemical factors (Rai et al., 2010). In relation to culture media, DCR (Gupta and Durzan, 1985), MS (Murashige and Skoog, 1962), and QL (Quoirin and Lepoivre, 1977) are commonly used basal media for *in vitro* regeneration of conifers (Arab et al., 2014; De Diego et al., 2010). Furthermore, the cytokinins added to the culture medium have a direct effect on the endogenous phytohormone balance, provoking a response to the induction of axillary shoot buds and affecting the organogenesis of the culture (Phillips, 2004; De Almeida et al., 2012).

Carbohydrates in plants are basic elements; they constitute substrates for respiration and are essential for many other processes related to plant development or gene expression, and in many species they favor rooting, acting mainly as a source of energy (Gibson, 2000; Calamar and De Klerk, 2002; Dewir et al., 2016).
Plant growth and development are also influenced by different physical factors, with light being one of the most important (Alallaq et al., 2020). The traditional light source used in *in vitro* culture in the growth chambers is fluorescent tubes (FL), with irradiances between 25 and 150 mmol $m^{-2}s^{-1}$ for a 16 h photoperiod (Larraburu et al., 2018). FL emit a broad light spectrum, and their physiological effects on plants are not specific (Rocha et al., 2010). Furthermore, the power consumption of FL is high as the heat emitted needs to be removed from growth

chambers using air conditioners, making the process expensive (Kulus and Woźny, 2020). Light-emitting diodes (LEDs) are available today as an alternative to conventional light sources for *in vitro* plant growth (Marín-Martínez et al., 2022), and they present advantages over FL such as small size, longer lifespan, less power consumption, high energy conversion efficiency, and adjustable light spectra (Río-Alvarez et al., 2014).

The analysis of the combined effects morphological (type of explants), chemical (culture media, sucrose concentrations), and physical factors (LED lights) at different micropropagation stages of adult trees has not been carried out in *Cryptomeria japonica*. Moreover, the use of ventilated culture containers is not widely used for conifer species. For these reasons, with the main objective of improving the micropropagation protocol for adult Japanese cedar, we focused on optimizing (1) the shoot induction stage using different types of explants, cytokinins, and culture media as well as (2) the rooting stage using different sucrose concentrations, Ecobox containers®, and light treatments (fluorescent versus LEDs).

2. MATERIALS AND METHODS

2.1. Plant Material

Actively growing *C. japonica* twigs were collected in October 2019 from two healthy >30-year-old adult trees located in Arkaute (Spain; 42°51′9.35″ N, 2°37′30.55″ W) to carry out Experiment 1 (Figure 1a). In October 2020, actively growing twigs were collected from four healthy >30-year-old adult trees located in Urnieta (Spain; 43°13′13.03″ N, 1°57′40.91″ W), and one adult tree (Spain; 43°13′50.32″ N, 1°59′25.37″ W) was chosen to carry out Experiment 2.

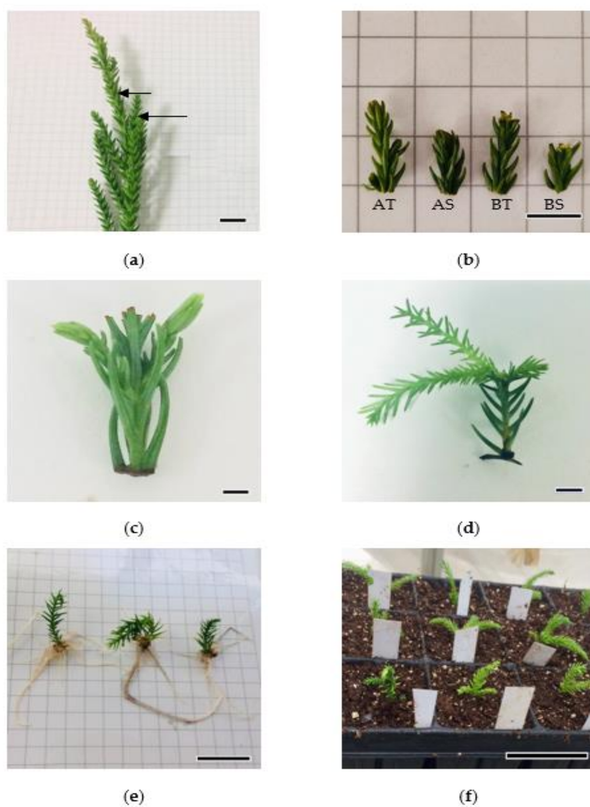

Figure 1. Plant material at different stages of *Cryptomeria japon*ica micropropagation process: (**a**) actively growing twigs used as starting explants; arrows show the explants used for *in vitro* culture establishment, bar = 30 mm; (**b**) explant type; apical explants > 1.5 cm (AT), apical explants < 1.0 cm (AS), basal explants > 1.5 cm (BT) and basal explants < 1.0 cm (BS), bar = 10 mm; (**c**) basal explants of 2.0 cm length after 4 weeks cultured in QL medium (Quoirin and Lepoivre, 1977) supplemented with 8.8 μM BA, bar = 30 mm; (**d**) elongated shoots after 6 weeks in hormone-free QL medium supplemented with 2 gL^{-1} activated charcoal, bar = 30 mm; (**e**) rooted shoots after six weeks in hormone-free QL medium supplemented with 2 gL^{-1} activated charcoal and under red LEDs, bar = 30 mm; (**f**) plantlets after four weeks in *ex vitro* conditions in the greenhouse, bar = 30 mm.

2.2. Sterilization

The plant material was first washed with commercial detergent, then rinsed under running water for 5 min immersed in 70% ethanol for 2 min, and then washed two times with sterile distilled water in the laminar flow unit. Finally, the actively growing twigs were disinfected in commercial bleach (30% v/v) (active chlorine 37 gL^{-1} sodium hypochlorite) for 20 min and rinsed three times in sterile distilled water for 5 min. each.

After the sterilization protocol, four types of explants were tested: apical explants >1.5 cm (AT), apical explants <1.0 cm (AS), basal explants >1.5 cm (BT), and basal explants < 1.0 cm (BS) (Figure 1b).

2.3. Micropropagation Process

2.3.1. Experiment 1

After sterilization, the four types of explants were cultured vertically on 25 × 145 mm test tubes with polypropylene caps (Lab Associates, Oudenbosch, Netherlands) containing 15 mL of bud induction medium (IM) (Supplementary Table S1). Three basal media were assayed: DCR (Gupta and Durzan, 1985), MS (Murashige and Skoog, 1962), and QL (Quoirin and Lepoivre, 1977). All media were supplemented with 3% (w/v) sucrose, 6-benzyladenine (BA, 8.8 μM, Duchefa Biochemie, Haarlem, Netherlands), and 8 gL^{-1} Difco Agar® (Becton and Dickinson, Madrid, España) granulated. The pH of all media was adjusted to 5.8, and then they were autoclaved at 121 °C for 20 min. All cultures were placed in the growth chamber at a photoperiod of 16 h with 120 μmol m^{-2} s^{-1} light intensity provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France) and a temperature of 21 ± 1 °C.

As soon as shoot induction was observed (after four weeks) (Figure 1c), four to five explants were transferred to baby food glass jars with MagentaTM b-cap lids filled with 25 mL of elongation medium (EM) (Supplementary Table S1). EM was composed of hormone-free DCR, MS, or QL supplemented with 2 gL^{-1} activated charcoal, 3% (w/v) sucrose, and solidified with 8.5 gL^{-1} Difco Agar® granulated; pH and autoclaving conditions were those mentioned for IM.

The shoots were transferred to fresh EM every six weeks. Shoots were cultivated individually in a fresh EM when they reached 10–15 mm (Figure 1d). The conditions in the growth chamber were the same as those described above.

2.3.2. Root Induction and Acclimatization of Rooted Plants

Elongated shoots of at least 20–25 mm long were used for root induction. Based on the results of Experiment 1, QL basal medium was selected. The explants were transferred to Ecoboxes (Eco2box/green filter, consisting of a polypropylene oval vessel with a "breathing" hermetic cover, Duchefa®) with 100 mL of root induction medium (RIM) (Supplementary Table S1), which was composed of half-strength macronutrient QL medium with 50 μM 1-naphthaleneacetic acid (NAA, Duchefa Biochemie, Haarlem, Neth-erlands), 8 gL^{-1} Difco Agar®, and 3% sucrose or 1.5% (w/v) sucrose. The pH and autoclaving conditions were those previously described. The shoots were placed under dim light for eight days. Then, two different light treatments were tested for four weeks: (A) white fluorescent light (FL) (color temperature 4000 K), 120 μmol $m^{-2}s^{-1}$ light intensity provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France); and (B) red light (peak wavelength 630 nm), 60 μmol $m^{-2}s^{-1}$ light intensity provided by adjustable LEDs (RB4K Grow Light LEDs). The photoperiod and the temperature of the growth chamber were the same as previously described.

After five weeks of culture in RIM, shoots were cultured for six weeks in Ecoboxes with 100 mL of root expression medium (REM) (Supplementary Table S1); this medium consisted of half-strength macronutrient QL medium supplemented with 2 gL^{-1} activated charcoal, 3% sucrose or 1.5% (w/v) sucrose, and 8.5 gL^{-1} Difco Agar®. Then, the rooted plants (Figure 1e) were planted *ex vitro*, transferring them to moist peat moss (Pindstrup, Aarhus, Denmark) with vermiculite at a proportion of 8:2 (v/v); acclimatization was carried out in a greenhouse under controlled conditions at a temperature of 21 ± 1 °C and progressively decreasing the humidity during a month from 95 to 80% (Figure 1f).

2.3.3. Experiment 2

Based on the results of Experiment 1, basal explants of >1.5 cm length and QL medium were selected to perform this experiment (Supplementary Table S2). The medium was supplemented with one of these three types of cytokinin (CK): BA, meta-topolin (m-T), or thidiazuron (TDZ, Duchefa Biochemie, Haarlem, Netherlands), all of them at 8.8 μM. The explants were placed in the growth chamber at the same conditions described above (Section 4.3.1. Experiment 1).

As soon as shoot induction was observed (after four weeks), four to five shoots were transferred into baby food glass jars with MagentaTM b-cap lids and 25 mL of EM (Supplementary Table S2), and they were subcultured every six weeks. Shoots that reached 10–15 mm were separated and individually cultivated in fresh EM. The growth chamber temperature and photoperiod were the same as those previously described (Section 4.3.1. Experiment 1).

2.3.4. Root Induction and Acclimatization of Rooted Plants

Shoots at least 20–25 mm long from the EM were employed for root induction. Based on the results of Experiment 1, the shoots were cultivated in Ecoboxes filled with half-strength macronutrient QL basal medium supplemented with 50 μM NAA, 1.5% (w/v) sucrose, and 8 gL^{-1} Difco Agar® (Supplementary Table S2). In addition, based on the results from Experiment 1, red light was selected for the rooting stage; the shoots were placed under dim light for eight days, followed by four weeks under a 16 h photoperiod with red light (peak wavelength 630 nm) and 60 $\mu mol\ m^{-2}s^{-1}$ light intensity provided by adjustable LEDs (RB4K Grow Light LEDs). After these five weeks, shoots were cultured for six weeks in Ecoboxes with 100 mL of REM (Supplementary Table S2). The photoperiod and the temperature of the growth chamber were the same as those described in previous sections. Then, rooted plants were acclimatized as described above (Section 4.3.2).

2.4. Data collection and Statistical Analysis

2.4.1. Experiment 1

Twenty-four to forty-eight test tubes and one explant per test tube (AT, AS, BT, or BS) per each tree (two trees) were cultured in each culture medium. After two months of culture, the contamination, survival, and shoot induction percentages for each condition tested were measured. In the case of the shoot induction (%) and the mean number of shoots per explant (NS/E), these were calculated with respect to the non-contaminated explants. The effect of the explant type and culture medium on survival and shoot induction (%) was analyzed using a logistic regression model, and when necessary, differences were assessed by Tukey's post hoc test (α = 0.05).

Data for the NS/E were analyzed by analysis of variance (ANOVA). When necessary, multiple comparisons were made using Tukey's post hoc test (α = 0.05).

After the root expression stage, data for the root induction percentage, the mean number of roots per explant (NR/E), and the length of the longest root (LLR) (cm) were recorded. A completely randomized design using seven to twenty-four plantlets per sucrose concentration and light treatment was performed.

The effect of the sucrose concentration and light treatment on root induction (%) was analyzed with a logistic regression model. Data for NR/E and LLR were analyzed by ANOVA, and when necessary, differences were assessed by Tukey's post hoc test (α = 0.05). To evaluate the effect of the sucrose concentration and light treatment on the acclimatization percentage, a logistic regression model was applied to plantlets after four weeks of growth in the greenhouse. Data processing was done using R Core Team software® (version 4.2.1, Vienna, Austria).

2.4.2. Experiment 2

Forty test tubes and one explant per test tube (BT) were cultured in each culture medium per tree (five trees). After two months of culture, the contamination, survival, and shoot induction percentages were recorded for each condition tested.

The shoot induction percentage (%) and the mean number of shoots per explant (NS/E) were calculated with respect to the non-contaminated explants after the elongation stage. The effect of the cytokinin type on survival and shoot induction (%) was analyzed using a logistic regression model; when necessary, differences were assessed by Tukey's post hoc test (α = 0.05).

Data for the NS/E were analyzed by analysis of variance (ANOVA), and when necessary, differences were assessed by Tukey's post hoc test (α = 0.05).

After the root expression stage, data for the root induction percentage, the mean number of roots per explant (NR/E), and the length of the longest root (LLR) (cm) were recorded. A completely randomized design using forty-one to fifty plantlets per cytokinin type applied during the shoot induction stage was performed.

The effect of the cytokinin type applied during the shoot induction stage on the root induction was analyzed using a logistic regression model. Data for NR/E and LLR were analyzed by ANOVA, and when necessary, differences were assessed by Tukey's post hoc test (α = 0.05). To evaluate the effect of the cytokinin type applied during the shoot induction stage on the acclimatization percentage, a logistic regression was used. As mentioned above, all data were processed using R Core Team software®.

3. RESULTS

3.1. Micropropagation Process

3.1.1. Experiment 1

The rates of contamination were registered eight weeks after sterilization, showing general values of 39%. Explants' survival was significantly affected by the explant type used; AS explants showed significantly higher survival rates (90%) than AT (62%) and BT (32%; Figure 2, Supplementary Table S3).

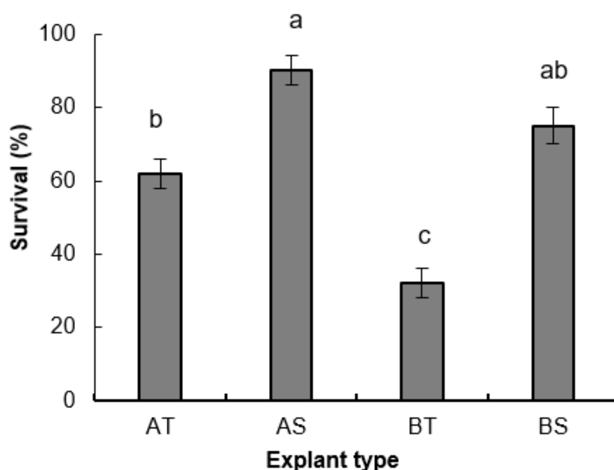

Figure 2. Survival (%) in different explant types of *Cryptomeria japonica* cultured in DCR medium (Gupta and Durzan, 1985), MS medium (Murashige and Skoog, 1962) and QL medium (Quoirin and Lepoivre, 1977). Apical explants >1.5 cm (AT), apical explants <1.0 cm (AS), basal explants >1.5 cm (BT), and basal explants <1.0 cm (BS). Data are presented as mean values ± S.E. Significant differences are indicated by different letters according to Tukey's post hoc test ($p < 0.05$).

The basal medium and the interaction between explant type and basal medium did not show significant differences for survival (%) (Supplementary Table S3). The survival percentage ranged from 57% in explants grown in MS and QL media to 63% in explants cultured in DCR medium.

When the variables explant type and basal medium and the interaction between them were analyzed after the induction stage, statistically significant differences were only found for the percentage of shoot induction depending on the explant type (Figure 3 and Supplementary Table S4). A significantly higher shoot induction percentage was obtained in AS (69%) and AT (56%), compared to the rest of the explants tested (Figure 3).

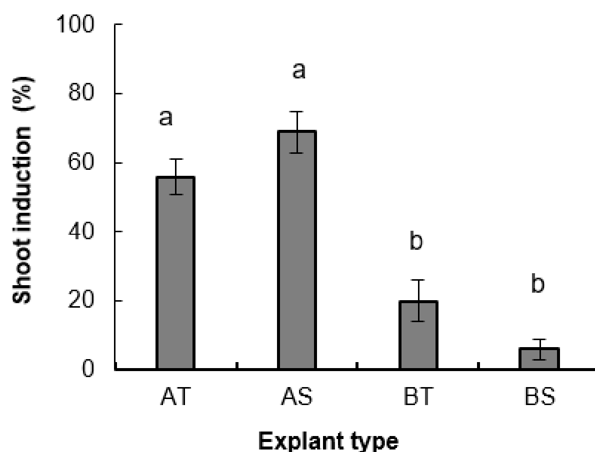

Figure 3. Shoot induction (%) in different explant types of *Cryptomeria japon*ica cultured in DCR medium (Gupta and Durzan, 1985), MS medium (Murashige and Skoog, 1962) and QL medium (Quoirin and Lepoivre, 1977). Apical explants >1.5 cm (AT), apical explants <1.0 cm (AS), basal explants >1.5 cm (BT), and basal explants <1.0 cm (BS). Data are presented as mean values ± S.E. Significant differences are indicated by different letters according to Tukey's post hoc test ($p < 0.05$).

The shoot induction percentage ranged from 38% in explants grown in DCR medium to 46% in explants cultured in QL medium. Explants developed in MS medium showed an intermediate value of shoot induction (43%).

Regarding the NS/E, significant differences were found for the variables explant type and basal medium and the interaction between them (Figure 4, Supplementary Table S4). BT explants cultured in QL medium produced a significantly higher response than the other explant and medium combinations tested (Figure 4). Based on the results of Experiment 1, basal explants of >1.5 cm length and QL medium were selected to carry out the micropropagation process in Experiment 2.

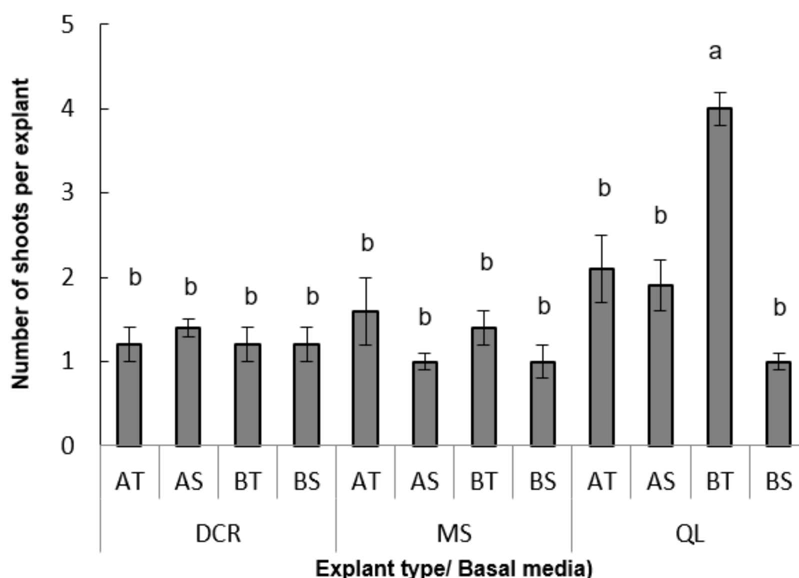

Figure 4. Number of shoots per explant in different explant types of *Cryptomeria japonica* cultured in DCR medium (Gupta and Durzan, 1985), MS medium (Murashige and Skoog, 1962) and QL medium (Quoirin and Lepoivre, 1977) supplemented with 6-benzyladenine (BA, 8.8 μM). Apical explants >1.5 cm (AT), apical explants <1.0 cm (AS), basal explants >1.5 cm (BT), and basal explants <1.0 cm (BS). Data are presented as mean values ± S.E. Significant differences are indicated by different letters according to Tukey's post hoc test ($p < 0.05$).

3.1.2. Root Induction and Acclimatization of Rooted Plants

When the effect of the sucrose concentration, the light treatment, or the interaction between them on the root induction (%) was analyzed, no statistically significant differences were observed (Supplementary Table S5). The root induction percentage ranged from 36% in shoots cultured in 3% sucrose concentration under

FL to 54% in shoots, independently of the sucrose concentration in the culture medium and exposure to red LEDs.

The root number was significantly affected by the light treatment applied in the rooting phase (Figure 5 and Supplementary Table S5). In this sense, explants exposed to red LEDs showed significantly higher NR/E (6.5 ± 0.5) than those under fluorescent light (2.7 ± 0.4) (Figure 5). The sucrose concentration and the interaction between sucrose concentration and light treatment did not show statistically significant differences (Supplementary Table S5). The NR/E ranged from 4.5 ± 0.6 for shoots grown with 1.5% sucrose to 5.4 ± 0.7 for shoots cultured in QL medium supplemented with 3% sucrose.

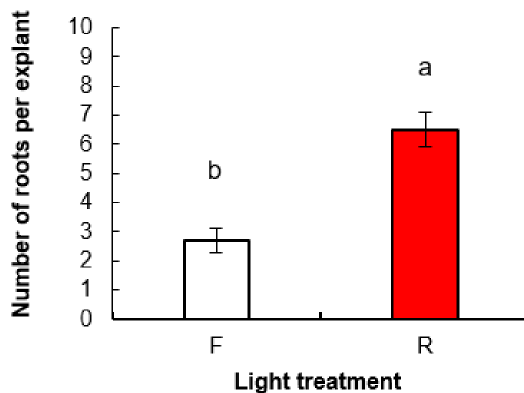

Figure 5. Number of roots per explant in shoots of *Cryptomeria japonica* cultured in QL medium (Quoirin and Lepoivre, 1977), supplemented with 3% (w/v) sucrose or 1.5% (w/v) sucrose under light treatments (fluorescent light (F) and red LEDs (R)). Data are presented as mean values ± S.E. Significant differences are indicated by different letters according to Tukey's post hoc test ($p < 0.05$).

The different sucrose concentrations (1.5% or 3.0%) and light treatments tested for shoot induction showed a statistically significant effect on LLR, whereas the

interaction between them did not have a significant effect (Figure 6 and Supplementary Table S5). A significantly higher LLR was observed in shoots cultured in the presence of 3% (w/v) sucrose compared with shoots grown at the lowest sucrose concentration (Figure 6a). In the same way, shoots exposed to fluorescent light showed significantly higher LLR than those under red LEDs (Figure 6b).

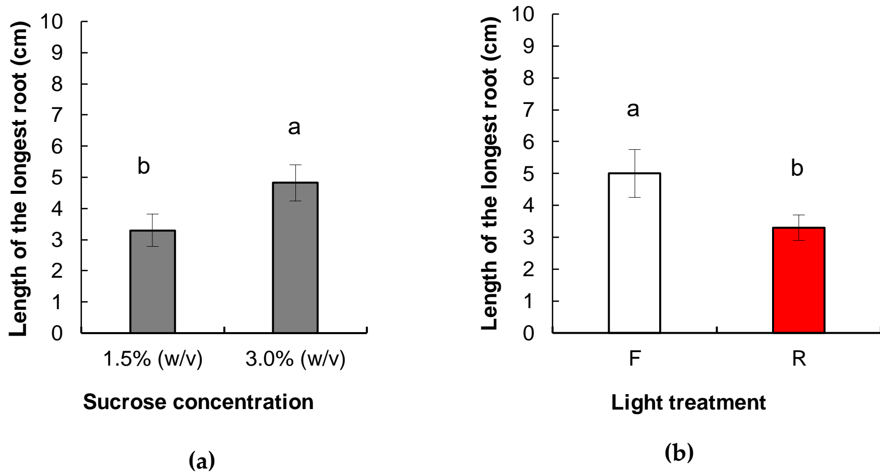

Figure 6. Length of the longest root in shoots of *Cryptomeria japonica* cultured in QL medium (Quoirin and Lepoivre, 1977), according to sucrose concentration (3% and 1.5% (w/v) (**a**) and light treatments (fluorescent light (F) and red LEDs (R)) (**b**). Data are presented as mean values ± S.E. Significant differences are indicated by different letters according to Tukey's post hoc test ($p < 0.05$).

When the effect of sucrose concentration and light treatment applied during the rooting phase on the acclimatization percentage of rooted shoots was analyzed, no statistically significant differences were observed (Supplementary Table S6). Statistically significant differences were found in the acclimatization percentage for the interaction between sucrose concentration and light treatment (Supplementary Table S6). Nevertheless, as the p-value was bordering on significance, Tukey's post

hoc test could not detect them. The acclimatization percentage ranged from 30% in shoots cultured in medium supplemented with 3% (w/v) and exposed to FL to 80% in those growing in the same sucrose concentration supplemented the culture medium under red LEDs.

3.2. Experiment 2

Four weeks after sterilization, the contamination rates were at 28%. No statistically significant differences were observed for the explant survival percentage considering the CK type tested (Supplementary Table S7). Explant survival percentages ranged from 66% in explants cultured in medium with m-T to 76% in explants grown with BA treatment.

The CK type showed a significant effect on the shoot induction (%) (Figure 7, Supplementary Table S8). A significantly higher shoot induction percentage was observed in explants induced with BA and m-T treatments (50% and 48%, respectively) than in explants grown on QL medium supplemented with TDZ.

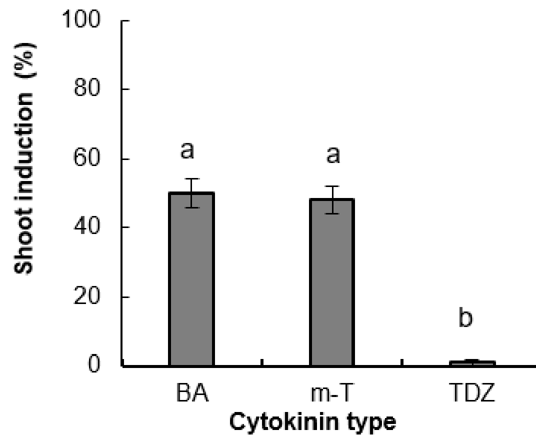

Figure 7. Shoot induction (%) in explants of *Cryptomeria japonica* cultured on QL medium (Quoirin and Lepoivre, 1977), supplemented with 6-benzyladenine (BA), meta-topolin (m-T), and thidiazuron (TDZ) (8.8 μM). Data are presented as mean values ± S.E. Significant differences are indicated by different letters according to Tukey's post hoc test ($p < 0.05$).

No statistically significant differences were observed when the effect of CK type on the NS/E was analyzed (Supplementary Table S8). It was not possible to obtain shoots from explants induced with TDZ treatment due to tissue necrosis. Explants induced with m-T and BA treatments produced 2.3 ± 0.1 and 2.4 ± 0.1 NS/E, respectively.

Regarding the root induction percentage, significant differences were observed depending on the CK used for shoot induction (Figure 8 and Supplementary Table S9). A significantly higher root induction percentage was recorded in shoots induced with m-T (54%) than in shoots grown with BA (33%) during the shoot induction stage (Figure 8).

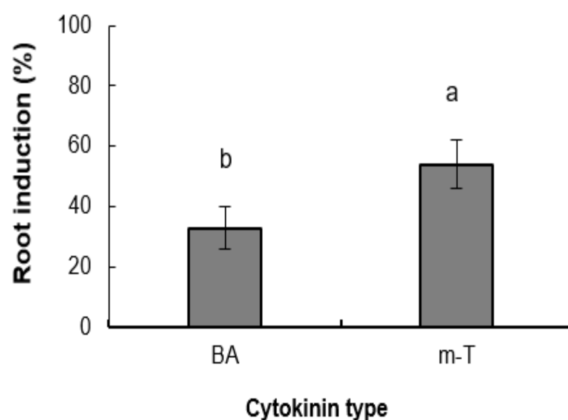

Figure 8. Root induction (%) in shoots of *Cryptomeria japonica* cultured in QL medium (Quoirin and Lepoivre, 1977), supplemented with 50 μM 1-naphthaleneacetic acid (NAA), according to cytokinin type (6-benzyladenine (BA) and meta-topolin (m-T) (8.8 μM) use during shoot induction stage. Data are presented as mean values ± S.E. Significant differences are indicated by different letters according to Tukey's post hoc test ($p < 0.05$).

When the effect of the CK type utilized for shoot induction was evaluated for the NR/E and LLR parameters, no statistically significant differences were observed (Supplementary Table S9). The NR/E was 3.1± 0.5 for shoots previously induced with BA and 3.9 ± 0.6 for those from m-T treatment.

Explants cultured with BA and m-T treatment during the induction stage showed a LLR of 3.3 cm ± 0.5 and 3.7 cm ± 0.3, respectively.

No statistically significant differences were observed for shoots coming from different CK treatments when acclimatization percentage was analyzed (Supplementary Table S10). The *ex vitro* survival rate was 92% for shoots induced with BA and 94% for those induced with m-T treatment.

4. DISCUSSION

As was reviewed in (Payghamzadeh and Kazemitabar, 2011), contamination is considered a crucial obstacle that prohibits the successful establishment of an aseptic *in vitro* culture. In this study, contamination rates ranging from 28% to 39% were obtained using sodium hypochlorite. In this sense, our work obtained lower contamination rates than those recorded by (Hine and Valverde, 2003) for *C. japonica*, who obtained 46% using calcium hypochlorite for surface sterilization of apical explants from adult trees. The sterilization protocol applied in Experiment 1 and Experiment 2 resulted in an optimal *in vitro* establishment of *C. japonica*. Therefore, our results suggest that it can be used for the establishment of cultures from other mature conifer explants (De Diego et al., 2010).

Factors such as the type, size, or age of the explant, the physiological state of the donor plant, and the type of disinfectant and its concentration can influence the effectiveness of the sterilization protocol (Da Silva el al., 2016). In our case, the survival percentage was significantly higher in AS explants in Experiment 1. A similar tendency was observed in *Cedar libani*, where shoot apices (<1.0 cm) from adult trees showed the best survival response (Renau-Morata et al., 2005). Similarly, in *Taxus mairei*, the highest survival percentage was obtained in small stems (<1.0 cm) from cutting (Chang et al., 2001). In our study, the smaller size and lower exposure of the explants' surface could have favored the culture establishment (Sathyagowri and Seran, 2011), as supported by (George and Debergh, 2008), who mentioned that smaller explants may be easier plant material to sterilize from microorganisms.

In our experiments, the efficiency of the organogenic response was assessed by considering the shoot induction percentage and the NS/E. In Experiment 1, the results showed statistically significant differences for AS and AT explants for shoot

induction percentage. In this sense, our study confirmed that the explant size is an important factor affecting axillary bud proliferation (Renau-Morata et al., 2005; George and Debergh, 2008), and probably, the larger explants have more mineral nutrient reserves and endogenous hormones to support the culture (Desai et al., 2015). Additionally, George and Debergh (2008) explained that bigger explants from extensive parts of the shoot apex or stem segments with lateral buds could have advantages over smaller explants. Furthermore, it is probable that the morphogenetic gradient, where variations in the levels of endogenous plant growth regulators such as auxins (from the basal to the apical explant region) (George and Debergh, 2008) are responsible for the different morphogenesis responses of the explants tested.

Cytokinins play several recognized roles in plant development, through the suppression of apical dominance and promotion of the development of axillary buds, the promotion of cell division, or the stimulation of plant protein synthesis (Arab et al., 2014; Rodríguez et al., 2022). BA is the most commonly used plant growth regulator; it is applied alone or in combination with other CK to promote *in vitro* shoot induction due to its effectiveness and affordability (Phillips and Garda 2019; De Diego et al., 2008; Bairu et al., 2007). During Experiment 2, explants cultured with BA or m-T showed an efficient organogenic response regarding shoot induction percentage, and although not significant, a slightly higher response in BT explants induced with BA was observed. Analogous results were obtained in several organogenesis protocols from adult trees of *Pinus pinea*, *P. radiata*, *Sequoia sempervirens*, and *P. halepensis*, where, to obtain an *in vitro* shoot response, BA at concentrations ranging from 4.4 to 50 μM was utilized (Cortizo et al., 2009; Montalbán et al., 2013; Rojas-Vargas et al., 2021; Pereira et al., 2021, Rojas-Vargas et al., 2022).

The highest NS/E was obtained in BT explants cultured in QL medium (Experiment 1). In accordance with this result, in previous experiments in our lab, the highest number of NS/E was obtained in *S. sempervirens* when explants bigger than 1.5 cm in length were used (Rojas-Vargas et al., 2021). Similarly, Hine and Valverde (2003) and Rafi and Salehi (2018) developed an *in vitro* protocol for *C. japonica* and *Cedrus deodara*, using explants of mature trees from 1.0 to 2.5 cm in length, respectively. On the other hand, NS/E was higher on basal QL medium than on basal MS and DCR medium. According to Maruyama et al., (2021), this may be attributable to a higher level of inorganic nitrogen present in those media when compared to QL medium. This hypothesis is supported by Tuskan et al., (1990) who explained that a nitrate excess could have a negative impact on the organogenic response. Similar to our experiment on *C. japonica*, the lower nitrogen content of QL medium promoted organogenesis in *Cedrus deodara* and *P. ponderosa* (Rafi and Salehi, 2018; Rojas-Vargas et al., 2023). Recently, for improving the micropropagation protocol of plants, artificial neural networks algorithms have been used to build models to determine the effect of mineral nutrients, vitamins, and plant growth regulators on several growth and quality parameters of micropropagated plants (Arteta et al., 2022).

Several studies attributed the improvement in multiplication rates or rooting percentages and the alleviation of physiological disorders to the use of topolins in plant tissue culture (Aremu et al., 2012). However, in our study, no significant differences for the effect of CK on NS/E were found, but a slightly higher response in explants cultured with BA was observed (Experiment 2). Analogous results were obtained in *P. radiata*, *S. sempervirens*, and *P. ponderosa*, where the induction of axillary buds was not improved when applying m-T instead of BA (Montalbán et al., 2011; Montalbán et al., 2013; Rojas-Vargas et al., 2021; Rojas-Vargas et al., 2023).

In recent years, TDZ has received more attention due to its ability to aid *in vitro* regeneration of woody plants (Cortizo et al., 2009; De Diego et al., 2010; Zarei et al., 2020), and it has been recommended in explants coming from adult trees to induce regeneration via axillary shoot proliferation and adventitious shoot organogenesis (Vinoth and Ravindhran, 2018). However, in our work, it was not possible to obtain shoots coming from explants induced with TDZ due to tissue necrosis. The reason for this result may be due to an inadequacies concentration of TDZ, as supported by (Dewir et al., 2018), who mentioned that the TDZ concentration and exposure time at their optimum depends on the species. Nevertheless, TDZ at low concentrations, in pulse treatments, or during short exposure periods can be effective in circumventing TDZ induced abnormalities such as tissue necrosis (Dewir et al., 2018).

Adventitious rooting can be influenced by physical and chemical factors, among them plant growth regulators, light quality, temperature, medium composition, and carbohydrates (Zarei et al., 2020). When root induction percentage was analyzed, no significant effect of sucrose concentration (1.5 or 3.0%) and light treatment was found. A similar result was observed in the organogenesis protocols of *C. japonica*, where the culture medium was supplemented with sucrose (from 1.5 to 3.0%) to obtain rooting (Hine and Valverde, 2003; Tsubomura and Taniguchi, 2008; Koguta et al., 2017).

As mentioned above, in the last few years, m-T has been proven as an alternative to conventional CK for *in vitro* propagation of plants (Gantait and Mitra, 2021). In this sense, the advantages of m-T to promote rhizogenesis have been described (Aremu et al., 2012). In Experiment 2, when we focused on the study of the effect of the cytokinin type on root induction, we found that shoots induced with m-T promoted the highest rooting percentage. Similar to our results, Naaz et al., (2019),

studying *Syzygium cumini*, reported that shoots induced with m-T increased rhizogenic competency compared with shoots coming from kinetin, 2-isopentyl adenine, or BA. Similarly, in *Caralluma umbellata*, shoots derived from a culture medium supplemented with m-T and NAA showed the highest *in vitro* rooting activity (Jayaprakash et al., 2023). In contrast to our work, in *S. sempervirens* and *Juniperus drupacea*, growing shoots with m-T did not improve the rooting response (Rojas-Vargas et al., 2021; Ioannidis et al., 2023). Summarizing, the effect of m-T to promote rhizogenesis at different concentrations may be species-specific and depends on the starting material used to establish the *in vitro* culture.

Plant growth and development are strongly influenced by the quality of the light in their environment (Sarropoulou et al., 2023). In the last few years, LEDs have shown a favorable response in *in vitro* culture when compared with the results obtained using fluorescent tubes (Chen et al., 2020; Gupta and Jatothu, 2013). Additionality, Ragonezi et al., (2010) mentioned that the light type and wavelength specificity influence adventitious rooting. In this sense, statistically significant differences in NR/E were found when shoots were cultured under red LEDs (Experiment 1). Similarly, in *C. japonica* and *Populus sieboldii* × *Populus grandidentata*, red LEDs showed a higher response to *in vitro* rooting (Tsubomura and Tanaguchi, 2008; Kobayashi et al., 2022). In *Pinus pseudostrobus* shoots exposed to red LEDs, the best rooting rates were observed at 30 days of evaluation (Marín-Martínez et al., 2022). Contrary to our results, *P. radiata* and *P. ponderosa* shoots grown under white fluorescent light and white LEDs, respectively, displayed the highest rooting responses (Rojas-Vargas et al., 2022; Rojas-Vargas et al., 2023). Regarding LLR, shoots under fluorescent light showed longer primary roots. Analogous results were observed by Ishii et al., (2011) in *C. japonica* and Rojas-Vargas et al., (2023) in *P. ponderosa,* where shoots growing under fluorescent light showed the longest root

length. Contrary to our result, the longest roots were recorded in plants of *P. radiata* and *P. pseudostrobus* exposed to red LEDs, (Marín-Martínez et al., 2022; Rojas-Vargas et al., 2022). Summarizing, different plant species respond differently, even when using the same light treatment, which may be due to the genotypic characteristics of the plants or the physiological state of the explants (Da Silva et al., 2016; Lai et al., 2022).

Regarding the acclimatization stage, no significant differences were observed for the effect of CK on acclimatization percentage, but a slightly higher response in explants cultured with m-T was observed. These results agreed with those observed in *Aloe polyphylla*, *S. cumini*, and *C. umbellate*, where the plantlets induced with m-T were successfully acclimatized (Bairu et al., 2007, Nazz et al., 2019, Jayaprakash et al., 2023). The reason for this may be due to the rapid uptake and transport of mT into the plant system and the production of reversibly sequestered metabolites (Jayaprakash et al., 2023).

5. CONCLUSIONS

The regeneration of *C. japonica* through micropropagation of adult trees using basal explants of >1.5 cm length was achieved and depended on physico-chemical factors. The optimal result in terms of shoot induction was obtained when basal explants were cultured in QL medium supplemented with BA treatment.

Our results suggest that the use of m-T and a 1.5% sucrose concentration favored root induction. In this sense, the use of red LEDs was better for the number of roots per explant. Finally, in the greenhouse, the shoots, independently of the cytokinins used in the shooting stage, showed high acclimatization success. In order to optimize the micropropagation efficiency in *C. japonica*, our results suggest the use of fluorescent light for the shoot induction stage and red LEDs for the rooting stage.

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CHAPTER 4

Testing Explant Sources, Culture Media, and Light Conditions for the Improvement of Organogenesis in *Pinus ponder*osa (P. Lawson and C. Lawson)

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ABSTRACT

Pinus ponderosa (P. Lawson and C. Lawson) is a commercial tree and one of the most important forest species in North America. Ponderosa pine suffers hardship when going through vegetative propagation and, in some cases, 15–30 years are needed to achieve full reproductive capacity. Based on previous works on *P. ponderosa* regeneration through *in vitro* organogenesis and trying to improve the published protocols, our objective was to analyze the influence of different types of explants, basal culture media, cytokinins, auxins, and light treatments on the success of shoot multiplication and rooting phases. Whole zygotic embryos and 44 μM 6-benzyladenine showed the best results in terms of explants survival. For shoot organogenesis, whole zygotic embryos and half LP (LP medium, Quoirin and Lepoivre, 1977, modified by Aitken-Christie et al. 1988) macronutrients were selected. A significant positive interaction between whole zygotic embryos and half LP macronutrients was found for the percentage of explants forming shoots. Regarding the light treatments applied, a significantly higher percentage of shoots elongated enough to be rooted was detected in shoots growing under blue LED at a light intensity of 61.09 μmol m^{-2} s^{-1}. However, the acclimatization percentage was higher in shoots previously cultivated under fluorescent light at a light intensity of 61.71 μmol m^{-2} s^{-1}. Anatomical studies using light microscopy and scanning electron microscopy showed the light treatments promoted differences in anatomical aspects in *in vitro* shoots; needles of plantlets exposed to red and blue LEDs revealed less stomata compared with needles from plantlets exposed to fluorescent light.

RESUMEN

Pinus ponderosa (P. Lawson y C. Lawson) es un árbol comercial y una de las especies forestales más importantes de América del Norte. El pino Ponderosa presenta dificultades en cuanto a propagación vegetativa y, en algunos casos, se necesitan entre 15 y 30 años para alcanzar la plena capacidad reproductiva. Basándonos en trabajos previos sobre regeneración de *P. ponderosa* a través de organogénesis *in vitro* y tratando de mejorar los protocolos publicados, nuestro objetivo fue analizar la influencia de diferentes tipos de explantos, medios de cultivo basal, citoquininas, auxinas y tratamientos de luz en el éxito de las fases de multiplicación y enraizamiento de brotes. Los embriones cigóticos completos y 44 µM de 6-benciladenina mostraron los mejores resultados en términos de supervivencia de los explantos. Para la organogénesis de los brotes, se seleccionaron embriones cigóticos completos y el medio de cultivo LP (medio LP, Quoirin y Lepoivre, 1977, modificado por Aitken-Christie et al. 1988) a la mitad de macronutrientes. Se encontró una interacción positiva significativa entre los embriones cigóticos completos y la mitad de los macronutrientes de LP para el porcentaje de explantos que forman brotes. Con respecto a los tratamientos de luz aplicados, se detectó un porcentaje significativamente mayor de brotes lo suficientemente alargados como para enraizarse, en brotes que crecieron bajo LED azul a una intensidad de luz de 61,09 µmol m^{-2} s^{-1}. Sin embargo, el porcentaje de aclimatación fue mayor en los brotes previamente cultivados bajo luz fluorescente a una intensidad lumínica de 61,71 µmol m^{-2} s−1. Los estudios anatómicos usando microscopía óptica y microscopía electrónica de barrido mostraron que los tratamientos con luz promovieron diferencias en aspectos anatómicos en brotes *in vitro*; las acículas de plántulas expuestas a LED rojos y azules revelaron menos estomas en comparación con las acículas de plántulas expuestas a luz fluorescente.

1. INTRODUCTION

Conifers cover approximately 39% of the world forests and are the best known and most economically important species among gymnosperms; with a total of around 615 species, including pines (*Pinus* spp.) (Armenise et al., 2012; Farjon, 2018).

Ponderosa pine (Western Yellow Pine) is the largest of the western pine species and is a major lumber tree in the Western North America; its natural range includes every state west of the Great Plains and north into Western Canada and south into Mexico (Ellis and Bilderback, 1991; Raish et al., 1997). In the West of the United States, it is considered one the most fire-resistant conifers. This species develops a protective outer corky bark early in life and presents long needles with high moisture content surrounding the terminal buds, which helps to protect the apical meristems, allowing branch tips to refoliate (Hall, 1980; Miller, 2000; Fitzgerald, 2005).

The species is a major source of timber; ponderosa pine forests are also important as wildlife habitats, for recreational use, and for esthetic values (Ellis and Bilderback, 1991; Raish et al., 1997). However, climate change conditions can provoke problems because this species is susceptible to diseases such as Diplodia tip blight (*Diplodia pinea*), Dothistroma blight (*Dothistroma pini*), and Elytroderma needle blight (*Elytroderma deformans*); although some authors consider ponderosa pine to be relatively disease resistant (Ellis and Bilderback, 1991; Ellis and Bilderback, 1984).

The time taken for conifers to reach sexual maturity is long, often 20–30 years; therefore, considerable time is required before superior offspring appear (Ellis and Bilderback, 1991; Ellis and Bilderback, 1984). Grafting, rooting cuttings, and tissue culture are vegetative propagation methods used to improve this process (Ellis and

Bilderback, 1991). In this sense, *in vitro* methods, including somatic embryogenesis or shoot organogenesis followed by rooting, provide valuable tools that can be used for the propagation of ponderosa pine (Phillips, 2004). Quite often, *in vitro* culture has low success rates of propagation due to the multitude of physico-chemical conditions influencing plant growth. Factors, such as the type and concentration of plant growth regulators, basal media, solidifying agents, type of explant, among others, can be manipulated to induce or optimize the whole process (Phillips, 2004; Kulus and Woźny, 2020).

Among these physico-chemical factors, light is one of the most important factors controlling plant growth and development (Alallaq et al., 2020). In general, fluorescent light (FL) has been used as the main source of lighting, with irradiances between 25 and 150 mmol m^{-2} s^{-1} for a 16 h photoperiod (Larraburu et al., 2018). However, the power consumption of this kind of lamp is high, making the process expensive, and culture rooms need to remove the heat emitted by them using air conditioners together with FL efficiency (Kulus and Woźny, 2020). For these reasons, the use of light emitting diodes (LEDs) is recommended as they present advantages over FL, such as durability, low power consumption, the possibility to fix specific wavelengths, smaller size, and negligible heat production (Fang and Jao, 2000).

Previous works dealing with the micropropagation *P. ponderosa* are scarce. Ellis and Biwerback (1984) studied multiple bud formations culturing zygotic embryos. Years later, Ellis and Judd (1987) analyzed protein profiles on bud-forming cotyledons. In 1990, Tuskan et al., (1990) investigated the influence of plant growth regulators, basal media and carbohydrate levels. Other authors investigated *in vitro* formation of axillary buds from immature shoots (Lin et al., 1991), and the influence of different culture media on bud induction in whole embryos and

cotyledons (Ellis and Bilderback, 1991). Finally, the effect of cytokinins on plastid development and photosynthetic polypeptides during organogenesis has also been investigated (Mazari and Camm, 1993).

In our lab, several studies have been developed to optimize somatic embryogenesis (Castander-Olarieta et al., 2022a) and organogenesis (Moncaleán et al., 2005; Montalbán et al., 2011) in many species of *Pinus*. Moreover, as a result of priming cells during the somatic embryogenesis process in conifers, different metabolic, epigenetic, and proteomic profiles related to abiotic stress tolerance have been described recently (Pereira et al., 2021a; Castander-Olarieta et al., 2022b; Do Nascimento et al., 2022). However, as far as we know, no work has been done in order to improve the micropropagation of *P. ponderosa* by testing the chemical composition of the culture media or physical factors, such as type of lights (fluorescent versus LEDs) and their effect on morphogenesis, tissue micromorphology, and the overall success of the process.

Considering the abovementioned information, the main objective of our study was to optimize the organogenesis process of *P. ponderosa* in order to develop an efficient protocol for *in vitro* propagation. To carry out this objective, we focused on improving (1) the multiplication phase using different types of explants, cytokinins, culture media, and light treatments, and (2) the rooting stage using different auxins and light treatments.

2. RESULTS

2.1. Induction of Organogenesis

2.1.1. Experiment 1

Contamination rates were registered after sterilization protocols A (21%) and B (16%). The sterilization protocol did not significantly affect the survival percentage.

Based on this result, protocol B was selected as the preferred sterilization method in Experiment 2.

When the type of initial explant was analyzed, a significantly higher survival percentage was observed when using whole zygotic embryos (89%) instead of isolated cotyledons (77%).

Explants' survival was also significantly affected by the 6-benzyladenine (BA) concentration used (Supplementary Tables S1 and S2); explants cultured in 44 µM BA showed significantly higher survival rates (86%) than those induced at 4.4 µM BA (Figure 1).

The interaction between BA concentration and culture medium, and between explant type and sterilization protocol did not reveal statistically significant differences (Supplementary Tables S1 and S2).

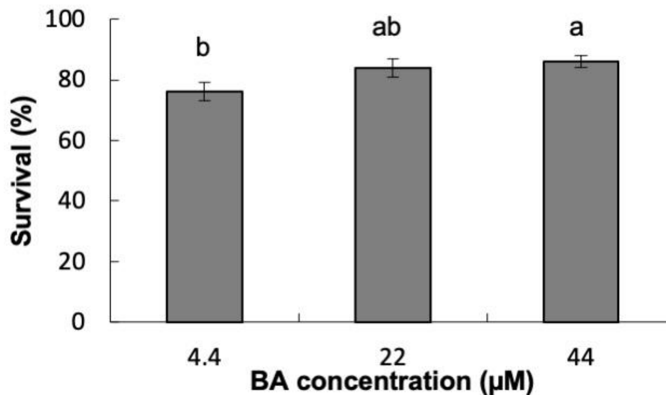

Figure 1. Percentage of survival of *Pinus ponderosa* explants cultured in LP or half LP macronutrients (Quoirin and Lepoivre (1977), modified by Aitken-Christie et al., (1988)), supplemented with 6-benzyladenine. Data are presented as mean values ± S.E. Different letters indicate significant differences according to Tukey's post hoc test ($p < 0.05$).

When considering all the variables analyzed (sterilization protocol, explant type, culture medium, and BA concentration) and the interaction between them, after four weeks of induction medium (IM), statistically significant differences were only found for the percentage of explants forming shoots (EFS) depending on the culture medium, the explant type and the interaction between them (Supplementary Table S3). Whole zygotic embryos cultured in half LP macronutrients (HLP) showed significantly higher EFS than both the embryos cultured in LP and cotyledons, regardless of the culture medium employed (Figure 2). When we used cotyledon as initial explants, buds developed slowly and failed to elongate. Based on these results, whole zygotic embryos and HLP were selected as the best conditions for shoot organogenesis in Experiment 2.

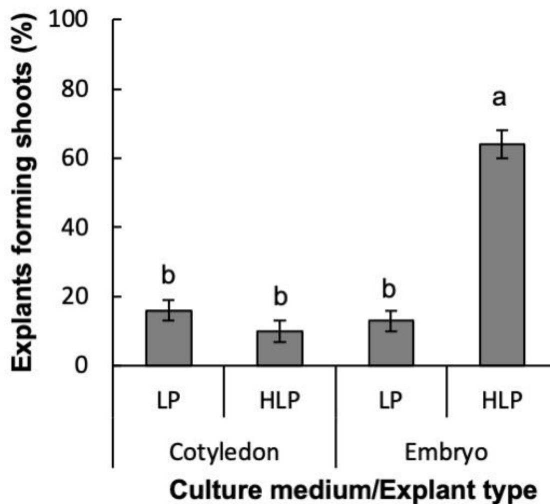

Figure 2. Percentage of explants forming shoots of *Pinus ponderosa* cotyledons or whole zygotic embryos cultured in LP or half LP macronutrients (HLP). Data are presented as mean values ± S.E. Different letters indicate significant differences according to Tukey's post hoc test ($p < 0.05$).

The highest number of shoots (3.31 ± 2.8) were found in whole zygotic embryos cultured in HLP supplemented with 22 µM BA.

2.1.2. Experiment 2

In this experiment, the contamination rate registered was 1%. As shown in Supplementary Table S4, statistically significant differences were found for the survival percentage according to cytokinin type and light treatment. However, Tukey's post hoc test did not detect these differences because the p-value was bordering on significance. The survival percentage was 93% in whole zygotic embryos cultured with meta-Topolin (m-T) in the culture medium and growing under blue LEDs. On the other hand, 100% survival was found in whole zygotic embryos growing under the remaining treatments tested.

After five weeks of culture in the induction medium (IM), no significant differences were found for the percentage of EFS according to the cytokinin type, the light treatment, or the interaction between them (Supplementary Table S5). The EFS ranged from 43% in whole zygotic embryos induced with m-T under white LEDs to 60% in whole zygotic embryos cultured with BA under blue and red LEDs, and whole zygotic embryos induced with m-T under FL.

Regarding the number of shoots per explant (NS/E), no significant differences were observed for the variables studied (cytokinin type and light treatment), or the interaction between them (Supplementary Table S6). The NS/E produced ranged from 5.40 ± 1.07 in whole zygotic embryos cultured with 13.1 µM m-T under FL to 9.11 ± 1.55 in whole zygotic embryos induced with 13.1 µM BA and exposed to red LEDs. However, at the end of the elongation stage of shoot organogenesis, a significantly higher percentage of shoots elongated enough to be rooted (PSR) was obtained in explants developed under blue LEDs (48%) than in those exposed to

red LEDs (16%); explants grown under white LEDs or FL showed intermediate values (Figure 3).

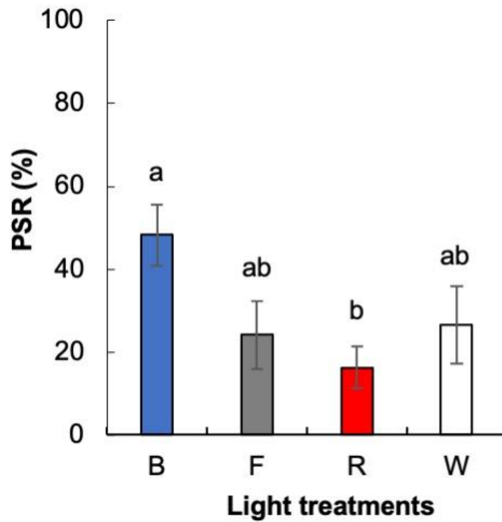

Figure 3. Percentage of shoots elongated enough to be rooted (PSR) of *Pinus ponderosa* growing on half LP macronutrients under light treatments (blue LEDs (B), fluorescent light (F), red LEDs (R), and white LEDs (W)). Data are presented as mean values ± S.E. Different letters indicate significant differences according to Tukey's post hoc test ($p < 0.05$).

On the other hand, the cytokinin type and the interaction between cytokinin type and light treatment did not have a significant effect on the PSR (Supplementary Table S7).

No statistically significant differences were observed in shoots exposed to different light treatments for root induction percentage (RI), number of roots per explant (NR/E), or the length of the longest root (LLR) (Supplementary Table S8). The RI percentage ranged from 3% in shoots cultured with 5 μM 1-naphthaleneacetic acid (NAA) to 18% in shoots grown with a mixture of 5 μM NAA and 5 μM indole-3-

butyric acid (IBA). Shoots induced with 10 μM NAA showed an intermediate value of RI (12%).

The highest response for root induction was obtained in shoots exposed to white LEDs and the lowest response was recorded in shoots under blue LEDs (Table 1). When shoots were exposed to white LEDs, the highest NR/E was obtained, and the lowest response was found in shoots under red LEDs. The longest primary roots (ranging from 2.78 to 2.15) were recorded in shoots exposed to white LEDs and FL, respectively. Shoots growing under red LEDs displayed the lowest response (Table 1).

Table 1. Root induction (%), number of roots per explant, and length of the longest root of *Pinus ponderosa* shoots cultured in half LP macronutrients according to light treatment (blue LEDs, red LEDs, white LEDs, and fluorescent light). Data are presented as mean values ± S.E.

Light Treatment	Root Induction (%)	Number of Roots per Explant	Length of the Longest Root (cm)
Blue LEDs	7 ± 30	1.71 ± 0.29	1.60 ± 0.77
Red LEDs	8 ± 40	1.25 ± 0.25	0.98 ± 0.34
White LEDs	19 ± 80	2.40 ± 0.87	2.78 ± 1.64
Fluorescent	8 ± 40	2.00 ± 0.71	2.15 ± 1.35

Acclimatized shoots were successfully obtained from shoots exposed to FL (50%), shoots under white LED (20%), and shoots exposed to blue LEDs (14%); it was not possible to acclimatize shoots grown under red LED.

2.2. Morphological Characterization of Needles

The effect of the chemical composition of the culture media and the physical factors on tissue micromorphology from needles of *in vitro* plantlets was studied, and then it was compared to the micromorphology from needles of *ex vitro* plants. In this way, adaxial and cross sections of *P. ponderosa* needles from plantlets cultured with different cytokinin types and light treatment were analyzed. Regardless of the cytokinin type employed, needles from plantlets did not show microscopic alterations in the internal structure and organization.

Adaxial needle surfaces from *in vitro* plantlets indicated the presence of stomata arranged in parallel and uniseriate bands, while the margin areas were serrated with spinose teeth (Figure 4a).

The longitudinal section of needles showed the parenchyma mesophyll cells and the large intercellular spaces between them. No structural changes in the mesophyll cell of needles from plantlets exposed to light treatments were found (Figure 4b). Furthermore, in the center of the needle cross section, differentiated vascular bundles with phloem and xylem were identified (Figure 4c).

Histological analysis of needles from *in vitro* plantlets showed that the epidermis presented a single-cell layer and numerous tannin-rich cells were spread among the mesophyll, where large intercellular spaces were common (Figure 4d). No clear differentiation of the endodermis and the vascular bundle was observed (Figure 4d).

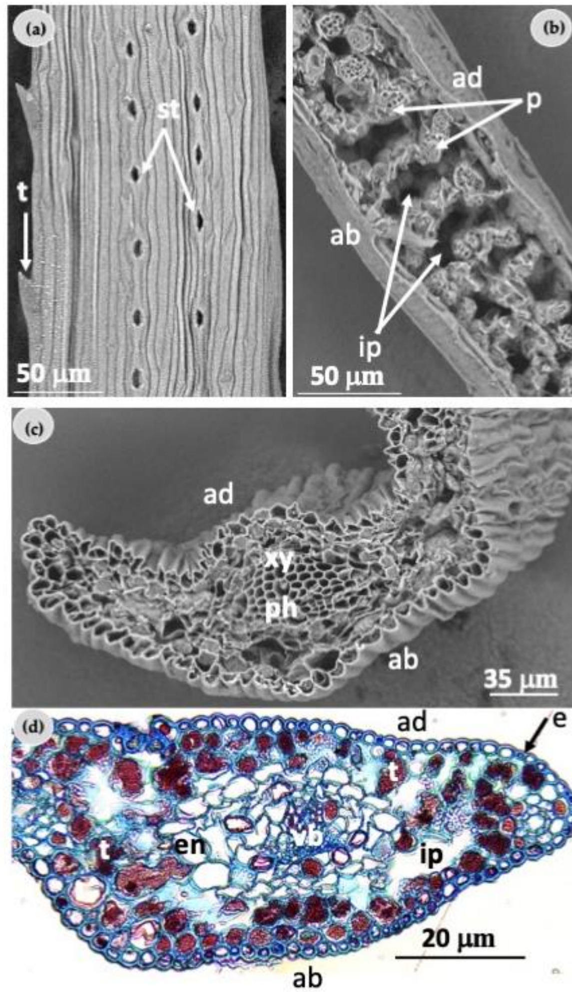

Figure 4. Scanning electron microscopy and histological observations of needles from *in vitro Pinus ponderosa* plantlets: (**a**) adaxial surface of a needle showing teeth (t) and two rows of stomata (st); (**b**) longitudinal section of a needle showing the parenchyma mesophyll cells (p) and the large intercellular spaces (ip) between them; (**c**) SEM observation of a needle cross section; (**d**) histological observation of needle cross section. There is poor differentiation of the vascular bundle (vb), phloem (ph), and xylem (xy) as well as of the endodermis (en). Tannin-rich cells (t) are spread among the mesophyll and large intercellular spaces (ip) are common. ab—abaxial surface; ad—adaxial surface; e—epidermis.

As a general trend, needles of plantlets exposed to blue and red LEDs showed less stomata compared with the stomata of needles from plantlets grown under FL (Figure 5a,c,h). In terms of arrangement and number of stomata, when the adaxial surface of a needle growing under FL was analyzed, a regular pattern in the needle was found (Figure 5h), such as mentioned above (Figure 4a).
The morphology of the stomata of needles from shoots grown under white LEDs (Figure 5e) was similar in the pattern of arrangement to those coming from needles of shoots grown under FL (Figure 5h). Regarding their shape, stomata were ovoidal in all light conditions. The scanning electron micrograph of adaxial needle surfaces showed spinose teeth at the margins (Figure 5a,c,e,f,h). No microscopic alterations in the morphological characterization of the spinose teeth of needles from plantlets grown under different light treatments were observed (Figure 5a,c,e,h).
The internal structure and organization of needles from plantlets was similar for all light treatments (Figure 5b,d,g,i).

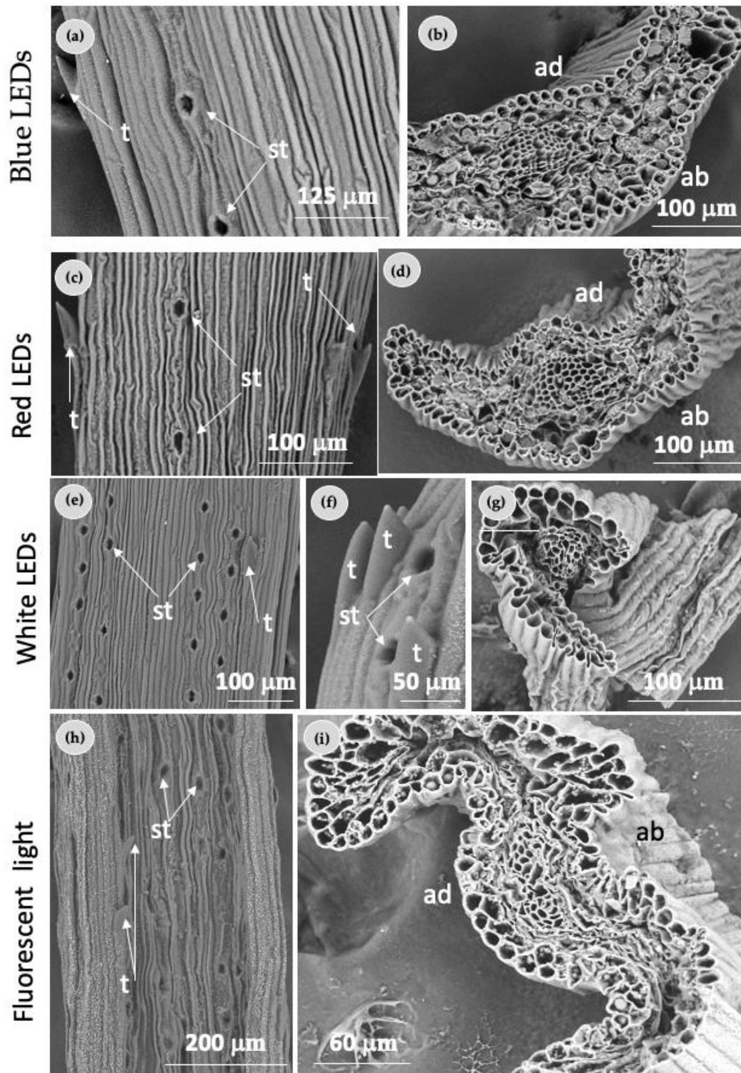

Figure 5. Scanning electron microscopy observations of needles from *in vitro Pinus ponderosa* plantlets exposed to different light treatments: (**a**) adaxial surface of a needle showing teeth (t) and two rows of stomata (st); (**b**) needle cross section; (**c**) adaxial surface of a needle showing teeth (t) and two rows of stomata (st); (**d**) needle cross section; (**e**) adaxial surface of a needle showing teeth (t) and two rows of stomata; (**f**) close view of the stomata. A tooth can also be observed; (**g**) needle cross section; (**h**) adaxial surface of a needle showing teeth (t) and two rows of stomata (st); (**i**) needle cross section. ab—abaxial surface; ad—adaxial surface.

SEM analysis showed similar anatomical aspects when needles from field-growing plants were compared with needles of in vitro plantlets (Figure 6a). The characterization of needles from field-growing plants revealed that the epidermal cells were arranged in a tightly packed single layer and the presence of a thin layer of cuticle on their outer surfaces was also observed (Figure 6a).

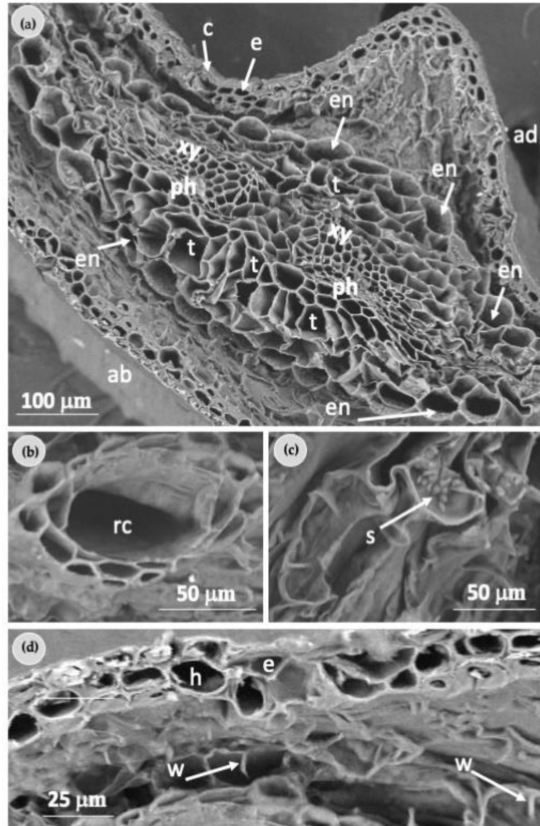

Figure 6. Scanning electron microscopy details of the organization of *Pinus ponderosa* needle from field-growing plants: (**a**) middle zone showing the organization of the vascular and surrounding tissues; (**b**) higher magnification of a resin duct (rc); (**c**) starch grains (s) in the parenchyma cells (transfusion tissue) surrounding the vascular bundles; (**d**) ridge-like invaginations (w) typical of the cell walls of the mesophyll parenchyma cells. ab—abaxial surface; ad—adaxial surface; c—cuticle; e—epidermis; en—endodermis; h—hypodermis; ph—phloem; rc—resin duct; t—transfusion tissue; xy—xylem.

The middle zone showed the organization of the vascular bundle and surrounding tissues; xylem, phloem, endodermis, and transfusion tissue were recognizable (Figure 6a).

The resin duct was generally localized adjacent to the epidermis and partially immersed in the mesophyll cells (Figure 6b). The presence of starch grains in the parenchyma cells (transfusion tissue) surrounding the vascular bundles was also distinguished (Figure 6c). The cell's morphology revealed the presence of ridge-like invaginations typical of the cell walls of the mesophyll parenchyma cells and the hypodermis was also identified (Figure 6d).

3. DISCUSSION

The establishment of an aseptic *in vitro* culture is one of the most important factors of plant tissue culture and the effective elimination of contamination contributes to the better development of the explants (Payghamzadeh and Kazemitabar, 2011; Pereira et al., 2021b). When introducing *P. ponderosa* material *in vitro*, contamination rates ranging from 1% to 16% were obtained using hydrogen peroxide (protocol B). In *P. radiata* (Montalbán et al., 2011, 2012), hydrogen peroxide was an effective agent for surface sterilization of seeds, obtaining contamination percentages below 7%. Our results suggest that the protocol used in this work was adequate for this species and can be used routinely, being a valid procedure for the propagation of other *Pinus* species as well. The most important initial step in the micropropagation process is an adequate sterilization protocol that minimizes tissue damage to achieve the greatest number of aseptic explants ready to be used in the next phases of micropropagation (De Souza et al., 2017; Pereira et al., 2021b).

As has been recently reviewed, factors such as explant source and plant growth regulators influence the *in vitro* regeneration of plants via organogenesis in different species (Parzymies, 2021; Tung et al., 2021; Rojas-Vargas et al., 2021). In this regard, other authors mentioned that cotyledon explants had several advantages over embryonic explants (Aitken et al., 1981; Saborio et al., 1997). First, when using cotyledons, the explant area that is exposed to the culture medium is bigger than when culturing whole zygotic embryos. Second, most seeds have at least eight cotyledons, which could be distributed among the various treatments (Aitken et al., 1981; Saborio et al., 1997). On the contrary, in *P. halepensis* Mill. when using whole embryos or cotyledons as initial explants, over 90% of the embryos gave rise to adventitious buds, whereas isolated cotyledons slowly developed adventitious buds and these buds failed to elongate (Lambardi et al., 1993). These results are in accordance with our study. When we used cotyledon as initial explants, buds developed slowly and failed to elongate. Additionally, De Diego et al., (2011) selected embryos as initial explants for *P. pinaster* and mentioned that this would increase the genetic diversity as well as considerably decrease the manual labor cost.

In our study, the capacity of explants to form shoots was significantly higher when whole zygotic embryos were cultured in HLP. Following the trend observed in previous studies (Ellis and Bilderback, 1984; Ellis and Judd, 1987; Ellis and Bilderback, 1991), significant differences in shoot induction rates were found when different culture media were tested for induction in *P. ponderosa*. Furthermore, several studies in *P. halepensis*, *P. pinaster*, *P. ayacahuite*, and *P. ponderosa* reported that salt concentration in the culture medium had an influence on morphogenesis (Ellis and Judd, 1987; Lambardi et al., 1993; Saborio et al., 1997; De Diego et al., 2011).

In woody species, it has been recognized that cytokinins are necessary to promote *in vitro* multiple shoot formation (Valverde-Cerdas et al., 2008; De Diego et al., 2011). Among cytokinins, BA is the most commonly used in plant tissue culture due to its effectiveness and affordability (Bairu et al., 2007).

In our experiments, BA at the concentrations tested (4.4, 22, and 44 μM) showed efficient organogenic response for NS, and, although not significant, a slightly higher response in whole zygotic embryos cultured in HLP supplemented with 22 μM BA was observed. A similar tendency was observed in several micropropagation protocols in *Pinus* where the culture medium was supplemented with BA (from 1 to 50 μM) to obtain bud induction (Sul and Korban, 2004; Moncaleán et al., 2005; Alonso et al., 2006; Montalbán et al., 2013; Pereira et al., 2021b; Rojas-Vargas et al., 2022).
When EFS and NSE were analyzed, no significant differences were found for the effect of BA or m-T, but a slightly higher response in explants cultured with BA was observed. BA in *in vitro* culture can cause hyperhydricity, shoot-tip necrosis, and histogenic instability (Chen et al., 2020). This was not the case in our work, where BA did not provoke *in vitro* abnormalities. In this sense, in the last years, many reports have described the use of topolins in plant tissue culture, because topolins improve parameters, such as multiplication rate, alleviating physiological disorders and increasing the rooting percentage (Aremu et al., 2012). In contrast to our study, in *Aloe polyphylla*, the use of m-T resulted in a high multiplication rate (Bairu et al., 2007). Likewise, Werbrouck et al., (1996) found better shoot–root balance when m-T was used in *Spathiphyllum floribundum*. In the same way, in *P. sylvestris* and *P. pinaster*, a higher rate of organogenic response was obtained by using m-T instead of BA (De Diego et al., 2010; De Diego et al., 2011). However, in other studies of our group, no improvement was found when using m-T instead of

BA for the induction of axillary shoots in *P. radiata* and *Sequoia sempervirens* (Montalbán et al., 2011, 2013; Rojas-Vargas et al., 2021). Summarizing, the effect of different cytokinins at different concentrations depends on the species and the initial explant used to initiate the *in vitro* culture.

The influence of light in regulating growth, development, and adventitious root formation has been confirmed in different studies (Chen et al., 2020; dos Santos et al., 2022). LED showed positive effects in *in vitro* plants responses, when used as an alternative to conventional lighting (Gupta and Jatothu, 2013; Chen et al., 2020). Several studies have indicated the stimulatory effect of red or blue LEDs on shoot organogenesis (Gupta and Agarwal, 2017). In this work, no statistically significant difference was found in EFS when whole zygotic embryos were cultured under different light treatments. In contrast to our work, in *Gerbera jamesonii in vitro* shoots growing under red LEDs displayed the greatest elongation rates (Pawłowska et al., 2018). Chen et al., (2020) in *Passiflora edulis* reported an increase in chlorophyll content and plantlet quality when plants were grown under red LEDs. Additionally, the highest stem length was observed in *in vitro* potato plantlets under red LEDs (Chen et al., 2020).

When studying the effect of the light treatment on PSR, shoots growing under blue LEDs showed the highest response. These results agree with those observed in *Curculigo orchioides* and *Zingiber officinale* where blue LEDs promoted *in vitro* shoot formation (Gupta and Sahoo, 2015; Gnasekaran et al., 2021). Furthermore, Chen et al., (2020) observed the greatest stem diameter and the highest health index in *Solanum tuberosum* when cultured under blue LEDs.

Studies performed in *Z. officinale* and *Phalaenopsis pulcherrima* indicated that LED treatments promoted *in vitro* root induction and development (Gnasekaran et al., 2021; Chashmi et al., 2022). In our experiments, when the LED was tested, no

significant differences were found for the RI, NR/E, and LLR, but a slightly higher response in shoots under white LEDs was found. In contrast, in *Z. officinale* and *Citronella mucronata* a higher rooting response was found when cultured under red or the combination of red and blue LEDs in a ratio of 1:2 (Gnasekaran et al., 2021; Guerra et al., 2022).

In *Cucumis metuliferus*, explants showed high root induction rates when exposed to white LEDs and in a mixed circuit of blue and red (Lai et al., 2022). Furthermore, *Cedrela odorata in vitro* cultures grown under white LEDs presented a higher number of roots formed (dos Santos et al., 2022). In this regard, Lai et al., explained that one reason for this result might be that white light provides with a wider variety of minor lights, which are also required by plants for optimal growth.

Contrary to our results, in *P. radiata*, *in vitro* shoots growing under FL showed high NR/E (Rojas-Vargas et al., 2022). Regarding LLR, shoots under white LEDs and FL light showed longer primary roots. A similar pattern was reported in *Handroanthus ochraceus* where plants showed higher root length values when exposed to low FL irradiances (Larraburu et al., 2018). In contrast, longer roots were obtained in plants of *P. radiata* and *Vitis ficifolia* growing under red LEDs (Poudel et al., 2008; Rojas-Vargas et al., 2022).
The light treatments tested promoted differences in anatomical aspects of the stomata in the needles of *P. ponderosa in vitro* shoots. The morpho-anatomic characteristics of needles from *in vitro* and *ex vitro* plants were similar and are in accordance with the study described for *P. halepensis* by (Boddi et al., 2002).

The teeth structures on the needle surface were morphologically similar to those found in primary needles of *P. halepensis* and *P. nigra* (Boddi et al., 2002; Mitić et al., 2017). In this sense, Boddi et al., (2002) described teeth or spine-like structures

in transverse sections as more elongated cells with cytoplasmic content differing from the adjacent epidermic cells for thicker walls; however, the relevance of these structures is unknown. A scanning electron micrograph revealed stomata arranged in parallel and as uniseriate bands, which is in accordance with the work described for *P. nigra* by Mitić et al., (2017). Similar to that observed in *P. halepensis*, (Boddi et al., 2002) large and ovoidal stomata were detected in this study. In contrast, stomata in primary needles of *P. canariensis* presented a volcanic shape (Stabentheiner et al., 2004).

SEM analysis revealed the presence of hypodermis in needles from field-growing plants. Contrary to our results, in *P. halepensis* seedlings, primary needles were characterized by the absence of a hypodermal cell layer and the epidermis being in direct contact with the mesophyll tissue, but these differences could be attributed to the fact that these analyses were performed in seedlings 22–24 weeks after emergence (Boddi et al., 2002).
LED sources improve the leaf anatomy of *in vitro* plantlets in Musa spp.; the abaxial epidermis was thinner than the adaxial epidermis (Vendrame et al., 2022). In *Chrysanthemun* leaves, the thickness of the adaxial epidermal cells increased under white LED; blue LED favored the anatomical development of the palisade parenchyma layer; and red LED reduced this parenchyma (Zheng and Van Labeke, 2018). Red LED also affected the epidermis morphology in *Solanum tuberosum* plantlets. Epidermises with greater thickness were induced and palisade parenchyma and spongy parenchyma were arranged neatly (Chen et al., 2020). However, LED illumination did not improve morpho-anatomic characteristics, including vascular bundles from *in vitro* cultures of *P. ponderosa*.

Silva et al., (2020), in *Pfaffia glomerata in vitro* plantlets, increased in the size of vascular bundles and vessel elements under combinations of red and blue LED. In

the same way, the central vascular bundle of plants from *Epidendrum fulgens* grown with natural ventilation under combinations of white/high blue, deep red/white/medium blue, and deep red/white/far red/medium blue LED had a higher content of sclerenchyma (Fritsche et al., 2022). Furthermore, Gnasekaran et al. (Gnasekaran et al., 2022) found that LED spectral quality alters plant chloroplast ultrastructure through the effects on starch accumulation in *Z. officinale*. Summarizing, the effect of different LEDs sources at different concentrations in morpho-anatomic characteristics seems to depend on the species cultured at *in vitro* conditions.

All these aspects could, to some extent, explain the results obtained in our study, where the basal media, cytokinins, the explant type, and the light quality influenced the organogenic process and root formation of *P. ponderosa* and their *ex vitro* acclimatization. Furthermore, our results demonstrated that different LEDs can be strategically used to improve micropropagation efficiency and reduce the costs of *in vitro* ponderosa pine plant production.

4. MATERIALS AND METHODS

4.1. Plant Material

P. ponderosa (P. Lawson and C. Lawson) seeds were obtained from 14-year-old trees grown in a clonal seed orchard of the Rotonda, Trevelin Experimental Field, (Argentina; 43°06′05″ N, 71°33′30″ W) (batch number: 25 U7519 JP) and they were also purchased from the Instituto Nacional de Tecnología Agropecuaria (Trevelin, Argentina).

4.2. Sterilization

Seeds were rinsed under running water for 5 min and sterilized following two different protocols: 1) commercial bleach 5% (active chlorine 37 gL^{-1} sodium hypochlorite) plus one drop of Tween 20® for 20 min, followed by three rinses in sterile distilled water for 5 min each; 2) 10% H_2O_2 (v/v) (30% active H_2O_2) plus one drop of Tween 20® for 20 min, followed by three rinses in sterile distilled water for 5 min each. The sterilization protocols were performed under sterile conditions in a laminar flow unit. Seeds were stored in moistened sterile filter paper at 4 °C in darkness. After four days, seed coats and megagametophytes were removed aseptically and two explant types (a) cotyledons (Figure 7a) or whole zygotic embryos (Figure 7b) were used.

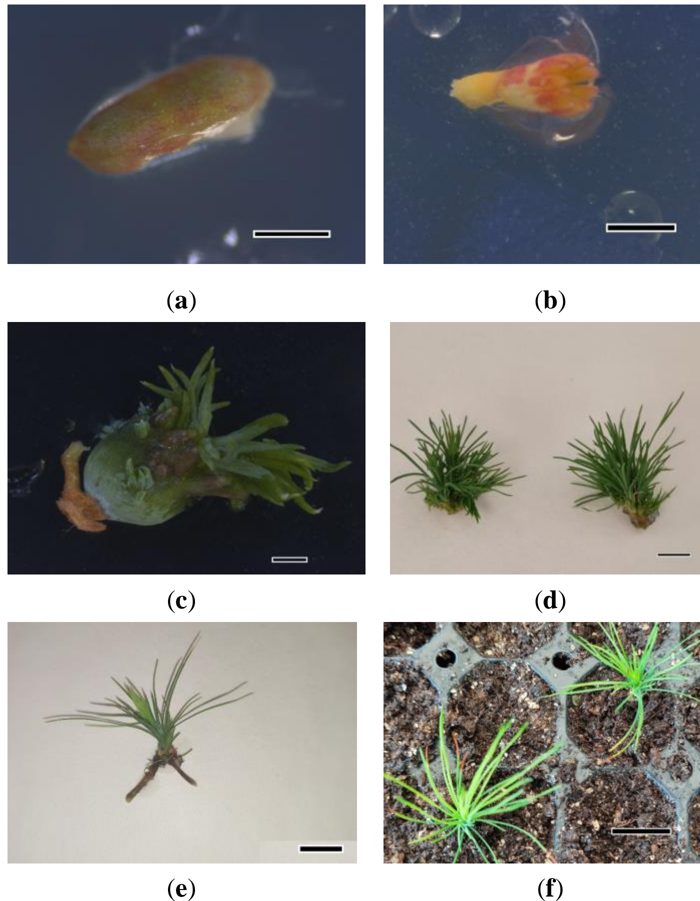

Figure 7. Plant material at different stages of *Pinus ponderosa* organogenic process: (**a**) cotyledon cultured for 2 weeks in LP (Quoirin and Lepoivre (1977) modified by Aitken-Christie et al. (1988) supplemented with 22 μM 6-benzyladenine (BA), bar = 1.5 mm; (**b**) whole zygotic embryo cultured for 1 week in LP supplemented with 22 μM BA, bar = 1.5 mm; (**c**) developing shoots from zygotic embryo cultured for 4 weeks in half LP macronutrients supplemented with 13.1 μM BA and exposed to red LEDs, bar = 3.0 mm; (**d**) shoots after 6 weeks in elongation medium, which consisted of hormone-free LP supplemented with 2 gL^{-1} activated charcoal, bar = 5.0 mm; (**e**) rooted shoots after 2 weeks in root induction medium, which consisted of half LP macronutrients supplemented with a mixture of 5 μM 1-naphthaleneacetic acid (NAA) and 5 μM indole-3-butyric acid (IBA), bar = 5.0 mm; (**f**) acclimatized shoots exposed to fluorescent light in *in vitro* conditions after 4 weeks in *ex vitro* conditions in the greenhouse, bar = 20 mm.

4.3. Organogenic Process

4.3.1. Experiment 1

After sterilization, explants (cotyledons or whole zygotic embryos) were cultivated on Petri dishes (90 × 15 mm) containing 20 mL of bud induction medium (IM) (Figure 7a,b). Two basal media were assayed: LP (Quoirin and Lepoivre (1977) modified by Aitken-Christie et al. (1988)) and HLP, which consisted of LP with half macronutrients. Both media were supplemented with 3% (w/v) sucrose and solidified with 8 gL^{-1} Difco Agar granulated. Moreover, three concentrations of BA (4.4, 22, and 44 μM) were evaluated. The pH of all media was adjusted to 5.8 before autoclaving (121 °C, 20 min). Cotyledons were excised from embryos and placed horizontally onto the induction medium; whole zygotic embryos were cultured in an inverted position with the cotyledons immersed in the induction medium.

As soon as bud induction was observed (after four weeks), the explants were transferred to Petri dishes containing 20 mL of elongation medium (EM). EM consisted of hormone-free LP or HLP supplemented with 2 gL^{-1} activated charcoal, 3% (w/v) sucrose, and was solidified with 8.5 gL^{-1} Difco Agar granulated. After 30 days in culture (when elongating needle fascicles were evident), explants were subcultured into baby food jars with Magenta TM b-cap lids containing 25 mL of LP or HLP elongation medium. The shoots were subcultured every six weeks into the same medium. When shoots were 10–15 mm long, they were separated and cultivated individually in a fresh medium.

All the cultures were laid on the growth chamber at a temperature of 21 ± 1 °C, at a 16 h photoperiod with 120 μmol m^{-2} s^{-1} light intensity provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France).

4.3.2. Experiment 2

Based on the results of Experiment 1, sterilization protocol B and whole zygotic embryos were used as initial explant and HLP was selected to carry out this experiment. Two types of cytokinins were tested: BA and m-T at the same concentration (13.1 μM). Three different light treatments were also tested for the conditions named above: A) blue light (peak wavelength 470 nm), 61.09 μmol m^{-2} s^{-1}; B) red Light (peak wavelength 630 nm), 61.09 μmol m^{-2} s^{-1}; and C) white light (color temperature 4000 K), 61.09 μmol m^{-2} s^{-1}, all of them provided by adjustable LEDs (RB4K Grow LEDs). As a control, whole zygotic embryos were cultured under cool white fluorescent light (FL) (TLD 58 W/33; color temperature 4100 K) at 61.71 μmol m^{-2} s^{-1} light intensity. The cultures were laid on the growth chamber at a temperature of 21 ± 1 °C for five weeks at a 16 h photoperiod.

As soon as bud induction was observed (after four weeks) (Figure 7c), the explants were transferred to Petri dishes with HLP elongation medium. After 30 days in culture (when elongating needle fascicles were evident), explants were subcultured into baby food jars with Magenta TM b-cap lids containing 25 mL of the same medium (Figure 7d). The shoots were subcultured every six weeks into the same medium. When shoots were 10–15 mm long, they were separated and cultivated individually. The different lighting conditions described previously were maintained in this phase.

4.3.3. Rooting and Adaptation

After the elongation phase, shoots of at least 20–25 mm long were used for root induction. The explants were cultivated into baby food jars with Magenta TM b-cap lids containing 25 mL of root induction medium, which consisted of HLP supplemented with three different auxins: A) 5 μMNAA; B) 10 μM NAA; and C) a

mixture of 5 μM NAA and 5 μM IBA; all media were supplemented with 3% (w/v) sucrose and solidified with 8.5 gL^{-1} Plant Agar®. The different light conditions were the same as described above. After four weeks of culture in medium A or two weeks in medium B and C, explants were cultured in baby food jars with Magenta TM b-cap lids containing 25 mL of root expression medium (REM), which consisted of hormone-free HLP supplemented with 2 gL^{-1} activated charcoal, 3% (w/v) sucrose, and 8.5 gL^{-1} Plant Agar® for six weeks. Growth chamber temperature and photoperiod were the same as those described above.

After six weeks in REM, explants with visible roots (Figure 7e) were transferred to wet peat moss (Pindstrup, Ryomgård, Denmark): vermiculite (8:2, v/v) and acclimatized in the greenhouse under controlled conditions at 21 ± 1 °C with progressively decreasing humidity for one month from 95% to 80% (Figure 7f). Prior to acclimatization, and following the procedure described in Castander et al., (2020), the plants that developed a poor root system were transferred to Ecoboxes (Eco2box/green filter: a polypropylene vessel with a "breathing" hermetic cover, Duchefa®, Duchefa Biochemie, Haarlem, Netherlands) containing perlite:peat (1:1, v/v) moistened with liquid HLP.

5. Morphological Characterization of Needles

5.1. Light Microscopy Analysis

For structural analysis after eighteen weeks in the elongation medium, 16 needles were used following the procedure described in Giacomolli et al., (2022). Two needles per cytokinin and light treatment were fixed in 0.2 M phosphate buffer (pH 7.2) and 2.5% paraformaldehyde for 48 h at 4 °C. Then, the samples were washed twice with 0.1 M phosphate buffer (pH 7.2) for 15 min and then dehydrated in ethanolic series (30%, 50%, 70%, 80%, 90%, 95%, and 100% v/v) (1 h each).

Subsequently, the samples were embedded in paraffin by means of dehydration with ethanol and Clear Rite™ and the consecutive immersion in liquid paraffin at 65 °C, as described in Rossi et al., (2006). The dehydration process was the following: ethanol 70% (v/v) for 120 min; ethanol 90% (v/v) for 90 min (2×); ethanol 95% (v/v) for 90 min; ethanol 100% (v/v) for 90 min (×2); ethanol: Clear Rite™ (1:1 v/v) and pure Clear Rite™ for 90 min (2×). Then, samples were embedded in paraffin wax at 65 ℃ for 120 min (2×) before the inclusion in paraffin molds. After paraffin inclusion (HistoDream EW, Milestone Medical Sorisole, Italy), sections of 8–10 μm were obtained in a rotary microtome (Microm HM 340E, Thermo Scientific, Waltham, USA) and transferred to microscope slides previously prepared with albumin glycerol and kept at 30 ℃ for 12 h. Next, the deparaffinization was performed with Clear Rite™ for 20 min, 100% ethanol for 4 min, and washing under running water. The samples were stained with an Astra Blue (0.15%) and Safranin (0.04%) water solution for 10 min; and finally, they were observed in an optical microscope (Leica DM 4000 B, Mannheim, Germany) and photos were taken with a Leica camera (Leica Application Suite version 4.13).

5.2. Scanning Electron Microscopy

For scanning electron microscopy (SEM), the analyses were performed following the procedure described in Giacomolli et al., (2022). Two needles per cytokinin and light treatment and two from adult trees were fixed in 0.1 M phosphate buffer (pH 7.2) and 2.5% paraformaldehyde for 24 h at 4 °C. After that, the samples were washed twice with 0.1 M phosphate buffer (pH 7.2) for 15 min and then dehydrated in a series of ethanol at different concentrations (30%, 50%, 70%, 80%, 90%, 95%, and 100% v/v) for 15 min each.

All the samples were cut into small pieces and prepared following the procedure described in Marques et al., (2021). Then, the samples were placed on carbon

stickers above metallic stubs, observed without further preparations in freeze conditions (−20 °C) at 10.0 kV, and were analyzed and photographed using a variable pressure scanning electron microscope (Flex SEM 1000, Hitachi, Tokyo, Japan).

6. Data Collection and Statistical Analysis

6.1. Experiment 1

Three Petri dishes and eight to ten explants per Petri dish (cotyledons or whole zygotic embryos) per sterilization protocol, were cultured in each culture medium and BA concentration. Contamination, survival, and explants forming shoots (EFS) percentages for each condition tested were measured after two months of culture. When the axillary shoots were isolated and cultured individually in an elongation medium, the EFS and the mean number of shoots were calculated with respect to the non-contaminated explants. A logistic regression model was used to analyze the effect of the sterilization protocol, explant type, culture medium, and BA concentration on survival and EFS (%). When necessary, Tukey's post hoc test (α = 0.05) was used for multiple comparisons.

6.2. Experiment 2

Five Petri dishes and eight whole zygotic embryos per Petri dish, cytokinin type, and light treatment were cultured in HLP. When the axillary shoots were isolated and cultured individually in the elongation medium, the EFS, the NS/E, and percentage of (PSR) out of the total number of shoots produced per explant were calculated. A logistic regression model was used to analyze the effect of the cytokinin type and light treatment on survival and EFS. When necessary, Tukey's post hoc test (α = 0.05) was used for multiple comparisons.

The confirmation of the homogeneity of variances and normality of the data on the NS/E and PSR were performed, and PSR was $\sqrt{x}$ transformed to meet homocedasticity. Data for the NS/E and PSR were analyzed by analysis of variance (ANOVA). When necessary, multiple comparisons were made using Tukey's post hoc test ($\alpha = 0.05$).

A completely randomized design was carried out using six to twenty plantlets per cytokinin type and light treatment per each auxin treatment. The RI percentage (RI), the mean NR/E, and the LLR (cm) were recorded after six weeks of culture in a root elongation medium.

To assess the effect of the cytokinin and light treatment on the RI, a logistic regression was performed. Data for NR/E and LLR were analyzed by ANOVA. When necessary, multiple comparisons were made using Tukey's post hoc test ($\alpha = 0.05$). The acclimatization percentage was calculated after four weeks under *ex vitro* conditions. The data were analyzed using R Core Team software® (version 4.2.1, Vienna, Austria).

7. CONCLUSIONS

The regeneration of *P. ponderosa* through organogenesis using whole zygotic embryos as explants was achieved. The best shoot induction percentage was obtained when whole zygotic embryos were cultured in half LP macronutrients. Following the novel use of LED lights for organogenesis, a higher number of rootable shoots were obtained when blue light was used. However, our results suggest that white LED could be more beneficial for rooting stages. Finally, in the greenhouse, the shoots cultured under fluorescent light showed the highest acclimatization success (50%). This may indicate that the use of different kinds of lights could be necessary during the process in order to optimize the organogenic

process in *P. ponderosa*. Concerning the anatomy studies, this was the first microscopy assessment of *in vitro* plantlets exposed to LEDs treatments. The needles from *in vitro* and *ex vitro* plants showed similar morpho-anatomic characteristics.

It would be interesting for future experiments to test other combinations and concentrations of auxins for rooting induction, and other LEDs, such as a combination of red and blue LEDs, to improve the rooting and acclimatization of shoots.

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CHAPTER 5

Optimization of the micropropagation of elite adult trees of *Sequoia sempervirens*: forest species of interest in the Basque Country, Spain

The content of this chapter corresponds to the published article "Rojas-Vargas, A.; Castander-Olarieta, A.; Montalbán, I.A.; Moncaleán, P. 2021. Optimization of the micropropagation of elite adult trees of *Sequoia sempervirens*: forest species of interest in the Basque Country, Spain. *Rev. Bionatura* 6, 1511–1519. https://doi.org/10.21931/RB/2021.01.01.11."

ABSTRACT

Forest trees are renewable sources of timber and other valuable non-timber products. Nowadays, the increase of population and demand of forestry products results in over exploitation of forestry. Therefore, there is an urgent need to produce elite plants with higher productivity under stress derived from climate change to have available to afforestation. For this reason, propagation methods should be improved in order to be more efficient in terms of quality and productivity.

The main species planted in the Basque Country is *Pinus radiata*; during the last three years *Pinus radiata* plantations have suffered a fungus attack affecting mainly needles until the death of the tree. This crisis is caused by the combined action of two fungi of the genus *Dothistroma* and *Lecanosticta acicola*, whose expansion seems to have been enhanced by weather-related factors, such as humid and hot summers. Although we have evidence of the presence of this disease in our mountains since 1942, for reasons that are not scientifically known today, the disease has had a very fast expansion with an aggressive effect. For the above, Basque Country forestry sector is looking for alternative species to be used in its plantations. Part of the forestry sector considers that *Sequoia sempervirens* could be a good choice for plantations. In addition, its high-quality wood and its tolerance to the attack of several pathogens and other diseases derived of climate change are characteristics that could confer some advantage over other forest species.

The main goal of this study was to optimize the micropropagation of adult elite trees of *Sequoia sempervirens*. To carry out this objective, the effect of 6-benzylaminopurine, meta-Topolin and kinetin, and 4 types of explant in the multiplication stage were analyzed. Furthermore, the effect of two types of auxins: 1-naphthalene acetic acid, indole-3-butyric acid and a mixture of both were evaluated on the induction of roots and their subsequent effect on the acclimatization process. The best multiplication index was obtained when 4.4 μM

6-benzylaminopurine and apical explants longer than 1.5 cm of length were used. The root induction percentage was 75% in the most responsive genotype analyzed when 4.4 μM 6-benzylaminopurine was used on induction stage and 50 μM of 1-naphthalene acetic acid was used for rooting. Finally, after 3 months in the greenhouse, the explants cultured with Kinetin and rooted in culture medium with indole-3-butyric acid showed highest acclimatization success (94%).

RESUMEN

Los árboles forestales son fuentes renovables de madera y otros productos no madereros valiosos. En la actualidad, el aumento de la población y la demanda de productos forestales se traduce en una sobreexplotación de los bosques. Por lo tanto, existe una necesidad urgente de producir plantas élite con mayor productividad bajo estrés derivado del cambio climático para tener disponibles para la reforestación. Por esta razón, se deben mejorar los métodos de propagación para que sean más eficientes en términos de calidad y productividad.

La principal especie plantada en el País Vasco es *Pinus radiata*; durante los últimos tres años las plantaciones de *Pinus radiata* han sufrido un ataque de hongos afectando principalmente a las acículas hasta la muerte del árbol. Esta crisis está provocada por la acción combinada de dos hongos del género *Dothistroma* y *Lecanosticta acicola*, cuya expansión parece haberse visto favorecida por factores relacionados con el clima, como veranos húmedos y calurosos. Aunque tenemos constancia de la presencia de esta enfermedad en nuestras montañas desde 1942, por razones que hoy no se conocen científicamente, la enfermedad ha tenido una expansión muy rápida con un efecto agresivo. Por lo anterior, el sector forestal del País Vasco está buscando especies alternativas para utilizar en sus plantaciones. Parte del sector forestal considera que *Sequoia sempervirens* podría ser una buena opción para las plantaciones. Además, su madera de alta calidad y su tolerancia al ataque de diversos patógenos y otras enfermedades derivadas del cambio climático son características que podrían conferirle alguna ventaja frente a otras especies forestales.

El objetivo principal de este estudio fue optimizar la micropropagación de árboles adultos de élite de *Sequoia sempervirens*. Para llevar a cabo este objetivo se analizó el efecto de 6-bencilaminopurina, meta-Topolin y kinetina, y 4 tipos de explantos en la etapa de multiplicación. Además, se evaluó el efecto de dos tipos de auxinas:

ácido 1-naftalenacético, ácido indol-3-butírico y una mezcla de ambos sobre la inducción de raíces y su posterior efecto sobre el proceso de aclimatación. El mejor índice de multiplicación se obtuvo cuando se utilizaron 6-bencilaminopurina 4,4 μM y explantos apicales de más de 1,5 cm de longitud. El porcentaje de inducción de raíces en el genotipo de mayor respuesta analizafo fue del 75% cuando se utilizó 6-bencilaminopurina 4.4 μM en la etapa de inducción y ácido 1-naftaleno acético 50 μM para el enraizamiento. Finalmente, después de 3 meses en invernadero, los explantos cultivados con Kinetina y enraizados en medio de cultivo con ácido indol-3-butírico mostraron el mayor éxito de aclimatación (94%).

1. INTRODUCTION

The Coast redwood or California redwood *Sequoia sempervirens* (D. Don.) Endl. (Taxodiaceae) is a valuable forest species and occurs naturally in Western North America, especially in California (Mihaljević et al., 1999; Meneguzzi et al., 2019).This species is the tallest tree on earth with a high volume of standing biomass, in some stands exceeding 3500 metric tons/hectare (Taha et al., 2014).

This conifer has been introduced and domesticated in European countries such as: Romania, Spain, France, Great Britain, Russia and Turkey, and it can be used for reforesting due to the quality of its wood as well as for ornamental purposes. In Spain, this species has been used by foresters for its productivity, its tolerance to the attack of several pathogens and other diseases. Moreover, its reforestation is recommended on valley bottoms and fotthills (Otazua, 2016).

Nowadays, redwood shows seed reproduction difficulties, displaying low germination and rooting rates, dormancy of the shoot and low seedling viability (Burns et al., 1990; Ozudogru et al., 2011). Therefore, biotechnological *in vitro* techniques of plant tissue culture as micropropagation, organogenesis, adventitious or axillary shoot/bud regeneration, shoot tip culture, micrografting and somatic embryogenesis, emerged as useful tools for the propagation and conservation of germoplasm (Vengadesan et al., 2002; Bonga, 2016; De Souza et al., 2017).

In the specific case of *Sequoia sempervirens*, there are few investigations on micropropagation, either through organogenesis or somatic embryogenesis. Arnaud et al., (1993) developed a protocol for micropropagation and rejuvenation of the species using direct organogenesis and somatic embryogenesis. Years later, Mihaljević et al., (1999) investigated the root formation in micropropagated shoots of *Sequoia sempervirens* using *Agrobacterium*. On the 21st century, Korban and Soul

(1994) developed a procedure for the micropropagation of juvenile and adult material and Lui et al., (2006) worked on the shoot regeneration and somatic embryogenesis from needles of redwood. The medium-term conservation of *Sequoia sempervirens* was investigated by Ozudogru et al., (2011), and Meneguzzi et al., (2019) evaluated shoot multiplication of two *Sequoia sempervirens* genotypes with addition of kinetin.

Taking into account all the above mentioned information, the objective of our study was to optimize the micropropagation process of elite selected adult trees of *Sequoia sempervirens*. We focused on improving 1) the multiplication phase using different types of explants and cytokinins 2) the rooting using different auxins and the subsequent acclimatization phase under *ex vitro* conditions.

2. MATERIALS AND METHODS

2.1. Plant material

Stem sections were collected from the basal parts of three different mother trees (7, 11 and 12) of *Sequoia sempervirens* located in Ataun, Gipuzkoa, Basque Country (Spain), at the geographic coordinates: 42° 58′ 41″ N and 2° 10′ 53″ O.

2.2. Shoot induction

In vitro microshoots growing in half strength ARN medium (Arnaud et al., 1993) were selected to carry out the experiments. Four types of explants were used: A) apical explants of 1.5 cm in length (AG), B) apical explants of 1.0 cm length (AP), C) basal explants of 1.5 cm length (BG) and D) basal explants of 1.0 cm in length (BP). Explants were cultured in glass jars (Merck) with 25 mL of ARN multiplication medium supplemented with 3% sucrose and 8 gL^{-1} Difco Agar®. The effect of different cytokinins for the induction of shoots was evaluated: A) 6-

benzylaminopurine (BAP), B) meta-Topolin (m-T) and D) kinetin (K) at a concentration of 4.4 μM. The pH of the medium was adjusted to 5.8 before autoclaving (121°C, 20 min). The explants were cultivated in the different induction media for four weeks in the growth chamber at a temperature of 21 ± 1°C, under 16-h photoperiod with 120 μmol m^{-2} s^{-1} of light intensity provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France).

2.3. Shoot elongation

The explants induced in the multiplication phase were cultivated for six weeks in shoot elongation medium. This medium was half strength ARN (Arnaud et al., 1993) supplemented with 2 gL^{-1} activated charcoal and solidified with 9 gL^{-1} Difco Agar®. The pH of the medium was adjusted to 5.8 before autoclaving (121°C, 20 min).

2.4. Root induction and acclimatization of plants

After the elongation phase, stems with at least 2.5 cm in length were used for root induction. Traceability of the explant was maintained according to its origin (genotype and cytokinin treatment used along the shoot induction stage). Subsequently, the stems were grown in root inducing medium (RIM), which consisted of 1/3 strength ARN basal medium supplemented with three different auxin treatments: A) 50 μM 1-naphthalene acetic acid (NAA), B) 50 μM indole-3-butyric acid (IBA) and C) a mixture of 40 μM NAA μM + 10 μM IBA. All the different media were solidified with 9 gL^{-1} Difco Agar®. The pH of the culture medium was adjusted to 5.8 before autoclaving (121°C, 20 min).

The stems were placed in the dark at 21 ± 1°C for 8 days. After this period, the stems were cultured in the root expression medium (REM), which consisted of 1/3 strength ARN basal medium (Arnaud et al., 1993) without plant growth regulators

and solidified with 9 gL^{-1} Difco agar®. The pH of the medium was adjusted to 5.8 before autoclaving (121°C, 20 min). The cultures were placed in the growth chamber at a temperature of 21 ± 1°C, under 16-h photoperiod at 120 μmol $m^{-2}s^{-1}$. After six weeks of culture in REM medium, explants with visible roots were transferred to wet peat: vermiculite mixture (2:1, v/v) and acclimatized in the greenhouse under controlled conditions at 21 ± 1°C and decreasing humidity progressively along one month from 95 to 80%.

2.5. Data collection and statistical analysis

To assess the effect of the genotype on each of the variables of this study an analysis of variance was performed (ANOVA), followed by a Tukey's post hoc test (α=0.05). The genotype had a significant effect in all variables studied. For this reason and in order to obtain robust conclusions, the genotype factor was introduced into all the models as a block variable to reduce variability and analyze the effect of the other variable factors (type of explant, cytokinin treatment, and auxin treatment) more accurately.

The shoot induction and the number of shoots per explant were recorded after six weeks. The experiment used a completely a randomized design with 25 explants per treatment. To assess the effect of the type of explant and the cytokinin treatment on the shoot induction percentage a logistic regression was performed, followed by a Tukey's post hoc test (α=0.05) for multiple comparisons. The number of shoots per explant variable did not fulfill homocedasticity and normality assumptions for ANOVA, then a Kruskal-Wallis was performed.

The root induction percentage, the number of roots per explant and the root length was recorded after six weeks in REM. A completely randomized design was carried

out using ten stems per cytokinin treatment. As exception, in genotype 7, four stems were employed when K was used in induction stage.

A logistic regression model was used to analyze the effect of the auxin and cytokinin treatment on the root induction percentage. Tukey's post hoc test (α=0.05) was used for multiple comparisons. The number of roots per explant variable did not fulfill homocedasticity and normality assumptions for ANOVA, then a Kruskal-Wallis was performed.

Data on the length of the longest root were square root transformed, and an ANOVA was performed, multiple comparisons were made using the Tukey's post hoc test (α=0.05)

After twelve weeks under *ex vitro* conditions the survival percentage was calculated. Survival percentage variable did not fulfill homocedasticity and normality assumptions for ANOVA, then a Kruskal-Wallis was performed.

3. RESULTS

3.1. Shoot induction

When the effect of the genotype on the shoot induction percentage was evaluated, genotypes 12 and 11 showed significantly higher percentages (98.98%) than genotype 7 (79%, Table 1). As shown in Table 1, the number of shoots obtained in genotype 12 (5.04) was significantly higher than those obtained in genotypes 11 and 7 (3.21 and 3.28, respectively).

Table 1. Shoot induction (%) and number shoots per explant from *Sequoia sempervirens* elite adult trees cultured in ARN medium (Arnaud et al., 1993).

	Genotype		
Variable	7	11	12
Shoot induction (%)	79.00± 0.03 [b]	98.98±0.01 [a]	98.98±0.01 [a]
Nº shoots /explant	3.28± 0.12 [b]	3.21±0.07 [b]	5.04±0.16 [a]

Data are presented as mean values ± S.E. Different letters within a row indicate significant differences (p<0.05).

The analysis of deviance of the factors studied showed a significant effect on both the shoot induction and the number of shoots per explant developed from each explant and cytokinin type. In the case of the number of shoots per explant there was a significant interaction between the explant type and the cytokinin type (Table 2).

Table 2. Analysis of deviance for shoot induction (%) and number shoots per explant from *Sequoia sempervirens* elite adult trees cultured in ARN medium (Arnaud et al., 1993).

		Shoot induction		**Nº shoots /explant**	
Source	df	**X^2 Test**	***p*-Value**	**X^2 Test**	***p*-Value**
Explant type (E)	3	382.39	0.00011*	23.63084	2.98290^{-5*}
Cytokinin type (C)	2	403.24	0.01861*	142.0222	$<2.0^{-16*}$
E x C	6	374.86	n.s	188.8565	$<2.0^{-16*}$

*Significant differences at p<0.05, n.s Non-significant at p<0.05, df Degrees of freedom.

Significantly higher shoot induction percentages were obtained in AP, BG and BP explants (between 91% and 94%) than in AG explants (84%) (Table 3).

Table 3. Shoot induction (%) in different explant types from *Sequoia sempervirens* elite adult trees cultured in ARN medium (Arnaud et al.; 1993) (apical explants of 1.5 cm in height (AG), apical explants of 1.0 cm height, C) basal explants of 1.5 cm height (BG) and basal explants of 1.0 cm in height (BP).

Explant type	Shoot induction (%)
AG	84.00± 0.02[b]
AP	94.00±0.02[a]
BG	91.00± 0.02[a]
BP	94.00±0.02[a]

Data are presented as mean values ± S.E. Different letters indicate significant differences (p<0.05).

When considering the effect of the cytokinin type on shoot induction percentages, shoots induced with m-T showed significantly higher induction percentages than shoots induced with K. Shoots induced in the presence of BAP displayed intermediate values (92%) (Table 4).

Table 4. Shoot induction (%) in different cytokinin type from *Sequoia sempervirens* elite adult trees cultured in ARN medium (Arnaud et al., 1993).

Cytokinin type	Shoot induction (%)
BAP	92.00± 0.02[ab]
K	87.00±0.02[b]
m-T	93.00± 0.01[a]

Data are presented as mean values ± S.E. Different letters indicate significant differences (p<0.05).

When evaluating the number of shoots per explant, a significant interaction between explant type and cytokinin was observed. The best results were obtained in AG, AP and BG explants induced with m-T and in AG explants induced with BAP (Figure 1). When BAP or m-T were used for induction, the worst results were

obtained in BP explants. The lowest response was observed in treatment K, independently of the explant type.

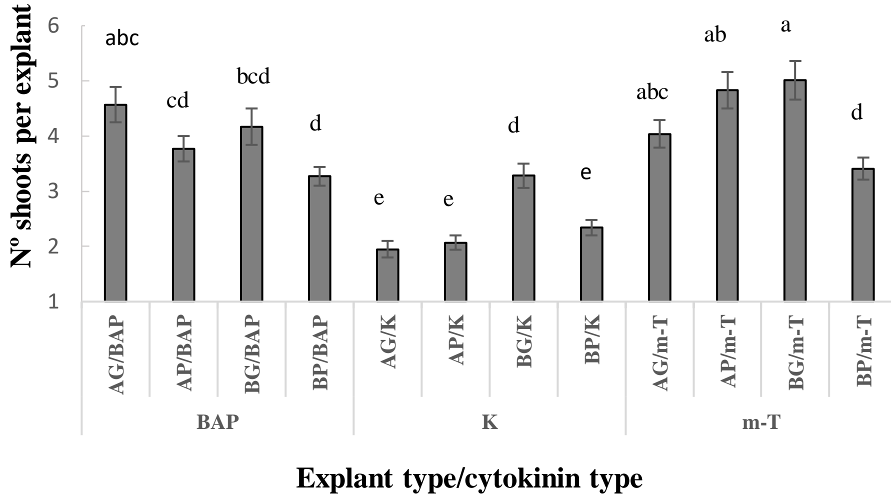

Figure 1. Number of shoots per explant in different explant types from *Sequoia sempervirens* elite adult trees cultured in ARN medium (Arnaud et al., 1993) supplemented with different cytokinins (BAP, m-T, K), (apical explants of 1.5 cm in length (AG), apical explants of 1.0 cm length (AP), basal explants of 1.5 cm length (BG) and basal explants of 1.0 cm in length (BP)).Data are presented as mean values ± S.E. Different letters indicate significant differences ($p<0.05$).

Despite there were no statistically significant differences between BG/m-T, AP/m-T, and AG/BAP, it is important to mention that the appearance of induced shoots was different Figure 2 (A-C). The shoots induced in presence of BAP and K were greener and more vigorous (Figure 2 A) than the explants cultured in m-T (2 B, C). These explants from m-T treatments were more yellowish and less vigorous (Figure 2 B and C). Therefore, AG explants cultured in presence of BAP showed the best results for shoot induction.

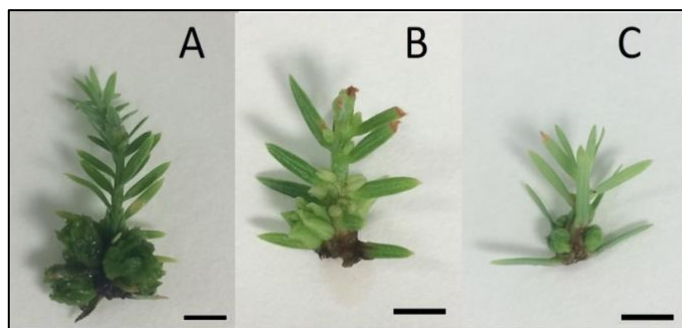

Figure 2. Shoot induction from *Sequoia sempervirens* elite adult trees cultured in ARN multiplication medium (Arnaud et al., 1993) cultured for 6 weeks: (A) apical explant > 1.5 cm of length (AG) of genotype 7 on ARN medium + 4.4 μM BAP, bar = 3 mm ; (B) basal explant > 1.5 cm of length (BG) of genotype 7 on ARN medium + 4.4 μM m-T, bar = 5 mm; (C) apical explant < 1.5 cm of length (AP) explant of genotype 7 on ARN medium + 4.4 μM m-T, bar = 3 mm.

Since AG explants showed the best appearance, a comparative figure was done to describe the aspect of regenerated shoots in the presence of cytokinins (Figure 3). The most vigorous shoots were observed in the presence of BAP and K (Figure 3 (A-C, G-H).

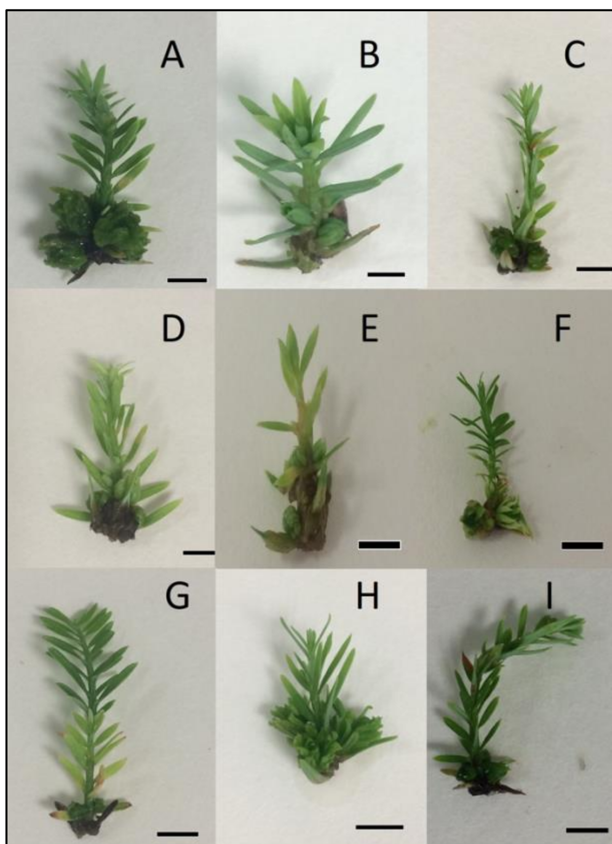

Figure 3. Shoot induction from *Sequoia sempervirens* elite adult trees cultured in ARN multiplication medium (Arnaud et al., 1993) cultured for 6 weeks: (A) apical explant > 1.5 cm of length (AG) of genotype 7 on ARN medium + 4.4 μM BAP, bar = 3 mm; (B) AG of genotype 11 on ARN medium + 4.4 μM BAP, bar = 3 mm; (C) AG explant of genotype 12 on ARN medium + 4.4 μM BAP, bar = 3 mm; (D) AG explant of genotype 7 on ARN medium + 4.4 μM m-T, bar = 3 mm; (E) AG explant > 1.5 of genotype 11 on ARN + 4.4 μM m-T, bar = 5 mm; (F) AG explant of genotype 12 on ARN medium + 4.4 μM m-T, bar = 5 mm; (G) AG of genotype 7 on ARN medium + 4.4 μM K, bar = 3 mm; (H) AG explant of genotype 11 on ARN medium +4.4 μM K, bar = 5 mm; (I) AG of genotype 12 on ARN medium + 4.4 μM KIN, bar = 3 mm.

3.2. Root induction

A significantly higher root induction percentage was obtained in genotype 12 (93%) (Table 5). The lowest response was shown by genotype 7 (31%) and intermediate values were recorded for genotype 11 (55%). Also, significantly higher number of roots per explant was observed in genotype 12 (4.73) when compared with genotype 7 and 11 (Table 5).

Table 5. Root induction (%), number roots per explant and length of longest root from *Sequoia sempervirens* elite adult trees cultured in ARN medium (Arnaud et al.,1993).

	Genotype		
Variable	7	11	12
Root induction (%)	31.25±0.04 ^{c}	55.42±0.04^{b}	93.47±0.02^{a}
Nº roots /explant	2.14±0.27^{b}	1.69±0.11^{b}	4.73±0.25^{a}
Length of longest root (cm)	2.95±0.21^{a}	3.73±0.33^{a}	2.18±0.12^{b}

Data are presented as mean values ± S.E. Different letters within a row indicate significant differences ($p<0.05$).

On the contrary, significantly longer primary roots were recorded for genotype 11 (3.73 cm, Figure 4A) and genotype 7 (2.95 cm, Figure 4B) than for genotype 12 (2.18 cm, Figure 4C).

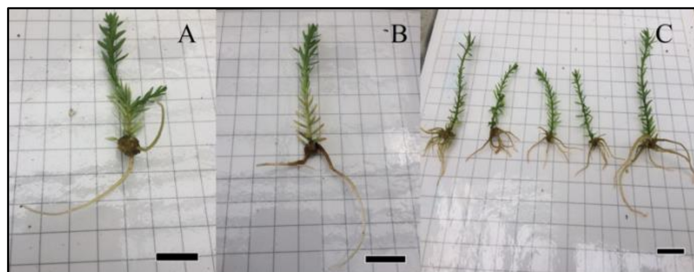

Figure 4. Root induction from *Sequoia sempervirens* elite adult trees micro shoots cultivated *in vitro* for 6 weeks on root expression medium (REM) (Arnaud et al., 1993): (A) explant of genotype 7 induced in root inducing medium (RIM) (Arnaud et al10) + 50 μM NAA (BAP applied in shoot induction stage), bar = 2 cm (B) explant of genotype 11 induced in RIM medium + 50 μM NAA (BAP applied in shoot induction stage), bar = 2 cm; (C) explant of genotype 12 induced in RIM medium + 50 μM NAA (BAP applied in shoot induction stage), bar = 2 cm.

The auxin type and cytokinin type used in multiplication stage showed a significant effect on the root induction percentage. Further, the cytokinin type showed a significant effect on the length of longest root (Table 6). Regarding the number of roots per explant, no significant differences were found for the variables tested.

Table 6. Analysis of deviance for root induction (%), number roots per explant and length of longest root from *Sequoia sempervirens* elite adult trees cultured in ARN medium (Arnaud et al., 1993).

Source	df	Root induction		Nº roots /explant		Length of longest root	
		X^2 Test	*p*-Value	X^2 Test	*p*-Value	*F* Test	*p*-Value
Auxin type (A)	2	510.96	0.01099*	3.42422	n.s	2.703	0.06913
Cytokinin type (C)	2	503.59	0.02503*	1.15589	n.s	5.469	0.00479*
A x C	4	496.71	n.s	11.81255	n.s	1.042	n.s

*Significant differences at $p<0.05$, n.s Non-significant at $p<0.05$, df Degrees of freedom.

As shown in Table 7, a significantly higher root induction percentage was obtained in the treatment with NAA (70%) than in treatments with IBA or IBA/NAA mixture. As mentioned before, there was no effect of the auxin treatment on the number of roots per explant (ranging from 2.98 to 3.94) or on the length of the primary root (ranging from 2.23 to 3.09 cm) (Table 7).

Table 7. Root induction (%), number of roots per explant and length of the longest root in *Sequoia sempervirens* elite adult trees cultured in ARN medium (Arnaud et al., 1993) supplemented with different auxin types (IBA, IBA/NAA or NAA).

	Auxin types		
Variable	**IBA**	**IBA/NAA**	**NAA**
Root induction per-cent	60.00±0.04^{b}	57.00±0.04^{b}	70.00±0.04^{a}
Nº roots /explant	2.98±0.25$^{n.s}$	3.94±0.35$^{n.s}$	3.30±0.26$^{n.s}$
Lenght of longest root (cm)	2.77±0.21 $^{n.s}$	2.23±0. 17$^{n.s}$	3.09±0.25$^{n.s}$

Data are presented as mean values ± S.E. Different letters within a row indicate significant differences ($p<0.05$).n.s Non-significant.

When evaluating the effect of the cytokinin type over root induction percentage significantly higher values were obtained from shoots induced in BAP treatment when compared with those induced in m-T and K treatment (Table 8).

As aforementioned, no significant differences were found for the number of roots per explant, independently of the shoot induction treatment applied (ranging from 3.11 to 3.89) (Table 8).

The longest primary roots (3.32 cm) showed significantly higher values when shoots were induced in BAP treatment (Table 8).

Table 8. Root induction (%), number roots per explant and length of longest root in different cytokinin types from *Sequoia sempervirens* elite adult trees cultured in ARN medium (Arnaud et al., 1993).

Cytokinin type applied in the root induction stage			
Variable	**BAP**	**m-T**	**K**
Root induction (%)	66.00±0.04[a]	58.00±0.04[b]	62.00±0.04[b]
Nº roots /explant	3.11±0.24[n.s]	3.89±0.33[n.s]	3.16±0.29[n.s]
Lenght of longest root (cm)	3.32±0.22[a]	2.39±0.21[b]	2.48±0.24[b]

Data are presented as mean values ± S.E. Different letters within a row indicate significant differences (p<0.05).n.s Non-significant.

3.3. Acclimatization of rooted microplants

The analysis of deviance for survival (%) of rooted shoots propagated *in vitro* showed a significant effect of the auxin type applied in the root induction stage and of the cytokinin type applied in the shoot induction phase (Table 9).

Table 9. Analysis of deviance for survival (%) of rooted shoots propagated *in vitro* coming from *Sequoia sempervirens* elite adult trees, after twelve weeks under *ex vitro* conditions.

		Survival (%)	
Source	df	**X^2 Test**	***p*-Value**
Auxin type (A)	2	244.40	5.679^{-5}*
Cytokinin type (C)	2	223.62	3.075^{-5}*
A x C	4	216.66	n.s

*Significant differences at p<0.05, n.s Non-significant at p<0.05, df Degrees of freedom.

When the effect of auxin type on the survival percentage of rooted shoots was analyzed, the presence of IBA in the rooting medium provoked a significantly higher survival percentage (94%) than the mixture IBA/NAA and NAA treatments (Table 10).

Table 10. *Ex vitro* survival (%) of *in vitro* rooted shoots from *Sequoia semper*virens elite adult trees, shoots were rooted in ARN medium (Arnaud et al., 1993) supplemented with different auxins (IBA, IBA/NAA or NAA).

	Auxin type		
	IBA	**IBA/NAA**	**NAA**
Survival %	94.00±0.03[a]	79.00±0.06[b]	68.00±0.05[b]

Data are presented as mean values ± S.E. Different letters indicate significant differences (p<0.05).

Regarding the effect of the cytokinin applied in the shoot induction stage as shown in Table 11, the shoots induced in K treatment showed a significantly higher *ex vitro* survival percentage than those induced in BAP or m-T treatments.

Table 11. *Ex vitro* survival (%) of *in vitro* rooted shoots from *Sequoia sempervirens* elite adult trees; shoots were propagated in ARN medium (Arnaud et al., 1993) supplemented with different cytokinins (BAP, m-T or K).

	Cytokinin type		
	BAP	m-T	K
Survival (%)	80.00±0.05[b]	65.00±0.05[b]	94.00±0.03[a]

Data are presented as mean values ± S.E. Different letters indicate significant differences by Tukey's post hoc test (p<0.05).

At the end of the experiment, the micropropagated plants from the three elite adult trees evaluated showed a normal development, good growth and uniformity after 5 months under *ex vitro* conditions in the greenhouse (Figure 5).

Figure 5. Plants of *Sequoia sempervirens* cultured *in vitro* after 5 months growing in *ex vitro* conditions in the greenhouse, bar = 4 cm.

4. DISCUSSION

The genotype is an endogenous factor that has an extremely important role for regenerative potential in the overall repeatability and reliability of tissue culture protocol (Coleman and Ernst, 1989) . In this study, the shoot induction, the number of shoots per explant, the root induction and the number of roots per explant were affected by the genotype of the explant. Our results are in concordance with the results reported by Meneguzzi et al, (2019), who observed different response among genotypes in shoot multiplication of *S. sempervirens*. In the same way, Sul and Korban (2005) found differences in shoot proliferation and shoot elongation of *S. sempervirens.*

The organogenesis processes are influenced by endogenous and exogenous factors (Sul and Korban , 2005; George et al., 2008; Aremu et al., 2012). There are different exogenous factors as the components in the tissue culture medium or the environmental conditions of culture. In this work we observed that the type of explant and chemical conditions in the culture medium (cytokinin and auxin type) had a significant effect in shoot induction and rooting response.

During the evaluation of the shoot induction rate, the explants with the longest length (AG, AP and BG) showed the best induction rate, and the highest number of shoots per explant were obtained. Similar results were found by Clapa et al., (2010) and Meneguzzi et al., (2020) who developed an *in vitro* protocol for *S. sempervirens* using shoot explants bigger than 1.5 cm of length. George et al., (2008) reported that larger explants coming from larger parts of shoot apex or stem segments bearing one or more lateral buds could show advantages over smaller size explants.

Several studies about tissue culture protocols have reported positive effects on parameters such as shoot proliferation, shoot multiplication rate, alleviating physiological disorders, better rooting and acclimatization when using topolins for shoot induction (Aremu et al., 2012; Moyo et al., 2018). In our study the explants induced in the presence of m-T exhibited the best results. In this sense, De Diego et al., (2010), obtained a high rate of organogenic response in adult buds of *Pinus sylvestris* using m-T and suggested that it could be used as an alternative cytokinin to BAP in micropropagation. Likewise, Bairu et al., (2007) obtained outstanding multiplication rates in *Aloe polyphylla* when used the same cytokinin. However, in the present study these explants from m-T treatments were more yellowish and less vigorous. In contrast, in *Prunus rootstoc*ks, *Pterocarpus marsupium* and *Corylus colurna* a positive effect of m-T on growth and quality of micropropagated shoots was found (Gentile et al., 2014; Gentile et al., 2017; Ahmad and Anis, 2019).

According to Sul and Korban (2005), Meneguzzi et al., (2020), Valverde et al.,(2004) and Montalbán et al., (2011) in forestry species, BAP stimulated the axillary bud breakage and shoot elongation. In our study, a similar result was obtained in the BAP treatment. It is important to mention that in the treatment with BAP the quality

of the shoots formed (robustness and color) was superior to those shoots generated in the presence of m-T. In conclusion, in our study BAP was the best cytokinin in terms of shoot induction and number of shoots per explant. This is in accordance with the results reported by Moncaleán et al., (2005), George et al.,(2008), Aremu et al., (2012) and Bairu et al., (2007) confirming that BAP is the most used cytokinin in micropropagation due to its effectiveness and affordability. Regarding K treatment, in this work we obtained the worst results in agreement with those found in *Barleria greenii* and *Eucalyptus globulus* (Bennett et al., 1994; Amoo et al., 2011).

In this research the NAA treatment showed the best result in root induction response. This is in agreement with the results reported in *Sequoia sempervirens*, *Pinus radiata*, *Pinus pinaster*, and other coniferous species (De Diego et al, 2008, 2011; Halmagyi et al., 2010; Montalbán et al., 2019; Ragonezi et al., 2010). It is important to mention that the rooting percentages obtained in the present studies were higher than those recorded by Huang et al., (1992) who obtained about a 30% of rooting competence using adult stem sections of *Sequoia sempervirens* in the presence of IAA/K. The number of roots per explant obtained in the IBA/NAA treatment presented the highest values. In species such as *Eucalyptus sideroxylon*, *Rosa hybrida* and *Citrus aurantiflolia* the best root induction was obtained when using a mixture of IBA/NAA. Nonetheless, this treatment did not lead to the highest values in the number of roots per explant (Kosh–Khui and Sink, 1982; Cheng et al., 1992; Al-Bahrany, 2002). The highest value in the length of longest root was also obtained in NAA treatment, being in accordance with the results obtained in *Citrus aurantifolia* (Al-Bahrany, 2002).

In our research the cytokinin type used in induction stage had a significant effect over root induction. Bairu et al., (2007) ,Werbrouck et al., (1996) and Aremu et al., (2012) found that m-T stimulated *in vitro* rooting activity, but in our work the m-T

treatment presented the worst results. In this sense, Bairu et al., (2008) and Escalona et al., (2003) obtained negative carryover effects on rooting at too high m-T levels, so it is possible that the m-T concentration applied in our cultures might have had a detrimental effect in the subsequent stages of development.

Regarding the acclimatization stage, the highest survival percentage was observed in plantlets rooted in the presence of IBA and developed in the presence of K. This result is in agreement with those found in *Eucalyptus globulus*, where low concentration of IBA showed the best survival rates; and in *Arachis paraguariensis* cultured in polyethylene terephthalate glycol vessel where IBA treatment was the best in survival (Bennett et al., 2003; Aina et al., 2015).

Aremu et al., (2012) explained that cytokinins generally have inhibitory effects on rooting, resulting in poor acclimatization rates afterwards. In this sense, the plants from m-T treatment showed the lowest survival. In contrast, several studies have shown that plantlets coming from m-T induction treatments have been successfully acclimatized (Bairu et al., 2007; Naaz et al., 2019).

In conclusion, the role of auxins and cytokinins in the micropropagation of different types of explants and their relationship with the survival and acclimatization of seedlings in *ex vitro* conditions was analyzed. The results of our study demonstrated that the apical explant ≥ 1.5 cm length, and BAP showed the best results in the shoot induction stage. Moreover, *in vitro* shoots rooted with IBA, led toa higher *ex vitro* survival. Finally, the results shown allow the development of forthcoming studies for large-scale propagation of this species in semi-solid systems and bioreactors.

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CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

In the last years the cultivated forest of *P. radiata* in the Basque Country has been affected by several diseases presumably due to climate change; in this scenario it is necessary to develop efficient propagation methods for alternative species to *P. radiata.* The best alternative to produce elite plants is the vegetative propagation. However, when plants show the desirable characteristics have reach maturity and propagation for traditional methods are imposible. For this reason, forestry countries combine traditional techniques with *in vitro* propagation methods to develop effective propagation systems.

In conifers, vegetative propagation is commonly used as a massive cloning method to obtain material from selected genotypes, to improve planting quality and to shorten the selection cycles for forest tree genetic improve programs (Niemi et al., 2004). Micropropagation is currently being used as effective method for mass cloning (Bonga, 2015), but in woody species it is widely recognized the difficulty of applying this technique; even more when explants from mature trees are used as initial explants (Giri et al., 2004).

In this context, the present study had the objective of developing *in vitro* propagation methods of different species considered as an alternative of *Pinus radiata* for Basque Country forests. Moreover, it contributes to the knowledge about how modifications applied in the physical and chemical environment during the different stages of micropropagation process of elite trees of *P. radiata*, *C. japonica*, *P. ponderosa* and *S. sempervirens* can affect the success of the *in vitro* culture process and the morphological characteristics and metabolite profile in the plantlets obtained. Adult *P. radiata* trees with apparent tolerance to needle blight disease can be used as a source of material to *in vitro* propagation. However, the collection date

and genotype of the explants had a significant effect on the *in vitro* response, being the percentage of explants forming shoots genotype-dependent. These results are in concordance with those carried out by Pereira et al., (2021) in *P. halepensis* and De Diego et al., (2008) in *P. pinaster* in which they showed that the genotype affected the *in vitro* organogenesis response.

The modifications of the chemical and physical environment influenced the success of the micropropagation process and regeneration efficiency of elite plantlets of *C. japonica*, *P. pond*erosa, *S. sempervirens* and adult *P. radiata*. Different concentrations and types of plant growth regulators had a significant effect in the *in vitro* morphogenesis of the abovementioned species.

The function of cytokinins has extensively been studied in *in vitro* culture and their role in the suppression of apical dominance and promotion of the development of axillary buds has been recognized (Arab et al., 2014; Rodríguez et al., 2022). In this sense, although different CKs have been tested, BA was the most effective for the *in vitro* shoot induction from explants of elite trees of *P. radiata*, *C. japonica*, *P. ponderosa* and *S. sempervirens*. In this regard, BA is the most commonly used in plant tissue culture due to its effectiveness and affordability to promote shoot induction (Bairu et al., 2007; De Diego et al., 2008; Phillips and Garda, 2019). In accordance with our results, Montalbán et al., (2013) and Pereira et al., (2021) also reported a higher *in vitro* shoot organogenesis response when BA was utilized in organogenesis protocols from adult trees of different *Pinus* species. Contrary to our work, Castro-Garibay et al., (2023) in *S. sempervirens* and Cortizo et al., (2009) in *P. pinea* adult trees obtained a better organogenic response when they used kinetin and thidiazuron, respectively. Furthermore, the use of a certain plant growth regulator during micropropagation process can not only improve the shoot induction but also have an effect on subsequent stages including acclimatization. Our work showed

different results in relation to the cytokinin applied along the induction period; we observed that a certain cytokinin in the induction phase caused an improvement in the rooting phase but another of the evaluated cytokinins improved the acclimatization percentages as we have assessed in this work, where shoots induced with m-T and rooted in NAA show significantly better *in vitro* root induction in *C. japonica*. Likewise, in *S. sempervirens* shoots induced with BA and rooted in NAA show significantly higher *in vitro* rooting; whereas plantlets induced in Kinetin and rooted in IBA show a significantly better acclimatization success.

In this study an efficient biotechnological tool for micropropagation of adult elite *C. japonica* and *S. sempervirens* trees has been developed. All propagation methods have limitations; in micropropagation these difficulties may include low regeneration, necrosis, inability to continue elongation and low adventitious root regeneration among others (Sarmast, 2018; Díaz-Sala, 2019; Von Aderkas and Bonga, 2000). Some modifications of the culture enviroment allowed to optimize the shoot and complex rooting stage (Diaz-Sala, 2019); so, higher rooting rates were obtained when IBA and NAA were utilized in plantlets of *C. japonica* and *S. sempervirens*, respectively. According to our results, Halmagyi et al., (2010) in *S. sempervirens* also obtained higher rooting rates using NAA. Contrary our results in *C. japonica*, Pereira et al., (2021) in *P. halepensis* reported that long exposure to IBA was not effective in inducing roots. These results demonstrated that the adventitious root regeneration also can be influenced by plant growth regulators among others chemical factors (Zarei et al., 2020) and that the effect of them is species dependent.

During this work, in *P. ponderosa* and *P. radiata* the obtention of a good adventitious root system was very difficult. According with Von Aderkas and Bonga, (2000), the *in vitro* conditions can influence the maintenance of juvenility of the tissue.

However, loss of organogenesis competence, progressive or sudden, reflects transition to the mature phase. For the above-mentioned reasons, the low rooting response of *P. ponderosa* and *P. radiata* may be due to an increase of tissue aging, limiting the *in vitro* organogenic response of the species (Von Aderkas and Bonga, 2000). In hybrid hazelnuts (*Corylus avellana* × *Corylus americana*) the use of a temporary immersion system significantly improved the *in vitro* rooting efficiency compared with semi-solid medium (Nicholson et al., 2020). Likewise, San José et al., (2020) in *Alnus glutinosa* obtained rooted plantlets using a temporary immersion system. In this context, these recent strategies for rooting, together with test other combinations and concentrations of auxins could be considered to improve *P. ponderosa* and *P. radiata* rooting in the future.

The application of different light treatments in shoot and root induction stage also provoked different responses in the *in vitro* response in all species studied. Our results are in accordance with the results reported by Alallaq et al., (2020) in *Picea abies*, who explain that the development and plant growth were influenced by different physical factors such us light as one of the most important. In this regard, *C. japonica* shoots produced a higher number of roots when they were grown under red LED lights. According to our results, Lotfi et al., (2019) reported a better rooting response *in vitro* in *Pyrus communis* plants when red LEDs were used. Likewise, *Protea cynaroides* rooting rates were the highest under red LEDs (Wu and Lin, (2012). However, this response seems to be also species dependent, for instance, in *Cucumis metuliferus* plantlets showed the best rooting response under white LEDs (Lai et al., 2022).

Significantly higher percentage of *P. ponderosa* shoots able to be rooted was detected in shoots growing under blue LEDs although the best rooting rate was observed in shoots growing under white LEDs. In this context, *Zingiber officinale* shoots exposed

to blue LEDs showed the highest shoot regeneration (Gnasekaran et al., 2021). Likewise, in *Solanum tuberosum* when shoots were cultured under blue LEDs the greatest stem diameter and the highest health index were obtained (Chen et al., 2020). In contrast, Santana Costa Souza et al., (2022) studying *Eucalyptus grandis* x *E. urophylla* obtained the highest organogenic response when the *in vitro* culture was exposed to FL. Likewise, Guerra et al., (2022) using white LEDs in *Citronella mucronata* trees obtained the lowest rooting rates. Summarizing, the effect of LEDs on the *in vitro* morphogenesis can be species dependent.

Higher root induction and number of roots per explant were observed in *P. radiata* shoots cultured under white FL. Similar results was reported by Alvarenga et al., (2015) in *Achilea millefolium* where higher rooting rates were obtained in *in vitro* plants growing under white FL. Contrary, in *P. cynaroides* and *Dendranthema* × *grandiflorum* the lowest rooting rates were obtained in plantlets growing under white FL (Wu and Lin, 2012; Gil et al., 2020). According to our results Gupta and Jatothu, (2013), also reported that at each stage of plant development the light requirements appeared to be different. In addition, even when applicating the same light treatments different species respond differently and may be associate with the genotypic characteristics or the physiological state of explants (Da Silva et al., 2016; Lai et al., 2022). Many reports have suggested that a combination of different types of light could be interesting to improve the micropropagation process (Diaz-Rueda et al. 2021; Dewir et al. 2023), so future experiments should be made to evaluate that combination effect.

Analysis of amino acids and carbohydrates during the rooting stage of *P. radiata* shoots were carried out in order to evaluate the effect of different physico-chemical factors along the organogenesis process. Fructose, glucose and sucrose levels were significantly different in the rooting stages considered. Carbohydrates in

micropropagation process are related to the maintenance of osmotic potential and to serve as a carbon source for developmental processes such as root formation (Yaseen et al., 2013; Moradi et al., 2017). In this work, shoots with better *in vitro* rooting ability grown in culture medium supplemented with 3% sucrose concentration presented higher fructose, glucose and sucrose content. Jo et al., (2009), in *Alocasia amazonica*, reported that the *in vitro* leaves showed higher glucose, fructose and sucrose content compared with *ex vitro* leaves. Also, Mingozzi et al., (2011) in *Cydonia oblonga* reported a higher content of sucrose, glucose and fructose in shoots cultured with higher sucrose concentration.

Amino acid acts as block units for proteins, osmolytes, signaling molecules, phytohormone precursors, cofactors and amino acid levels readjust both nutritional and metabolic cell responses (Wu, 2009). In this study, higher threonine and tyrosine levels were obtained in shoots with displaying the worst results in rooting percentage. Joshi et al., (2010) and Muthuramalingam et al., (2018) reported that threonine metabolites are involved in plant growth and development, cell division and responses to abiotic stresses. However, in our work, threonine level did not favor the plant growth and development. In this sense, Sarrobert et al., (2000) in an *Arabidopsis thaliana* mutant suggested that the excess of endogenous threonine is directly or indirectly toxic for rooting. Regarding to tyrosine, in *Alternanthera brasilian* acts as precursor of secondary metabolites such as betacyanin (Silva et al., 2005). Furthermore, the tyrosine metabolism pathway acts as starting point for the synthesis of tyrosine-derived metabolites essential to plant survival, for example such as tocopherols, plastoquinone and ubiquinone (Xu et al., 2020).

On the other hand, it is widely recognized that the physico-chemical microenvironment of vessels used in micropropagation can induce anatomical, morphological and physiological abnormalities in plantlets (Shekhawat et al, 2017).

Our study reported the first microscopy assessment of *in vitro* plantlets from *P. ponderosa* exposed to LEDs treatments. Our analyses concluded that light treatments promoted differences in anatomical aspects of *in vitro* shoots; needles of micropropagated plants exposed to red and blue LEDs showed less stomata compared with needles from plantlets exposed to FL. In this way, Díaz-Rueda et al., (2021) reported minimal stomatal density when 70% red and 30% blue LEDs with 34 umol $m^{-2}s^{-1}$ of light irradiance were used in *in vitro* culture of *Olea europaea.* Studes carried out in white strawberry (*Fragaria x ananassa*) demonstrated that the stomata of the seedlings exposed to blue LED resulted damaged compared those from white, red, blue, and red+blue LEDs (Pang et al., 2023). As reported by Franco-Navarro et al., (2019) a lower stomatal frequency could reduce water loss through leaf transpiration, and improved the *ex vitro* acclimatization. Further research is needed in our species to confirm this hypothesis.

Our work demonstrated that plant tissue culture could be used as a complementary cloning method of elite trees of *C. japonica*, *P. ponderosa*, *P. radiata* and *S. sempervirens* in future breeding program in order to improve the Basque forest health in the current climate change scenario.

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CONCLUSIONS

CONCLUSIONS

1. The collection date and the interaction between genotype and BA concentration have a significant effect on the percentage of explants forming shoots of adult *Pinus radiata* trees organogenesis. Shoots cultured in rooting medium supplemented with 3% sucrose and exposed to white FL show a significant higher root induction response.

2. The profile of carbohydrates and aminoacids in *in vitro* shoots of *Pinus radiata* is influenced by the rooting medium. Fructose, glucose and sucrose contents increase in shoots during root induction stage. Significantly higher threonine and tyrosine levels are present in shoots after root expression stage.

3. *Cryptomeria japonica* basal explants from adult trees longer than 1.5 cm produce a significantly higher number of shoots than the rest of explants evaluated. QL medium supplemented with BA is the best chemical environment for the success of organogenic response.

4. *Cryptomeria japonica* explants induced with m-T and rooted with 1.5% sucrose display the best results in terms of root induction. Then, plantlets exposed to red LEDs show a significantly higher number of roots.

5. *Pinus ponderosa* whole zygotic embryos are the best initial explants to obtain developed shoots. *Pinus ponderosa* explants growing under blue LEDs produce a significantly higher number of shoots with rooting capacity; while plantlets grown under FL light during *in vitro* culture increase the acclimatization success.

6. LEDs show an effect in *Pinus ponderosa* needles decreasing the number of stomata in relation to FL.

7. *Sequoia sempervirens* needs different chemical environment in the *in vitro* stage depending on the objective of the process (improve the rooting or acclimatization stage).

SUPPLEMENTARY MATERIALS

CHAPTER 2

Influence of Physico-Chemical Factors on the Efficiency and Metabolite Profile of Adult *Pinus radiata* D. Don Bud Organogenesis

SUPPLEMENTARY MATERIALS CHAPTER 2

Table S1. Statistical analysis for explants forming shoots [EFS (%)] in different collection dates from *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988).

Source	df	Explants forming shoots [EFS (%)]	
		X^2 Test	***p*-Value**
Collection dates	5	776.71	≤0.05*

*Significant differences at $p<0.05$, df Degrees of freedom.

Table S2. Statistical analysis for explants forming shoots [EFS (%)] from nine different genotypes of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with 6-benzyladenine (BA) (22 and 44 μM).

Source	df	EFS (%)	
		X^2 Test	***p*-Value**
Genotype (G)	8	795.04	≤0.05*
BA concentrations (B)	1	794.21	>0.05 n.s
G x B	8	745.98	≤0.05*

*Significant differences at $p<0.05$, n.s Non-significant at $p<0.05$, df Degrees of freedom.

Table S3. ANOVA for number of shoots formed per explant (NS/E) from *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with 6-benzyladenine (BA) (22 and 44 μM).

Source	df	NS/E	
		***F* Test**	***p*-Value**
BA concentrations	1	0.06	>0.05 [n.s]

[n.s] Non-significant at $p<0.05$, df Degrees of freedom.

Table S4. Statistical analysis for root induction (%), number roots per explant, and length of longest root from *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) according to 6-benzyladenine (BA) concentrations and light treatments applied in the root induction phase.

Source	df	Root induction		N^0 roots/explant		Length of the longest root	
		X^2 Test	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
BA concentrations (B)	1	251.24	>0.05 [n.s]	0.83	>0.05 [n.s]	0.31	>0.05 [n.s]
Light treatments (L)	1	243.56	≤0.05*	2.94	>0.05 [n.s]	2.27	>0.05 [n.s]
B x L	1	242.87	>0.05 [n.s]	0.75	>0.05 [n.s]	16.60	≤0.05*

*Significant differences at $p<0.05$, [n.s] Non-significant at $p<0.05$, df Degrees of freedom.

Table S5. Statistical analysis for root induction (%), number roots per explant, and length of longest root from *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) according to and 6-benzyladenine (BA) and sucrose concentrations applied in the root induction phase.

		Root induction		N^0 roots/explant		Length of the longest root	
Source	df	X^2 **Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
BA concentrations (B)	1	251.24	>0.05 n.s	0.72	>0.05 n.s	0.25	>0.05 n.s
Sucrose concentrations (S)	1	242.33	≤0.05*	0.06	>0.05 n.s	0.95	>0.05 n.s
B x S	1	242.10	>0.05 n.s	0.32	>0.05 n.s	5.43	≤0.05*

*Significant differences at $p<0.05$, n.s Non-significant at $p<0.05$, df Degrees of freedom.

Table S6. Statistical analysis for root induction (%), number roots per explant, and length of longest root from *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) according to light treatments and sucrose concentrations applied in the root induction phase.

		Root induction		N^0 roots/explant		Length of the longest root	
Source	df	X^2 **Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Light treatment (L)	1	236.52	≤0.05*	4.82	≤0.05*	0.99	>0.05 n.s
Sucrose concentrations (S)	1	243.38	≤0.05*	0.11	>0.05 n.s	0.86	>0.05 n.s
L x S	1	232.75	>0.05 n.s	0.92	>0.05 n.s	3.28	>0.05 n.s

*Significant differences at $p<0.05$, n.s Non-significant at $p<0.05$, df Degrees of freedom.

Table S7. ANOVA for carbohydrates content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with a mixture of 5 μM 1-Naphthaleneacetic acid (NAA) and 10 μM indole-3-butyric acid (IBA) according to 6-benzyladenine (BA) concentrations and light treatments.

Carbohydrates	df	Source					
		BA concentrations (B)		Light treatments (L)		B x L	
		***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Fructose	1	0.04	>0.05 $^{n.s}$	0.90	>0.05 $^{n.s}$	0.002	>0.05 $^{n.s}$
Galactose	1	1.41	>0.05 $^{n.s}$	0.83	>0.05 $^{n.s}$	2.65	>0.05 $^{n.s}$
Glucose	1	0.16	>0.05 $^{n.s}$	0.94	>0.05 $^{n.s}$	0.14	>0.05 $^{n.s}$
Mannitol	1	0.66	>0.05 $^{n.s}$	0.04	>0.05 $^{n.s}$	0.71	>0.05 $^{n.s}$
Sucrose	1	1.99	>0.05 $^{n.s}$	1.19	>0.05 $^{n.s}$	0.009	>0.05 $^{n.s}$

$^{n.s}$ Non-significant at *p*<0.05, *Significant differences at *p*<0.05, df Degrees of freedom.

Table S8. ANOVA for carbohydrates content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with a mixture of 5 μM 1-Naphthaleneacetic acid (NAA) and 10 μM indole-3-butyric acid (IBA) according to 6-benzyladenine (BA) and sucrose concentrations.

Carbohydrates	df	Source					
		BA concentrations (S)		Sucrose concentrations (B)		B x S	
		***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Fructose	1	0.07	>0.05 $^{n.s}$	14.04	≤0.05*	0.01	>0.05 $^{n.s}$
Galactose	1	1.39	>0.05 $^{n.s}$	1.90	>0.05 $^{n.s}$	1.24	>0.05 $^{n.s}$
Glucose	1	0.25	>0.05 $^{n.s}$	13.73	≤0.05*	0.01	>0.05 $^{n.s}$
Mannitol	1	0.76	>0.05 $^{n.s}$	3.61	>0.05 $^{n.s}$	0.20	>0.05 $^{n.s}$
Sucrose	1	4.08	>0.05 $^{n.s}$	23.42	≤0.05*	1.05	>0.05 $^{n.s}$

$^{n.s}$ Non-significant at *p*<0.05, *Significant differences at *p*<0.05, df Degrees of freedom.

Table S9. ANOVA for carbohydrates content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with a mixture of 5 μM 1-Naphthaleneacetic acid (NAA) and 10 μM indole-3-butyric acid (IBA) according to light treatments and sucrose concentrations.

Carbohydrates	df	Source					
		Light treatment (L)		Sucrose concentrations (S)		L x S	
		***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Fructose	1	1.39	>0.05 n.s	13.71	≤0.05*	0.59	>0.05 n.s
Galactose	1	0.54	>0.05 n.s	2.24	>0.05 n.s	0.97	>0.05 n.s
Glucose	1	1.31	>0.05 n.s	12.86	≤0.05*	0.06	>0.05 n.s
Mannitol	1	0.10	>0.05 n.s	3.49	>0.05 n.s	0.08	>0.05 n.s
Sucrose	1	3.37	>0.05 n.s	9.83	≤0.05*	2.50	>0.05 n.s

n.s Non-significant at $p<0.05$, *Significant differences at $p<0.05$, df Degrees of freedom.

Table S10. ANOVA for carbohydrates content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with 2 gL^{-1} activated charcoal, according to 6-benzyladenine (BA) concentrations and light treatments.

Carbohydrates	df	Source					
		BA concentrations (B)		Light treatments(L)		B x L	
		***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Fructose	1	0.23	>0.05 n.s	0.51	>0.05 n.s	0.68	>0.05 n.s
Galactose	1	0.64	>0.05 n.s	1.09	>0.05 n.s	0.22	>0.05 n.s
Glucose	1	1.22	>0.05 n.s	0.95	>0.05 n.s	1.28	>0.05 n.s
Mannitol	1	0.43	>0.05 n.s	0.00	>0.05 n.s	2.96	>0.05 n.s
Sucrose	1	2.19	>0.05 n.s	0.82	>0.05 n.s	0.88	>0.05 n.s

n.s Non-significant at $p<0.05$, *Significant differences at $p<0.05$, df Degrees of freedom.

Table S11. ANOVA for carbohydrates content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with 2 gL^{-1} activated charcoal, according to 6-benzyladenine (BA) concentrations and sucrose .

Carbohydrates	df	Source					
		BA concentrations (B)		Sucrose concentrations (S)		B x S	
		***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Fructose	1	0.24	>0.05 $^{n.s}$	1.71	>0.05 $^{n.s}$	0.005	>0.05 $^{n.s}$
Galactose	1	0.61	>0.05 $^{n.s}$	0.16	>0.05 $^{n.s}$	0.10	>0.05 $^{n.s}$
Glucose	1	1.17	>0.05 $^{n.s}$	0.56	>0.05 $^{n.s}$	0.80	>0.05 $^{n.s}$
Mannitol	1	0.39	>0.05 $^{n.s}$	0.28	>0.05 $^{n.s}$	0.64	>0.05 $^{n.s}$
Sucrose	1	2.16	>0.05 $^{n.s}$	0.83	>0.05 $^{n.s}$	0.56	>0.05 $^{n.s}$

$^{n.s}$ Non-significant at $p<0.05$, *Significant differences at $p<0.05$, df Degrees of freedom.

Table S12. ANOVA for carbohydrates content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with 2 gL^{-1} activated charcoal, according to light treatments and sucrose concentrations.

Carbohydrates	df	Source					
		Light treatments (L)		Sucrose concentrations (S)		L x C	
		F Test	*p*-Value	*F* Test	*p*-Value	*F* Test	*p*-Value
Fructose	1	0.65	>0.05 $^{n.s}$	2.26	>0.05 $^{n.s}$	0.006	>0.05 $^{n.s}$
Galactose	1	0.79	>0.05 $^{n.s}$	0.13	>0.05 $^{n.s}$	0.01	>0.05 $^{n.s}$
Glucose	1	1.23	>0.05 $^{n.s}$	1.01	>0.05 $^{n.s}$	0.41	>0.05 $^{n.s}$
Mannitol	1	0.006	>0.05 $^{n.s}$	0.20	>0.05 $^{n.s}$	0.19	>0.05 $^{n.s}$
Sucrose	1	1.27	>0.05 $^{n.s}$	1.48	>0.05 $^{n.s}$	1.36	>0.05 $^{n.s}$

$^{n.s}$ Non-significant at $p<0.05$, *Significant differences at $p<0.05$, df Degrees of freedom.

Table S13. ANOVA for amino acid content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with a mixture of 5 μM 1-Naphthaleneacetic acid (NAA) and 10 μM indole-3-butyric acid (IBA) according to 6-benzyladenine (BA) concentrations and light treatments.

		Source					
		BA concentrations (B)		Light treatments (L)		B x L	
Free amino acid	df	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Alanine	1	0.09	>0.05 [n.s]	0.19	>0.05 [n.s]	0.32	>0.05 [n.s]
Arginine	1	0.01	>0.05 [n.s]	0.81	>0.05 [n.s]	0.21	>0.05 [n.s]
Asparagine	1	0.81	>0.05 [n.s]	0.51	>0.05 [n.s]	1.50	>0.05 [n.s]
Aspartic acid	1	0.10	>0.05 [n.s]	2.36	>0.05 [n.s]	0.96	>0.05 [n.s]
Cystine	1	0.91	>0.05 [n.s]	2.34	>0.05 [n.s]	0.90	>0.05 [n.s]
Glutamic acid	1	0.28	>0.05 [n.s]	2.10	>0.05 [n.s]	0.15	>0.05 [n.s]
Glutamine	1	0.97	>0.05 [n.s]	0.11	>0.05 [n.s]	0.16	>0.05 [n.s]
Glycine	1	0.006	>0.05 [n.s]	0.86	>0.05 [n.s]	0.17	>0.05 [n.s]
Hydroxyproline	1	0.35	>0.05 [n.s]	0.27	>0.05 [n.s]	1.02	>0.05 [n.s]
Histidine	1	0.56	>0.05 [n.s]	0.24	>0.05 [n.s]	0.03	>0.05 [n.s]
Isoleucine	1	1.36	>0.05 [n.s]	0.02	>0.05 [n.s]	0.35	>0.05 [n.s]
Leucine	1	1.57	>0.05 [n.s]	0.06	>0.05 [n.s]	0.18	>0.05 [n.s]
Lysine	1	0.40	>0.05 [n.s]	0.001	>0.05 [n.s]	0.08	>0.05 [n.s]
Methionine	1	1.05	>0.05 [n.s]	0.10	>0.05 [n.s]	1.52	>0.05 [n.s]
Phenylalanine	1	2.99	>0.05 [n.s]	0.013	>0.05 [n.s]	1.35	>0.05 [n.s]
Proline	1	0.06	>0.05 [n.s]	0.82	>0.05 [n.s]	0.01	>0.05 [n.s]
Serine	1	1.28	>0.05 [n.s]	0.74	>0.05 [n.s]	1.04	>0.05 [n.s]
Threonine	1	1.64	>0.05 [n.s]	1.25	>0.05 [n.s]	0.34	>0.05 [n.s]
Tryptophan	1	1.14	>0.05 [n.s]	0.13	>0.05 [n.s]	0.22	>0.05 [n.s]
Tyrosine	1	3.53	>0.05 [n.s]	0.002	>0.05 [n.s]	0.72	>0.05 [n.s]
Valine	1	2.23	>0.05 [n.s]	0.67	>0.05 [n.s]	0.27	>0.05 [n.s]

[n.s] Non-significant at $p<0.05$, df Degrees of freedom.

Table S14. ANOVA for amino acid content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with a mixture of 5 µM 1-Naphthaleneacetic acid (NAA) and 10 µM indole-3-butyric acid (IBA) according to 6-benzyladenine (BA) and sucrose concentrations.

Free amino acid	df	Source					
		BA concentrations (B)		Sucrose concentrations (S)		B x S	
		***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Alanine	1	0.09	>0.05 [n.s]	1.23	>0.05 [n.s]	0.77	>0.05 [n.s]
Arginine	1	0.02	>0.05 [n.s]	1.20	>0.05 [n.s]	2.03	>0.05 [n.s]
Asparagine	1	0.84	>0.05 [n.s]	2.93	>0.05 [n.s]	0.03	>0.05 [n.s]
Aspartic acid	1	0.08	>0.05 [n.s]	0.001	>0.05 [n.s]	0.00	>0.05 [n.s]
Cystine	1	0.82	>0.05 [n.s]	0.01	>0.05 [n.s]	0.69	>0.05 [n.s]
Glutamic acid	1	0.27	>0.05 [n.s]	0.95	>0.05 [n.s]	1.09	>0.05 [n.s]
Glutamine	1	0.98	>0.05 [n.s]	0.00	>0.05 [n.s]	0.52	>0.05 [n.s]
Glycine	1	0.006	>0.05 [n.s]	1.38	>0.05 [n.s]	0.45	>0.05 [n.s]
Hydroxyproline	1	0.35	>0.05 [n.s]	0.001	>0.05 [n.s]	1.31	>0.05 [n.s]
Histidine	1	0.57	>0.05 [n.s]	0.10	>0.05 [n.s]	0.63	>0.05 [n.s]
Isoleucine	1	1.42	>0.05 [n.s]	0.23	>0.05 [n.s]	1.12	>0.05 [n.s]
Leucine	1	1.67	>0.05 [n.s]	0.68	>0.05 [n.s]	1.01	>0.05 [n.s]
Lysine	1	0.46	>0.05 [n.s]	0.70	>0.05 [n.s]	2.85	>0.05 [n.s]
Methionine	1	0.94	>0.05 [n.s]	0.13	>0.05 [n.s]	0.01	>0.05 [n.s]
Phenylalanine	1	3.22	>0.05 [n.s]	0.16	>0.05 [n.s]	2.95	>0.05 [n.s]
Proline	1	0.06	>0.05 [n.s]	0.28	>0.05 [n.s]	0.58	>0.05 [n.s]
Serine	1	1.32	>0.05 [n.s]	0.97	>0.05 [n.s]	1.60	>0.05 [n.s]
Threonine	1	1.59	>0.05 [n.s]	0.40	>0.05 [n.s]	0.47	>0.05 [n.s]
Tryptophan	1	1.18	>0.05 [n.s]	0.04	>0.05 [n.s]	1.13	>0.05 [n.s]
Tyrosine	1	3.73	>0.05 [n.s]	0.00	>0.05 [n.s]	2.04	>0.05 [n.s]
Valine	1	2.26	>0.05 [n.s]	1.04	>0.05 [n.s]	0.28	>0.05 [n.s]

[n.s] Non-significant at p<0.05, df Degrees of freedom.

Table S15. ANOVA for amino acid content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with a mixture of 5 µM 1-Naphthaleneacetic acid (NAA) and 10 µM indole-3-butyric acid (IBA) according to light treatments and sucrose concentrations.

		Source					
		Light treatments (L)		Sucrose concentrations		L x S	
Free amino acid	df	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Alanine	1	0.18	>0.05 n.s	1.41	>0.05 n.s	0.50	>0.05 n.s
Arginine	1	0.85	>0.05 n.s	0.86	>0.05 n.s	0.00	>0.05 n.s
Asparagine	1	0.68	>0.05 n.s	2.62	>0.05 n.s	0.99	>0.05 n.s
Aspartic acid	1	2.14	>0.05 n.s	0.06	>0.05 n.s	0.01	>0.05 n.s
Cystine	1	1.86	>0.05 n.s	0.007	>0.05 n.s	0.00	>0.05 n.s
Glutamic acid	1	2.31	>0.05 n.s	0.60	>0.05 n.s	0.53	>0.05 n.s
Glutamine	1	0.05	>0.05 n.s	0.002	>0.05 n.s	0.38	>0.05 n.s
Glycine	1	0.89	>0.05 n.s	1.11	>0.05 n.s	0.42	>0.05 n.s
Hydroxyproline	1	0.34	>0.05 n.s	0.01	>0.05 n.s	1.91	>0.05 n.s
Histidine	1	0.17	>0.05 n.s	0.14	>0.05 n.s	0.000	>0.05 n.s
Isoleucine	1	0.06	>0.05 n.s	0.18	>0.05 n.s	0.77	>0.05 n.s
Leucine	1	0.01	>0.05 n.s	0.60	>0.05 n.s	0.54	>0.05 n.s
Lysine	1	0.009	>0.05 n.s	0.60	>0.05 n.s	0.000	>0.05 n.s
Methionine	1	0.15	>0.05 n.s	0.18	>0.05 n.s	0.15	>0.05 n.s
Phenylalanine	1	0.003	>0.05 n.s	0.14	>0.05 n.s	0.000	>0.05 n.s
Proline	1	0.82	>0.05 n.s	0.17	>0.05 n.s	1.30	>0.05 n.s
Serine	1	0.91	>0.05 n.s	0.67	>0.05 n.s	0.74	>0.05 n.s
Threonine	1	0.93	>0.05 n.s	0.23	>0.05 n.s	1.002	>0.05 n.s
Tryptophan	1	0.21	>0.05 n.s	0.08	>0.05 n.s	0.22	>0.05 n.s
Tyrosine	1	0.01	>0.05 n.s	0.00	>0.05 n.s	0.83	>0.05 n.s
Valine	1	0.43	>0.05 n.s	0.82	>0.05 n.s	1.13	>0.05 n.s

n.s Non-significant at $p<0.05$, df Degrees of freedom.

Table S16. ANOVA amino acid content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with 2 gL^{-1} activated charcoal according to 6-benzyladenine (BA) concentrations and light treatments.

		Source					
		BA concentrations (B)		Light treatments (L)		B x L	
Free amino acid	df	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Alanine	1	1.45	>0.05 n.s	0.09	>0.05 n.s	1.36	>0.05 n.s
Arginine	1	0.01	>0.05 n.s	1.31	>0.05 n.s	1.64	>0.05 n.s
Asparagine	1	0.48	>0.05 n.s	0.82	>0.05 n.s	0.44	>0.05 n.s
Aspartic acid	1	0.21	>0.05 n.s	0.21	>0.05 n.s	0.004	>0.05 n.s
Cystine	1	0.11	>0.05 n.s	0.04	>0.05 n.s	0.54	>0.05 n.s
Glutamic acid	1	0.49	>0.05 n.s	0.50	>0.05 n.s	4.10	>0.05 n.s
Glutamine	1	1.04	>0.05 n.s	0.49	>0.05 n.s	0.30	>0.05 n.s
Glycine	1	0.29	>0.05 n.s	0.04	>0.05 n.s	0.01	>0.05 n.s
Hydroxyproline	1	2.66	>0.05 n.s	0.05	>0.05 n.s	0.03	>0.05 n.s
Histidine	1	0.03	>0.05 n.s	0.02	>0.05 n.s	1.12	>0.05 n.s
Isoleucine	1	0.01	>0.05 n.s	0.97	>0.05 n.s	0.006	>0.05 n.s
Leucine	1	0.10	>0.05 n.s	0.66	>0.05 n.s	0.02	>0.05 n.s
Lysine	1	1.55	>0.05 n.s	0.53	>0.05 n.s	1.38	>0.05 n.s
Methionine	1	0.77	>0.05 n.s	0.18	>0.05 n.s	0.006	>0.05 n.s
Phenylalanine	1	0.46	>0.05 n.s	1.43	>0.05 n.s	0.01	>0.05 n.s
Proline	1	0.001	>0.05 n.s	0.31	>0.05 n.s	0.006	>0.05 n.s
Serine	1	0.01	>0.05 n.s	1.03	>0.05 n.s	0.01	>0.05 n.s
Threonine	1	0.23	>0.05 n.s	0.02	>0.05 n.s	0.06	>0.05 n.s
Tryptophan	1	2.06	>0.05 n.s	0.47	>0.05 n.s	0.18	>0.05 n.s
Tyrosine	1	0.10	>0.05 n.s	0.24	>0.05 n.s	1.21	>0.05 n.s
Valine	1	0.46	>0.05 n.s	0.37	>0.05 n.s	0.15	>0.05 n.s

n.s Non-significant at $p<0.05$, df Degrees of freedom.

Table S17. ANOVA for amino acid content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with 2 gL^{-1} activated charcoal according to 6-benzyladenine (BA) and sucrose concentrations.

Free amino acid	df	Source					
		BA concentrations (B)		Sucrose concentrations (S)		B x S	
		***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Alanine	1	1.52	>0.05 $^{n.s}$	1.37	>0.05 $^{n.s}$	0.98	>0.05 $^{n.s}$
Arginine	1	0.01	>0.05 $^{n.s}$	0.08	>0.05 $^{n.s}$	2.95	>0.05 $^{n.s}$
Asparagine	1	0.48	>0.05 $^{n.s}$	0.88	>0.05 $^{n.s}$	0.43	>0.05 $^{n.s}$
Aspartic acid	1	0.22	>0.05 $^{n.s}$	0.99	>0.05 $^{n.s}$	0.12	>0.05 $^{n.s}$
Cystine	1	0.11	>0.05 $^{n.s}$	1.53	>0.05 $^{n.s}$	0.29	>0.05 $^{n.s}$
Glutamic acid	1	0.44	>0.05 $^{n.s}$	2.03	>0.05 $^{n.s}$	0.47	>0.05 $^{n.s}$
Glutamine	1	1.01	>0.05 $^{n.s}$	0.11	>0.05 $^{n.s}$	0.001	>0.05 $^{n.s}$
Glycine	1	0.30	>0.05 $^{n.s}$	0.76	>0.05 $^{n.s}$	0.17	>0.05 $^{n.s}$
Hydroxyproline	1	5.28	≤0.05*	2.38	>0.05 $^{n.s}$	9.10	≤0.05*
Histidine	1	0.04	>0.05 $^{n.s}$	2.22	>0.05 $^{n.s}$	0.41	>0.05 $^{n.s}$
Isoleucine	1	0.01	>0.05 $^{n.s}$	2.50	>0.05 $^{n.s}$	0.08	>0.05 $^{n.s}$
Leucine	1	0.10	>0.05 $^{n.s}$	0.82	>0.05 $^{n.s}$	0.02	>0.05 $^{n.s}$
Lysine	1	1.62	>0.05 $^{n.s}$	0.05	>0.05 $^{n.s}$	2.86	>0.05 $^{n.s}$
Methionine	1	0.77	>0.05 $^{n.s}$	0.06	>0.05 $^{n.s}$	0.03	>0.05 $^{n.s}$
Phenylalanine	1	0.44	>0.05 $^{n.s}$	0.16	>0.05 $^{n.s}$	0.74	>0.05 $^{n.s}$
Proline	1	0.002	>0.05 $^{n.s}$	3.03	>0.05 $^{n.s}$	0.59	>0.05 $^{n.s}$
Serine	1	0.01	>0.05 $^{n.s}$	3.67	>0.05 $^{n.s}$	0.11	>0.05 $^{n.s}$
Threonine	1	0.31	*>0.05 $^{n.s}$	6.09	≤0.05*	0.34	>0.05 $^{n.s}$
Tryptophan	1	2.30	>0.05 $^{n.s}$	0.16	>0.05 $^{n.s}$	2.80	>0.05 $^{n.s}$
Tyrosine	1	0.13	>0.05 $^{n.s}$	7.86	≤0.05*	0.75	>0.05 $^{n.s}$
Valine	1	0.48	>0.05 $^{n.s}$	1.10	>0.05 $^{n.s}$	0.19	>0.05 $^{n.s}$

*Significant differences at $p<0.05$, $^{n.s}$ Non-significant at $p<0.05$, df Degrees of freedom.

Table S18. ANOVA amino acid content of *Pinus radiata* D. Don cultured in LP medium (Quoirin and Lepoivre, 1977) modified by Aitken-Christie et al., (1988) supplemented with 2 gL^{-1} activated charcoal according to light treatments and sucrose concentrations.

Free amino acid	df	Source					
		Light treatments (L)		Sucrose concentrations (S)		L x S	
		***F* Test**	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Alanine	1	0.41	>0.05 $^{n.s}$	1.09	>0.05 $^{n.s}$	0.53	>0.05 $^{n.s}$
Arginine	1	1.31	>0.05 $^{n.s}$	0.21	>0.05 $^{n.s}$	1.21	>0.05 $^{n.s}$
Asparagine	1	1.03	>0.05 $^{n.s}$	0.57	>0.05 $^{n.s}$	0.68	>0.05 $^{n.s}$
Aspartic acid	1	0.42	>0.05 $^{n.s}$	1.15	>0.05 $^{n.s}$	9.30	≤0.05 *
Cystine	1	0.07	>0.05 $^{n.s}$	1.38	>0.05 $^{n.s}$	0.43	>0.05 $^{n.s}$
Glutamic acid	1	0.57	>0.05 $^{n.s}$	1.55	>0.05 $^{n.s}$	0.07	>0.05 $^{n.s}$
Glutamine	1	0.13	>0.05 $^{n.s}$	1.24	>0.05 $^{n.s}$	0.72	>0.05 $^{n.s}$
Glycine	1	0.01	>0.05 $^{n.s}$	0.87	>0.05 $^{n.s}$	1.58	>0.05 $^{n.s}$
Hydroxyproline	1	0.23	>0.05 $^{n.s}$	2.23	>0.05 $^{n.s}$	0.00	>0.05 $^{n.s}$
Histidine	1	0.03	>0.05 $^{n.s}$	2.63	>0.05 $^{n.s}$	2.24	>0.05 $^{n.s}$
Isoleucine	1	1.45	>0.05 $^{n.s}$	2.81	>0.05 $^{n.s}$	6.16	≤0.05 *
Leucine	1	0.71	>0.05 $^{n.s}$	0.87	>0.05 $^{n.s}$	4.34	>0.05
Lysine	1	0.77	>0.05 $^{n.s}$	0.00	>0.05 $^{n.s}$	1.28	>0.05 $^{n.s}$
Methionine	1	0.30	>0.05 $^{n.s}$	0.01	>0.05 $^{n.s}$	0.74	>0.05 $^{n.s}$
Phenylalanine	1	1.56	>0.05 $^{n.s}$	0.14	>0.05 $^{n.s}$	7.22	≤0.05 *
Proline	1	0.40	>0.05 $^{n.s}$	3.05	>0.05 $^{n.s}$	2.57	>0.05 $^{n.s}$
Serine	1	1.38	>0.05 $^{n.s}$	4.06	>0.05 $^{n.s}$	4.29	≤0.05 $^{n.s}$
Threonine	1	0.07	>0.05 $^{n.s}$	6.68	≤0.05 *	4.18	≤0.05 *
Tryptophan	1	0.74	>0.05 $^{n.s}$	0.01	>0.05 $^{n.s}$	0.11	>0.05 $^{n.s}$
Tyrosine	1	0.40	>0.05 $^{n.s}$	11.88	≤0.05 *	11.31	≤0.05 *
Valine	1	0.29	>0.05 $^{n.s}$	1.22	>0.05 $^{n.s}$	2.59	>0.05 $^{n.s}$

$^{n.s}$ Non-significant at $p<0.05$, *Significant differences at $p<0.05$, df Degrees of freedom.

CHAPTER 3

Adult trees *Cryptomeria japonica* (Thunb. ex L.f.) D. Don Micropropagation: Factors Involved in the Success of the Process

SUPPLEMENTARY MATERIALS CHAPTER 3

Table S1. Variations of basal DCR (Gupta and Durzan, 1985), MS (Murashige and Skoog, 1962) and QL (Quoirin and Lepoivre, 1977) media tested at different stages of *Cryptomeria japonica* micropropagation process, Experiment 1.

Stage	Basal medium	PGRs (µM)	Sucrose (w/v)	AC (g L^{-1})	Agar (g L^{-1})
Induction	DCR	BA (8.8)	3%	-	Difco Agar® granulated (8)
Induction	MS	BA (8.8)	3%	-	Difco Agar® granulated (8)
Induction	QL	BA (8.8)	3%	-	Difco Agar® granulated (8)
Elongation	DCR	-	3%	(2)	Difco Agar® granulated (8.5)
Elongation	MS	-	3%	(2)	Difco Agar® granulated (8.5)
Elongation	QL	-	3%	(2)	Difco Agar® granulated (8.5)
Rooting	Half strength macronutrients QL	NAA (50)	3%	-	Difco Agar® granulated (8)
Rooting	Half strength macronutrients QL	NAA (50)	1.5%	-	Difco Agar® granulated (8)
Root expression	Half strength macronutrients QL	-	3%	(2)	Difco Agar® granulated (8.5)
Root expression	Half strength macronutrients QL	-	1.5%	(2)	Difco Agar® granulated (8.5)

PGRs, plant growth regulators; BA,6-benzyladenine; NAA, 1-naphthalenacetic acid; AC, activated charcoal.

Table S2. Variations of basal QL (Quoirin and Lepoivre, 1977) medium tested at different stages of *Cryptomeria japonica* micropropagation process, Experiment 2.

Stage	Basal medium	PGRs (μM)	Sucrose (w/v)	AC (g L^{-1})	Agar (g L^{-1})
Induction	QL	BA (8.8)	3%	-	Difco Agar® granulated (8)
Induction	QL	m-T (8.8)	3%	-	Difco Agar® granulated (8)
Induction	QL	TDZ (8.8)	3%	-	Difco Agar® granulated (8)
Elongation	QL	-	3%	(2)	Difco Agar® granulated (8.5)
Rooting	Half strength macronutrients QL	NAA (50)	1.5%	-	Difco Agar® granulated (8)
Root expression	Half strength macronutrients QL	-	1.5%	(2)	Difco Agar® granulated (8.5)

PGRs, plant growth regulators; BA,6-benzyladenine; m-T, meta-topolin; thidiazuron, TDZ; NAA, 1-naphthalenacetic acid; AC, activated charcoal.

Table S3. Statistical analysis for the survival (%) showed in *Cryptomeria japonica* per explant type (apical explants of >1.5 cm in length, apical explants of <1.0 cm length, basal explants of >1.5 cm length, and basal explants of <1.0 cm in length) and basal media (DCR [Gupta and Durzan, 1985], MS [Murashige and Skoog, 1962] and QL [Quoirin and Lepoivre, 1977]).

Source	df	Survival (%)	
		X^2 Test	***p*-Value**
Explant type (E)	3	492.11	<0.05*
Basal media (B)	2	490.60	≥0.05 $^{n.s}$
B x C	6	490.60	≥0.05 $^{n.s}$

*Significantly different at p<0.05, $^{n.s}$ Non-significant at p<0.05, df Degrees of freedom.

Table S4. Statistical analysis for shoot induction (%) and number of shoots per explant showed in *Cryptomeria japonica* per explant type (apical explants of >1.5 cm in length, apical explants of <1.0 cm length, basal explants of >1.5 cm length, and basal explants of <1.0 cm in length) and basal media (DCR [Gupta and Durzan, 1985], MS [Murashige and Skoog, 1962] and QL [Quoirin and Lepoivre, 1977]).

Source	df	Shoot induction (%)		Nº shoots /explant	
		X^2 Test	***p*-Value**	***F* Test**	***p*-Value**
Explant type (E)	3	266.95	<0.05*	4.31	<0.05*
Basal media (B)	2	266.00	≥0.05 n.s	9.09	<0.05*
E x B	6	256.35	≥0.05 n.s	2.59	<0.05*

*Significantly different at p<0.05, n.s Non-significant at p<0.05, df Degrees of freedom.

Table S5. Statistical analysis for root induction (%), number roots per explant and length of longest of *Cryptomeria japonica* shoots cultured in QL medium [Quoirin and Lepoivre, 1977]), supplemented with 3% (w/v) sucrose or 1.5% (w/v) sucrose, according to light treatment.

Source	df	Root induction (%)		Nº roots /explant		Length of longest root	
		X^2 Test	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Sucrose concentration (S)	1	152.78	≥0.05 n.s	1.38	<0.05 n.s	4.13	<0.05*
Light treatment (L)	1	149.44	≥0.05 n.s	22.70	<0.05*	4.8	<0.05*
S x L	1	149.44	≥0.05 n.s	0.01	≥0.05 n.s	0.30	≥0.05 n.s

*Significantly different at p<0.05, n.s Non-significant at p<0.05, df Degrees of freedom.

Table S6. Statistical analysis for the survival (%) of rooted shoots propagated *in vitro* coming from *Cryptomeria japonica* adult trees, after four weeks under *ex vitro* conditions.

Source	df	Survival (%)	
		X^2 Test	***p*-Value**
Sucrose concentration (S)	1	67.94	≥0.05 [n.s]
Light treatment (L)	1	67.07	≥0.05 [n.s]
S x L	1	60.17	$<0.05^*$

*Significantly different at $p<0.05$, [n.s] Non-significant at $p<0.05$, df Degrees of freedom.

Table S7. Statistical analysis for the survival (%) showed in *Cryptomeria japonica* explants (basal explants of >1.5 cm length) cultured in QL medium [Quoirin and Lepoivre, 1977]), supplemented with 6-benzyladenine (BA), meta-Topolin (m-T) and thidiazuron (TDZ) (8.8 µM).

Source	df	Survival (%)	
		X^2 Test	***p*-Value**
Cytokinin type	2	703.95	≥0.05 [n.s]

[n.s] Non-significant at $p<0.05$, df Degrees of freedom.

Table S8. Statistical analysis for shoot induction (%) and number of shoots per explant showed in *Cryptomeria japonica* explants (basal explants of >1.5 cm length) cultured in QL medium [Quoirin and Lepoivre, 1977]), supplemented with 6-benzyladenine (BA), meta-Topolin (m-T) and thidiazuron (TDZ) (8.8 µM).

Source	df	Shoot induction (%)		Nº shoots /explant	
		X^2 Test	***p*-Value**	***F* Test**	***p*-Value**
Cytokinin type (E)	1	405.51	$<0.05^*$	0.19	≥0.05 [n.s]

*Significantly different at $p<0.05$, [n.s] Non-significant at $p<0.05$, df Degrees of freedom.

Table S9. Statistical analysis for root induction (%), number root per explant and length of longest root showed in *Cryptomeria japonica* explants (basal explants of >1.5 cm length) cultured in QL medium [Quoirin and Lepoivre, 1977]), supplemented with 50 μM 1-naphthaleneacetic acid (NAA), according to cytokinin type (6-benzyladenine (BA), meta-Topolin (m-T) and Thidiazuron (TDZ)) (8.8 μM).

Source	df	Root induction		Nº root /explant		Length of longest root	
		X^2 Test	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Cytokinin type	1	118.52	<0.05*	0.70	≥0.05 n.s	0.49	≥0.05 n.s

*Significantly different at $p<0.05$, n.s Non-significant at $p<0.05$, df Degrees of freedom.

Table S10. Statistical analysis for the survival (%) showed in *Cryptomeria japonica* explants (basal explants of >1.5 cm length) cultured in QL medium [Quoirin and Lepoivre, 1977]), supplemented with 50 μM 1-naphthaleneacetic acid (NAA), according to cytokinin type (6-benzyladenine (BA) and meta-Topolin (m-T) (8.8 μM)).

Source	df	Survival (%)	
		***X^2* Test**	***p*-Value**
Cytokinin type	1	14.53	≥0.05 n.s

n.s Non-significant at $p<0.05$, df Degrees of freedom.

CHAPTER 4

Testing Explant Sources, Culture Media, and Light Conditions for the Improvement of Organogenesis in *Pinus ponder*osa (P. Lawson and C. Lawson)

SUPPLEMENTARY MATERIALS CHAPTER 4

Table S1. Statistical analysis for the survival (%) showed in explants *of Pinus ponderosa* per 6-benzyladenine (BA) (4.4, 22 and 44 μM) and cultured media (LP or half LP macronutrients) (Quoirin and Lepoivre, 1977), modified by Aitken-Christie et al., (1988).

Source	df	Survival (%)	
		X^2 Test	***p*-Value**
BA concentration (B)	2	600.83	≤0.05*
Culture medium (C)	1	608.99.	>0.05 n.s
B x C	1	597.87	>0.05 n.s

*Significantly different at $p<0.05$, n.s Non-significant at $p<0.05$, df Degrees of freedom.

Table S2. Statistical analysis for the survival (%) showed in explants of *Pinus ponderosa* per explant type (cotyledons or whole zygotic embryos) and sterilization protocol [5% commercial bleach and 10% H_2O_2 (v/v)].

Source	df	Survival (%)	
		X^2 Test	***p*-Value**
Explant type (E)	1	596.21	≤0.05*
Sterilization protocol (S)	1	593.11	>0.05 n.s
E x S	1	592.96	>0.05 n.s

*Significantly different at $p<0.05$, n.s Non-significant at $p<0.05$, df Degrees of freedom.

Table S3. Statistical analysis for the explants forming shoots (EFS) (%) showed in explants of *Pinus ponderosa* per 6-benzyladenine (BA) (4.4, 22 and 44 μM), culture media (LP or half LP macronutrients) (Quoirin and Lepoivre, 1977), modified by Aitken-Christie et al., (1988), explant type (cotyledons or whole zygotic embryos) and sterilization protocol [5% commercial bleach and 10% H_2O_2 (v/v)].

Source	df	EFS (%)	
		X^2 Test	***p*-Value**
BA concentration (B)	2	496.36	>0.05 n.s
Cultured media (C)	1	498.04	≤0.05*
Explant type (E)	1	534.93	≤0.05*
Sterilization protocol (S)	1	534.93	>0.05 n.s
E x S	1	496.36	>0.05 n.s
E x C	1	454.59	≤0.05*
C x S	1	453.84	>0.05 n.s
B x E	2	453.54	>0.05 n.s
B x S	2	452.56	>0.05 n.s
B x C	2	452.18	>0.05 n.s
E x C x S	1	448.81	>0.05 n.s
B x E x S	2	448.19	>0.05 n.s
B x C x E	2	443.74	>0.05 n.s
B x C x S	2	441.34	>0.05 n.s
B x C x E x S	2	436.19	>0.05 n.s

*Significantly different at $p<0.05$, n.s Non-significant at $p<0.05$, df Degrees of freedom.

Table S4. Statistical analysis for the survival (%) showed in explants of *Pinus ponderosa* per cytokinin type and light treatment cultured in half LP macronutrients (Quoirin and Lepoivre, 1977), modified by Aitken-Christie et al., (1988).

Source	df	Survival (%)	
		X^2 Test	***p*-Value**
Cytokinin type (C)	1	29.206	≤0.05*
Light treatment (C)	3	21.311	≤0.05*
C x L	3	21.311	>0.05 n.s

*Significantly different at $p<0.05$, n.s Non-significant at $p<0.05$, df Degrees of freedom.

Table S5. Statistical analysis for the explants forming shoots (EFS) (%) showed in explants of *Pinus ponderosa* cultured in half LP macronutrients (Quoirin and Lepoivre, 1977), modified by Aitken-Christie et al., (1988) supplemented with 6-benzyladenine (BA) and *meta*-Topolin (*m*-T) according to light treatment.

Source	df	EFS (%)	
		X^2 Test	***p*-Value**
Cytokinin type (C)	1	393.51	>0.05 n.s
Light treatment (C)	3	392.40	>0.05 n.s
C x L	3	391.23	>0.05 n.s

n.s Non-significant at p<0.05, df Degrees of freedom.

Table S6. ANOVA for total number of shoots produced per initial explant (NS/E) showed in explants of *Pinus ponderosa* cultured in half LP macronutrients (Quoirin and Lepoivre, 1977), modified by Aitken-Christie et al., (1988) supplemented with 6-benzyladenine (BA) and *meta*-Topolin (*m*-T) (13.1 μM) according to light treatment.

Source	df	NS/E	
		***F* Test**	***p*-Value**
Cytokinin type (C)	1	0.31	>0.05 n.s
Light treatment (C)	3	1.00	>0.05 n.s
C x L	3	1.07	>0.05 n.s

*Significantly different at p<0.05, n.s Non-significant at p<0.05, df Degrees of freedom.

Table S7. ANOVA for percentage of shoots elongated enough to be rooted (PSR) (%) showed in explants of *Pinus ponderosa* cultured in half LP macronutrients (Quoirin and Lepoivre, 1977), modified by Aitken-Christie et al., (1988) supplemented with 6-benzyladenine (BA) and *meta*-Topolin (*m*-T) (13.1 μM) according to light treatment.

Source	df	PSR (%)	
		***F* Test**	***p*-Value**
Cytokinin type (C)	1	0.02	>0.05 [n.s]
Light treatment (C)	3	3.94	≤0.05*
C x L	3	0.21	>0.05 [n.s]

*Significantly different at $p<0.05$, [n.s] Non-significant at $p<0.05$, df Degrees of freedom.

Table S8. Statistical analysis for root induction (%), number root per explant and length of longest root showed in explants of *Pinus ponderosa* cultured in half LP macronutrients (Quoirin and Lepoivre, 1977), modified by Aitken-Christie et al., (1988) according to light treatment.

Source	df	Root induction		Nº root /explant		Length of longest root	
		X^2 Test	***p*-Value**	***F* Test**	***p*-Value**	***F* Test**	***p*-Value**
Light treatment (L)	3	132.79	>0.05 [n.s]	0.66	>0.05 [n.s]	0.42	>0.05 [n.s]

[n.s] Non-significant at $p<0.05$, df Degrees of freedom.

Development of biotechnological tools for the *in vitro* propagation of elite trees for the Basque Country forest

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