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Index

I. Abstract

Since the implementation of the Kyoto Protocol, European governments are urged to increase forest areas trough afforestation/reforestation and to improve the efficiency of forest systems in terms of biomass to avoid or stop processes of desertification. This fact is due to the crucial role played by forests in the response and mitigation of climate change effects along with the maintenance and protection of biodiversity and natural resources. Moreover, increasing of world population followed by a huge demand for tree products and the expected climate warming, accompanied with an increase frequency and magnitude of extreme climate events, make the improvement of tree productivity a critical need.

Somatic embryogenesis that allows the regeneration, under controlled conditions, of a new plant from small explants of tissues, organs or plant cells, is a biotechnological tool with potential for large-scale clonal propagation. In order to establish an efficient regeneration protocol for *Pinus halepensis*, a native Conifer from the Mediterranean area largely used for afforestation/reforestation, the effects of physical and chemical environments at initiation phase of this species SE were tested.

To assess if different temperatures and/or water availability conditions during initiation of embryonal masses affect the rate of initiation, proliferation, and/or maturation, and the number of embryos produced, different concentrations of gellan gum (2, 3, and 4 g L^{-1} Gelrite®) were added to the initiation medium and the explants (megagametophytes containing immature zygotic embryos) were stored at three different temperatures (18, 23, and 28 ºC). It was found that environmental conditions during the initiation stage of *Pinus halepensis* somatic embryogenesis influence the success of initiation and proliferation. Induction of somatic embryogenesis with lower water availability (4 g L^{-1} of gellan gum) increases initiation rates and medium culture at 23 ºC during the initiation stage increases the success of initiation and proliferation. There was no effect of initiation conditions on the maturation rate and somatic embryos could be obtained in all treatments tested, indicating that plants can be obtained from extreme conditions of induction (28 ºC and 4 g L^{-1}). The epigenetic "memory" provoked by the environment were these plants have been initiated may allow them to better resist to global drying and warning predictions, continuing *Pinus halepensis* to be a wide spread species and an effective choice for afforestation.

Keywords: Embryonal Masses, Somatic Embryos, Temperature, Water availability

Abbreviations: Abscisic Acid (ABA), Embryogenic cell lines (ECLs), Embryonal masses (EMs), Embryogenic Tissue (ET), N^6-benzylaminopurine (BAP), Somatic Embryogenesis (SE), Zygotic Embryogenesis (ZE), 2,4-Dichlorophenoxyacetic acid (2,4-D)

II. Resumo

Desde a implementação do Protocolo de Quioto que os governos europeus são instados a aumentar as áreas florestais através de florestamento/reflorestamento e a melhorar a eficiência dos sistemas florestais em termos de biomassa para evitar ou interromper processos de desertificação. Este facto deve-se ao papel crucial desempenhado pelas florestas tanto na resposta e mitigação dos efeitos das alterações climáticas como na manutenção e proteção da biodiversidade e dos recursos naturais. Por outro lado, o aumento da população mundial, seguido por uma enorme demanda por produtos das árvores, e o aquecimento global esperado, acompanhado pelo aumento de frequência e magnitude de eventos climáticos extremos, fazem o aumento da produtividade das árvores uma necessidade crítica.

A embriogénese somática permite a regeneração, sob condições controladas, de uma nova planta a partir de pequenos explantes de tecidos, órgãos ou células vegetais, sendo uma ferramenta biotecnológica com potencial de propagação de clones a grande escala. De modo a estabelecer um protocolo de regeneração eficiente para *Pinus halepensis*, uma conífera nativa da região mediterrânica largamente utilizada para florestamento/reflorestamento, os efeitos dos ambientes físicos e químicos na fase de iniciação da embriogénese somática desta espécie foram testados.

Para avaliar se diferentes temperaturas e/ou disponibilidades de água durante a iniciação das massas embriogénicas afetam as taxas de iniciação, proliferação, e/ou de maturação e o número de embriões produzidos, diferentes concentrações de goma gellan (2, 3, e 4 g L^{-1} de Gelrite®) foram adicionados ao meio de iniciação e os explantes (megagametófitos contendo embriões zigóticos imaturos) foram armazenados a três temperaturas diferentes (18, 23, e 28 ºC). Verificou-se que as condições ambientais durante a fase de iniciação da embriogénese somática de *Pinus halepensis* influenciam o sucesso das fases de iniciação e proliferação: a indução de embriogénese somática a menor disponibilidade de água (4 g L^{-1} de goma gellan) aumenta as taxas de iniciação e o armazenamento do meio de cultura na fase de iniciação a 23 ºC aumenta o sucesso de iniciação e proliferação. Não houve efeito das condições de iniciação sobre a taxa de maturação e os embriões somáticos podem ser obtidos em todos os tratamentos testados, podendo-se, assim, obter plantas a partir de condições extremas de indução (28 ° C e 4 g L^{-1}). A "memória" epigenética provocada pelo ambiente onde estas plantas foram induzidas pode permitir-lhes uma maior resistência ao aquecimento e à seca previstas, permitindo que *Pinus halepensis* continue a ser uma espécie com uma grande distribuição e uma escolha eficaz para a reflorestação.

Palavras-chave: Disponibilidade de Água, Embriões Somáticos, Massas Embriogénicas, Temperatura

Abreviaturas: Ácido Abscísico (ABA), Ácido 2,4-Diclorofenoxiacético (2,4-D), Embriogénese Somática (SE), Embriogénese Zigótica (ZE), Linhas de Células Embriogénicas (ECLs), Massas Embriogénicas (EMs), N^6- benzilaminopurina (BAP), Tecido Embriogénico (ET)

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1. Introduction

1.1. Contextualization of the work

As far as we know, the only study conducted so far in somatic embryogenesis (SE) of *Pinus halepensis* Mill. was carried out by Montalbán et al. (2013). The present work is focused on increasing the knowledge about SE process in this species and it has been developed in NEIKER-TECNALIA, Spain, in collaboration with the Centre for Functional Ecology of the University of Coimbra, Portugal.

NEIKER-TECNALIA is a public agricultural research center, dependent of the Basque Government, in which previous works were performed in order to optimize the process of SE in *Pinus radiata* (Montalbán et al., 2010; 2012; 2015) and *Pinus halepensis* (Montalbán et al., 2013). NEIKER-TECNALIA team has been focused in improving this biotechnological tool to be able to apply it in genetic improve programs, afforestation/reforestation and restoration and conservation of species.

NEIKER-TECNALIA and University of Coimbra started collaborating in 2013 in a project in which the main objective was the study of proteins involved in the success of *Pinus radiata* SE.

1.2. Biotechnological tools: *in vitro* culture

Forests are essential components of natural landscapes that protect land and water resources, maintain biodiversity and help to mitigate the effects of climate change. Moreover, forests provide raw materials, some tree species are used as feedstock for bioenergy production and provide social and environmental benefits, in addition to supplying a wide range of commercial products such as wood, latex and resins among others (Harfouche et al., 2011). European forests are shared by over 10 million owners who, along with countless workers in rural areas and processing industries, depend on the sustainable management of forests for their income. In this regard, since the Kyoto Protocol (www.apambiente.pt) governments are urged both to increase forest areas through afforestation and reforestation processes and to improve the efficiency of forest systems in terms of biomass to avoid or stop processes of desertification. In this context, marginal lands can be used for forestry, thus increasing employment in rural areas and helping to avoid migration to over populated cities and the consequent environmental problems.

World population is increasing at an alarming rate, resulting in a huge demand for tree products such as wood for construction materials, fuel and paper, fruits, oils, medicines, etc.

(Jain and Gupta, 2005). Also, expected climate warming may increase the frequency and magnitude of extreme climatic events that will negatively affect tree growth and vigor (Sánchez-Salguero et al., 2012) and, as consequence, the fate of many forest ecosystems will depend on the relationship between the rate of the climate changes to the rate of adaptation to such events (Klein et al., 2011).

Considering the above mentioned issues, there is an immediate need to improve tree productivity. However, the limited effectiveness of the conventional breeding methods combined with the long life cycles of trees has resulted in poor genetic improvement of most species used in forestry programs (Jain and Gupta, 2005).

Traditional breeding methods and conventional forestry practices have significantly contributed to the improvement of forest tree species in the past and will continue to have a substantial impact; nonetheless, biotechnology is one more tool that utilizes fundamental discoveries in the field of plant tissue culture for clonal forestry, gene transfer techniques, molecular biology and genomics which provides an extended platform for improvement of traits that have previously been considered difficult to manage (Nehra et al., 2005).

In vitro culture generally refers to a set of techniques of plant propagation in which a new plant is regenerated from small portions (called explants) of tissues, organs or plant cells, under aseptic and controlled conditions (Davis and Becwar, 2007). These cultures, maintained in sterile conditions, can be used for various purposes such as plant cloning, secondary metabolite production, genetic transformation, protoplast fusion or haploid production. Any of these different goals requires different culture conditions and different composition of culture media: hence, they have to be adjusted in order to achieve those different objectives (Canhoto, 2010).

In vitro cloning depends of two main factors namely the ability of plant cells to express their totipotency and the culture media, in particular, the presence of plant growth regulators (Montalbán, 2010). As stated by Canhoto (2010) in the course of ontogenesis, the formation of different tissues involves cell division and specialization, a process called differentiation. However, if we proceed to the culture of a fragment from a differentiated plant organ, in optimal conditions, at culture medium we found that certain cells undergo a reverse process, called dedifferentiation, and can return to a meristematic state. Since the pioneer works of Skoog and Miller (1957) that showed that the differentiation of roots and shoots in tobacco pith tissue cultures was a function of the auxin-cytokinin ratio, and that organ differentiation could be regulated by changing the relative concentrations of the two substances in the medium, it is well known that a combination of auxins and cytokinins controls the organogenic behavior of

tissues in culture. Although this general principle applies to a great number of species, the concentrations and types of auxins and cytokinins in the culture media must be optimized for each species or even cultivars or genotypes.

1.3. Somatic embryogenesis

Sexual propagation implies the formation of a new entity, the zygote, which from successive divisions will give different cell types. However, this process may be initiated *in vitro* from cells that have high embryogenic potential, usually cells as zygotic embryos or young leaves or other juvenile organs. More specific cell types such as microspores and protoplasts are also able to undergo embryogenesis (Williams and Maheswaran, 1986). These somatic cells after the perception of an exogenous stimulus, accompanied by the induction of gene transcription required for embryogenesis and metabolism reorganization, enter a dedifferentiation process, that reprogram the genome and unable these cells to further express totipotency allowing the formation of a new sporophyte through embryogenesis (Smertenko and Bozhkov, 2014; Stasolla and Yeung, 2003).

Usually, the cultivation of these explants in a media containing the appropriate balance of plant growth regulators induces the proliferation of embryogenic callus cultures that will later originate somatic embryos. This type of SE, with an intermediary stage of callus formation, is called indirect somatic embryogenesis. By contrast, the formation of somatic embryos without passing through a callus stage is called direct SE (Smertenko and Bozhkov, 2014).

The sequence of events for SE as a morphogenic phenomenon is frequently expressed as discrete phases or steps that are characterized by distinct biochemical and molecular events. The first phase of SE is the induction stage in which differentiated somatic cells acquire embryogenic competence (Zavattieri et al., 2010). This step is followed by the expression or initiation of SE in which competent cells or proembryos start developing. Finally, during maturation, somatic embryos anticipate germination by desiccation and reserve accumulation (Jiménez, 2001). Embryogenic competence is expressed at the level of a single somatic cell that has the ability of differentiating into embryos if it receives inducers of differentiation (Fehér et al., 2003). As reviewed by Zavattieri et al. (2010), there are two main categories of inductive conditions which allow differentiated cells to develop into competent dedifferentiated cells: plant growth regulators (internal and/or external cellular levels) and stress factors such as osmotic shock, starvation, water stress, heavy metal ions, pH, heat or cool shock treatments, hypoxia, antibiotics, ultraviolet radiation, and mechanical or chemical treatments.

Although the first steps of SE may differ from zygotic embryogenesis (ZE), the further stages of embryogenesis are identical in the two types of embryogenesis (Fig. 1).

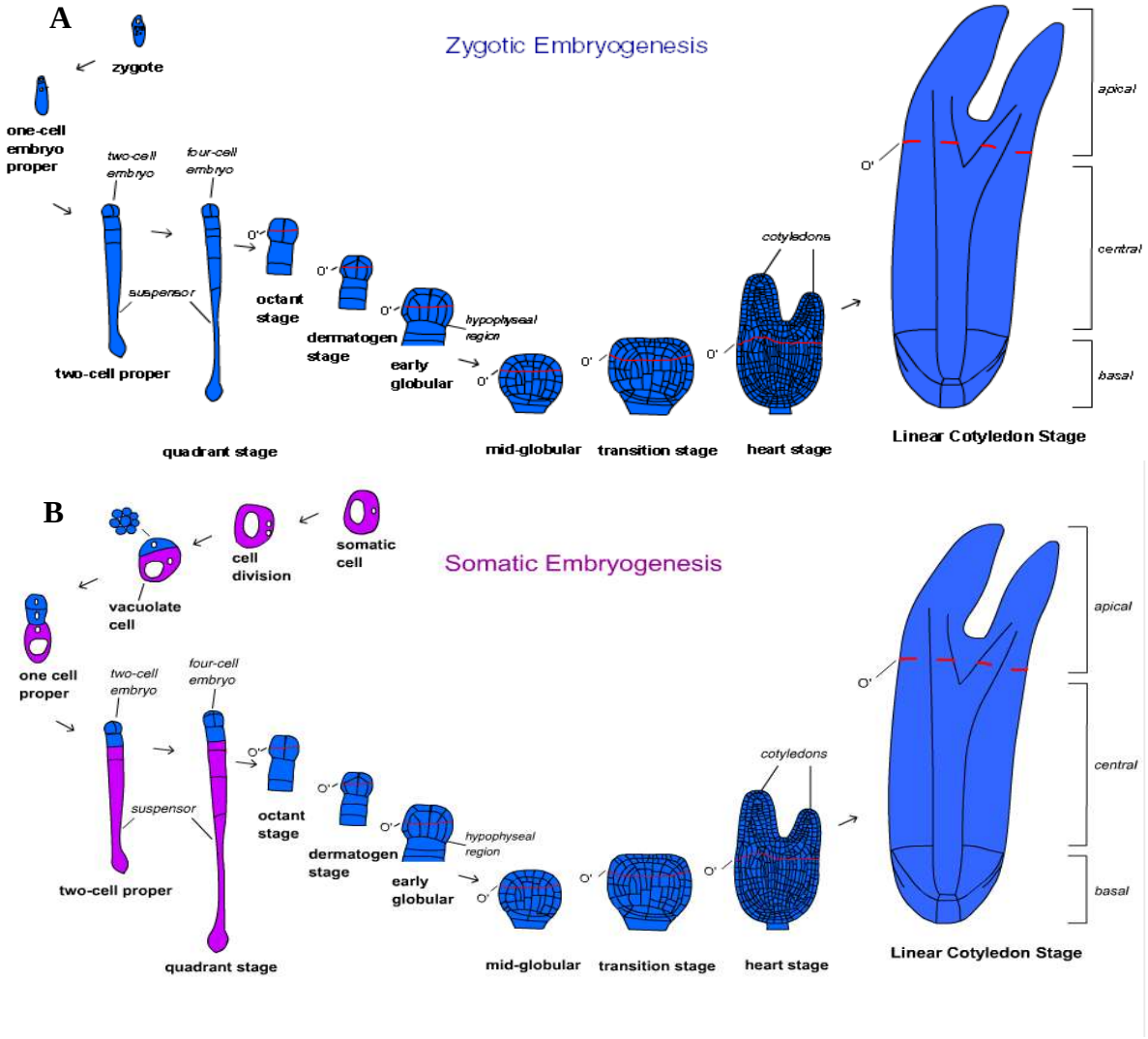

Figure 1: Comparison between zygotic and somatic embryogenesis: **(A)** different steps of ZE; **(B)** different stages of SE, process that begins with a sequence of divisions of a somatic cell followed by the same steps as ZE. (from: passel.unl.edu)

SE brings great advantages and applications to biotechnology presenting a large-scale propagation system for superior clones with high multiplication potential. Molecular tools for tree improvement such as marker-assisted selection, genetic engineering and functional genomics allow the selection of specific genotypes and phenotypes in order to improve the quality and productivity of crops making possible the formation of genetically modified crops (Charity et al., 2005). In addition, cryoconservation may be applied to embryogenic tissue (ET)

in order to preserve his potentiality and the combination of both techniques makes the development of high value forestry achievable (Weng et al., 2010).

1.3.1. Somatic embryogenesis in conifers

The first hints of SE in gymnosperms occurred in the late 1970s and early 1980s with the descriptions of embryo-like structures (Durzan and Chalupa, 1976; Durzan and McClenahan, 1980), but it was only a few years later that mature somatic embryos, able to convert into plantlets, were first generated in a conifer species from immature embryos of *Picea abies* (Chalupa, 1985; Hakman et al., 1985). Since then, the tremendous effort in the development of methods for the induction and maturation of somatic embryos has resulted in a large number of conifer species showing the ability to generate somatic embryos in culture (Stasolla et al., 2002; Montalbán et al., 2010; Montalbán et al., 2013).

Plant propagation through SE in conifers led to a significant interest in establishing efficient regeneration protocols for this group of plants with high economical importance throughout the world. Nonetheless, some difficulties were found such as the long life span of these species, the limited embryogenic competence of adult tissues and recalcitrance, which together impair an effective application of this cloning method (Bonga et al., 2010)

The establishment and improvement of existing protocols in order to adapt the conditions for each species has been the solution used to improve the efficiency of SE. In some species a successful induction of ET only occurs from juvenile tissue such as immature somatic embryos (Silva and Malabadi, 2012).

The choice of the explants used to induce SE is of major importance. In *Pinus* spp., immature embryos at the pre-cotyledonary stage of development or mature cotyledonary embryos are usually more effective (Hargreaves et al., 2009; Montalbán et al., 2012). In addition to whole embryos, cultures of female gametophytes or cotyledon segments have been also utilized (Stasolla and Yeung, 2003). SE has also been achieved from shoot buds of 10 years-old *Picea glauca* trees obtained from somatic embryos (Klimaszewska et al., 2011). In some studies, *in vitro* shoot cultures have been used as a source of explants. These cultures enable better control of the growing conditions of stock material, avoid difficulties associated with possible differences in the physiological state of the explants, and guarantee a supply of an unlimited number of explants independently of the season (Conde et al., 2004; San-José et al., 2010; García-Mendiguren et al., 2015)

Depending on the species, several basal media have been utilized during the induction process: MS medium (Murashige and Skoog, 1962) in *Abies cephalonica* by Krajnáková et al. (2008); MSG medium (Becwar et al., 1990) in *Larix* species by Tretyakova and Barsukova (2012); EDM (Walter et al., 1998) in *Pinus radiata* by Montalbán et al. (2012); and DCR (Gupta and Durzan, 1985) in *Pinus halepensis* by Montalbán et al. (2013).

A combination of auxin and cytokinin seem to be necessary to initiate ET. The most used auxin is 2,4-dichlorophenoxyacetic acid (2,4-D) (Fehér, 2008) and the most used cytokinins are N^6-benzylaminopurine (BAP), kinetin, and zeatin (Jiménez and Thomas, 2005).

Other factors, such as pH (Tautorus et al., 1991), agar (García-Mendiguren et al., personal communication), carbon source (Salajova and Salaj, 2005), nitrogen level (Bozhkov et al., 1993), and utilization of activated charcoal (Van Winkle and Pullman, 2005) may affect the induction of embryogenic tissue.

Further proliferation of ET occurs by transferring it to a maintenance medium, gelled or liquid, again with auxins and cytokinins, to continue cleavage polyembryony and somatic embryo multiplication (Pullman and Bucalo, 2011).

This step occurs in the dark and requires regular sub-culturing either to avoid depletion of nutrients/plant growth regulators or to limit the accumulation of toxic compounds in the culture medium. The ability of the tissue to withstand storage without losing embryogenic ability and being contaminated is critical for providing material for the propagation of clones with selected traits (Pullman and Bucalo, 2014).

SE maturation is usually achieved on a basal medium of the same composition but in which auxins and cytokinis are removed and abscisic acid (ABA) included, the concentrations depending on the species (Jiménez and Thomas, 2005). Water availability, which can be reduced through manipulation of the culture medium by osmotic or physical means (increasing the concentration of the gelling agent), is another factor that was found as critical to promote development of large numbers of somatic embryos (Klimaszewska et al., 2007).

Germination of mature somatic embryos is carried out most frequently on a semi-solid medium without plant growth regulators. It has been found beneficial for some species to culture the somatic embryos in darkness or dim light for the first 7-10 days before exposure to light (Klimaszewska et al., 2007). In several species of *Pinus* it is also beneficial to place somatic embryos horizontally on the medium and then tilt the Petri dishes vertically to 45 or 60º so the roots can develop on medium surface (Montalbán et al., 2010; Montalbán et al., 2013). Once the somatic embryos have converted to plantlets, they are transferred to the same

substrate as used for seedlings and grow under controlled greenhouse conditions (Montalbán et al., 2011).

1.4. Aleppo Pine (*Pinus halepensis* Mill.)

Pinus halepensis Mill. (Fig. 2A), commonly referred to as Aleppo Pine, is a monoecious member of Pinaceae.

Female cones present a biannual maturation with a limited development in the first summer and autumn and a second phase of development, after fertilization, on the second summer. Mature female cones (Fig. 2B) are brown, symmetrical and pedunculated, with 6-12 x 3,5-4,5 cm and appear alone or in clusters (2-3), while male cones are smaller, 3-4 x 5-8 mm, dingy and grouped in large numbers (Fig. 2C). The same plant may present cones in different development stages (Puértolas Simón et al., 2012).

Aleppo pine is a medium size tree, until 20-22 m high, with a light grey trunk and, despite the strong apical dominance at young ages, with an irregular shaped crown. The needles (Fig. 2D) appear in groups of two, are flexible, green and 6-12 cm long (Puértolas Simón et al., 2012).

This specie is native from the Mediterranean area and is widespread from Spain to Algeria (Botella et al., 2010) where, due to its very specific temperature and precipitation requirements, its distribution is generally confined (Klein et al., 2011). In Southern Europe occurs in several countries such as like Spain, France, Italy and Greece and in Northern Africa in Morocco, Algeria and Tunisia (Fig. 3A).

In Spain it is largely distributed, occupying an approximate surface of 1.500.000 ha, being autochthonous and abundant in Balearic Islands and by the Mediterranean coast. Due to its important ecological plasticity it has been intensively used for afforestation in north-western areas (Botella et al., 2010) extending to other autonomous regions as the Basque Country, Navarre and Castile and Leon (Fig. 3B). In Portugal *Pinus halepensis* is neither native nor abundant but some specimens have been introduced near Lisbon and in the Centre, near Figueira da Foz (Fig. 3C).

Figure 2: Morphological characteristics of *Pinus halepensis* Mill. (**A)** Aleppo pine tree with irregular shaped crown; (**B)** two clustered mature female cones; (**C)** several clusters of male cones; (**D)** detail of the needles. (from: arboretum.arizona.edu; www.botanical-online.com; ichn.iec.cat; keyserver.lucidcentral.org)

This species has eco-physiological characteristics as fast-growing radical system, a water-save strategy and resistance to water-drought interaction that allow it to live in a wide variety of soils, being mostly found in limestone substrates with high pH and in shallow steep soils, were others arboreal species have difficulties to establish (Puértolas Simón et al., 2012).

Its regeneration capacity after fires has been extensively exploited throughout most of the mediterranean area (Osem et al., 2013). This species, like most of the Mediterranean conifers, has a mandatory regeneration by seed, since there is no regrowth from the trunk and branches. These seeds are resistant to high temperatures, even requiring this stimulus to germinate, and thermal shocks and the presence of inhibitors seem to improve the germination rates (Calvo et al., 2013).

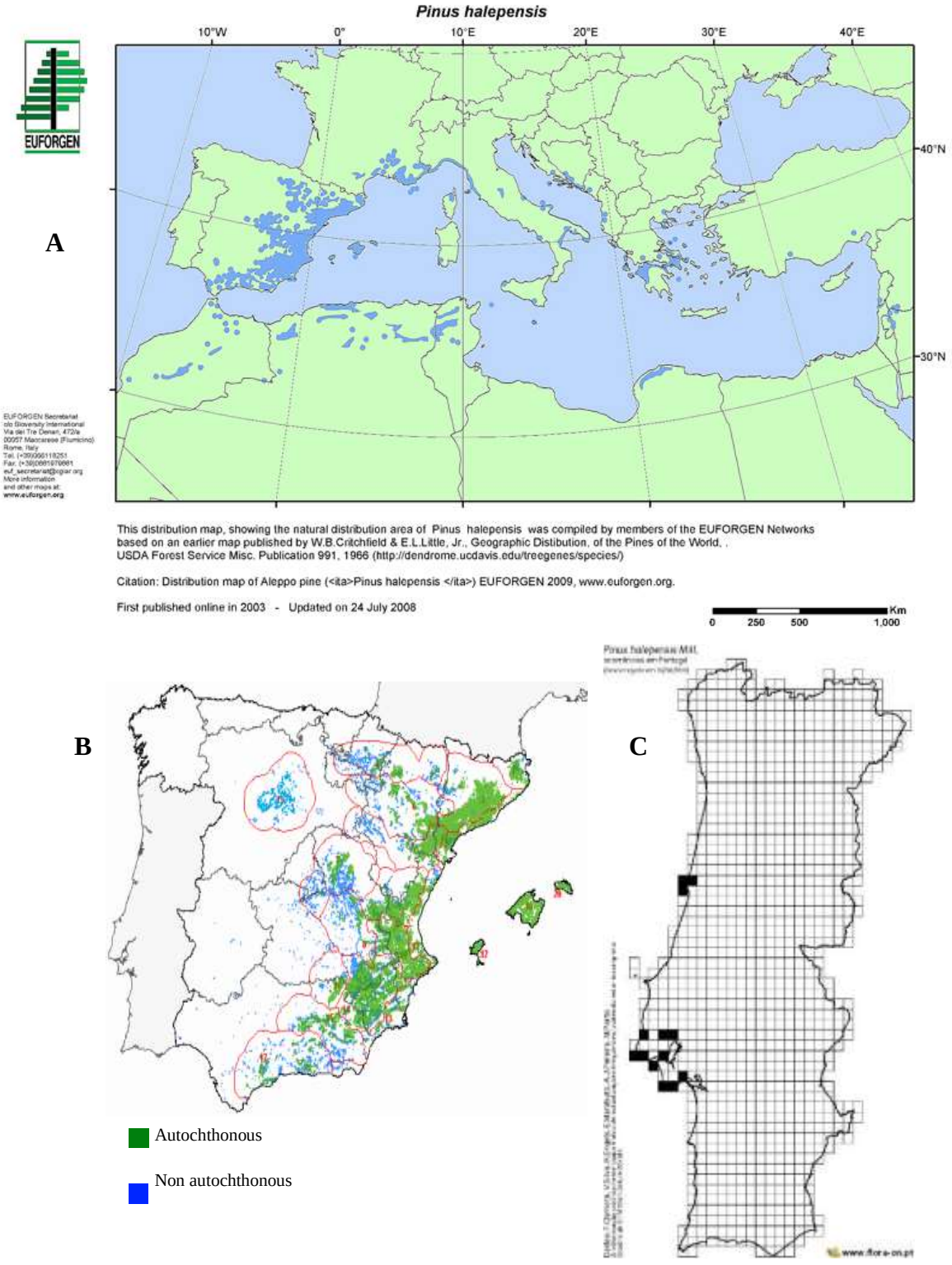

Figure 3: Geographical distribution of *Pinus halepensis* Mill. (**A)** natural distribution area, mostly confined in Mediterranean, at Southern Europe and Northern Africa; **(B)** distribution in Spain where the specie occupies a large surface specially at mediterranean coast; **(C)** occurrence of introduced specimens in Portugal. (from: euforgen.org; especiesforestales.com; flora-on.pt)

Aleppo pine is a pioneer species and it has been suggested that the introduction of *P. halepensis* into degraded areas could facilitate the long-term colonization and development of late-successional species (Montero and Alcanda, 1993; Gil and Aránzazu, 1993).

For all the above mentioned reasons, Aleppo pine has been described of a great economic importance. Also, considering the global drying and warning predictions there is some concern about the physiological ability of *P. halepensis* to be used in large afforestations in the future (Oliveras et al., 2003; Maestre and Cortina, 2004).

1.5. Objectives

It is generally known that adjustments of tissue culture media can result in higher success of SE initiation frequencies (Montalbán et al., 2012). In this sense some studies have focused on improving initiation and proliferation through testing different media (Zhang et al., 2007), plant growth regulator concentrations (Choudhury et al., 2008), sugar types (Salajová and Salaj, 2005) or gelling agent concentrations (Li et al., 1998), but few studies have focused on temperature and water availability.

Taking all the above mentioned data into account, the aim of this work was to analyze the effect of physical and chemical conditions at initiation stage of *Pinus halepensis* SE on the final success of the process. Thus, the first objective of this work was to assess if different temperatures and/or water availability conditions during initiation of EMs affect the rate of initiation and/or proliferation stages. Our second objective was to test if maturation rates, number and quality of embryos produced are affected by different environmental conditions at the earliest stage of SE process.

2. Material and Methods

2.1. Plant material

The plant material used was obtained from five open-pollinated families (8, 9, 12, 13, 14) belonging to a seed orchard established by NEIKER-TECNALIA at Berantevilla, Álava, Basque Country (Spain). One-year-old green female cones, enclosing immature seeds of *Pinus halepensis,* from each of the selected trees were sampled weekly, from the 7th of July to 15th August 2014. Cones were stored at 4 ºC until October following the method described by Montalbán et al. (2015).

2.2. Analysis of the developmental stage of zygotic embryos

Embryo morphological stage from the cones stored at the different collection dates was analyzed following Montalbán et al. (2012). Briefly, ten megagametophytes per family, from each collection date, were isolated from the immature seeds, the embryo removed and immediately stained with 2 % (w/v) acetocarmine directly on glass slides for 4 min. The samples were further rinsed with sterile distilled water and observed with a Zoom Stereomicroscope (Nikon SMZ1000). The stages of embryo development were recorded using a high resolution microscope camera (Nikon DS-Fi1c).

The proper stage was that corresponding to early cleavage polyembryony and the first ‘‘bullet’’ stages with a dominant embryo (Montalbán et al., 2012).

2.3. Initiation of embryogenic cell lines

To induce embryogenic cell lines (ECL) green cones (9 x 3 cm) were first sprayed with 70 % (v/v) ethanol and cut in four pieces to remove all immature seeds. These were surface sterilized in H_2O_2 10 % (v/v) with two drops of Tween 20® for 10-15 min, and then rinsed three times with sterile distilled H_2O, in the laminar flow unit under sterile conditions. Whole megagametophytes containing immature embryos were excised aseptically and placed horizontally onto the medium. The culture conditions were those described by Montalbán et al., (2013). Briefly, induction DCR medium (Gupta and Durzan, 1985) supplemented with 3 % (w/v) sucrose and a combination of 9.0 µM 2,4-D and 2.7 µM kinetin was used. The pH was adjusted to 5.7 prior autoclaving and to increase or reduce water availability of the medium, three different concentrations of gellan gum were added (2, 3 and 4 g L^{-1} Gelrite®). After autoclaving the medium at 121 °C for 20 min, a filter-sterilized solution with adjusted pH to

5.7 containing EDM amino acid mixture (Walter et al., 2005) (550 mg L^{-1} L-glutamine, 525 mg L^{-1} asparagine, 175 mg L^{-1} arginine, 19.75 mg L^{-1} L-citrulline, 19 mg L^{-1} L-ornithine, 13.75 mg L^{-1} L-lysine, 10 mg L^{-1} L-alanine and 8.75 mg L^{-1} L-proline) was added to the cooled medium. The Petri dishes containing 20 mL of culture medium and 8 megagametophytes were stored randomly on the shelves of the growth chamber at three different temperatures (18, 23 and 28 ºC) in darkness.

The experiment comprised nine different treatments (Fig. 4), five seed families and 3-6 Petri dishes per seed family and treatment.

2.4. Water availability assessment of initiation media

Water availability of the initiation media was assessed by placing one autoclaved filter paper disc (Whatman no. 2) in the surface of initiation medium (composition described in section 2.3) with the three concentrations of gellan gum (2, 3 and 4 g L^{-1}). First, each one of the used Petri dishes containing the media was weighed. Then, a filter paper disc was placed onto the medium and the weight of the Petri dish was assessed again. Petri dishes were sealed with cling film and 3-4 replicas for gellan gum concentration were incubated for six weeks under each one of the three tested temperatures (18, 23 and 28 ºC), comprising the 9 different treatments tested. Filter paper discs were subsequently weighed and the amount of water (mg) absorbed by the filter paper was calculated.

2.5. Proliferation of ECLs

After 5-10 weeks in the initiation media, proliferating embryonal masses (Ems) with an approximate diameter of 16 mm were separated from the megagametophytes and sub-cultured on the proliferation medium. This medium had a composition similar to that used in the initiation stage. All ECLs were placed onto a medium with 3 g L^{-1} Gelrite® in the first subculture. During the next subcultures, EMs were cultured on a medium containing 4.5 g L^{-1} Gelrite®. Samples were subcultured every two weeks, stored in the dark at the same temperature, 23±1 ºC, and laid out randomly on the shelves of the growth chamber.

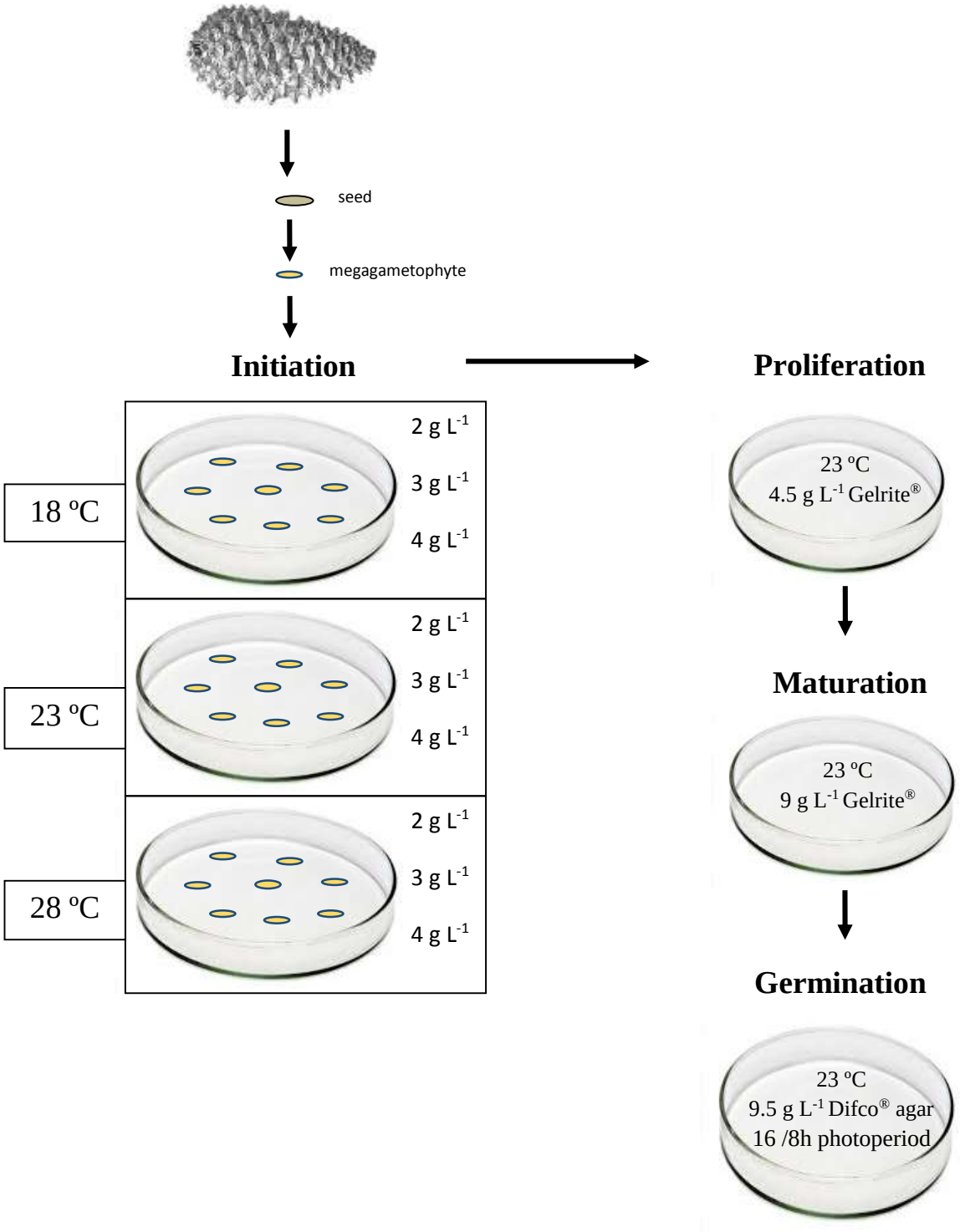

Figure 4: Schematic representation of the culture conditions used to induce somatic embryogenesis in *Pinus halepensis*. After collection and sterilization of the seeds, megagametophytes were extracted and placed on a DCR medium (Gupta and Durzan, 1985) supplemented with 3 different Gelrite®concentrations (2, 3, 4 g L^{-1}) that were stored at 3 different temperatures (18, 23 and 28 ºC). EMs proliferation, somatic embryo maturation and germination were carried out under the same culture conditions.

2.6. Maturation of ECLs

ET was suspended in 20 mL of liquid growth regulator-free DCR medium (Gupta and Durzan, 1985). In order to homogenize the suspension it was manually shaken for a few seconds. Then a 5 mL aliquot containing 70-80 mg fresh weight, were transferred with a sterile 10 mL pipette and poured onto a filter paper discs (Whatman no.2) upon a Büchner funnel that was connected to a side-arm flask by a black rubber adaptor. A vacuum pulse was applied for 10 s and the filter paper with the attached ET was transferred to a Petri dish (90 x 20 mm) containing 40 mL of maturation medium. This medium was the DCR medium (Gupta and Durzan, 1985) supplemented with 60 g L^{-1} sucrose, 75 µM ABA, the same aminoacid mixture used in the earlier stages, and a higher concentration of gellan gum (9 g L^{-1} Gelrite®).

All ECLs were cultured in the dark, at 23±1 ºC and randomly placed on the shelves of the growth chamber. Ten ECLs per treatment and four Petri dishes per ECL were tested.

2.7. Germination of ECLs

Mature somatic embryos were collected and cultured on half strength LP medium [(Quoirin and Lepoivre 1977) modified by Aitken-Christie et al. (1988)] and supplemented with 2 g L^{-1} activated charcoal and 9.5 g L^{-1} Difco® agar granulated. Twenty mature embryos per Petri dish were cultured and maintained at 21±1 ºC under a 16-h photoperiod at 120 µM m^{-2} s^{-1} provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France).

2.8. Data collection and statistical analysis

After the assess of the normal distribution and variance homogeneity, a factorial ANOVA was considered to evaluate the effect of the temperature and gellan gum concentrations at the water availability of the media. When significant differences between the levels of the factor variables were detected, a post hoc test, Tukey HSD, was carried out to find out which values were statistically different.

After 5-10 weeks in the initiation medium, the number of initiated EMs was registered and the initiation percentage per Petri dish was evaluated.

Following three subcultures, actively growing EMs were recorded as established cell lines and proliferation percentage per Petri dish was calculated.

A logistic regression was considered to assess the effect of mother tree, temperature and gellan gum concentration on the initiation and proliferation rates. The mother tree was introduced in the model as a block variable to reduce variability. Temperature and gellan gum concentration were considered as factor variables, each one with three levels. Interactions between temperature and gellan gum concentration were also taken into account in the model. When significant differences among the levels of the factor variables were detected, estimable linear functions of model coefficients were computed (McCulloch and Searle, 2001) to find out which levels significantly differed. The P-values of the multiple comparisons were conveniently adjusted following Benjamini-Yekutieli method (Benjamini and Yekutieli, 2001).

After 4 months of the beginning of the maturation stage, the number of ECLs able to differentiate somatic embryos was calculated. The percentage of maturation and the number of mature somatic embryos per gram were calculated. A factorial ANOVA was considered to evaluate the effect of the temperature and gellan gum concentrations, in initiation phase, at the number of mature somatic embryos per gram.

3. Results

3.1. Analysis of the developmental stage of zygotic embryos

Study of the developmental stage of the embryos isolated from cones collected weekly from the 7th to 28th July 2014 showed that cones collected before 28th July contained megagametophytes with embryos not yet formed. To initiate embryogenic cultures, megagametophytes containing embryos between stage 2 (Fig. 5A) and stage 4 (Fig. 5B) were used. Embryos at stage 2 showed elongated and separated suspensors and several proembryos whereas in stage 4, first ‘‘bullet’’ stage, one of the embryos was more developed than the others.

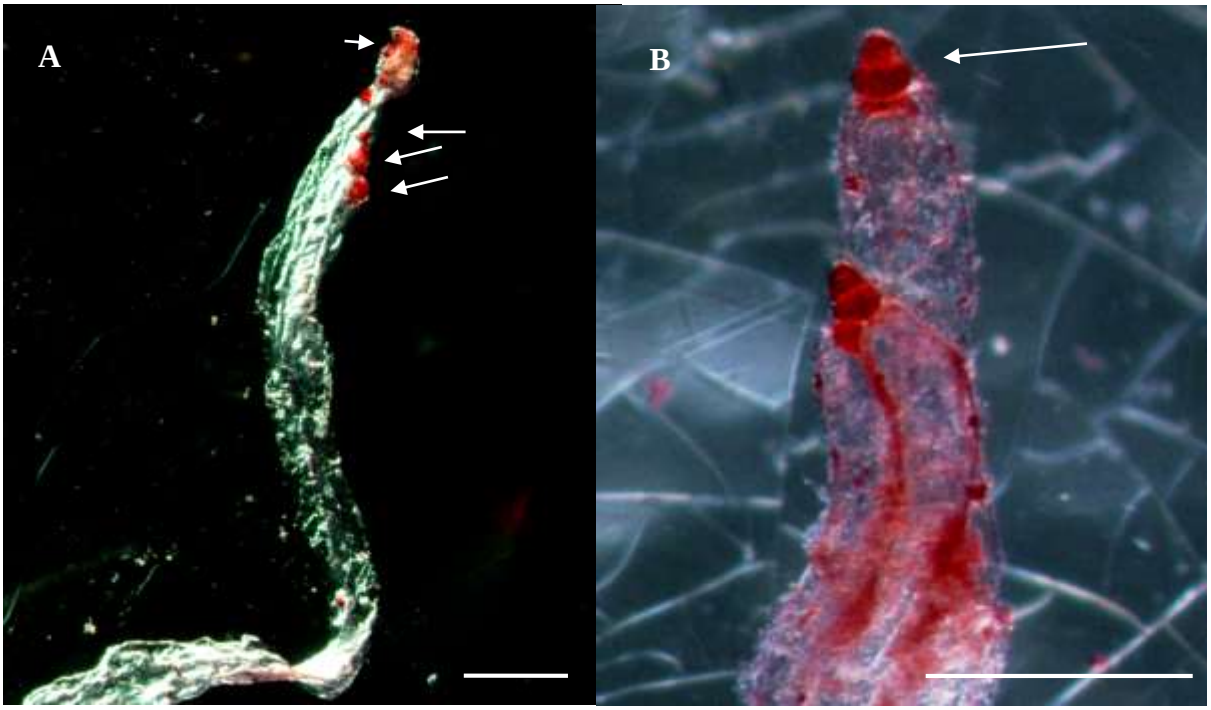

Figure 5: *Pinus halepensis* zygotic embryos. **(A)** Developmental stage 2, showing the long suspensor cells and several pro-embryos (arrows); **(B)** Developmental stage 4, first ‘‘bullet’’ stage, with a well-developed embryo (white arrow). Bars = 2 mm.

3.2. Water availability assessment of initiation media

The statistical analysis made to assess if different temperatures and gellan gum concentrations affected the water availability of DCR medium showed that there is only significant differences among the levels of gellan gum (P-value < 0.01) and the effect of the temperature and the double interaction among temperature and gellan gum were not statistically significant (Table 1).

The highest water availability (0.646 mg) was obtained with DCR medium supplemented with 2 g L^{-1} Gelrite® (Table 1; Fig. 6). A significant statistical difference was found between this and DCR media supplemented with 3 and 4 g L^{-1} Gelrite®, with 0.631 mg and 0.625 mg respectively (Table 1; Fig. 6).

Table 1: ANOVA for water availability (mg) of DCR medium supplemented with different gellan gum concentrations (2, 3 and 4 g L^{-1}) and stored at different temperatures (18, 23 and 28 ºC).

Source	*df*	Mean square	F	P-value
Temperature	2	8.64E-006	0.046	n.s
Gellan gum	2	0.001	5.892	< 0.01
Temperature: Gellan gum	4	0.000	0.721	n.s.

n.s.: not statistically significant different

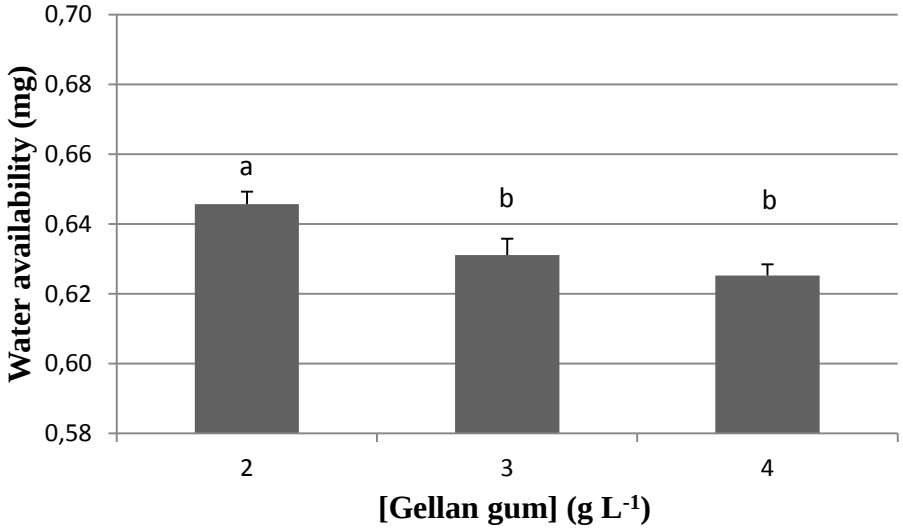

Figure 6: Water (mg) availability of DCR medium supplemented with different gellan gum concentrations (2, 3 and 4 g L^{-1}). Values are the average of 33 records ± SE. Different letters show significant differences at the significance level of α=0.05. Pair wise comparisons have been assessed with the post hoc test Tukey HSD.

3.3. Initiation of ECLs

In spite of all cones had the same morphological appearance some were infested with fungi a situation that may have contributed to the 14 % of explants contamination observed during the initiation of embryogenic cultures.

EMs started to appear at one pole of the megagametophyte (Fig. 7A) after about 3 to 5 weeks of culture (Fig. 7B). Two types of EMs were found concerning their proliferation capacity. Some showed a slow growth rate and never grew beyond a certain limit and megagametophytes showing this type of EMs were not considered as induced (Fig. 7C). Explants in which the EMs showed a strong proliferative ability and that reached at least 16 mm (Fig. 7D; Fig. 8) were considered as induced (39 %) and this EMs were further transferred to the proliferation medium.

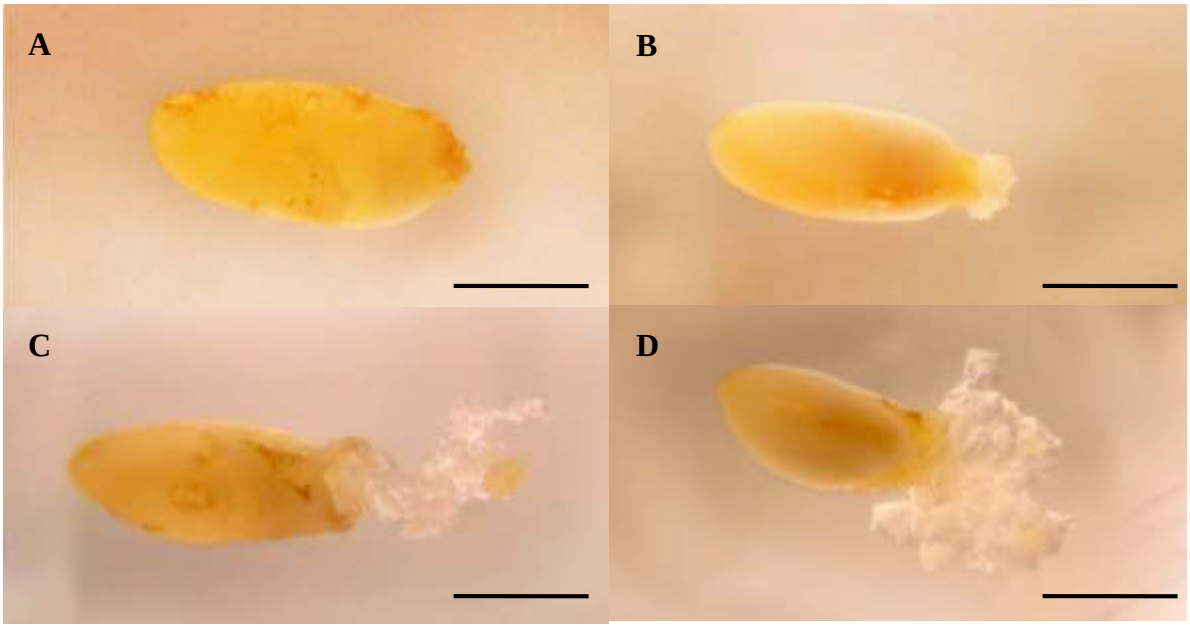

Figure 7: Induction of embryonal masses in megagametophytes of *P. halepensis* cultured on DCR medium. **(A)** Non induced explants **(B)** Megagametophyte showing an embryogenic mass at one pole **(C)** Embryogenic mass presenting a slow rate of proliferation **(D)** High-proliferative embryonal mass which was further transferred to the proliferation medium. Bars = 1cm

Figure 8: High proliferative embryonal mass induced in megagametophyte of *P. halepensis* cultured on DCR medium, which was further transferred to the proliferation medium. Bar = 1cm.

Statistical differences among the levels of seed family (P-value < 0.001), temperature (P-value < 0.001) and gellan gum concentration (P-value < 0.001) were found. Nonetheless, the double interaction among temperature and gellan gum was not statistically significant (Table 2).

When seed provenance was tested the results showed that differences occurred between families (Table 2). Seeds from families 9 and 12 showed the highest rates of induction (51 % and 47 % respectively) whereas family 14 gave the poorest results (Fig. 9).

Regarding culture temperature, the results indicated that the highest rates of induction (53 %) were obtained in megagametophytes cultured at 23 ºC (control temperature). Lower (18 ºC) or higher (28 ºC) temperatures greatly reduced somatic embryogenesis induction with the results decreasing to 36 % and 29 %, respectively (Fig. 10).

Gellan gum concentration (2, 3 and 4 g L^{-1}) is also a parameter that influences explant´s behavior (Table 2; Fig. 11). In this case, the highest level of response was obtained when megagametophytes were cultured on a medium supplemented with 4 g L^{-1} Gelrite® (49 %). Lower concentrations (2 and 3 g L^{-1}) significantly reduced EMs formation.

Table 2: Logistic regression analysis for proportion of initiation in *P. halepensis* megagametophytes induced on DCR medium obtained from five different seed families (8, 9, 12, 13 and 14), supplemented with different gellan gum concentrations (2, 3 and 4 g L^{-1}) and stored at different temperatures (18, 23 and 28 ºC).

Source	*df*	χ^2-test	P-value
Seed family	4	41.227	< 0.001
Temperature	2	49.075	< 0.001
Gellan gum	2	20.744	< 0.001
Temperature: Gellan gum	4	0.299	n.s.

n.s.: not statistically significant different

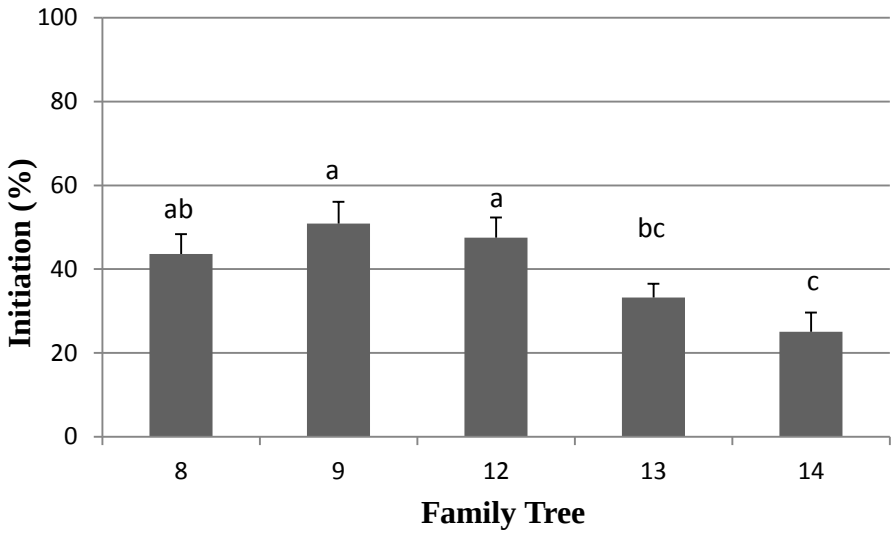

Figure 9: Induction of embryonal masses in megagametophytes from different tree families cultured on the induction medium. Each value is the mean ±SE of 3 to 6 replicas. About 250 explants were tested per family tree and the results taken after 5-10 weeks of culture. Different letters show significant differences at the significance level α=0.05. P-values for the pair wise comparisons have been conveniently adjusted following the Benjamini-Yekutieli method.

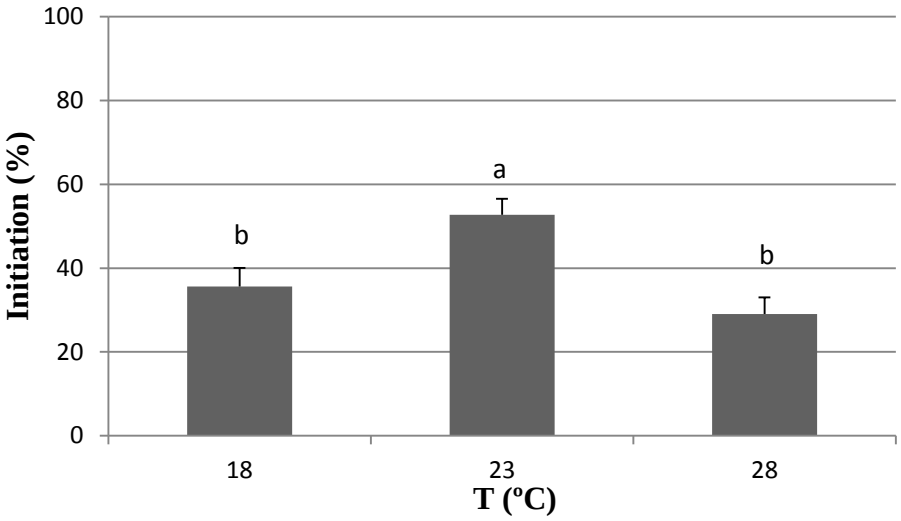

Figure 10: Induction of embryonal masses in megagametophytes on the induction medium cultured at different temperatures. Each value is the mean ±SE of 60 replicas. About 437 explants were tested per temperature and the results taken after 5 to 10 weeks of culture. Different letters show significant differences at the significance level α=0.05. P-values for the pair wise comparisons have been conveniently adjusted following the Benjamini-Yekutieli method.

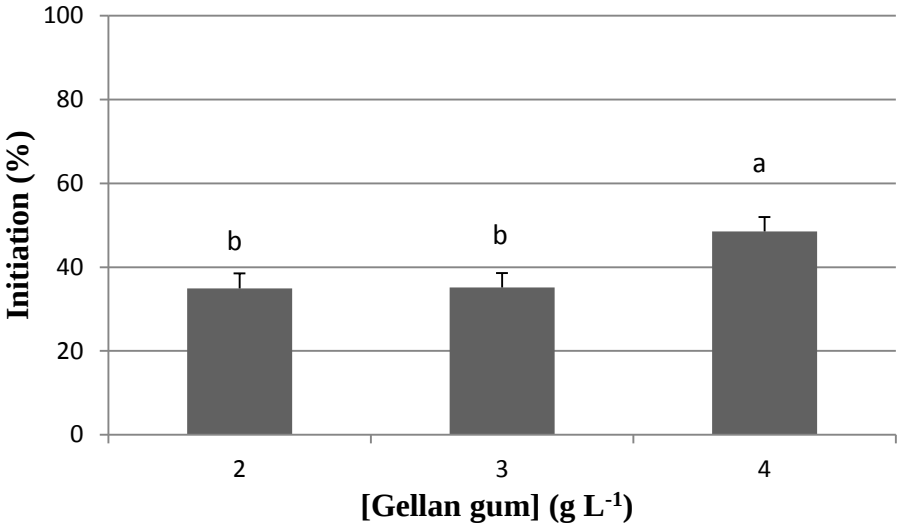

Figure 11: Induction of embryonal masses in megagametophytes on the induction medium cultured containing different gellan gum concentrations. Each value is the mean ±SE of 60 replicas. About 437 explants were tested per gellan gum concentration and the results taken after 5 to 10 weeks of culture. Different letters show significant differences at the significance level α=0.05. P-values for the pair wise comparisons have been conveniently adjusted following the Benjamini-Yekutieli method.

3.4. Proliferation of ECLs

Although initial cell proliferation was a preliminary indicator of a positive response it was found, as previously stated, that not all initiated EMs were able to keep on proliferating. The percentages of explants showing cell proliferation were calculated taking into account the number of initiated explants (449) and not the initial number of megagametophytes cultured. Following three subculture periods of 2 weeks in the same conditions, actively growing EMs were recorded as established cell lines and a total of 69 % of proliferation (Fig. 12) was obtained.

As during the induction stage, the effects of temperature, seed family and gellan gum on cell proliferation were analyzed. The data obtained showed that significantly statistical differences occurred, specially, when temperature was the parameter tested (Table 3). Seed family and gellan gum concentration as well as the interaction between temperature and gellan gum did not influence the response in the conditions tested (Table 3).

The highest proliferation (%) of EMs was achieved at 23 ºC (control temperature) (78 %). A statistical difference was found between this temperature and 18 ºC in which the explants showed the lowest proliferation success (59 %). The temperature of 28 ºC (67 %) did not show statistically significant differences when compared with lower temperatures (Table 3; Fig. 13).

Table 3: Logistic regression analysis for proportion of proliferation in *P. halepensis* megagametophytes induced on DCR medium obtained from five different seed families (8, 9, 12, 13 and 14), supplemented with different gellan gum concentrations (2, 3 and 4 g L^{-1}) and stored at different temperatures (18, 23 and 28 ºC).

Source	*df*	χ^2-test	P-value
Seed family	4	2.8040	n.s.
Temperature	2	10.9679	< 0.01
Gellan gum	2	0.6629	n.s.
Temperature: Gellan gum	4	8.6404	n.s.

n.s.: not statistically significant different

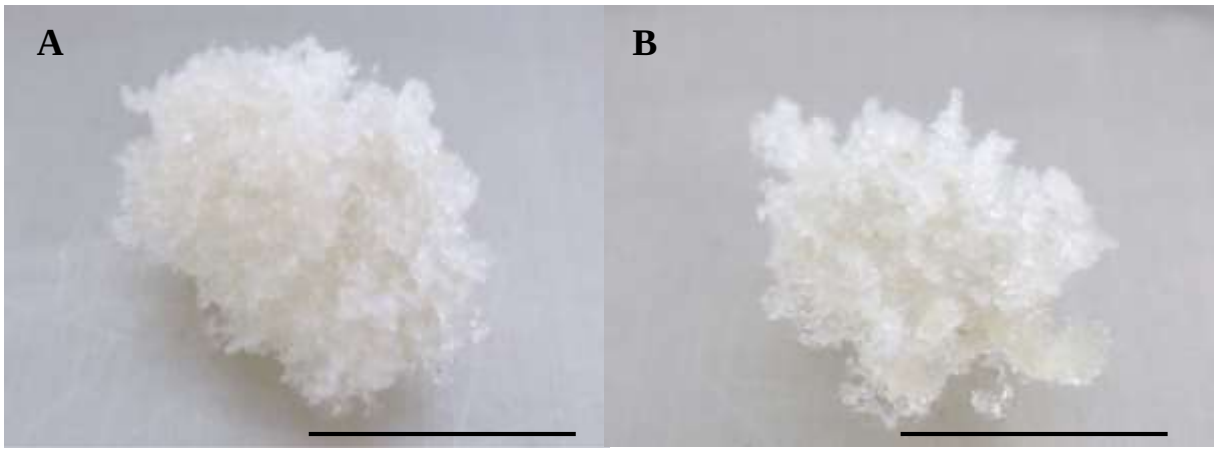

Figure 12: Two examples of established cell lines. (A): ECL induced on DCR medium cultured at 18 ºC and supplemented with 2 g L^{-1} Gelrite®; (B) ECL induced on DCR medium cultured at 23 ºC and supplemented with 2 g L^{-1} Gelrite®. Bars = 2cm.

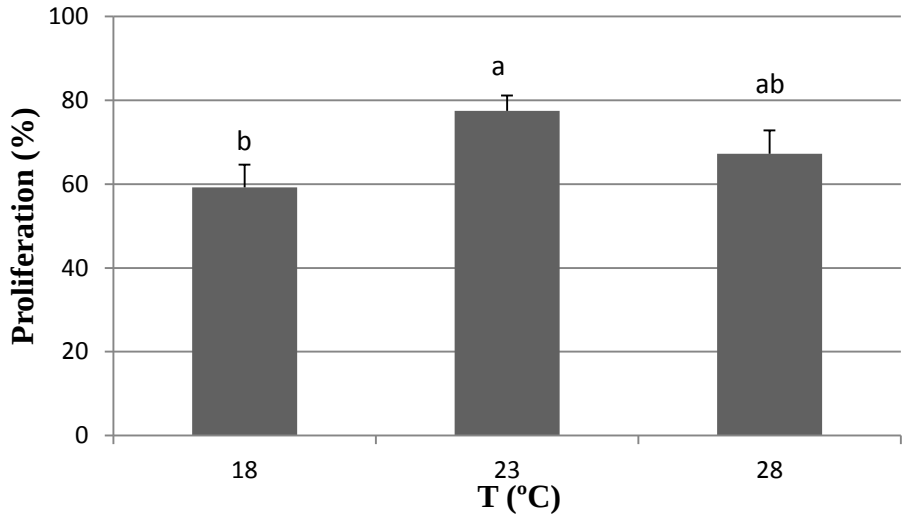

Figure 13: Proliferation of embryonal masses in megagametophytes induced on medium cultured at different temperatures are the average of 449 records ± SE and the results taken after 3 subcultures, periods of 2 weeks. Different letters show significant differences at the significance level α=0.05. P-values for the pair wise comparisons have been conveniently adjusted following the Benjamini-Yekutieli method.

3.5. Somatic embryo maturation

Proliferating ECLs were placed onto maturation medium (Fig. 14A), during at least four months to achieve somatic embryo development (Figs. 14A-C) and maturation (Fig. 14D). All 90 ECLs transferred to development/maturation conditions produced mature somatic embryos (100 %). A statistical analysis was carried out to assess if the number of mature somatic embryos per gram was affected by different temperature and gellan gum concentration of initiation medium. The results showed that there are no significant differences between temperatures, gellan gum concentrations or double interactions between these two variables (Table 4).

The highest number of embryos per gram was achieved at an initiation temperature of 18 ºC (2683) and the lowest at 28 ºC (2229). Regarding the gellan gum concentrations, ECLs induced at 4 g L^{-1} produced the highest number of somatic embryos (2640), while the worst results were obtained with 3 g L^{-1} (2285).

Table 4: ANOVA for 90 ECLs somatic embryos per gram in *P. halepensis* megagametophytes cultured on DCR medium supplemented with different gellan gum concentrations (2, 3 and 4 g L^{-1}) and stored at different temperatures (18, 23, and 28 ºC).

Source	*df*	Mean square	F	P-value
Temperature	2	1697029	0.7565	n.s
Gellan gum	2	1051635	0.4688	n.s
Temperature: Gellan gum	4	4724639	2.1060	n.s.

n.s.: not statistically significant different

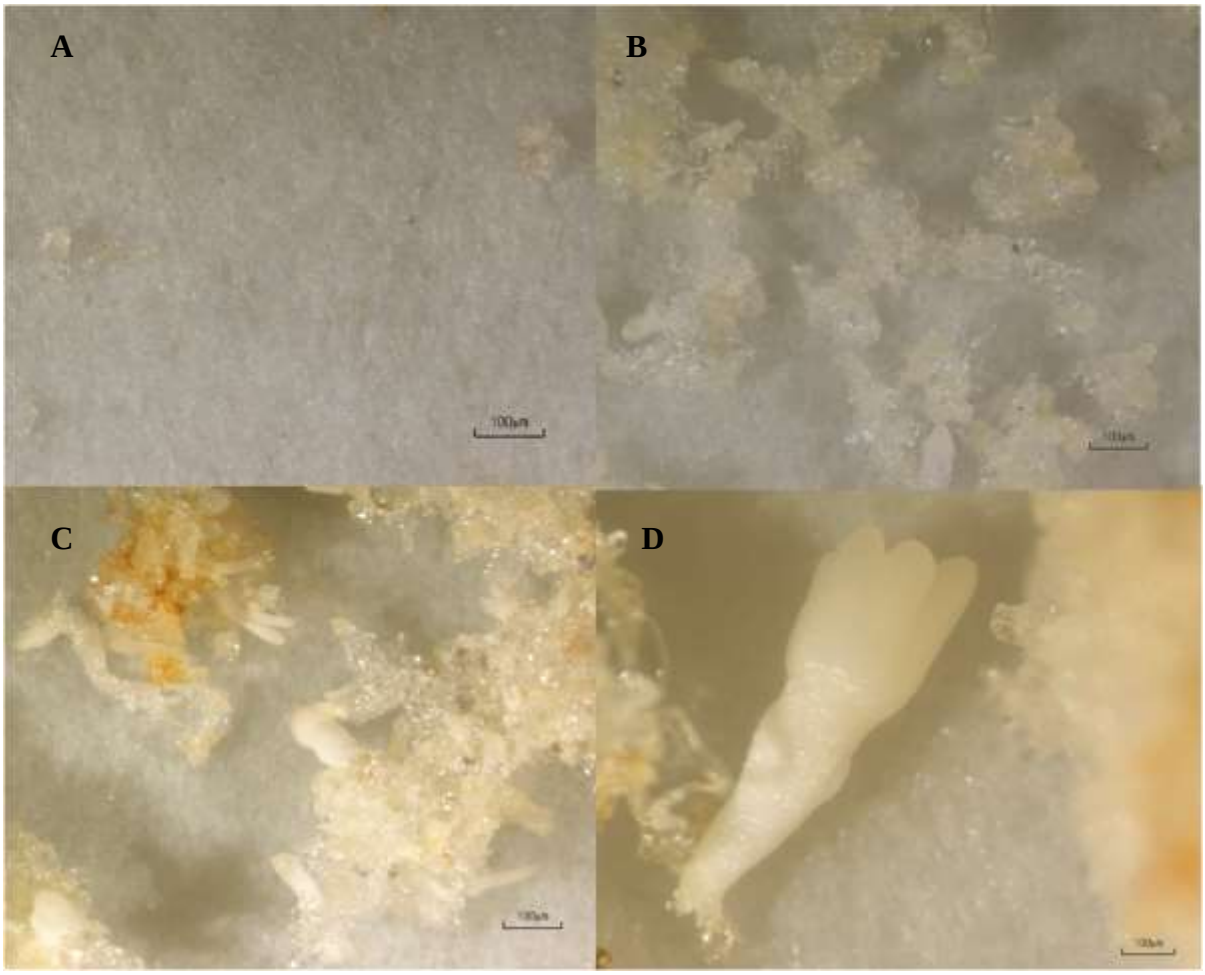

Figure 14: Development of *Pinus halepensis* ECLs cultured on maturation DCR medium. **(A)** ECLs immediately transferred to maturation medium; **(B)** ECLs cultured for 1 month on maturation medium; **(C)** ECLs cultured for 2 month on maturation medium, showing the formation of pro-embryos; **(D)** ECLs cultured for 4 month on maturation medium, showing a mature embryo with cotyledons.

4. Discussion

The aim of this work was to analyze the effect of some physical and chemical conditions in the initiation stage of *Pinus halepensis* somatic SE on the final success of the process. Different temperatures and water availability were tested at the earlier phase of the process and the standard conditions were maintained in the subsequent ones. SE of *Pinus halepensis* has been successfully induced and these results showed that both the induction rates of ET and EMs proliferations were affected by the factors tested.

Several basal media and factors of the culture conditions have been used in pine SE (Bozhkov et al., 1993; Ramarosandratana et al., 2001; Carnero et al., 2009) but very few were focused on temperature and water availability. The first report in which both temperature and gellan gum variations, and their interaction, have been tested in initial stages of the SE, was carried out by García-Mendiguren et al. in *Pinus radiata* (personal communication).

The initiation of ET was significantly affected by the genotype of the *P. halepensis* donor tree. Similar results were reported in a previous study in this species (Montalbán et al., 2013) and in other *Pinus* species (Hargreaves et al., 2009; Montalbán et al., 2012; Lelu-walter et al., 2006; Klimaszewska et al., 2007), indicating that there is a strong genetic influence on the initiation success using current SE technologies. However, this effect was not observed in the next stages of SE, contrary to previous work on the same species performed by Montalbán et al. (2013), where proliferation of ET was greatly affected by tree origin. Since the effect of the mother plant cannot be equally reproduced unless the same mothers are used for a future experiment, its effect was only studied individually and the double interactions between the mother and the other factors were not taken into account.

Interestingly, the genetic background of the explant is not the only factor influencing a successful induction of SE. Due to the longevity of their sessile life, trees have developed systems to modify their phenotype to tolerate abiotic factors, among them weather changes during the year. Thus, temporal and spatial variation in environmental conditions can lead to adaptations of plant populations (Yakovlev et al., 2011). Stress is one of the principal causes for a cell or tissue to reprogramming itself to express the embryogenic pathway. The response to stress conditions depends on the level of stress and the physiological state of the cells: extreme stress may cause the cells to die if they are unable to tolerate this stress level (Zavattieri et al., 2010; Bonga et al., 2010). Epigenetic memory marks are established in response to the temperature conditions prevailing during zygotic and SE (Yaklovev et al., 2014) and epigenetic mechanisms like DNA methylation and histone modifications have a key role in plants response to stress (Boyko and Kovalchuk, 2008).

In the present work, three different temperatures (18, 23, and 28 ºC) were tested and the highest initiation percentage of embryogenic induction (53 %) was obtained when cultures were maintained at 23 ºC (control temperature). The initiation rates of the lowest temperature (18 ºC), with 36 %, and the highest (28 ºC), with 29 %, were significantly lower. These results contrast to those reported in *Pinus radiata* by García-Mendiguren et al. (personal communication) in which the lowest initiation temperature (18 ºC) led to significantly better initiation rates. Increase of the optimal temperature may be related with the fact that *Pinus halepensis* grows in the hotter parts of the Mediterranean coast (Botella et al., 2010), where forest fires are frequent, and *Pinus radiata* is usually found in mild climate regions of New Zealand, Canada, Chile and Australia (Cerda et al., 2002).

The strength of gelling agents seems to play an important role in the initiation medium, as it affects water availability to the cultures (Choudhury et al., 2008). In order to test the effect of water availability, different concentration of gellan gum (2, 3, and 4 g L^{-1}) in the initiation medium were tested. Some authors have shown that when gellan gum increases the initiation of embryogenesis is negatively affected. As examples can be referred the observations of Harry and Thorpe (1991) in *Picea rubens*, Becwar et al. (1995) in several *Pinus* species, Winkle and Pullman (2005) using liquid medium in *P. taeda*, and Pullman an Skyrabina (2007) also with liquid medium in *P. taeda*. On the contrary, the present work showed that with lower water availability (higher concentration of gellan gum), higher the initiation rates are: the highest success of initiation was obtained when megagametophytes were cultured in a medium supplemented with 4 g L^{-1} Gelrite® (49 %). Similar results were obtained by García-Mendiguren et al. (personal communication), and Pullman and Johnson (2002).

Initiation of SE may not result in establishment of an embryogenic cell line because EMs may cease to proliferate after a few time of culture. Thus, when assessing the success rate it is important to distinguish between the initial outgrowth/extrusion observed in the explants and further continuous growth from these initial mitotic divisions (Klimaszewska et al., 2007). The results obtained in this work showed that from the different conditions tested during the proliferation stage, the only factor that had a significant effect was temperature.

EMs initiated at 23 ºC proliferated significantly better than those initiated at 18 ºC, which showed the lowest proliferation rate. Although at 28 ºC the proliferation success was lower than at 23 ºC, no statistically significant differences between them were found. These results may be compared with those obtained by Kvaalen and Johnsen (2007) in *Picea abies*, in which a decrease in the culture temperature from 23 to 18 °C, significantly reduced the growth of embryogenic tissue when this was measured 6 weeks after the temperature treatment

started. However, in *Pinus radiata*, García-Mendiguren et al. found out that an initiation temperature of 28 °C increased the proliferation rates (personal communication). Taken together, these data show that different species seem to have distinct behaviors when submitted to different conditions of temperature during proliferation of ET, what can be related not only with the genetic characteristics of the explant but also with the epigenetic memory caused by particular conditions that the donor trees suffered during their life cycle.

Regarding the effect of initiation conditions on maturation rates, number and quality of embryos produced, the results showed that there are no significant differences among different temperatures as well as water availability. All the 90 ECLs submitted to maturation produced mature somatic embryos and there were no differences in the number of embryos produced per treatment. Once again, this is different from what was found in *P. radiata* in which the conditions applied during the early stages of the embryogenic process affected positively the number of embryos produced, since explants initiated at 28 ºC produced a higher number of mature somatic embryos (García-Mendiguren et al., personal communication). Nonetheless, despite the fact that there were no statistically significant differences on the number of somatic embryos produced, it should be noted that the highest number of somatic embryos was not achieved at 23 ºC, were initiation and proliferation rates were higher, but at 18 ºC. These results are in accordance with Fehér (2014) who reviewed that the selective pressure of temperature may lead to lower rates of initiation but higher rates of maturation.

Overall, it was found that environmental conditions during the initiation stage of *Pinus halepensis* SE influence the success of initiation and proliferation. Induction of SE with lower water availability (4 g L^{-1} of gellan gum) increases initiation rates; additionally, medium culture at 23 ºC during the initiation stage increases the success of initiation and proliferation. A combination of these two conditions enhances the number of ECLs obtained, and, as consequence, allows a higher number of plants obtained.

Even though there was no effect of initiation conditions on the maturation rate, the fact that mature somatic embryos could be obtained in all treatments tested indicates that plants will be obtained from extreme conditions of induction (28 ºC and 4 g L^{-1}). This is particularly relevant in the framework of the earlier observations carried out in Norway spruce (Yakovlev et al., 2011). In this species, the temperature that prevails in the induction phase of embryogenesis causes changes in gene expression that could enable seedlings to memorize the environmental influence and thus fine-tune the regulation of adaptive performance for many years in this species. Taking those data into account, it is expected that the same could occurs in *P. halepensis* and that the somatic embryos obtained in the present work could, eventually,

become plants able to resist on extreme environment with higher temperatures and less water availability.

5. Concluding remarks

This work showed that somatic embryogenesis is a biotechnological tool that can be successfully applied as a large-scale propagation system in Aleppo Pine, a species largely distributed in the Mediterranean area with eco-physiological features that make it extensively used for afforestation, especially, in degraded areas with poor soils.

It was found that initiation stress conditions like water availability and temperature affect the rates of induction and proliferation of explants and, as so, the number of plants produced at the end of the process. On the other hand, it was possible to obtain mature somatic embryos from all treatments, allowing the production of a large number of plants from extreme conditions. These plants induced from high temperatures and low water availability may, in the future, be able to better resist to global drying and warning predictions, continuing to be a wide spread species and an effective choice for afforestation.

A further study and analysis of the effect caused by different initiation treatments on the subsequent phases like germination and acclimatization, under controlled greenhouse conditions, will be carried out. Also, a study of the proteins involved during the process will be done, as collaboration between NEIKER-TECNALIA and the Centre for Functional Ecology of the University of Coimbra, in order to have a general idea of its effects throughout the process.

Since all different steps of somatic embryogenesis influences the final success, studies concerning the effect of different environmental conditions in the proliferation and maturation phases are currently going on to create an improved and efficient multi-step protocol for this species.

Adaptability evaluation to different temperatures and water availability in ex vitro conditions, and physiological characterization of clones coming from embryogenic cell lines obtained in different environments together with their behavior in different controlled environments (different temperatures and irrigation levels) will be made to confirm if there are differences in the adaptability of the plants obtained.

Lastly, an effective cryoconservation protocol for *Pinus halepensis* embryogenic tissue could enable the preservation of its potentiality, and, as consequence, a continuous production of selected elite clones.

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