UNIVERSIDADE D
COIMBRA

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IN VITRO MORPHOGENESIS ASSAYS IN *PINUS HALEPENSIS* MILL.

Dissertação no âmbito do Mestrado em Biodiversidade e Biotecnologia Vegetal orientada pelo Professor Doutor Jorge Manuel Pataca Leal Canhoto e apresentada ao Departamento de Ciências da Vida.

Agosto de 2019

Faculdade de Ciências e Tecnologias da Universidade de Coimbra

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Resumo

Pinus halepensis é uma conífera naturalmente presente na bacia Mediterrânica que tem sido usada para programas de florestamento e reflorestamento de áreas marginais e submarginais. A importância e a necessidade desses programas têm crescido nos últimos anos devido às crescentes ameaças às florestas, como a desflorestação e a alta demanda por serviços florestais. Além disso, o crescimento populacional e o desenvolvimento económico têm pressionado o mundo a aumentar a produção e usar menos terra e recursos, enquanto as alterações climáticas e suas consequências colocam em causa o futuro dos ecossistemas florestais.

A embriogénese somática é uma técnica crucial para o melhoramento de coníferas, pois para além de fornecer embriões geneticamente idênticos, também é possível desenvolver variedades mais produtivas e tolerantes. No entanto, protocolos para esta técnica em coníferas ainda precisam ser otimizados para aplicações comerciais. Neste trabalho, diferentes explantes foram testados para induzir embriogénese somática, assim como tratamentos de choque para converter calos não embriogénicos em embriogénicos, e estudos histológicos em cones femininos jovens.

Embriões zigóticos no estado cotiledonar, inteiros ou transversalmente divididos ao meio; escamas e segmentos de cones femininos não fertilizados, foram cultivados em variações de meio DCR de indução. Nenhum desses explantes originou tecido embriogénico detetável, embora calos obtidos de cones não fertilizados fossem homogéneos e semelhantes entre si, enquanto calos obtidos de embriões maduros apresentavam áreas distintas no mesmo tecido. Calos não embriogénicos foram expostos a 100 µM de 2,4-D, pH 4, pH 10, 0,3 M de sacarose e 0,15 M de sacarose mais 0,15 M de manitol, durante 1, 2, 4 e 8 dias, mas nenhum desses tratamentos converteu o calo para embriogénico. O genótipo da planta mãe deve ser levado em consideração e novas composições de meio de indução e outras moléculas devem ser investigadas para melhorar a indução da embriogénese somática em *P. halepensis*.

Palavras-chave: explante, histologia, indução, não embriogénico, *Pinus halepensis*.

Abreviaturas: 6-Benzilaminopurina (BAP); Ácido 1-Naftalenoacético (NAA); Ácido 2,4-Diclorofenoxiacético (2,4-D); Cinetina (KIN); Reguladores de crescimento (PGR).

Abstract

Pinus halepensis is a conifer naturally present in the Mediterranean basin that has been used for afforestation programs and reforestation of marginal and sub marginal areas. The importance and necessity of these programs have grown in the past years due to the increasing threats on forests, such as deforestation and high demand on forest services. Besides this, population growth and economic development has pushing the world to increase production and using less land and resources while climate change and its consequences puts in cause the future of forest ecosystems.

Somatic embryogenesis is a crucial technique for conifers improvement, it can not only provide genetically identical embryos, but also develop more productive and tolerant varieties. However, protocols for this technique in conifers still need to be optimized for commercial applications. In this work, different explants were tested in order to induce somatic embryogenesis, so as shock treatments to convert non-embryogenic *callus* into embryogenic, and histologic assays on young female cones.

Zygotic embryos in cotyledonary stage, whole or transversely divided in halves; scales and sections of unfertilized young female cones, were cultured in variations of DCR induction medium. None of these explants originated detectable embryogenic tissue, although *callus* obtained from unfertilized cones were homogenic and similar among them, while *callus* obtained from mature embryos presented distinct areas in the same tissue. Non-embryogenic *calluses* were exposed to 100 µM 2,4-D, pH 4, pH 10, 0.3 M sucrose and 0.15 M sucrose plus 0.15 M mannitol, for 1, 2, 4 and 8 days, but none of these treatments converted the *callus* to embryogenic. The genotype of the mother plant must be taken in consideration and new induction medium compositions and other molecules must be investigated in order to improve induction of somatic embryogenesis in *P. halepensis*.

Keywords: explant, histology, induction, non-embryogenic, *Pinus halepensis*.

Abbreviations: 1-Naphthaleneacetic acid (NAA); 2,4-Dichlorophenoxyacetic acid (2,4-D); 6-Benzylaminopurine (BAP); Kinetin (KIN); Plant growth regulators (PGR).

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1. Introduction

1.1. Contextualization of the work

This project results from a partnership between the Center of Functional Ecology of the University of Coimbra, Portugal, and Neiker-Tecnalia, Spain. This collaboration has the objective to study somatic embryogenesis in woody plants, particularly in coniferous like *Pinus radiata*, *Pinus halepensis*, among others, so these species can be applied in restoration and afforestation/reforestation programs, conservation of species and also in genetic improvement programs.

To this date, there are only a few studies of the somatic embryogenesis in *Pinus halepensis*, they were carried by Montalbán *et al.* (2013) and Pereira *et al.* (2015 to 2017). Due to this information and the collaboration referred above it was intended to continue this partnership by optimizing steps of the protocol of somatic embryogenesis and understand the morphogenic behaviour of in vitro cultures of this pine.

1.2. *Pinus halepensis* Mill.

Pinus halepensis Mill. (Fig. 1A), also commonly referred to as Aleppo pine, is a monoecious gymnosperm that belongs to the Pinaceae family.

In natural conditions, this species can reach 20 m in height and 150 cm diameter of the trunk (Mauri *et al*., 2016). Features a crown broadly conical to dome-shaped, a greyish bark and light green needles arranged in groups of two (between 6 and 12 cm long) (Fig. 1), with age the crown will flatten and open, and the bark will turn to reddish-brown and fissure (Talavera *et al*., 1999; Mauri *et al*., 2016).

It has cones of both sexes in separate structures; the female cones have a biennial maturation period and in the same tree cones in different phases of development can be found (Fig. 1B, 1C & 1E). The female cone usually appears alone or in clusters of 2 or 3, and when mature are brown, pedunculated and with 6-12 x 3,5-4,5 cm (Fig. 1E) (Simón *et al*., 2012). In the first summer and autumn, these cones will have limited development, only after fertilization will happen the second phase of development, during the following summer (Simón *et al*., 2012). The male cones are smaller (3-4 x 5-8 mm), brownish-yellow and grouped in large

Figure 1. Morphological characteristics of *Pinus halepensis* Mill. (**A**) Allepo pine tree (**B**) & (**C**) Young female cones (**D**) Cluster of male cones (**E**) Almost mature female cone (from: jb.utad.pt)

numbers when mature (Fig. 1D) (Simón *et al*., 2012). The seeds are usually 5-6 mm long and the natural regeneration of *P. halepensis* depends only upon them. The seeds are resistant to high temperatures and their germination can be improved with thermal shocks, what can be an ecological strategy to colonize an area following forest fires (Skordilis & Thanos, 1997; Calvo *et al.*, 2013).

Pinus halepensis is naturally present in the Mediterranean basin (Fig. 2B), being more abundant in the western Mediterranean, grows mainly on calcareous soils and mostly in habits of lower altitudes and lower arid or semiarid to humid bioclimates, due to his temperature and precipitation requirements (Escudero *et al.*, 1999; Klein *et al*., 2011; Mauri *et al*., 2016). In some areas, the distribution *P. halepensis* is restricted to fire-prone areas, and fire can play an important part in maintaining this species position in the ecosystem (Hanley *et al.*, 1998; Calvo *et al.*, 2013). In Portugal is an exotic species and its present in places closer to the coast, like in Figueira da Foz, near Lisbon and in some areas of the Algarve. (Fig. 2A).

This pine is of great interest, since it possess a thermophile behaviour which makes it one of the most drought resistant pines, and a xerophytic behaviour, possessing a highly plastic hydraulic system and the capacity to maintain a good canopy seed bank under xeric conditions (Tapias *et al*., 2004; Klein *et al*., 2011; Montalbán *et al*., 2013). His root system is highly branched and fast growing; these characteristics, combined with his water-saving strategy,

allow the Aleppo pine to thrive in limestone with high pH (Simón *et al*., 2012; Montalbán *et al*., 2013).

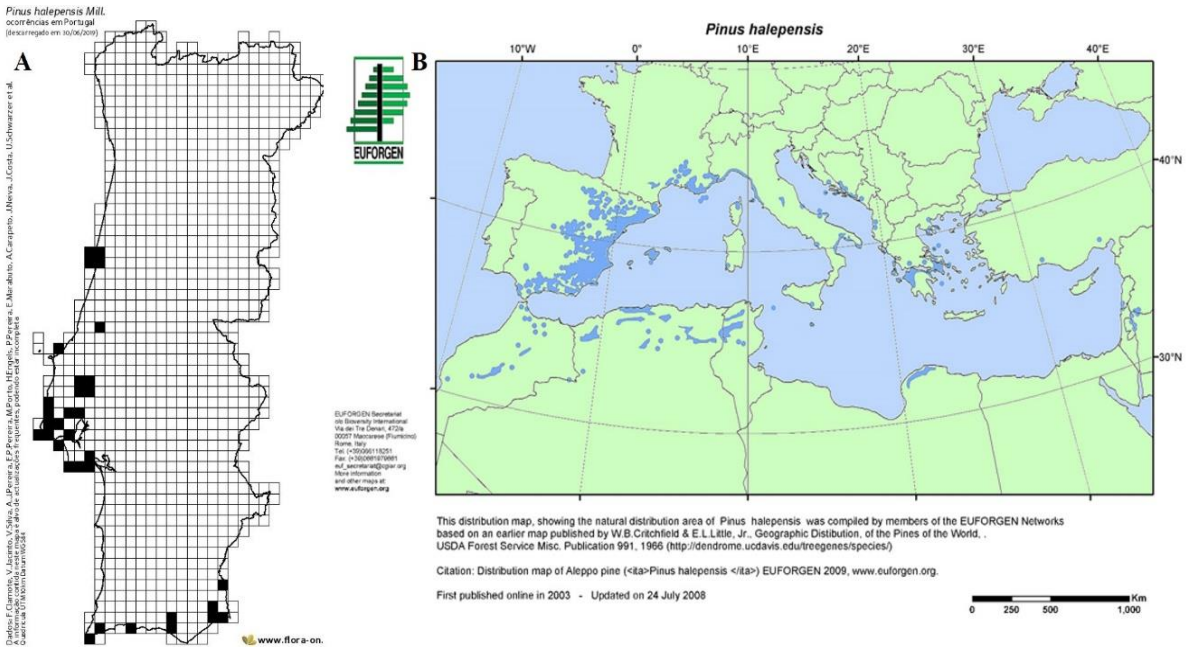

Figure 2. Geographic distribution of *Pinus halepensis* Mill. (**A)** Occurrence of introduced trees in Portugal, more confined to the coastal zone (**B)** Ordinary distribution in the Mediterranean area in Europe (from: flora-on.pt, euforgen.org)

P. halepensis is also a fire resilient tree, it presents strategies like an early reproduction and a heavy production of serotinous cones that will be an advantage in post-fire regeneration (Escudero *et al.*, 1999; Tapias *et al*., 2004; Mauri *et al*., 2016).

In some occasions, this species is used to avert soil erosion on dry slopes and to amend water infiltration on mountainous hills, but studies are not clear about this ability (Mauri *et al*., 2016). Another feature of interest is that some authors defend that although it can be a host, *P. halepensis* has a moderate resistance to *Bursaphelenchus xylophilus*, the pine wilt nematode that has been devastating pine forests worldwide (Evans *et al*., 1996; Futai, 2013).

For the above reasons, this species has been used for afforestation programs since the 1920s, targeting soil protection and windbreaks near the coast, and is indicated for reforestation of marginal and sub marginal areas due to its capacity to thrive in poor soils and its ability to withstand higher temperatures and reduced levels of water in the soil (Montalbán *et al.*, 2013; Osem *et al.*, 2013; Mauri *et al*., 2016). In addition, is also used for firewood and as raw material for the paper and pulp industry (Mauri *et al*., 2016).

With the increase of problems driven from the advance of global warming, like the increase of droughts and forests fires, *P. halepensis* can become an essential species in these scenarios, in order to maintain biodiversity and mitigate desertification. In 2008, Garzon *et al*. concluded that in scenarios of reduced rainfall, warming and intensification of summer drought, *P. halepensis* appeared to be capable of increasing its occupied area.

1.3. Biotechnological tools

Forests and their productivity have been and are extremely important to current societies, human history and overall the planet (Boisvenue & Running, 2006). In Europe, forest and other wooded lands occupy more than 43% of the land, hosting a dominant part of Europe's terrestrial biodiversity (Bastrup-Birk *et al*., 2016). Despite this, currently, forests deal with increasing pressure, mostly anthropogenic, from fragmentation, climate change, loss of biodiversity and expansion of urban areas, while the demand on forest services are increasing every day accompanied by rapid deforestation (Harfouche *et al.*, 2011; Bastrup-Birk *et al*., 2016). Forests have a lot of important roles, such as the protection of land and water resources, mitigation of the increasing CO_2 levels and climate change, maintaining biodiversity, capturing and storing carbon to provide bio-fuel, and raw materials for diverse purposes, like building and construction, furniture, production of energy and the making of paper (Walter, 2004; Harfouche *et al.*, 2011; Bastrup-Birk *et al*., 2016). Many of these products can be produced simultaneously, and trade-offs can occur mostly between commercial and non-commercial products (Bastrup-Birk *et al*., 2016).

Nowadays the world is facing huge population growth and economic development, which can be a challenge with the continuous decrease of cultivated areas, shortage of resources and urban growth (Campbell *et al.*, 2003; Canhoto, 2010). In the near future, there will be an increase in the demand for forest products accompanied by a demand to conserve forest ecosystems, the world will need to produce more with less land and water (Campbell *et al.*, 2003; Canhoto, 2010).

However, this isn’t the only problem that our society might face, climate change has been identified as one of the biggest environmental, social and economic threats, and forests are especially sensitive to these changes, due to the long life-span of trees that do not allow them to rapidly adapt (Linder *et al.*, 2010; Portuguese Environment Agency, 2019). The Mediterranean region will be particularly vulnerable to climate changes, with strong drought

effects and an increase of extreme events like storms, flooding, fires and heat waves being expected in the years ahead (Linder *et al.*, 2010; Sarris *et al.*, 2011). These changes can have strong negative effects on the growth of pine species, although in the northern Mediterranean basin, the recent increase in the minimum temperature seems to improve the growth of *P. halepensis* (Sarris *et al.*, 2011; Sánchez-Salguero *et al.*, 2012). The future of most forest ecosystems will depend on how fast the climate changes happen and how fast can forests adapt to these changes, the problem is that the impacts of environmental changes are uncertain and the multiple threats to forest ecosystems can act independently or in combination (Boisvenue & Running, 2006; Linder *et al.*, 2010; Klein *et al.*, 2011).

It is imperative to increase the productivity of trees while conserving ecosystems and reduce the environmental impacts of the agricultural activity; the world needs to reforest and establish managed plantations and one way of meeting these demands is through the use of biotechnological tools, since the classical techniques of breeding to genetic manipulation of plants, and plant cloning (Campbell *et al.*, 2003; Canhoto, 2010; Harfouche *et al.*, 2011). *In vitro* culture can be defined as a tissue culture technique in which a plant can be regenerated from small portions of tissue, plant cells or organs, under aseptic and controlled conditions that can be later used to obtain certain products, new characteristics or to perform certain functions (Davis & Becwar, 2007; Canhoto, 2010). With the appropriate culture medium and conditions, like mineral elements, vitamins, amino acids, hormones, carbohydrates, physical factors, it is possible to observe *in vitro* dedifferentiation, a process that will cause an organized structure to change and lead to the formation of a *callus*, in which the cells are relatively uniform (Canhoto, 2010). These conditions will vary depending on the species or the desired results.

The use of these techniques has enormous potential, they can not only develop more productive plants but also develop varieties more tolerant to water and salt stress, which can have important effects on the conservation of resources and the use of land (Canhoto, 2010). Nowadays, clonal propagation has been used to establish superior individuals, but there's still a lot of work and investigation to do in this matter (Campbell *et al.*, 2003).

The conventional techniques of plant biotechnology can provide clones with a genotype of interest and genetically modified plants (Canhoto, 2010). They have been used for millions of years and have granted a significant improvement to plant biotechnology in the past, but these techniques are no longer sufficient to meet the requirements of today's modern societies (Nehra *et al.*, 2005; Canhoto, 2010; Harfouche *et al.*, 2011). The difficulties of conventional

techniques are the reduced number of obtained plants, the slowness of the process due to the long reproductive cycles and juvenile periods of most tree species, the struggle in achieving notable improvements to complex traits, and in some cases the availability of the means necessary for the accomplishment of this processes (Nehra *et al.*, 2005; Canhoto, 2010; Harfouche *et al.*, 2011). With the use of plant biotechnology tools, like *micropropagation techniques*, one can obtain clones with a genotype of interest and also genetically modified plants with fewer difficulties, less vegetal material, faster and more efficiently (Canhoto, 2010). Biotechnology can also provide a better understanding of the genome organization, functioning of genes, morphogenesis processes and other methods that will contribute to the study and comprehension of molecular, biochemical and physiological mechanisms that will improve biotechnology development (Nehra *et al.*, 2005; Canhoto, 2010).

1.4. Somatic embryogenesis

One of the methodologies used in the field of plant biotechnology is somatic embryogenesis, which can be defined as the development of embryos from somatic cells. These embryos, morphologically identical to their zygotic counterparts (Fig. 3), may have a unicellular or multicellular origin (Chawla, 2002; Smertenko & Bozhkov, 2014;). The main differences between zygotic and somatic embryos have to do with their origin and their formation site, which will affect only their first stages of development, after that somatic embryos will go through the same development stages as zygotic embryos and, when mature, somatic embryos do not need desiccation and dormancy periods (Fig. 3; Canhoto, 2010; Smertenko & Bozhkov, 2014). These similarities suggest that the genetic control is similar in zygotic and somatic embryogenesis (Smertenko & Bozhkov, 2014).

Somatic embryogenesis involves a series of phases that can be identified by different molecular and biochemical events (Zavattieri *et al.*, 2010). The first step on somatic embryogenesis is the induction phase and the success of this phase will be essential for the entire process (Stasolla & Yeung, 2003). In this phase, differentiated somatic cells, from a leaf or stem segment, zygotic embryos, ovules, seedlings, protoplasts or microspores, gain embryogenic competence whether directly, if they form directly in the explant used, or indirectly, when there is *callus* formation (Jiménez, 2001; Zavattieri *et al.*, 2010). This phase requires the reprogramming of cells by an appropriate external stimulus that will lead to a

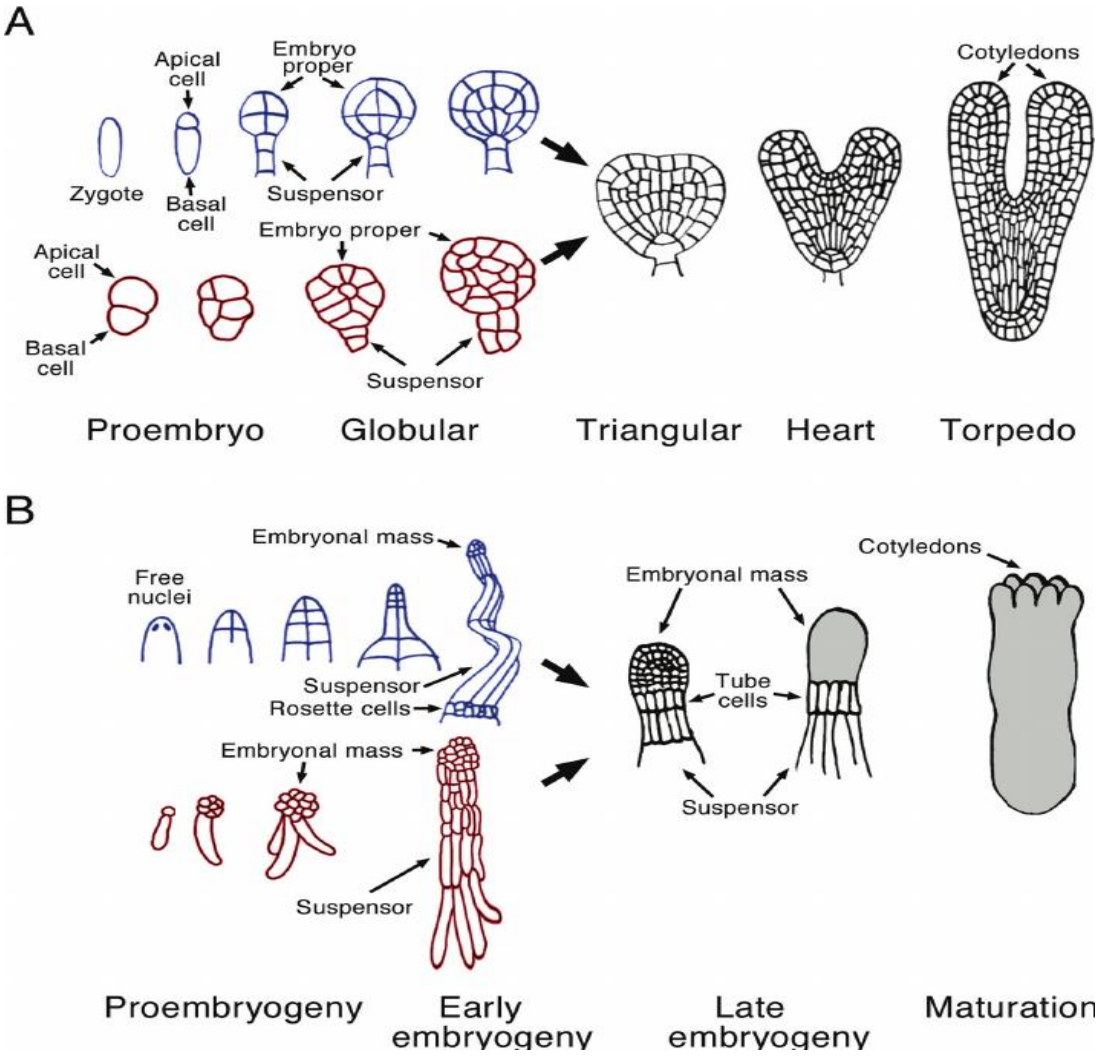

Figure 3. Comparison of zygotic embryogenesis (blue) and somatic embryogenesis (red) in (**A**) angiosperms and (**B**) gymnosperms, in both processes the initial phases present their differences and distinct origins but further in the embryogenesis both go through the same phases, having almost no differences between the somatic embryo and the zygotic embryo. (from: Smertenko & Bozhkov 2014, © Springer-Verlag Berlin Heidelberg)

change in the gene expression and consequently establish cell lineages with different morphology, gene transcription pattern and developmental fate (Litz, 1993; Zavattieri *et al.*, 2010; Smertenko & Bozhkov, 2014). The induction phase depends on many conditions and factors, and these will vary between species, although some species in particular trees, have shown some recalcitrance to somatic embryogenesis (Litz, 1993). While some authors sustain that the stage of development, preconditioning, and genotype are the factors that will most affect the induction, others advocate that induction is more dependent on the induced stimulus, like the concentration and type of plant growth regulators (PGR) or stress factors such as culture medium pH, osmotic shocks, water stress, among many others (Jiménez, 2001; Zavattieri *et al.*, 2010; Smertenko & Bozhkov, 2014). In some species, the selection of somatic embryogenesis

conditions was based on trial and error experiments and are not clear what are the right conditions to induce somatic embryogenesis and what changes must happen in the somatic cell in order to become embryogenic (Jiménez, 2001).

After the induction phase followed by proliferation of the embryogenic tissue, the next phases are embryo development and maturation, and then embryo germination and conversion into plants (Stasolla & Yeung, 2003). Has stated by Smertenko & Bozhkov (2014), these next phases are autoregulatory and can carry with none or minimal contributions from external stimuli. However, it should be noted that the induction phase and the remaining phases appear to be independent of each other and consequently are influenced by different factors (Jiménez, 2001).

In the last years, there has been an effort to develop and optimize protocols for different phases of somatic embryogenesis, mainly because this process holds a notable role in clonal propagation (Stasolla & Yeung, 2003). With somatic embryogenesis is possible to achieve variable objectives, from artificial seeds, long-term germplasm storage, induced dormancy, cryopreservation, cold and dry storage to morphological, biochemical and physiological studies, making it a versatile tool and useful for the development of new technologies (Tautorus *et al.*, 1991; Jiménez, 2001; Corredoira *et al.*, 2019).

1.4.1. Somatic embryogenesis in conifers

Somatic embryogenesis has been a crucial tool for conifers improvement, especially in pines (Lelu-Walter *et al.*, 2016; Trontin *et al.*, 2016b). With this technique, it's possible to select and mass propagate elite genotypes, what can be very useful in industrial production and plantation forestry (Lelu-Walter *et al.*, 2016; Egertsdotter, 2018). But regenerating through somatic embryogenesis in conifers can be a difficult task, mainly because various species can be recalcitrant to in vitro conditions (Stasolla *et al.*, 2002). Over the last few decades, protocols for somatic embryogenesis in most species of conifers have been developed, however, in some cases, there are problems in the maturation of the embryogenic tissue into embryos, the initiation rate is insufficient, there is a reduced efficiency in the germination process and culture survival is often poor (Montalbán *et al.*, 2010; Montalbán *et al.*, 2012; Montalbán *et al.*, 2013; Pullman & Bucalo, 2014).

However, the main issue in regenerating conifers is with the selection of the explant used in the initiation phase. For most conifers, juvenile tissues like immature zygotic embryos

are the most used, although, in some species is possible to induce somatic embryogenesis from vegetative shoot apices (Malabadi & Van Staden, 2005), secondary needles of mature pines (Malabadi & Nataraja, 2007), mature zygotic embryos (Gupta & Durzan, 1986), excised cotyledons (Krogstrup, 1986), intact female gametophytes containing immature zygotic embryos (Becwar *et al*., 1990) and seedlings (Attree *et al*., 1990) (Stasolla *et al.*, 2002; Montalbán *et al.*, 2011; Silva & Malabadi, 2012). The major problem of using immature zygotic embryos, besides the narrow competence window of this explant, is that it is the result of a sexual crossing, therefore, it is not possible to capture the genetic gain, which will imply that we have to perform test periods on the clones produced while the embryogenic cultures are cryopreserved (Klimaszewska & Cyr, 2002; San-José *et al.*, 2010; Montalbán *et al.*, 2011). In the case of *Pinus halepensis*, studies have been based on the culture of megagametophytes containing immature pre-cotyledonary zygotic embryos for initiating somatic embryogenesis (Montalbán *et al.*, 2013; Pereira, 2015).

Due to these difficulties, protocols for somatic embryogenesis in some conifers still need to be optimized in order to turn the initiation rate sufficient for commercial applications. However, somatic embryogenesis is a good multi-propagation process, especially when combined with other technologies, such as cryopreservation, and a good system for genetic transformation (Montalbán *et al*., 2012).

In the case of conifers, stages of somatic embryogenesis usually depend on the result of the previous stage, and every stage has is different difficulties, therefore, the choice of the explant in the right phase and the appropriate medium will be important not only in induction but also in the following phases (Klimaszewska & Cyr, 2002). There are several basal mediums that can be used in conifers. In the case of *Pinus halepensis*, the DCR medium (Gupta & Durzan, 1985; Montalbán *et al.*, 2013) is usually applied. The medium will be supplemented with organic nitrogen sources, a low percentage of sucrose, growth regulators and agar, usually gellan gun (Stasolla *et al.*, 2002; Klimaszewska *et al.*, 2007). Other factors, like pH, agar, nitrogen level and light regime will also affect somatic embryogenesis (Stasolla *et al.*, 2002). Plant growth regulators are a very important factor because they will promote the transition of phases, although non-hormonal stimuli, such as stress factors can also have the same effect (Pullman & Bucalo, 2014).

In the induction phase, it is necessary a combination of auxins, usually 2,4-dichlorophenoxyacetic acid (2,4-D), and cytokinins (Stasolla *et al.*, 2002; Feher, 2008).

Asymmetrical cell divisions in the embryogenic tissue will define the initiation of a somatic embryo formation and after 2 to 16 weeks at 22-25 ºC in the dark, there will be a visible growth of embryogenic tissue, which is constituted of early-stage embryos or pro-embryos multiplicating though budding and cleavage (Timmis, 1998; Klimaszewska & Cyr, 2002; Stasolla *et al.*, 2002; Jiménez & Thomas, 2005; Klimaszewska *et al.*, 2007). The rate of somatic embryogenesis initiation will depend on the developmental stage of the chosen explant (Klimaszewska & Cyr, 2002).

When the forming embryogenic mass reaches a few millimetres in diameter, they need to be subcultured onto a solid or liquid medium of composition similar to the induction medium (Timmis, 1998; Stasolla *et al.*, 2002). These cultures are maintained in the dark, at 22-25 ºC and subcultured every 10-14 days (Stasolla *et al.*, 2002). Prolonged subculture is associated with changes in the embryogenic potential and genetic instability, so in order to prevent this, the tissues can be stored and maintain their juvenility and genetic fidelity (Klimaszewska & Cyr, 2002; Stasolla *et al.*, 2002; Egertsdotter, 2018).

In order to initiate the maturation phase, auxins and cytokinins need to be removed and abscisic acid (ABA) added with an increase in the osmolarity (Stasolla & Yeung, 2003). This will inhibit cleavage polyembryony and promote the proper development of the embryos (Timmis, 1998). Some protocols can implement a pre-maturation step, low light intensity or activated charcoal and maltose to improve results (Timmis, 1998; Klimaszewska & Cyr, 2002). During this phase, the embryo increments in size, initially presenting a globular head and filamentous aspect but after 6 to 7 weeks it's possible to see cotyledons, 5 to 8 depending on the species, arise from the proximal portion of the embryos (Timmis, 1998; Stasolla *et al.*, 2002). The resulting embryos of the maturation phase will be morphological mature and resemble mature zygotic embryos, however, they present a smaller and less defined shoot apical meristem (Klimaszewska & Cyr, 2002; Stasolla & Yeung, 2003).

If at the end of the maturation phase the water content of the mature embryos is not sufficiently low there is a need for a desiccation period that will increase germination and conversion to plantlets (Klimaszewska & Cyr, 2002; Stasolla *et al.*, 2002). A desiccation period will reduce the water content and help mature embryos reach physiological maturity (Klimaszewska & Cyr, 2002; Stasolla *et al.*, 2002). Completely matured embryos are defined by distinct root and shoot apical meristems (Stasolla *et al.*, 2002). After this step, somatic embryos are normally germinated in a hormone-free gelled medium, that contains a low sucrose

concentration and might contain activated charcoal and a source of organic nitrogen (Timmis, 1998; Klimaszewska & Cyr, 2002; Stasolla *et al.*, 2002). On the first weeks, light intensity should be low and then slowly increased (Klimaszewska & Cyr, 2002). After 12 to 16 weeks is expected the development of needles, elongation of an epicotyl and sufficient growth to be transplanted to soil on a greenhouse, with humid shaded conditions, for an acclimation period prior to transfer to the desired site (Klimaszewska & Cyr, 2002; Stasolla *et al.*, 2002).

1.5. Objectives

Studies on somatic embryogenesis of *Pinus halepensis* have focused on testing different temperatures and water availability in initiation (Pereira *et al.*, 2016) and proliferation (Pereira *et al.*, 2017) phases; how different environmental conditions in early stages of somatic embryogenesis influences maturation rates, number and quality of embryos (Pereira, 2015); determinating the proper immature zygotic embryo collection time and induction medium (Montalbán *et al.*, 2013), and testing the effect of activated charcoal, sucrose and nitrogen source in the maturation and conversion into plantlets medium (Montalbán *et al.*, 2013). Although there are still a lot of aspects to improve in the several steps of somatic embryogenesis in *Pinus halepensis*, the explant used is still a limiting step in multi-propagation of this pine. Eliminating the narrow competence window and/or maintain the genetic material of the mother plant would undoubtedly improve somatic embryogenesis. In order to overcome these limitations, it is not only important to test new explants but also to study the development of the zygotic embryo and try to work with the results of the tested explants.

Taking this into account, the purpose of this work was to improve the initiation phase in somatic embryogenesis and expand the knowledge about the embryogenic process in *Pinus halepensis*. Therefore, the first objective was to induce embryogenesis with different types of explants at different development stages in different mediums. The second objective of this work was the treatment of non-embryogenic *calli* with auxins, sucrose, mannitol, and variations of pH in order to stimulate somatic embryogenesis. The third and final objective of this work was to characterize phases of the embryogenic process by optical microscopy and to study cones and their evolution in order to observe at what point the embryo begins to develop.

2. Materials and Methods

2.1. Initiation assays of embryogenic cell lines

2.1.1. Plant material

The plant material used was collected from several wild trees free-pollinated near the city of Figueira da Foz, Portugal, (Latitude: 40,1507 and Longitude: -8,8187) between October 2018 and May 2019. During this time female cones in different stages of development were found and collected. Their morphology at the time of gathering can be seen in figure 4. The collected cones were stored at 4 ºC until they were used, for a period that never exceeded 2 months.

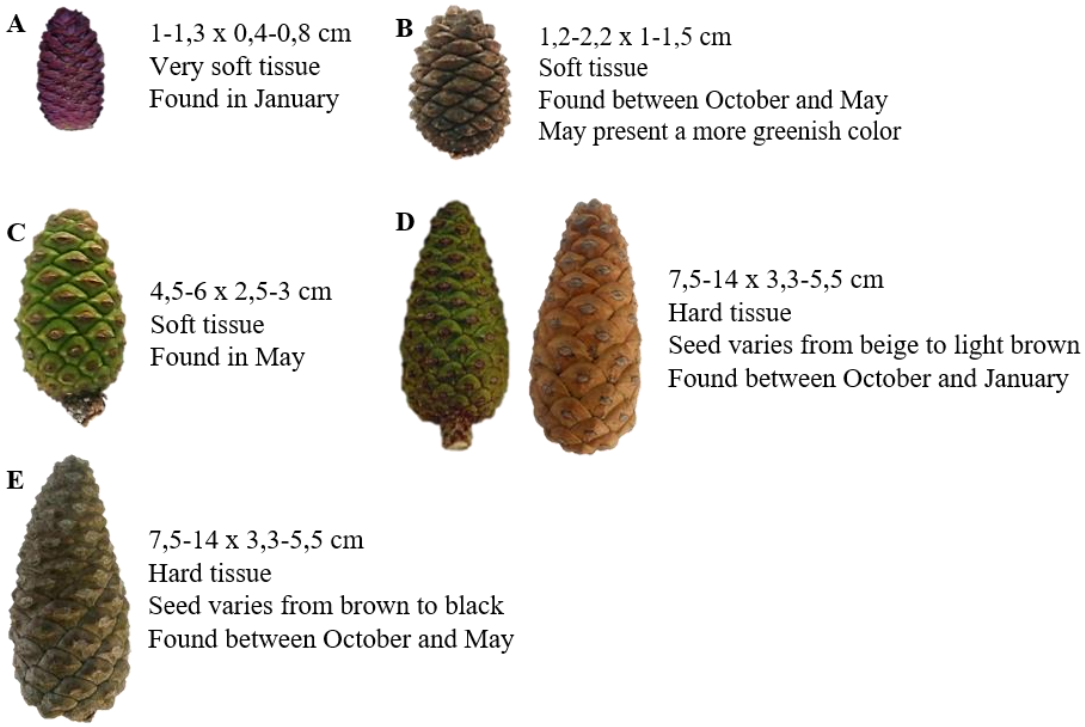

Figure 4. Morphogenic characteristics of female cones of *Pinus halepensis* collected between October 2018 and May 2019.

2.1.2. Analysis of the developmental stage of zygotic embryos

For the cones in figures 4D and 4E, few megagametophytes were isolated from the seeds and then the embryo removed. The embryos were observed with a Zoom Stereomicroscope and all of them were in the cotyledonary stage, and in some cases, it was even easy to distinguish the cotyledons with naked eye.

For the cones at stages illustrated by the figures 4B and 4C, due to their soft tissue and smaller portions, histological assays were carried out to identify their developmental stage. In the brownish smaller cones (Fig. 4B), the megagametophyte appeared to be not developed yet, therefore the purple younger cones (Fig. 4A) are in a more precocious stage of development. In the cones of figure 4C the megagametophyte was already formatted and so the archegonia.

2.1.3. Initiation of cell lines

2.1.3.1. Cotlyledonary stage embryos as explants

For the larger cones with embryos at cotyledonary stage (Fig. 4D) collected in October, their surface was sprayed with 70% (v/v) ethanol and then divided in four pieces in order to isolate all the seeds. Under a laminar flow, the seeds were submerged in H_2O_2 10% (v/v) with one or two drops of Tween 20®, depending on the quantity of seeds, for approximately 12 min and washing with sterile distilled water for three or more times. Under aseptic conditions, the megagametophytes were removed from the seed and then the intact embryo isolated and placed horizontally on the medium (Fig. 5A). The culture conditions used were those described by Montalbán *et al*. (2013) and by Pereira (2015). These culture conditions include DCR induction medium (Gupta and Durzan, 1985) and four variations of this medium. All the media were supplemented with 3% (w/v) sucrose, 3.5 g/L Gelrite® and the pH was adjusted to 5.7 before autoclaving. The five mediums tested differed from each other in the concentration of Kinetin (KIN) and 2,4-Dichlorophenoxyacetic acid (2,4-D) used: medium 1 2.7 µM KIN and 18 µM 2,4-D; medium 2 1.35 µM KIN and 9 µM 2,4-D; medium 3 1.35 µM KIN and 18 µM; medium 4 had the ordinary combination used in induction DRC medium, 2.7 µM KIN and 9 µM 2,4-D; finally medium 5 had no PGR added (Fig. 5B). All the media were autoclaved at 121 ºC for 20 min and after a filter-sterilized solution of EDM amino acid mixture (Walter *et al.*, 2005) is added to the warmer medium. Ten embryos were placed per Petri dish (Fig. 5B) containing approximately 20 mL of induction medium and stored in a growth chamber at about 23 ºC, in darkness. Four replicas were tested in each treatment.

Using cones with embryos at the same development stage, other two experiments were carried out using the same sterilization process and the same media as described before. In the first one, there were used cones collected in May (Fig. 4E) and was tested a modification of the DCR medium (DCR IM) based on IM medium used by Park *et al.* (2010) to induce somatic embryogenesis in shoot buds of *Pinus contorta*. This DCR medium has the regular composition of DCR medium, and 20 µM 2,4-D, 25 µM 1-Naphthaleneacetic acid (NAA), 9 µM 6-

Benzylaminopurine (BAP), 90 µM maltose, 2 mg/L glycine and 1.5 g/L of gelrite® (Fig. 5C). In this case seven embryos were used per Petri dish (Fig 5C) and three replica per treatment. In the second one, there were utilized cones collected in November (Fig. 4D) and the isolated embryos were longitudinally cut in half and each part placed separately and horizontally in the medium (Fig. 5D). In each Petri dish five halves of the embryos were cultured, and the five DCR induction media (1,2,3,4 and 5) (Fig. 5D) and four replica per treatment were evaluated.

2.1.3.2. Tissues of unfertilized young cones as explant

For the brownish smaller cones collected in November and May (Fig. 4B), these were sterilized using the same method has describe in previous sections. In aseptic conditions, the scales were isolated from the cone and the woodier part cut out (Fig. 5E). The tested media were induction DCR medium (for the ones collected in November) and the same five mediums (DRC induction mediums 1, 2, 3, 4 and 5) used in the previous experiment (for the ones collected in May) (Fig. 5F). There were used three cut scales per Petri dish (Fig. 5F) and for the DCR medium seven replicas were used whereas for the five different treatments four replica were tested per treatment.

For the purplish smaller cones collected in January (Fig. 4A), they were first sprayed with 70% (v/v) ethanol and then horizontally sliced (Fig 5G) in laminar flow chamber. Each Petri dish containing DRC induction medium had three sections of the cones (Fig. 5G) and three replicas were tested.

2.1.4. Proliferation of cell lines

After 4 to 13 weeks in the DRC initiation medium, the originated embryonal masses that had approximately 10 mm of diameter were separated from the cultivated explant and subcultured, initially on DRC induction medium and on the next subcultures in DRC proliferation media. These media are very similar to the induction media, maintaining 2.7 µM KIN and 9 µM 2,4-D and the same pH, sucrose concentration and EDM mixture. The only difference was that DCR proliferation media have 4.5 g/L of gelrite®. These samples were subcultured each two weeks and stored in the dark at 23 ºC.

2.2. Assays to convert non-embryogenic cell lines to embryogenic

In this experience there were used non-embryogenic cell lines resulted from the previous assays. H18-28, a non-embryogenic cell line, resulted from a whole embryo cultivated in induction media 2, was cultured in DCR proliferation medium with 0.3 M of sucrose or DCR proliferation media containing 0.15 M of sucrose plus 0.15 M of mannitol, for one, two, four and eight days of incubation, and then transferred to regular DCR proliferation media. For each treatment three replicas were made and each Petri dish had two *callus* clusters with approximately 1.5 cm diameter.

Another assay was carried out using non-embryogenic cell lines resulted from scales cultivated in DCR induction media. Two *callus* clusters with approximately 1.5 cm were cultured on DCR proliferation media with 100 µg/L 2,4-D, DRC proliferation media with pH 4 and DRC proliferation media with pH 10 for the same incubation times and replicas as in the previous described assay.

2.3. Histological assays

For the histological assays, ovuliferous scales of small brownish cones (Fig. 4B) and the seeds of green cones (Fig. 4C) were fixed for 24 h at room temperature in 100% glacial acetic acid. The samples were additionally dehydrated in a rising ethanol series followed by Clear Rite™ (ethanol 70% 2x, 90% 2x, 95%, 100% 2x, 100%+ Clear Rite™, pure Clear Rite™ 2x) and embedded in paraffin wax at 65 ºC. The samples are then oriented and placed in molds containing paraffin at 65 ºC. Included material was obtained following cooling of the paraffin at room temperature. Sections of approximately 10 µm were obtained in a rotary microtome and transferred first into water and later on to microscope slides previously prepared with glycerine albumin. These microscope slides were transferred to an environmental chamber for 12 h and then submitted to a dewaxing with Clear Rite™, 100% ethanol and finally a quick washing under running water. After this step the samples are stained with 0.2% (w/v) toluidine blue for 1 h at room temperature and washed again in running water. The samples were observed in an optical microscope and photos taken with a Nikon DS-Fi3 camera using the NIS-Elements software (version 4.60).

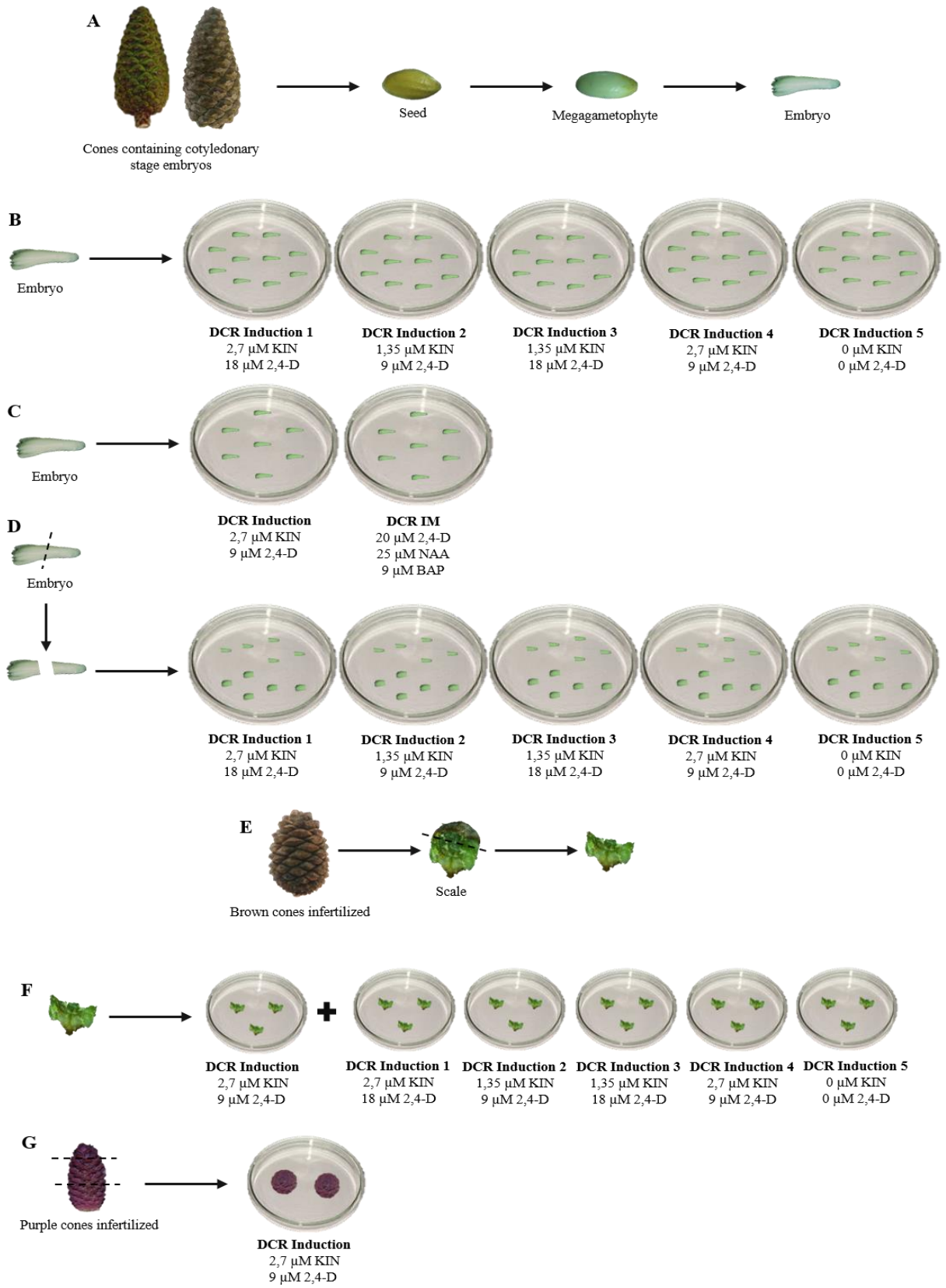

Figure 5. Schematic representation of the procedures and culture conditions utilized to induce cell lines in the Allepo pine. (**A**) The collected cones were sterilized and the embryo, intact or cut in halves, was isolated from the seed and placed in DCR media supplemented with different concentrations of 2,4-D and Kinetin (**B**), (**C**) and (**D**). (**E**) Younger brownish cones were also sterilized, their scales isolated and cultivated in DCR medium with the same characteristics has before (**F**). (**G**) Young purple cones were sterilized, cut into horizontal sections and cultivated in DCR induction media.

3. Results

3.1. Initiation of cell lines

3.1.1. Cotyledonary stage embryos as explants

For the experiment in which cotyledonary embryos were tested in five different media (Fig. 5B), *callus* proliferation was observed within just a week in 64.5% of the cultured explants, usually with *callus* appearing closer to the colyledonary region (Fig. 6A; Table 1). These *calluses* were generally white and, in some regions, appeared to be slightly filamentous (Fig. 6A). In all the media tested the cotyledons enlarged and some gained a light green colour (Fig. 6A) while the radicle zone seemed to be proliferating into a soft tissue. Embryos in the DCR induction medium 5 begun to germinate. The cotyledons in this media gained a green colour and increase in size together with the hypocotyl and radicle (Fig. 6C and 6D). In some cases, there was proliferation of *callus* also in the hypocotyl (Fig. 6C) or, in other cases, a change of colour to pink/purple (Fig. 6D). After four weeks of culture the developing *callus* started to turn brown. When this happened, they were subcultured in the initiation media.

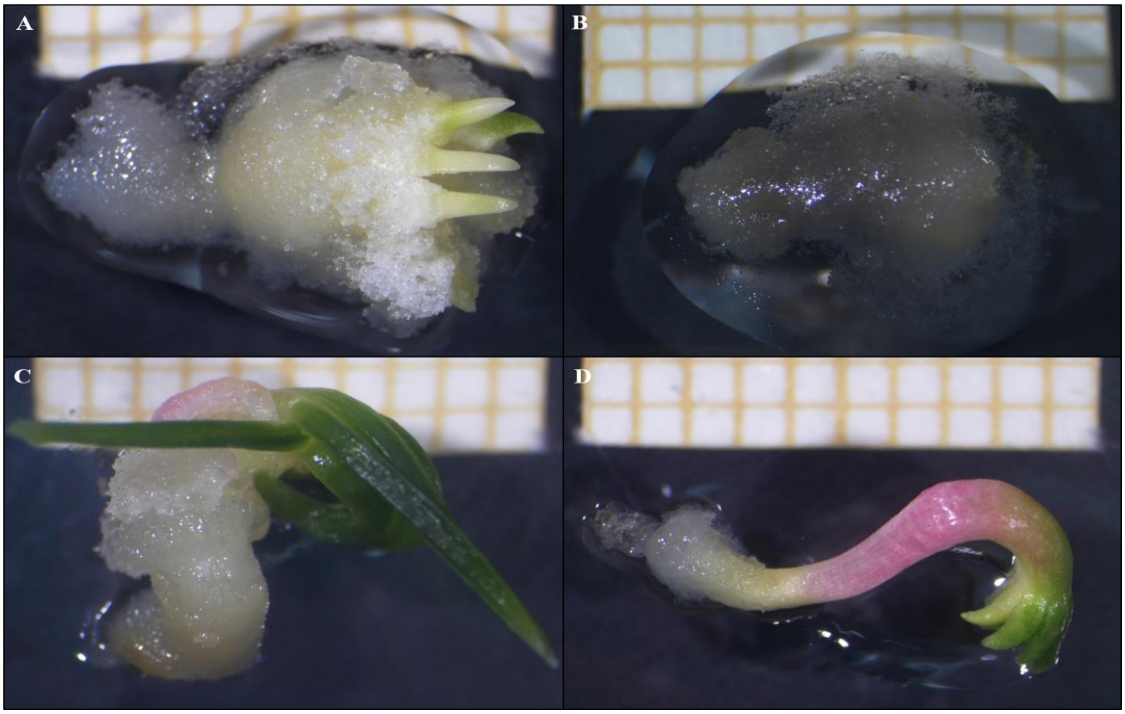

Figure 6. Cotyledonary stage embryos of *Pinus halepensis* cultivated in variations of DCR induction medium. (**A**) embryo in DCR medium 1 showing proliferation of white *callus* closer to the cotyledons (**B**) non induced embryo (**C**) embryo in DCR medium 5 showing signs of germination and *callus* proliferation in the hypocotyl (**D**) embryo in DCR medium 5 showing signs of germination with pink/purple hypocotyl.

When cotyledonary embryos were tested in DCR induction media and DCR IM (Fig. 5C), the response of the embryos in the induction media was similar to the embryos cultivated in DCR induction medium 4 of the previous experiment (Fig. 7C) with 95.24% of the induced explants producing *callus* with the same characteristics as observed in the previous described experiment (Fig. 7C; Table 1). In the DCR IM, the embryos seemed to expand but only 4.76% of the induced explants showed *callus* formation (Fig. 7A and 7B; Table 1). These developing *calluses* also started to turn brown after four weeks but, in this case, they were not subcultured and stayed on induction media for eight weeks. After this time most of the developed *callus* had turned brown and there wasn't formation of new structures.

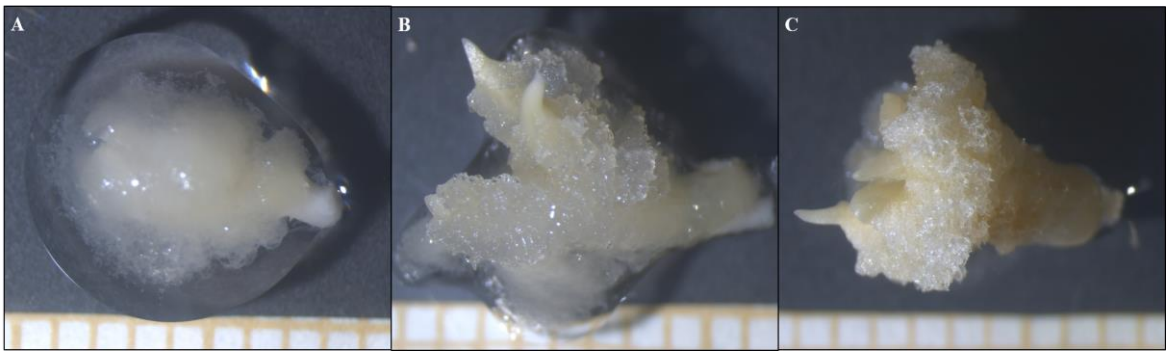

Figure 7. Cotyledonary stage embryos of *Pinus halepensis* cultivated in DCR induction media (**C**)(**D**) and DCR IM media (**A**)(**B**). (**A**) non induced embryo in DCR IM medium (**B**) embryo in DCR IM showing slight proliferation of white *callus* (**C**) embryo in DCR induction media with white *callus* proliferation closer to the cotyledon region.

In the assay in which the cotyledonary embryos were transversally cut and cultivated in five induction media (Fig. 5D), it was also possible to see, within a few days, white *callus* proliferating, mainly in the cotyledonary half (Fig. 8B). In this case, 95% of the explants formed *callus* (Table 1). In these explants cotyledons also slightly elongate and gained a light green colour (Fig. 8E). On medium 5 cotyledon elongation was particularly evident, the cotyledons showed a vivid green colour and had *calli* proliferating in the region were the cut was made (Fig. 8A). In the radicle halves *callus* formation was rarely observed (Fig. 8C), with only 13% of the explants showing cell proliferation (Table 1). On medium 5 most of the radicle halves gained a brown colour (Fig. 8H) but, in some explants, white *calli* could be seen closer to the region where the cut was made (Fig. 8D). This *callus* also started to turn brown after four weeks of culture.

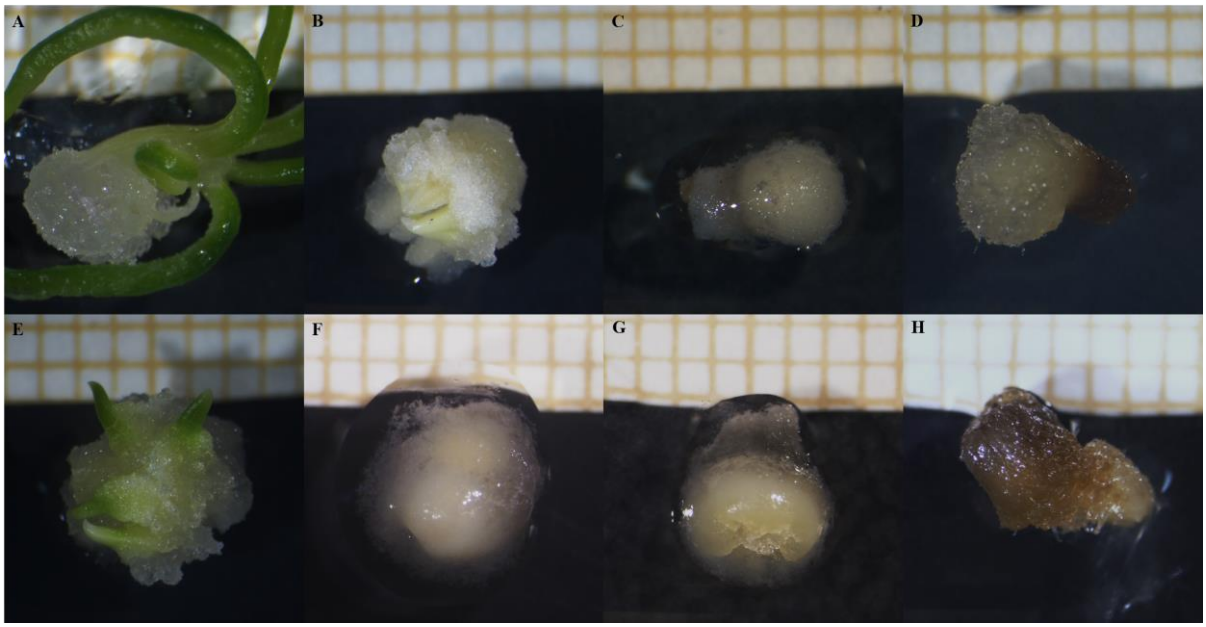

Figure 8. Halves of cotyledonary stage embryos of *Pinus halepensis* cultivated in variations of DCR induction media. (**A**) cotyledonary halve cultured in induction medium 5 with white *callus* proliferating in the region where the cut was made and elongated green cotyledons (**B**) Cotyledonary halve cultured in medium 3 with white *callus* proliferating (**C**) radicular halve non induced (**D**) radicular halve cultivated in medium 5 with slight proliferation of *callus* (**E**) cotyledonary halve in medium 2 showing *callus* proliferation and green cotyledons (**F**) non induced cotyledonary halve (**G**) radicular halve in medium 4 with slight *callus* proliferation (**H**) brown radicular halve in medium 5.

In all of the explants were white *calli* proliferated, this one was usually growing attached to the cotyledons and in some of the explants the *calli* cease to proliferate (Fig. 9C). The ones that showed continuous proliferation, after reaching a few millimetres, were transferred to DRC induction media, to promote *callus* growth. After 15 days on these media the *calluses* were cultured on DRC proliferation media. Once in induction media, the cultivated *callus* started to lose the white and translucent colour and the filamentous aspect and gained a more yellowish colour (Fig. 9A). Some *callus* presented regions that ceased to proliferate and regions that kept on proliferating. In these cases, the zones that kept proliferating were isolated and subcultured into the same medium. Due to contamination the experiments with the whole embryos (Fig. 5B) and cut embryos (Fig. 5D) cultured in the five induction media, 42,5% of the induced explants were lost. This problem had more consequences for the cut embryos, because when it occurred, most of the *calli* derived from whole embryos had already been subcultured, which was not the case for cut embryos.

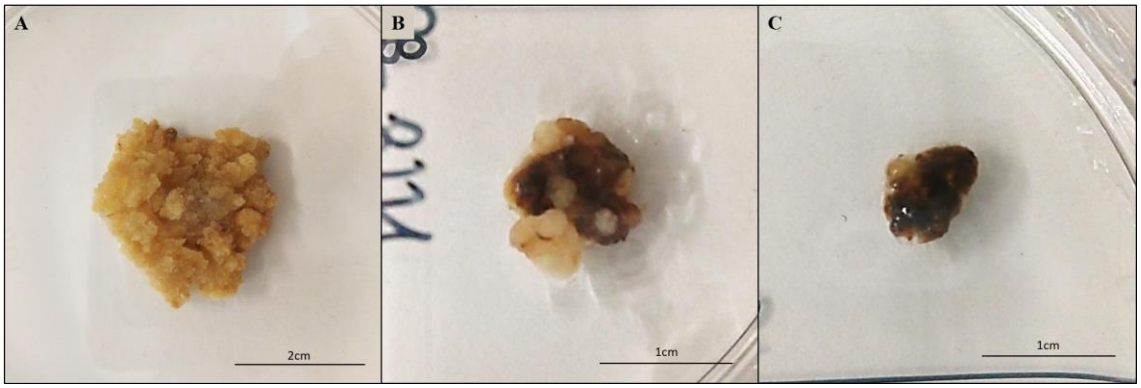

Figure 9. *Callus* obtained from cotyledonary stage embryos of *Pinus halepensis*. (**A**) yellowish *callus* with ordinary proliferation rates (**B**) *callus* with proliferating regions and non-proliferating regions (**C**) *callus* that cease to proliferate.

3.1.2. Tissues of unfertilized young cones as explant

When cut scales of young brownish cones were cultivated (Fig. 5F) *callus* proliferation could be observed in all the explant after one week of culture (Fig. 10). The explants cultivated on DCR induction media, displayed some contamination but, even so, all the scales showed *callus* formation (Fig. 10A). The explants cultured on the five induction media, some explants also contaminated but all exhibited white *callus* proliferating (Fig. 10B; Table 1) except for the ones on media 5, that showed now response at all (Fig. 10C; Table 1).

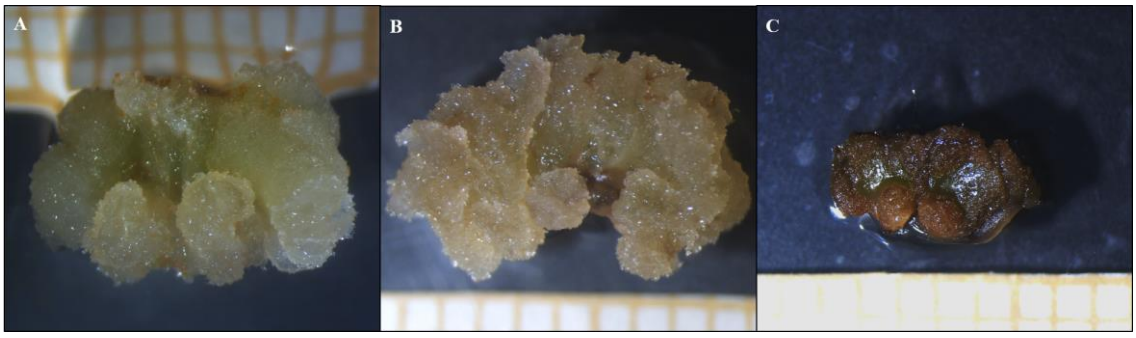

Figure 10. Induced scales of non-fertilized young cones of *Pinus halepensis*. (**A**) scale in DCR induction media (**C**) scale in DCR medium 1 (**D**) non induced scale in DCR medium 5.

In the experiment with the young purple cones (Fig 5G) it was more frequent for the explants to contaminate and only after six weeks it was possible to observe *callus* proliferating in all of the induced explants (Table 1). This *callus* has a more yellowish colour and grew attached to the inner face of the scale rather on the cone axis and the woodier part of the scale (Fig. 11). Once the *callus* proliferated a few millimetres, these were subcultured into proliferation media.

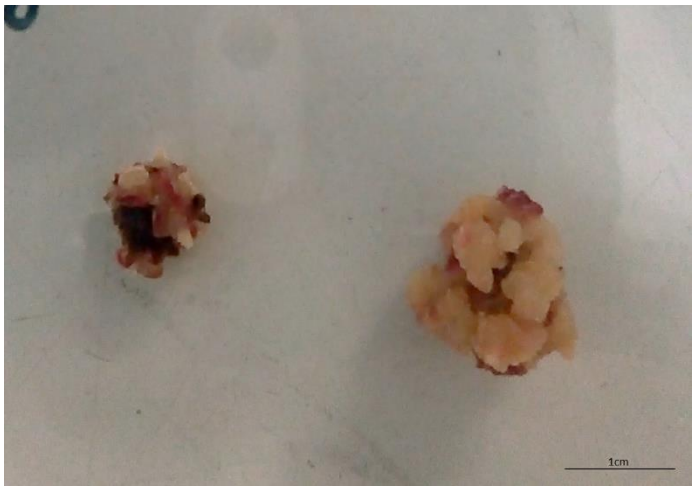

Figure 11. Induced sections of young unfertilized cones of *Pinus halepensis* in DCR induction media.

Table 1. Resume of the percentage of explants that formatted *callus* in all the assays in this work.

Culture media		**Percentage of explants that formatted *callus***				
		Mature embryos	Cotyledons from mature embryos	Radicles from mature embryos	Scales from unfertilized cones	Sections of unfertilized cones
DCR ind. 1	2.7 µM KIN 18 µM 2,4-D	60	85	0	100	-
DCR ind. 2	1.35 µM KIN 9 µM 2,4-D	77.5	95	15	100	-
DCR ind. 3	1.35 µM KIN 18 µM 2,4-D	82.5	95	100	100	-
DCR ind. 4	2.7 µM KIN 9 µM 2,4-D	87.5[1] 95.24[2]	100	100	100[3]	100
DCR ind. 5	0 µM KIN 0 µM 2,4-D	0.75	100	30	0	-
DCR IM	20 µM 2,4-D 25 µM NAA 9 µM BAP	4.76	-	-	-	-

[1] Percentage obtained when mature embryos were cultivated in five variations of DCR induction media

[2] Percentage obtained when mature embryos were cultivated in DCR induction media and DCR IM

[3] Percentage obtained when scales from unfertilized cones were cultivated only in DCR induction media and in the five variations of DCR induction media

3.2. Proliferation of cell lines

As stated before, not all the induced material kept proliferating, especially in the experiments with cotyledonary stage embryos. In these experiments, staining with 2% (w/v) acetocarmine on the squashed *calli* revealed that these were non-embryogenic (Fig. 12), and some of them had different morphologic characteristics, such has variations in colour (from light yellow to dark brown), texture and consistence; and proliferation rates, some cell lines duplicate size in 15 days while others proliferated very slowly. *Calli* which lost their ability to proliferate began to gain a darker colour, became harder to desegregate and some formed rigid clusters within the *callus*, whereas *calli* with greater proliferative capacity had a light yellowish colour, were softer and easier to disaggregate from each other; however, some had regions of the *callus* where whitish-appearing filamentous *callus* could be observed. This type of *callus* was too small to be isolated, so when it was subcultured with the remaining callus, it became yellowish like the rest of the *callus*.

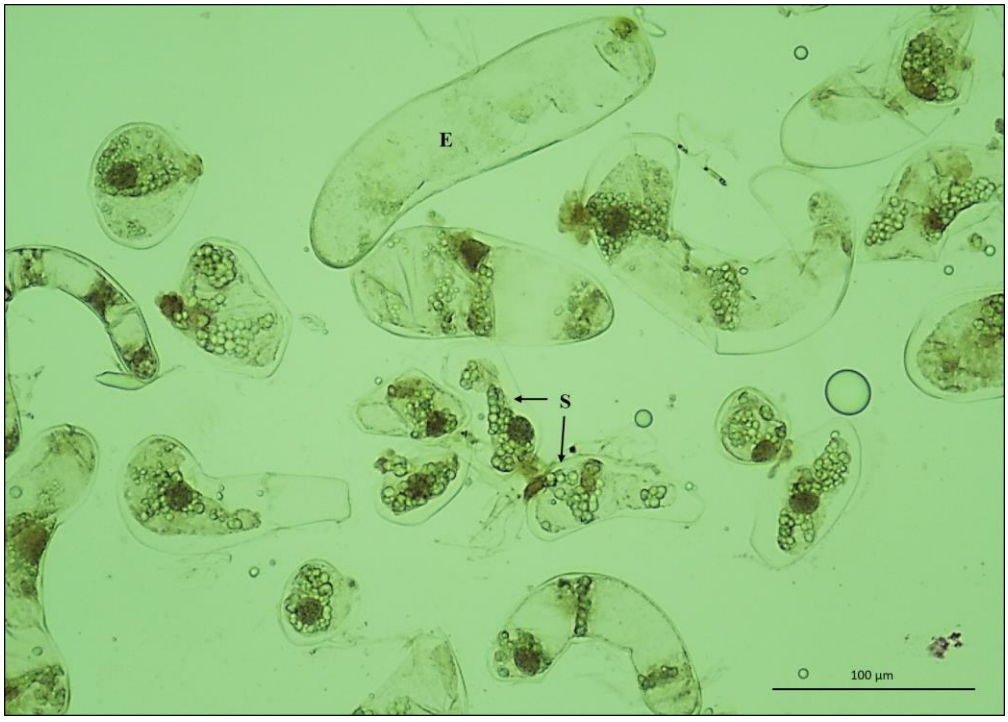

Figure 12. Squash of *calli* resulting from mature zygotic embryos of *P. halepensis* cultured in DCR proliferation media stained with 2% acetocarmine (w/v). The *callus* seems to have a mixture of elongated cells (**E**) and smaller cells with a lot of starch vesicles (**S**).

In the case of *callus* resulting from young cones (Fig 5F and 5G), these had similar morphology, light yellow and easy to disaggregate; and high proliferation rates. Acetocarmine 2% (w/v) staining also revealed that this *callus* was non-embryogenic (Fig. 13). However, after

a few subcultures in DCR proliferation medium, these started to also lose proliferative capacity and started to darkening as well, but they did not get rigid as the previous ones.

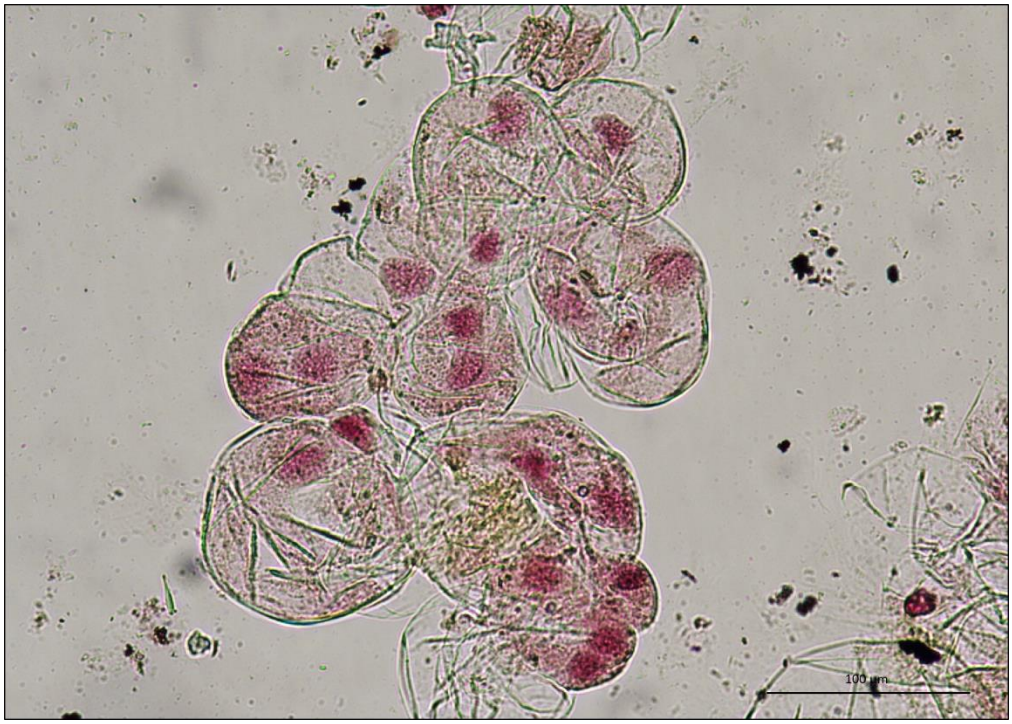

Figure 13. Squash of *calli* resulting from unfertilized scales from young cones of *P. halepensis* cultured in DCR proliferation media stained with 2% acetocarmine (w/v). Irregular shaped cells seem to form aggregates.

3.3. Assays to convert non-embryogenic cell lines to embryogenic

Shock treatments with different pH and 2,4-D concentrations, showed that in some experiments morphologic change of the *callus* occurred. In the case of 2,4-D shocks, with increasing days of treatment it was notorious that the *callus* grew darker and proliferated less. However, after a few days subculture on DCR proliferation medium, white zones appeared. These zones were isolated, subcultured, and after 15 days they returned to a yellowish colour and proliferation rate similar to that presented before the auxinic shock. In shocks with pH 4 and pH 10 these did not darken but became harder and difficult to disaggregate, specially the *callus* from the pH10 shocks, even after some subcultures in DCR proliferation media.

Regular acetocarmine staining (2% w/v) confirmed that these cell lines remained non-embryogenic but some *callus* showed a good proliferation rate, light yellow colour and stayed easy to desegregate. These were the cell lines submitted to pH4 for two days and cell lines submitted to 100 µg/L 2,4-D for one, two, four and eight days.

When stress conditions were induced by sugars, such as sucrose and mannitol, these also had influence on the morphologic characteristics of the *callus*, some became softer and easier to disaggregate, but acetocarmine staining (2% w/v) confirmed that this *callus* remained non-embryogenic (Fig. 14B). These *calli* also started to have zones that were light yellow and other zones more brownish (Fig. 14A). No relevant differences were observed between the different times of incubation in the shock medium. Although the *callus* remained non-embryogenic, after two months of being subcultured back to DCR proliferation media, the *callus* maintained the aspect and proliferation rates that had before submitted to the shock treatments.

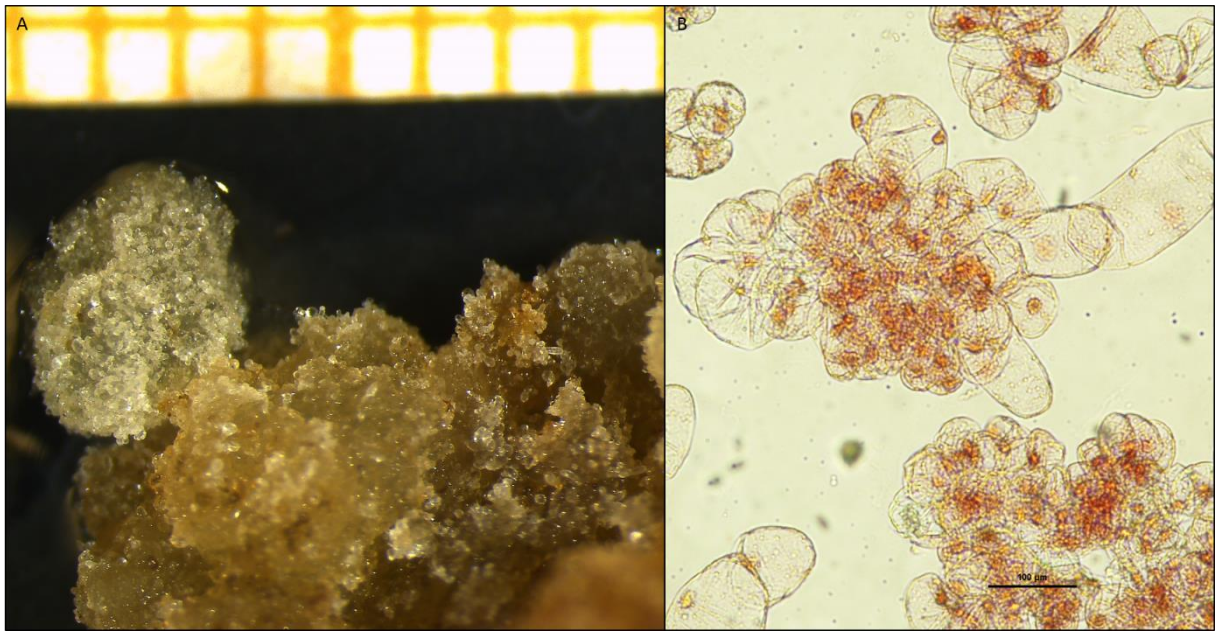

Figure 14. Non-embryogenic *callus* obtained from mature zygotic embryos of *P. halepensis* cultures in DCR proliferation media that were submitted to 0.3 M of sucrose for 4 days and then subcultured back to DCR proliferation media. (**A**) close-up of non-embryogenic *calli* (**B**) squash with 2% acetocarmine (w/v), the cells appear to form disorganized clusters.

3.4. Histological assays

The sections of ovuliferous scales of young brownish cones (Fig. 4B) showed these were in an early stage of the ovule development, prior to the megagametophyte formation, where the nucellus occupies most of the ovule (Fig. 15).

Sections in seeds of green cones (Fig. 4C) showed the megagametophyte containing two archegonia, but it could not be concluded whether fertilization was occurred or not (Fig. 16).

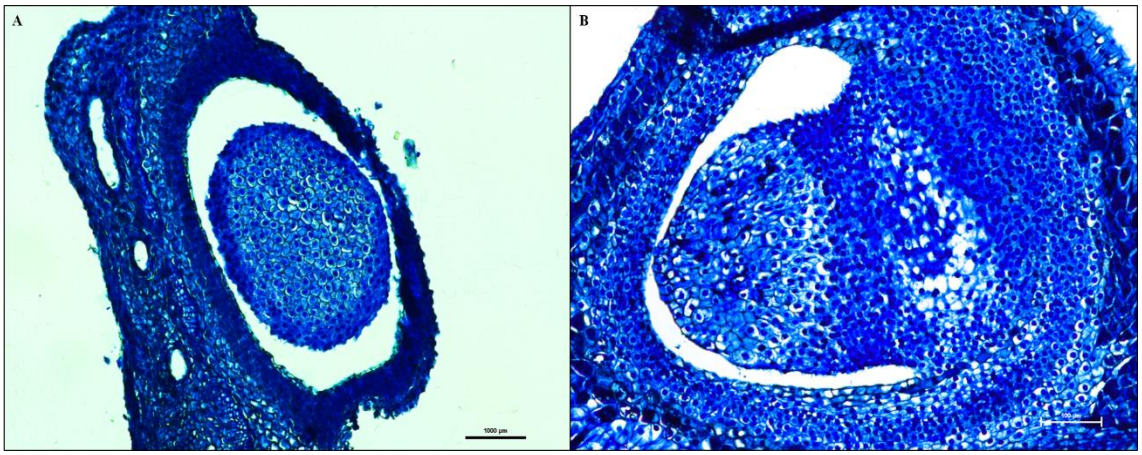

Figure 15. Analysis of the development stage of ovules of young cones of *Pinus halepensis*. (**A**) Longitudinal radial and (**B**) transversal cut of ovuliferous scale.

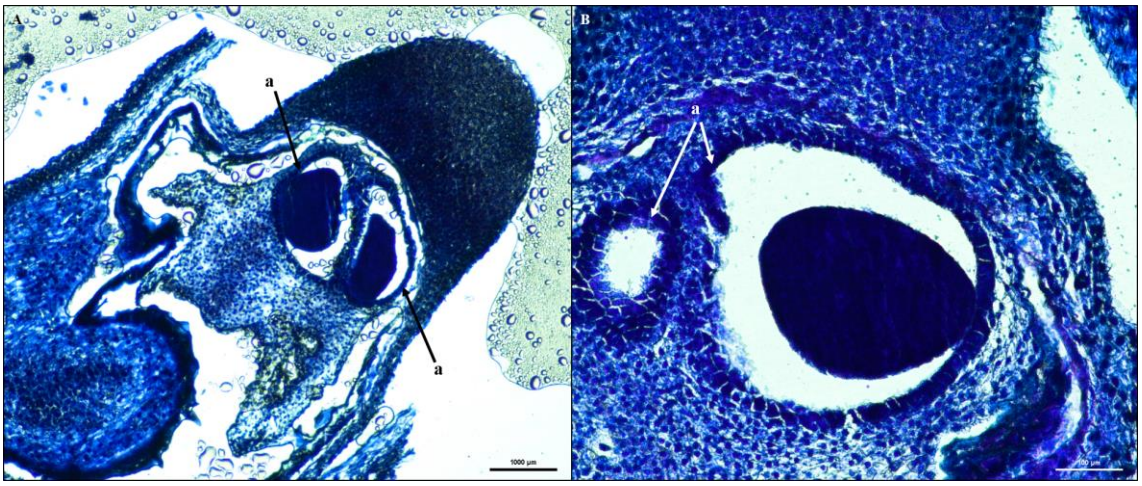

Figure 16. Analysis of the development stage of seeds of *Pinus halepensis*. (**A**) transversal cut of seed with two archegonia (**a**) (**B**) transversal cut of seed with two archegonia (**a**).

4. Discussion

Relatively to the assays with embryos in the mature stage, there are no evidence of this type of explant being used in *P. halepensis*, however, it has been used with success in other trees within the Pinaceae family, but it's not a very popular explant choice in the *Pinus* genus (Tautorus *et al.*, 1991). Although, in most species were it was possible to induce somatic embryogenesis from this type of explant, the authors defend that the frequency of initiating embryogenic cell lines is much lower than when immature zygotic embryos are used (Tautorus *et al.*, 1990; Lelu *et al.*, 1994; Garin *et al.*, 1998; Find *et al.*, 2014; Isah, 2016; Salaj *et al.*, 2019), and unable to use in practical applications (Klimaszewska *et al.*, 2007). Comparatively with zygotic embryos at more precocious developmental stages that have been used for somatic embryogenesis induction, mature somatic embryos can be used at any time of the year since seeds can be maintained for large periods. This is not the case for earlier stages that are available only for short periods of time and could not maintained for large periods for further utilization. However, being formed by more specialized cells, mature zygotic embryos are more difficult to embark into an embryogenic pathway that earlier stages. In any case, somatic embryogenesis induction from zygotic embryos does not assure the genetic uniformity of the plantlets obtained and could not be considered true cloning process.

In assays in which mature zygotic embryos of *Pinus* sp. have been used as explant for somatic embryogenesis induction, the response was slight similar to the data obtained in this work, although some different basal media and hormones combinations have been tested. Most works describe a growth of the cotyledons and hypocotyl, accompanied by the formation of white filamentous *callus* that often and rapidly turn brown, while the radicle region usually produces friable *callus* that later softened and degenerate (Garin *et al.*, 1998; Salajova *et al.*, 1999; Find *et al.*, 2014).

In Pine species, the genotype of the explant seems to influence the potential to induce somatic embryogenesis, which can explain the different responses obtained in this work (Isah, 2016). Radojevic *et al.* (1998) pointed out in their work with mature embryos of *P. nigra* that female cones collected at the same time might not be in the same physiological stage due to conifers irregular reproductive habits, which can also explain the difference between the embryos cultivated. Besides these factors, the plant regulators balance in the medium and the origin of the seeds also will have an impact on inducing somatic embryogenesis (Isah, 2016).

In some cases, the solution to obtain embryogenic masses can be the use of other PGRs than auxinas and/or cytokinins, Thus, in their work with *Pinus caribaea*, Malabadi *et al.* (2011) were able to induce embryogenic lines from zygotic mature embryos using 24-epiBrassinoline in the induction medium.

The time when the obtained *callus* is isolated and cultivated in proliferation media can be an issue to take in order. In the works of Garin *et al.* (1999) with mature zygotic embryos of *Pinus strobus* and Find *et al.* (2014) with cotyledonary zygotic embryos of *Pinus radiata*, embryogenic tissues only started to appear after 8 and 10 weeks (respectively), and usually at the surface of a filamentous *callus* formed first. In our experiments, most of the initial filamentous white *callus* was subcultured after five weeks, however, in the case of the mature embryos cultivated in DCR induction media and DCR IM, these were left on these initiation media for 8 weeks and, even so, no signs of somatic embryo formation could be detected.

The use of segmented mature zygotic embryos to induce somatic embryogenesis, divided in cotyledon and radicle halves, had not been used in *P. halepensis* and only a few references are available in the literature concerning the use of these explants among the Pinaceae family (Tautorus *et al.*, 1991). When compared with the culture of whole zygotic embryos, the culture of half embryos showed that most of the *callus* obtained originated from the cotyledon halves, which was expected since in the whole embryos, most of the *calli* forms around the cotyledonary region. Cutting the embryos seems to improve the amount of explants that produce *callus* (in the case of cotyledon halves), probably has a result of the medium composition combined with this stress factor. Some radicle halves also formed non-embryogenic *callus*, although this response can also be seen in experiments carried by Radojevic *et al.* (1998), it can also be due to the place where the excision was made. Like the experiments before, there wasn't possible to distinguish two types of *callus* proliferating in the explant.

In the work of Radojevic *et al.* (1998) with *P. nigra*, embryogenic tissue appears only in excised cotyledons, even when these are cultivated in medium without PGRs and radicle halves usually originate embryogenic *callus*, but with time, non-embryogenic *callus* started proliferating around the embryogenic *callus*. Other works with *P. nigra* carried out by Klubicová *et al.* (2017) the cotyledon explants only originated non-embryogenic *callus*. In the *Larix* genus, the works of Lelu *et al*. (1994) revealed that a pre-treatment with BAP, sucrose

and light exposure increases the frequency of excised cotyledons capable of forming embryogenic masses.

The *callus* obtained from mature embryos, either whole or in halves, had initially distinct characteristics from each other. While some were formed by hard clusters of tightly aggregate cells others were soft and friable, but after some subcultures in proliferation media, most of the *callus* started to indurate and change colour to brown, some sooner than others. Interestingly, some *callus* had both of the characteristics mentioned above, while in others these differences in *callus* structure was very distinguishable in others appeared to be dappled. However, even the *callus* that were lighter and easier to desegregate did not showed the morphological characteristics observed by Montalban *et al.* (2013) and Pereira *et al.* (2017) in *P. halepensis callus* that were embryogenic. Acetocarmine staining also did not revealed any clues of somatic embryo formation.

There are no reports of the morphologic characteristics of non-embryogenic *callus* in *Pinus halepensis,* but in other *Pinus* species this type of *callus* is usually characterized as a white yellowish friable tissue containing spherical cells with prominent nuclei (Klublicová *et al.*, 2017; Salaj *et al.*, 2019). These cells do not show any evidence of polarity, do not evolve into proembryogenic masses and, after a certain period of culture, *callus* tend to get dark and necrotic (Bravo *et al.*, 2017; Klubicová *et al.*, 2017). These features are common the *callus* obtained in this work, although some differences in texture, proliferation rates and colour were found between the non-embryogenic *callus* obtained. This could be due to the differences in the genotype among the obtained non-embryogenic *callus*, that will lead to different levels of hormones, amino acids, phenolic compounds, among others, that will result in different behaviours and morphologic characteristics of the cells. In embryogenic masses, differences among the same cell line can be due to a prolonged subculture in the proliferation state, which is usually associated with genetic alterations, differences in the proliferation rate and morphologic characteristics (Dunstan *et al.*, 1993; Egertsdotter, 2018). Consequences of a prolonged subculture not only justifies the difference between the *calli* in proliferation media but also the different responses obtained among the same cell line in the shock assays with 2,4-D, extreme pH values and sugars.

Other explanation for the morphologic differences observed within a same *callus*, could be that they are a mixture of embryogenic masses and non-embryogenic *callus*, where this last on is more abundant and due to the loss of embryogenic capacity by embryogenic lines in

mature zygotic embryos. Previous reports of somatic embryogenesis induction in Pinaceae from mature embryos, either whole or in halves, have shown the formation of two distinct *callus*, embryogenic and non-embryogenic (Gupta & Durzan, 1986; Tautorus *et al.*, 1990; Lelu *et al.*, 1993; Salajova *et al.*, 1999;). In some of these reports, the embryogenic and non-embryogenic *callus* were mixed being hard to separate from each other (Gautier *et al.*, 2016). In others cases a particular type of *callus* is confined to a specific area and easy to distinguish from the other (Durzan & Gupta, 1987). Non-embryogenic *callus* reduces the proliferation of embryogenic masses and repeated subcultures are also associated with the loss of the embryogenic capacity, especially in *Pinus* (Gautier *et al.*, 2016; Klubicová *et al.*, 2017). However, in our case, neither the histological assays with acetocarmine staining nor the morphogenic characteristics gave any evidence of the formation of an embryogenic tissue in the proliferating cell lines.

Relatively to the assays with scales and sections of unfertilized cones, the histological studies on the scales of brown small female cones seemed to reveal that these cones are in a state where the megagametophyte was not yet formed, indicating that the originated *callus* derive from nucellus or other diploid tissues involving the ovule and the scales, being of the same genotype that the original explant. The induction of somatic embryogenesis from this tissues can have great potential in conifer biotechnology, mainly because the obtained embryos will be clones of the mother tree, giving insights about the characteristics and performance of the resultant plantlets, reduce the costs and time of the delivery of elite plants (Lelu-walter *et al.*, 2016), and opening a door for a more effective genetic transformation. There aren't any reports of regeneration via somatic embryogenesis from nucellus, non-fertilized ovules or integuments in conifers (Bonga, 2017), but it has been successful in other woody plants such has *Citrus* and *Castanea*, but even in these species, the frequency of inducing somatic embryogenesis is low and dependent of the genotype of the explant (Sauer & Wilhelm, 2005; Corredoira *et al.*, 2019). In this experiment, the *callus* obtained from cone explants had similar morphologic characteristics and proliferation rates, suggesting that these could be clones but all of the *callus* were non-embryogenic. This problem could be addressed by testing new induction mediums with different combinations of PGRs, sucrose or other components. Trontin *et al.* (2016a) in is works with other adult explants from Pine trees, found a solution by applying a pre-treatment based on DCR induction media modified. This medium had activated charcoal and no PGRs, the explants would stay in this media for 3 days and stored at 2º-4ºC (Trontin *et al.*, 2016a). Although the results so far obtained did not showed any evidence of embryogenic

callus formation, tissue proliferation was achieved. This type of assays must be pursued because they pave the way for a true cloning of coniferous trees.

Relatively to the shock assays with 2,4-D, extreme pH values, and sugars, these types of treatments are usually used to induce cellular stress, which is known to play a part in the acquirement of embryogenic aptitude in woody plants (Isah, 2016). Even though some of the treated *callus* showed some morphologic differences after the treatment, acetocarmine squash revealed that none of the non-embryogenic *callus* turned embryogenic. The observed morphological differences also fit the doubt discussed above: are these differences due to genetic alterations, derived from prolonged subculture, among the same cell line; or is the *callus* is a mixture between embryogenic and non-embryogenic *callus*.

In the non-embryogenic *callus* submitted to 100 µM 2,4-D, as the incubation days increased, the *callus* became brown and their proliferation capacity diminished, which could indicate that this treatment induced too much stress on the *callus* (Moon *et al.*, 2014) impairing an embryogenic behaviour. However, once back into DCR proliferation media, light yellow *callus* begun to proliferate again, with similar aspect and proliferation rate as before the shock. Treatments with high concentrations of 2,4-D are known to induce embryogenesis, cell division and differentiation (Pasternak *et al.*, 2002; Feher *et al.*, 2003; Moon *et al.*, 2014), although in conifers, higher of lower concentrations of this hormone can lead to the induction of non-embryogenic tissue (Silva & Malabadi, 2012).

The shocks with pH values of 4 and 10 did not lead to any change in the embryogenic capacity, and the *callus* submitted to the treatments started to form hard clusters and proliferating less as the incubation days increased. In one way, pH medium can change nutrient availability, cell metabolism, hormone uptake, determine cell differentiation pathways and influence the induction of somatic embryogenesis (Pasternak *et al.*, 2002; Feher *et al.*, 2003; Pullman & Johnson, 2009), but in another way, studies carried out by Pullman *et al.* (2005) with *Pinus taeda* and *Pseudotsuga menziesii* showed that initiation of somatic embryogenesis may be inhibited if pH levels are outside the range of 4.8-6.1.

Osmotic shocks are also known to allow differentiated cells develop into competent dedifferentiated cells (Zavattieri *et al.*, 2010; Silva & Malabadi, 2012), but besides this, sucrose in high concentrations can promote embryogenic transition (Feher, 2003), and mannitol is proven to have better results than sucrose in some species and a longer osmotic effect

(Thompson *et al.*, 1986; Lipavská & Konrádová, 2004). In this work, shock treatments with 0.3 M sucrose and 0.15 M sucrose plus 0.15M mannitol also didn't turn the non-embryogenic *callus* to embryogenic and didn't lead to big changes in the *callus* aspect and proliferation rates.

5. Concluding remarks

One of the objectives of this work was to investigate more explants that could be used for somatic embryogenesis initiation in *Pinus halepensis*, and although none of the explants gave origin to embryogenic masses, it is important to remark that most of these explants showed response to *in vitro* conditions.

Initiating somatic embryogenesis from zygotic embryos at immature pre-cotyledonary stage really narrows the time when female cones can be collected, so initiating somatic embryogenesis from mature zygotic embryos and unfertilized young cones would be very useful to the whole process due to the fact that these explants can be found most of the year and that using unfertilized young cones would be a big time and money saver in elite clones production for afforestation programs with *P. halepensisis*. Taking this to account, it's important to try new induction media conditions, mainly focusing on the PGRs concentration since these play an important part in acquire embryogenic capacity. Furthermore, the mother trees used in this project weren't used in other studies, so we don't know if their genotype is prone or not to initiating somatic embryogenesis.

Expanding the knowledge about non-embryogenic *callus* and their differences compared with embryogenic *callus* is also of great interest. Non-embryogenic *callus* occurrence is common in *Pinus*, and sometimes this *callus* can appear mixed with embryogenic tissue, leading to less maturation and conversion rates. Studying their differences among proteins and hormone levels, can provide tools to make a more effective treatment when non-embryogenic *calli* are found.

Most of the culture conditions used in somatic embryogenesis were based on trial and error experiments, so in order to keep improving this process in conifers it's important to work and investigate all the steps of the protocol for somatic embryogenesis in order to obtain the greatest possible yield in each step of the protocol. For this it's not only important to work in inducing more suitable explants or in converting non-embryogenic *callus* to embryogenic, but also in medium and environmental conditions, cryopreservation protocols and genetic gain in *Pinus halepensis*.

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