



SHORT COMMUNICATION

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Pinus canariensis plant regeneration through somatic embryogenesis

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Abstract

Aim of the study: To develop an efficient method to regenerate plants through somatic embryogenesis of an ecologically relevant tree species such as *Pinus canariensis*.

Area of study: The study was conducted in the research laboratories of Neiker-Tecnalia (Arkaute, Spain).

Material and methods: Green cones of *Pinus canariensis* from two collection dates were processed and the resulting immature zygotic embryos were cultured on three basal media. The initiated embryogenic tissues were proliferated testing two subculture frequencies, and the obtained embryogenic cell lines were subjected to maturation. Germination of the produced somatic embryos was conducted and acclimatization was carried out in a greenhouse under controlled conditions.

Main results: Actively proliferating embryogenic cell lines were obtained and well-formed somatic embryos that successfully germinated were acclimatized in the greenhouse showing a proper growth.

Research highlights: This is the first report on *Pinus canariensis* somatic embryogenesis, opening the way for a powerful biotechnological tool for both research purposes and massive vegetative propagation of this species.

Key words: acclimatization; Canary Island pine; micropropagation; embryogenic tissue; somatic embryo.

Abbreviations used: embryogenic tissue (ET); established cell line (ECL); somatic embryogenesis (SE); somatic embryos (Se's).

Authors' contributions: PM, IM and ACO conceived and planned the experiments. ACO performed the experiments. ACO wrote the manuscript and all authors provided critical feedback and helped shape the research, analyses and manuscript.

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Introduction

The Canary Island pine (*Pinus canariensis* Chr. Sm. Ex DC) is a subtropical species endemic to the western Canary Islands, growing across contrasting habitats, from xeric conditions to areas with > 1200 mm of annual rain, and from the sea level up to 2400 m (López De Heredia *et al.*, 2014). Despite the well-known great adaptability of this species to drought conditions and resistance to forest fires, exemplified by xeromorphic needles, serotinous cones and deep tap roots, the warm-

ing trend derived from climate change, coupled with reduced precipitation, is expected to constraint tree growth and tree ring width (Brito *et al.*, 2016). Furthermore, more frequent intense wildfires have been demonstrated to cause long-term changes in the composition of soil and negatively affect the vitality of adult trees and the establishment of new plantlet (Durán *et al.*, 2010; Otto *et al.*, 2010).

Ecologically it is a relevant species because it colonizes volcanic soils where no other tree species can compete, thus maintaining soil stability on the higher

slopes of the islands. Besides, *P. canariensis* is an important timber-producing species in the Canary Islands, employed in cabinetmaking and outdoor carpentry thanks to its rot-proof nature. Growth is rapid and the heart-wood is of extremely high quality, which promoted its introduction as a forest and ornamental species to several continents, becoming a successful forest tree in South Africa (Martinez Pulido *et al.*, 1990). Therefore, the development of a rapid clonal propagation method would be of considerable value.

Successful micropropagation and regeneration of plantlet using organogenic techniques from cotyledonary explants have already been achieved (Martinez Pulido *et al.*, 1990; 1992; 1994). However, as far as we know, there are no studies of somatic embryogenesis (SE) in this species. SE is the most efficient biotechnological approach for conifer clonal propagation and it can be combined with cryopreservation, becoming this technique a useful tool to increase the availability of elite plant material. Furthermore, recent research has demonstrated that the culture conditions during SE can determine both the success of the process (García-Mendiguren *et al.*, 2016; Pereira *et al.*, 2016; Castander-Olarieta *et al.*, 2019) and the behaviour of the generated plants *ex vitro* (García-Mendiguren *et al.*, 2017).

Considering the abovementioned information, the aim of this work was to evaluate the feasibility of SE in *P. canariensis* using immature zygotic embryos as initial explants.

Materials and methods

One-year-old green female cones of *P. canariensis* were collected in July and September 2018 from open-pollinated trees in Orio (Gipuzkoa, Spain; latitude: 43°29'63"N, longitude: 2°08'54"W, elevation: 296m). Immature megagametophytes were extracted and sterilized following Montalbán *et al.*, (2014) and the developmental stage of the zygotic embryos was determined using a Leica DMS 1000 microscope (Montalbán *et al.*, 2012).

In order to induce the initiation of embryogenic tissue (ET), three media were tested: EDM initiation medium (Walter *et al.*, 2005), DCR initiation medium (Gupta & Durzan 1985) and a modified MCM medium (Bornman 1983). EDM initiation medium was supplemented with 4.5 µM 2,4-dichlorophenoxyacetic acid and 2.7 µM 6-benzylaminopurine, while the other two media were supplemented with 9 µM 2,4-dichlorophenoxyacetic acid and 2.7 µM kinetin. All media were solidified with 3.5 gL⁻¹ gellan gum (Gelrite®) and, after autoclaving, EDM amino acid mixture was added (Walter *et al.*, 2005). Eight megagametophytes per Petri dish and five Petri dishes per initiation medium and collection time were cultured,

comprising a total number of 240 initial explants. All the megagametophytes were kept at 23°C in darkness and after 12 weeks on the initiation medium, initiation rates were calculated. These incubation conditions were maintained during proliferation and maturation.

Proliferation of ET was carried out using EDM proliferation medium, increasing the gellan gum concentration to 4.5 gL⁻¹. In this case, small parts of about 0.5 cm in diameter from the generated embryonal masses were divided in different Petri dishes (>3); one part was subcultured every two weeks while the other was subcultured monthly. Maturation was carried out using EDM basal medium supplemented with 60 µM abscisic acid and 9 gL⁻¹ gellan gum following Montalbán *et al.*, (2010); 90 mg of ET inoculum per Petri dish, two established cell lines (ECL) and five replicates per ECL were employed. After 14 weeks, the maturation success was evaluated and the number of mature somatic embryos (Se's) per gram of ET was calculated.

Germination and acclimatization in the greenhouse followed the procedure described by Montalbán & Moncaleán (2018). The Se's were germinated on Petri dishes with half-strength macronutrients LP medium (1/2 LP, Quoirin & Lepoivre 1977 modified by Aitken-Christie *et al.*, 1988) supplemented with 2 gL⁻¹ activated charcoal and 9 gL⁻¹ gellan gum (Difco® Agar granulated). After nine weeks, germination rates were evaluated and successfully germinated seedlings were subcultured to glass jars with medium of the same composition. After another nine weeks, the somatic plants were transferred to individual pots containing peat: vermiculite (8:2, v/v) and acclimatized in a greenhouse under controlled conditions (T = 23 ± 3°C and RH = 70 ± 5%) and regular watering.

Prior to acclimatization, the plants that had not developed a proper root system were transferred to a O118/80+OD118 microbox (SacO₂) containing perlite:peat (7:3, v/v) moistened with 1/2 LP liquid medium supplemented with 1 µM 1-naphthalenacetic acid and 0.5 µM indol-3-butyric acid. Seven weeks later they were carefully removed from the microbox, the rooting rate was evaluated, and all plants were acclimatized in the greenhouse as previously described.

The results of all the experiments were analysed by ANOVA. However, for both the number of initiations and Se's, the analysis of variance did not fulfil the normality hypothesis, and thus, a Kruskal-Wallis test was performed.

Results and discussion

The microscopic analysis of the zygotic embryo along the different collection times revealed the pres-

ence of several developmental stages. All the megagametophytes from cones collected in July enclosed zygotic embryos at early polyembryony, as described by Montalbán *et al.*, (2012). Only a few suspensor cells were visible, along with very tiny pro-embryo heads, without a clear dominance of any of them. However, the cones collected in September presented a broader range of developmental stages. Most excised embryos showed well-formed cotyledons, but some examples of polyembryony and embryos at bullet stage were also observed (Fig. 1).

Regarding the initiation results, no significant differences were observed between the two collection times ($p = 0.152$). Initiation percentage for explants collected in July was 0.8%, and 3.3% for explants collected in September. This low initiation percentages could be explained by the developmental stages in which most zygotic embryos were at the moment of harvesting, the same stages that led to the lowest initiation percentages in other pine species such as *Pinus radiata* (Montalbán *et al.*, 2012).

In relation to the initiation media tested, no significant differences could be detected. Nonetheless, the ET initiated on DCR did not continue to proliferate. Surprisingly, no initiations could be observed in MCM basal medium, which was found to be the best for organogenesis in this species (Martinez Pulido *et al.*, 1990).

As a result, we decided to continue our experiments using EDM basal medium. 66% of the proliferated ET led to ECLs, presenting a spiky morphology, with big proembryogenic areas and long suspensors, which resembled their counterparts in *P. pinaster* and *P. pinea* (Breton *et al.*, 2005; Carneros *et al.*, 2017) (Fig. 2A). However, the proembryogenic areas showed a darker, yellowish tone, as compared with other *Pinus* species, such as radiata pine or Aleppo pine, whose ET show a clear white aspect (Montalbán *et al.*, 2012; 2013). It is worth mention-

ing that ECLs subcultured monthly showed a more vigorous growth when compared with the same ECLs subcultured fortnightly.

All the ECLs obtained were subjected to maturation and all of them produced Se's. The results from the maturation stage revealed that the ECL had a significant effect on the success of the process ($p < 0.05$). The least productive ECLs presented one somatic embryo (2 Se's per gram of ET), while the most productive one gave rise to 11 well-formed Se's (24 Se's per gram of ET). No overgrowth of ET was observed, as occurred in other pine species when employing higher amounts of ET inoculum (Montalbán *et al.*, 2010), and the Se's obtained differed both in size and shape from their zygotic counterparts as they were smaller and had a shorter hypocotyl and cotyledons (Fig. 1C and 2B). They had a white to yellowish colour, except for 16% of Se's that presented a greenish colour. In parallel, some aberrant Se's were detected (48% of all the Se's) (Fig. 2C).

The results on maturation presented are encouraging as all the ECLs subjected to maturation produced somatic embryos. However, the number of Se's per gram of ET in this experiment is quite low as compared with SE in other *Pinus* species. Therefore, further experiments should focus on obtaining a greater number of ECLs to assure a broader genetic diversity, along with the modification of some components of the culture medium, such as sugars, amino acids or gellan gum concentrations (Garin *et al.*, 2000). As it has been demonstrated in other *Pinus* species, the culture conditions at different stages of SE have a determinant effect in the success of the whole process (Montalbán *et al.*, 2016). Recently, it has been also demonstrated that the application of stressful conditions at initiation (Pereira *et al.*, 2016; Castander-Olarieta *et al.*, 2019) and proliferation stage (Pereira *et al.* 2017) can have an impact on the efficiency and productivity of the process in *P. halepensis* and *P. radiata*.

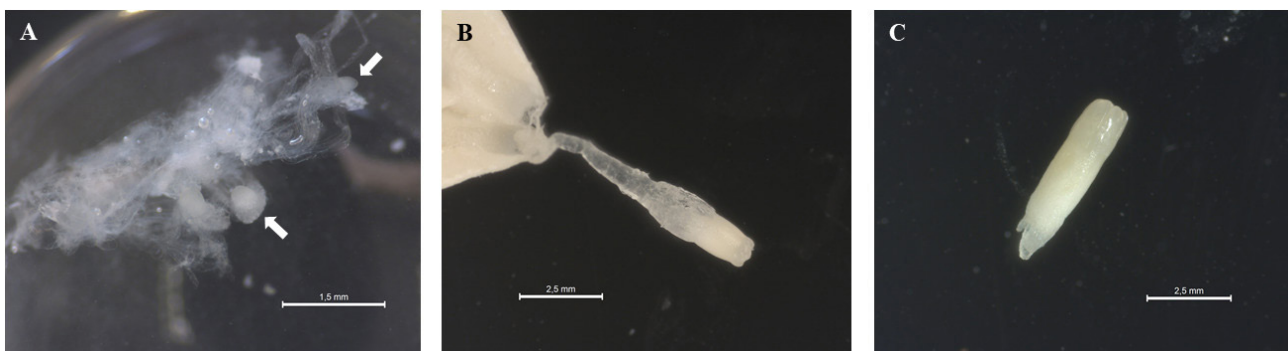


Figure 1. Zygotic embryo developmental stages in megagametophytes of *P. canariensis* collected in September 2018. (A) Example of polyembryony. Multiple pro-embryo heads are visible (arrows). (B) Bullet-shaped embryo with a forming epicotyl. (C) Completely developed cotyledonary embryo.

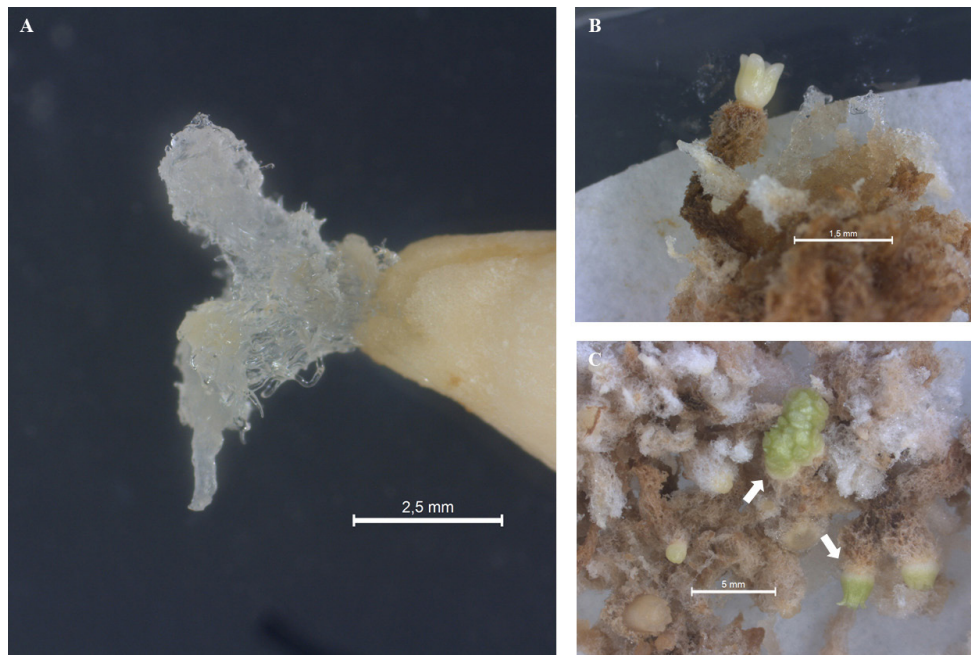


Figure 2. Somatic embryogenesis in *P. canariensis*. (A) Initiation of embryogenic tissue in EDM medium. Note the big yellowish proembryogenic areas and the long suspensor cells. (B) Detail of a well-formed somatic embryo with visible big cotyledons. (C) Multiple aberrant somatic embryos (arrows) presenting an abnormal shape and colour.

Ten out of twelve embryos (83%) germinated properly (Fig. 3A). The Se's that presented a greenish tone showed a decreased vigour and an altered development of the aerial part, so they could not be acclimatized. It is also noticeable that despite a proper early growth of the root system, after the first subculture in the same germination medium, root growth was restricted in some seedlings. Nonetheless, the application of 1 μM 1-naphthalenacetic acid and 0.5 μM indol-3-butyric acid in the microbox resulted in 40% of root growth recovery (Fig. 3B). Furthermore, even the seedlings

that did not show root growth after this treatment presented small nodules at the base of the hypocotyl. In that regard, Martínez Pulido *et al.* (1990) showed that porous substrates, such as peat mixed with vermiculite or perlite, are better substrates for rooting than media solidified with gellan gum.

All the germinated seedlings transferred to the greenhouse (10) were properly acclimatized. After 3 months, they showed a well-developed aerial part, ranging from 3 cm to 4 cm and a deep root mass (Fig. 3C).

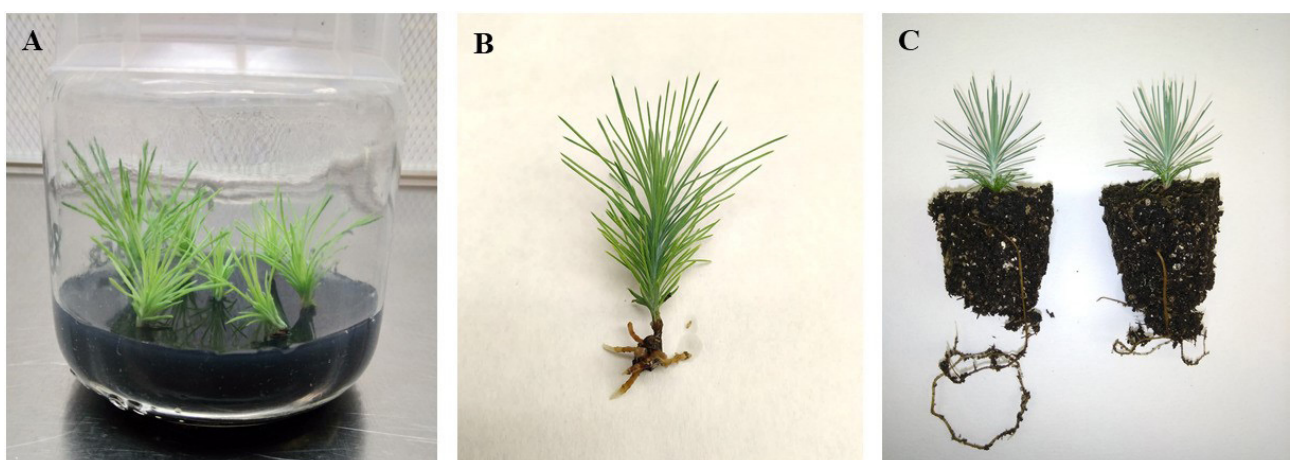


Figure 3. (A) Canary island pine somatic seedlings after first subculture on half-strength LP germination medium supplemented with activated charcoal. (B) Detail of the multiple developing roots of a somatic plant after 7 weeks treatment in a microbox with high auxin concentration. (C) Three-month-old somatic seedlings grown under greenhouse conditions.

To the best of our knowledge this is the first report on Canary Island pine plantlet regeneration through SE. This work confirmed the feasibility of SE in *P. canariensis* and laid the foundations for the development of an efficient micropropagation system that could be used as a powerful biotechnological tool if combined with traditional breeding-techniques.

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