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Regeneration of *Pinus halepensis* (Mill.) through Organogenesis from Apical Shoot Buds

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Abstract: Organogenesis and somatic embryogenesis have been widely applied as the two main regeneration pathways in plant tissue cultures. However, recalcitrance is still the main restriction in the clonal propagation of many woody species, especially in conifers. They undergo a "phase change" that leads to significant loss of vegetative propagation capacity, reducing the aptitude of tissues and organs to be regenerated in vitro beyond this point. In line with this, the in vitro regeneration of mature conifer trees has been a long-cherished goal in many laboratories worldwide. Based on previous works in *Pinus* species regeneration from adult trees, we now present data about the culture of apical shoot buds in an attempt to induce organogenesis and somatic embryogenesis to clone mature trees of Aleppo pine (*Pinus halepensis*). Reinvigorated axillary shoots were submitted to conditions usually applied to induce somatic embryogenesis through the manipulation of culture media, including the use of auxins such as 2,4-Dichlorophenoxyacetic acid and 1-Naphthaleneacetic acid, cytokinins (6-benzyladenine and kinetin), and phytosulfokine (50, 100, and 200 nM). Although somatic embryos could not be obtained, an embryogenic-like tissue was produced, followed by the emergence of actively proliferating non-embryogenic calli. Variations in the consistence, texture, and color of non-embryogenic calli were observed; especially those arising in the media containing phytosulfokine. Reinvigorated shoots, induced by 22 or 44 µM 6-benzyladenine, were obtained through organogenesis and acclimatized, and phenotypically normal plants were obtained.

Keywords: Aleppo pine; conifers; phytosulfokine; plant growth regulators; rooting

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1. Introduction

Approximately 52% of the land surface is occupied by forests [1]. Among trees, conifers are particularly important since they are by far the largest and most diverse gymnosperm group, covering approximately 60% of the forested areas of the world [2]. In vitro propagation techniques have been widely applied as a model system for plant regeneration analysis and as a large-scale propagation system for coniferous cloning [2,3]. Aleppo Pine (*Pinus halepensis* Mill.) is native to the Mediterranean region, thriving in the driest and warmest areas due to its tolerance to high temperatures and drought stress [4,5], which makes it a potential alternative for reforestation in the climate change scenarios predicted for large areas of the globe in the near future [6,7].

Multicellular organisms harbor multiple types of tissues, each consisting of cells with particular features and functions. However, in some cases, cell specificity can be totally

or partially lost and the cells return to a juvenile proliferating state usually known as dedifferentiation [8]. Cellular plasticity defines the competence of differentiated cells to switch their differentiation process and to acquire new fates. This loss of a specialized state previously acquired during development has been one of the central concepts in plant regeneration [9].

In vitro regeneration of plants can be accomplished through two different pathways: shoot organogenesis and somatic embryogenesis (SE) [10,11]. Organogenesis relies on the de novo formation of a shoot that requires further rooting to develop into an entire plant. Somatic embryogenesis is a more direct pathway of regeneration since bipolar structures possessing a root and shoot meristem are formed. However, SE induction is mostly restricted to juvenile embryonary organs and, in most cases, immature zygotic embryos have been used to produce somatic embryos in coniferous and other trees [12]. Regeneration from embryonary explants is less valuable for cloning because the process rules out the selection of specific traits that can only be seen when trees have entered the mature phase. However, the switch of a developmental program in adult cells remains a difficult obstacle to overcome in many species, especially in forest trees [13]. Thus, recalcitrance is still the main restriction for the clonal propagation of elite trees [12,14].

The direct induction of axillary shoot buds and somatic embryo formation are both morphogenic pathways highly controlled by exogenous plant growth regulators added to the culture media and their interaction with endogenous phytohormones [15]. The balance between auxin and cytokinin and changes to their composition and ratio in culture media have been found to determine the morphogenic competence of cultured explants [16]. Variations in this ratio are frequently the primary empirical approach to the optimization of in vitro cultures. However, many species do not respond to this common approach and require additional physical or chemical stimuli [10]. For example, it has been shown that the presence of phytosulfokine in the culture medium stimulates the initial steps of cellular dedifferentiation even at nanomolar concentrations, significantly increasing cell proliferation and callus growth [17]. Endogenous and environmental factors, such as genotype, excision tissue and timing, phenology, and tree maturation are other factors influencing in vitro regeneration [12].

Several protocols for adult pine organogenesis have been established in our lab [18–21], and SE has been successfully reported in various *Pinus* species using juvenile material as the initial explant [22–24]. Despite all the progress that has been made in the practical applications of somatic embryogenesis induction, the results concerning adult cloning are still scarce. Moreover, cloning from adult trees would substantially reduce field testing and the time for breeding [14]. Therefore, the cloning of adult trees is still being attempted in numerous laboratories.

In Aleppo pine, the first record of organogenesis was reported using mature zygotic embryos as explants [25]. More recently, our team successfully developed the first SE protocol using immature megagametophytes as explants for this species [26].

Considering the reasons mentioned above, the main goal of this work was to successfully develop protocols for in vitro regeneration (organogenesis and SE), using apical shoot buds as explants, which could allow for the selection and cloning of mature trees of Aleppo pine.

2. Materials and Methods

2.1. Plant Material

2.1.1. Induction of Organogenesis

Four 20-year-old adult zygotic trees (17.3, 17.4, 17.5 and 18.1) from Manzanos (Spain; 42°44′29″ N, 2°52′35″ W) and four 4-year-old juvenile somatic trees (H8, H29, H32, and H5) planted at Neiker, Arkaute (Spain; 42°51′08.5″ N, 2°37′37.1″ W) were selected in 2017, and apical shoot buds were collected between January and March. In 2018, the apical shoot buds from five 20-year-old adult trees (P1, P5, P6, P7, and P8) were collected between November and January near Figueira da Foz (Portugal; 40°09′02.5″ N, 8°49′07.3″ W).

The apical shoot buds were stored in polyethylene bags at 4 °C for a maximum of 10 days until their use. For superficial cleaning, buds were washed with a commercial detergent, rinsed under running water for 5 min, and then immersed in ethanol 96% for 1 min. Afterward, explants in the first sample collection (2017) underwent three different sterilization protocols: (A) surface sterilization in commercial bleach, 1:1 diluted with sterile water plus two drops of Tween 20® (Scharlab, Barcelona, Spain) for 15–20 min before being rinsed three times with sterile distilled H_2O; (B) surface sterilization in commercial bleach, 1:1 diluted with sterile water plus two drops of Tween 20® for 15 min, rinsed one time with sterile distilled H_2O before being immersed in a silver nanoparticle solution (Argovit®, Vector Vita LLC, Novosibirsk, Russia) (200 mg L^{-1}) for 10 min, and then rinsed three times with sterile distilled H_2O; (C) surface sterilization in a silver nanoparticle solution (Argovit®, Vector Vita LLC, Novosibirsk, Russia) (200 mg L^{-1}) for 15 min before being rinsed three times with sterile distilled H_2O. All sterilization protocols were performed under sterile conditions in a laminar flow unit. Sterilization protocol (A) was used for all the explants in the second 2017 collection and thereafter in the 2018 collection.

2.1.2. Attempts to Induce Embryogenic Tissue

Six 4-year-old juvenile somatic trees (H29, H32, H13, H18, H5, and H42) planted at Neiker, Arkaute (Spain; 42°51′08.5″ N, 2°37′37.1″ W) and two 20-year-old adult zygotic trees (17.3 and 17.4) from Manzanos (Spain; 42°44′29″ N, 2°52′35″ W) were selected in 2017, and the apical shoot buds were collected between January and March. The storage, cleansing, and sterilization of the apical shoot buds were performed as described in Section 2.1.1. Twelve reinvigorated axillary shoots (2.0–3.0 cm) from two genotypes previously obtained through organogenesis were also selected for embryogenic tissue induction.

2.2. Organogenesis

2.2.1. Axillary Shoot Induction, Growth and Elongation

Scales from apical shoot buds (0.5–3 cm length) (Figure 1a,b) were removed, and the buds were cut transversely with a surgical scalpel blade into slices 0.3–0.8 cm thick (Figure 1c). Four to five bud slices were cultured in 90 × 14 mm Petri dishes containing twenty milliliters of O1 induction medium (Table 1). Two different concentrations of 6-benzyladenine (BA; 11 and 22 µM), were tested in the first sample collection of 2017. Five to six Petri dishes per BA treatment and genotype were cultured. The explants were maintained at 23 °C under a 16 h photoperiod at 100 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TFL 58 W/33; Philips, France).

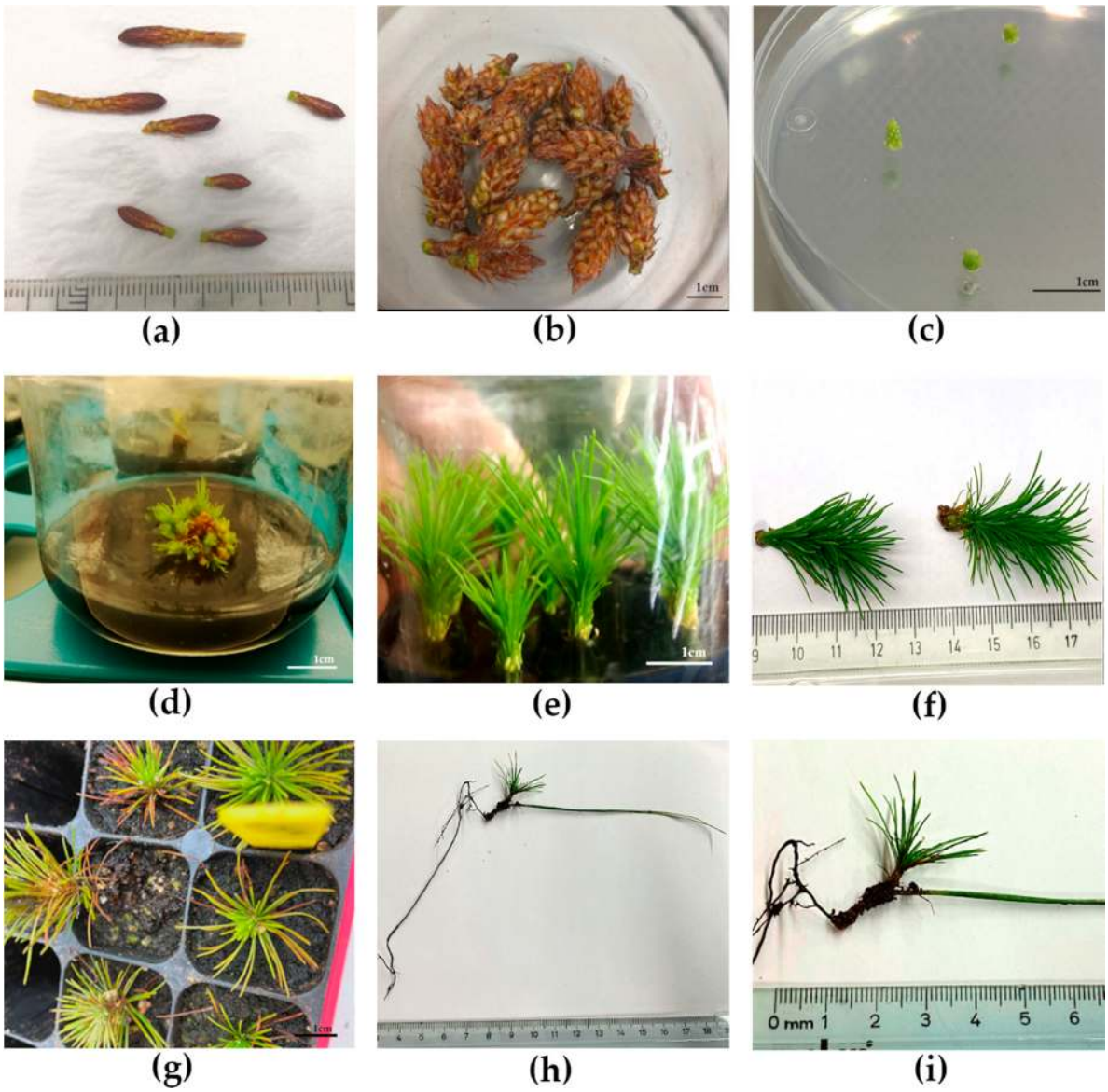


Figure 1. Plant material at different stages of the organogenic process: (**a**) apical shoot buds with different sizes and totally closed scales; (**b**) apical shoot buds at an advanced developmental stage with open scales; (**c**) bud slices in the induction medium; (**d**) shoot organogenesis in a bud slice cultured in the elongation medium; (**e**) axillary shoots separately cultured in the elongation medium; (**f**) shoots with no roots immediately before acclimatization; (**g**) acclimatized shoots; (**h**) acclimatized shoot removed from the container to display the ex vitro developed roots; (**i**) closer view of the latter acclimatized shoot.

Table 1. Variations of basal DCR medium [27] used along different stages of *P. halepensis* organogenesis (O1–O3) and embryogenic tissue induction (S1–S10).

Medium	PGRs (μM)	AC (g L^{-1})	Others (g L^{-1})	Agar (g L^{-1})	pH [1]	[28] EDM Amino Acid Mixture [2]	Phytosulfokine (nM) [2]
O1	BA (11,22,44)	-	Sucrose (30)	Difco® granulated agar (9)	5.8	-	-
O2	-	(2)	Sucrose (30)	Difco® granulated agar (9.5)	5.8	-	-
O3	IBA (7)	-	Sucrose (30)	Difco® granulated agar (9.5)	5.8	-	-
S1 [29]	-	(3)	Maltose (32)	Gelrite® (2)	5.7	-	-
S2	BA (9) 2,4-D (20) NAA (25) [2]	-	Maltose (32)	Gelrite® (1.5)	5.7	yes	(50)
S3	-	-	Sucrose (30)	-	5.7	-	-
S4	BA (9) 2,4-D (20) NAA (25) [2]	-	Maltose (32)	Gelrite® (2.5)	5.7	yes	(50)
S5	BA (9) 2,4-D (20) NAA (25) [2]		Maltose (32)	Gelrite® (2.5)	5.7	yes	(100)
S6	-	(10)	Sucrose (60)	-	5.7	-	-
S7	BA (9) 2,4-D (20) NAA (25) [2]	-	Maltose (32) PVP (0.2)	Gelrite® (2.5)	5.7	yes	(100)
S8	ABA (80) [2]		Sucrose (68) Casein hydrolysate (1) Glutamine (0.5) [2]	Gelrite® (10)	5.7	yes	-
S9	ABA (120) [2]		Sucrose (68) Casein hydrolysate (1) Glutamine (0.5) [2]	Gelrite® (12)	5.7	yes	-
S10	BA (9) 2,4-D (20) NAA (25) [2]	-	Maltose (32)	Gelrite® (1.5)	5.7	yes	(200)
S11	2,4-D (9) Kinetin (2.7)		Sucrose (30)	Gelrite® (3.5)	5.7	yes	-

PGRs, plant growth regulators; AC, activated charcoal; BA, 6-benzyladenine; IBA, indole-3-butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, 1-naphthaleneacetic acid; PVP, polyvinylpyrrolidone; ABA, abscisic acid. [1] Adjusted before autoclaving at 121 °C for 20 min. [2] Filter-sterilized and added to the medium after autoclaving.

After 35–50 days, the elongated needles were cut off and the explants were transferred to the same medium to promote axillary bud growth. At this point, since the explants cultured in the medium with 11 μM of BA did not show the expected response, they were transferred to a new O1 induction medium with 44 μM of BA. For the samples from the second and third collection in 2017 and all three collections in 2018, the initial BA concentrations tested were 22 and 44 μM of BA. Overall, a total of 1511 bud slices were cultured.

When the needle fascicles emerged, the explants with axillary buds were transferred to glass jars with the O2 elongation medium (Table 1; Figure 1d). Once axillary bud growth was evident and shoots were 0.5 cm long, they were separated and cultured individually in a fresh elongation medium (Figure 1e). The part of the explant that had secondary needles was separated, the secondary needles were cut, and the explants were returned to the O1 medium to promote re-induction.

2.2.2. Root Induction

Shoots 2.0–3.0 cm long were transferred to the O3 root induction medium (Table 1). Three to four shoots per five replicates of five genotypes (H8, H32, 17.3, P1, and P8), comprising a total of 89 explants, were tested for root induction. After four weeks of culture, shoots were transferred into a fresh O2 medium to promote root growth. None of the shoots developed visible roots, so they were retransferred to the O3 medium for two weeks. After this time, no roots were developed in vitro (Figure 1f). Despite that fact, shoots were directly transferred ex vitro to sterile peat:perlite (3:1, v/v) and acclimatized under controlled conditions (Figure 1g). During ex vitro conditions, roots spontaneously developed, and four months later the percentage of acclimatized plantlets was recorded.

2.2.3. Data Collection and Statistical Analysis

At the time that axillary shoots were isolated and cultured individually in the elongation medium, the percentage of explants forming shoots (EFS) (%) and the mean number of shoots formed per explant (NS/E) were calculated with respect to the non-contaminated explants. Following the confirmation of the homogeneity of variances and the normality of the samples, an unpaired *t*-test analysis [GraphPad Prism 8.4.1 (676) (GraphPad Software Inc., California, CA, USA)] was performed in order to identify possible differences in these two variables regarding explant induction at 22 and 44 μM of BA. Each measurement was made by considering the mean data collected for each genotype from different sample collections (sample collections with 100% contamination were not considered), comprising a total of 20 replicates per BA treatment.

Regarding possible differences in acclimatization percentages between genotypes, since there was no homogeneity of variances between samples, a Kruskal–Wallis test was applied using the percentage of successfully acclimatized plants counted after four months.

2.3. Attempts to Induce Embryogenic Tissue

2.3.1. Apical Shoot Buds as Initial Explants

Scales from the apical shoot buds (0.5–3 cm length) were removed, and the buds were cut transversely with a surgical scalpel blade into 0.3–0.8 cm thick slices that were first cultured in the S1 medium (Table 1). Two to five Petri dishes per genotype and five bud slices per Petri dish, comprising a total of 490 bud slices, were cultured and maintained for three days, at dark conditions at 4 °C. Afterward, the explants were cultured in the S2 induction medium (Table 1) and the cultures were maintained, at dark conditions at 23 °C.

After 3–5 weeks in the S2 induction medium, half of the embryogenic-like proliferating calli were directly transferred to the proliferation medium, and half were detached from the bud slices. The detachment was performed by resuspending the explants in the S3 medium (Table 1), in 50 mL centrifuge tubes, and vigorously shaking them by hand for a few seconds. Thereafter, a 5 mL aliquot was poured onto a filter paper disc (Whatman no. 2.7 cm) in a Büchner funnel, and a vacuum pulse was applied for 10 s [30]. Filters containing the tissue were then poured into the S4 and S5 proliferation media (Table 1). Cultures were subcultured every 3–5 weeks and cultured in the dark, half at 23 and the other half at 28 °C. Embryogenic-like tissue that presented a similar morphology to an embryogenic callus was selected and stained with 2% (w/v) acetocarmine and observed using a Leica DMS1000 (Leica Microsystems, Wetzlar, German) and a Nikon ECLIPSE 80 i (Nikon Corporation, Tokyo, Japan).

After 2–3 subcultures in the S4 and S5 proliferation medium, all samples were resuspended, using the resuspension method described above, in the S6 medium (Table 1) before being transferred to the S7 pre-maturation medium (Table 1). Samples were cultured at 23 °C, in the dark, for 5–7 weeks.

With the embryogenic-like tissue, an attempt at tissue maturation was made and induction was carried out in the S8 and S9 maturation media (Table 1). Eight Petri dishes per sample, containing 60 mg of tissue each, were cultured in the dark for 18 weeks.

2.3.2. In Vitro Axillary Shoots as Initial Explants

Needles of the axillary shoots reinvigorated from organogenesis were cut before culture without damage to the apical meristem, and seven to nine transversal shoot slices (0.2–0.5 cm) per explant were cultured in three different induction media: S2, S10, and S11 (Table 1). Two to six Petri dishes per induction medium were cultured and maintained, in the dark, at 23 °C. The calli were subcultured in the same induction medium two times, after 5 and 10 weeks from the beginning of induction.

3. Results

3.1. Organogenic Process

A total of 1010 bud slices were cultured at different induction media in 2017. The contamination rates obtained in the first sample collection of 2017 were 48.2% for protocol (A), 49.1% for (B), and 89.0% for (C). Protocol (A) was selected for the rest of the experiment, and a total contamination rate of 43.9% was obtained during this experiment, with 567 living explants remaining. In 2018, 501 bud slices were cultured, and a total of 242 explants remained after contamination.

The reinvigorated shoots were obtained from both of the BA treatments (22 and 44 μM), from adult zygotic trees, and juvenile somatic trees. Higher numbers of EFS (%) and the NS/E were obtained from explants cultured in the induction medium supplemented with 44 μM BA. No statistically significant differences were found for EFS (%) (Table 2). However, significant differences were obtained for the NS/E, since treatment at 44 μM BA (5.79) led to more than double the NS/E than treatment with 22 μM BA (2.87) (Tables 2 and 3). A total of 683 shoots were obtained from the 159 explants induced (4.3 shoots per explant).

Table 2. *T*-test analysis of variance for explants forming shoots (EFS) (%) and NS/E (number of shoots formed per explant) of *Pinus halepensis* apical shoot buds induced under two different concentrations of BA (22 and 44 μM L^{-1}) and Kruskal–Wallis analyses for ex vitro rooting of five different genotypes (H8, H32, 17-3, P1, P8).

		Source	
***t*-Test**	**d*f***	***t***	***p* Value**
EFS	36	1.28	n.s. [1]
NS/E	34	2.12	0.0411
Kruskal-Wallis	**d*f***	**X^2 Test**	***p*Value**
Ex vitro Rooting	4	2.613	n.s.

[1] not statistically different.

Table 3. Values for EFS (%) (explants forming shoots) and NS/E (number of shoots formed per explant) for *Pinus halepensis* apical shoot buds induced under two different concentrations of BA (22 and 44 μM).

Treatment	EFS (%)	NS/E
22 μM L^{-1} BA	21.47 ± 4.70 [a]	2.87 ± 0.51 [b]
44 μM L^{-1} BA	30.90 ± 5.58 [a]	5.79 ± 1.2 [a]

Data are presented as mean values ± SE. Significant differences at $p < 0.05$ within a column are indicated by different letters.

No data could be obtained from families 17.5, H29, H5, or P7 since 100% of the cultured explants were contaminated. The rates of contamination were noticed to be related not only to the condition of the initial explant but, also, to its development stage. When explants consisted of apical shoot buds at an advanced developmental stage with open scales (Figure 1b), higher contamination rates were observed, as in the third collection of both P5 and P6, when a 100% rate of contamination was recorded.

A representation of the mean results obtained for each genotype per BA treatment for EFS (%) and NS/E can be found in Table 4.

Table 4. Values for EFS (%) (explants forming shoots) and NS/E (number of shoots formed per explant) for *Pinus halepensis* apical shoot buds from different genotypes, induced under two different concentrations of BA (22 and 44 µM).

Genotype	EFS (%)		NS/E	
	22 µM	**44 µM**	**22 µM**	**44 µM**
H32	25 ± 0	33.3 ± 0	4.5 ± 0	8 ± 0
H8	0 [1]	11.1 ± 0	0 [1]	22 ± 0
17.3	41.2 ± 10.0	59.2 ± 8.3	3.85 ± 1.0	5.2 ± 1.1
17.4	14.3 ± 5,4	33.3 ± 7.2	2.5 ± 0.9	3 ± 1.1
18.1	13.66 ± 3.8	29.7 ± 9.8	1.7 ± 0.5	2.4 ± 0.5
P1	0 [1]	39.9 ± 16.4	0 [1]	9.8 ± 2.2
P5	14.8 ± 0.4	19.54 ± 1.3	3 ± 0	3.7 ± 0.24
P6	12.0 ± 4.2	11.1 ± 3.93	1.67± 0	11.33 ± 0
P8	38.5 ± 0	25.0 ± 0	6.8 ± 0	5.3 ± 0

Data are presented as mean values ± SE. [1]: Contaminated.

None of the shoots cultured in the root-induction medium developed roots in vitro. Nevertheless, acclimatized ex vitro true-to-type plants from five different genotypes (H8, H32, 17.3, P1, and P8) were successfully obtained (Figure 1g), since ex vitro roots developed (Figure 1h,i). Regarding the acclimatization percentages, no statistically significant differences were found between the different genotypes (Table 2). However, the mean percentage was 65% for genotype H8, while in the other four genotypes the mean percentages were between 20 and 25% (Figure 2).

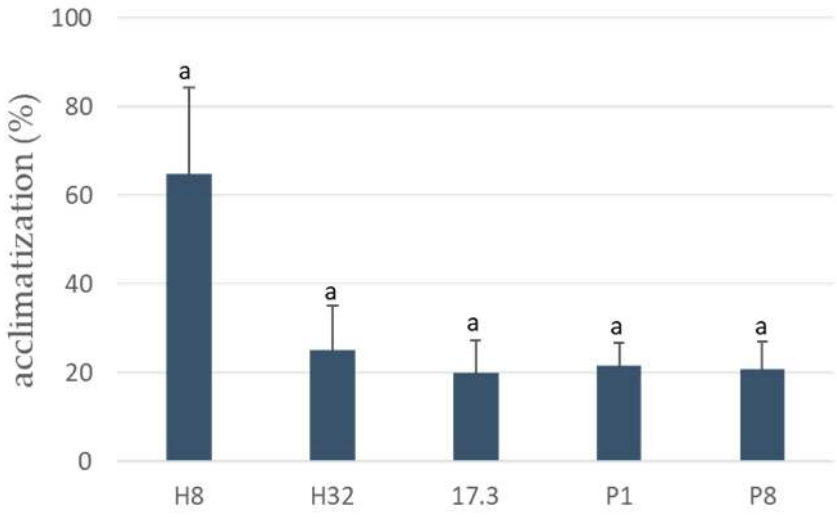


Figure 2. Acclimatization percentages of five different genotypes (H8, H32, 17.3, P1, and P8). The letter "a" indicates that there are no statistical differences between genotypes.

3.2. Attempts to Induce Embryogenic Tissue

A total of 490 bud slices from apical shoot buds were submitted to SE induction and a contamination rate of 61% was obtained. Of the 191 non-contaminated explants, 94.2% were induced and able to produce embryogenic-like tissue.

Bud slices from the juvenile somatic trees were cultured in their growth position in the S2 induction medium (Figure 3a) and approximately one week after culture, white-green soft embryogenic-like tissue begun to appear, first at the wounded areas and then throughout the explant (Figure 3b). Explants subcultured directly in the proliferation media presented tissues with a watery texture at first, which started to stiffen and to acquire a yellowish color through subculturing (Figure 3c). When the embryogenic-like tissue was detached from the explant and subcultured in filter paper, a more compact tissue was observed. Nevertheless, clusters with both brown, hard to disaggregate tissue, and

whiter, softer tissue could be observed in the S4 and S5 proliferation media (Figure 3d,e), with some calli showing a texture and color similar to the embryogenic ones (Figure 3e). However, acetocarmine staining (2% *w*/*v*) (Figure 3f,g) indicated that this callus remained non-embryogenic.

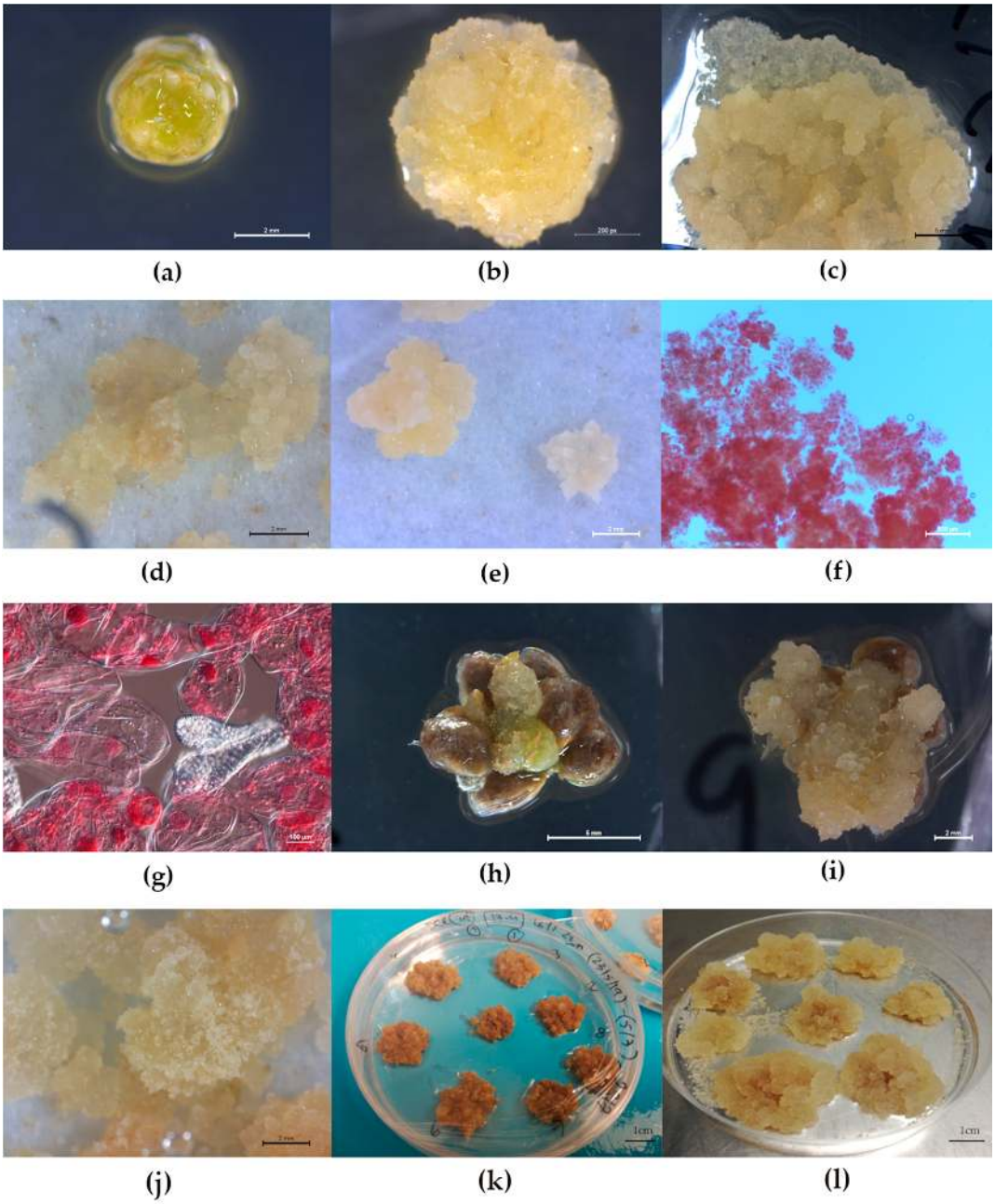


Figure 3. Induction response of apical shoot buds and axillary shoots to somatic embryogenesis (SE): (**a**) bud slice in the SE induction medium; (**b**) tissue development throughout the explant; (**c**) later proliferation of directly subcultured non-embryogenic callus; (**d**) filtered cultured tissue on the proliferation medium with 50 nM phytosulfokine; (**e**) example of non-embryogenic calli with distinct morphology in the same sample: the callus at the bottom right has more similarities with the embryogenic one; (**f**) cells collected from the lower cluster represented above, stained with acetocarmine (2% *w*/*v*) and observed using a Leica DMS1000, showing non-polarized cells; (**g**) observation of previous cells using a Nikon ECLIPSE 80 i, demonstrating the non-embryogenic state of the calli; (**h**) bud slice in the SE induction medium with tissue development in the upper wounded area of the explant; (**i**) tissue development progress in the upper area of the explant with no tissue development throughout the whole explant; (**j**) filtered cultured tissue proliferating at 28 °C; (**k**) non-embryogenic calli formed in the S11 induction medium; (**l**) non-embryogenic calli formed in the S10 induction medium.

Different from the above-mentioned explants, apical shoot buds with open scales (represented in Figure 1b) first developed a white-green soft embryogenic-like tissue at the upper wounded area, which then, growing only at this location of the explant, started to stiffen and acquire a yellowish color before the first subculture (Figure 3h,i). When comparing the differences obtained from the calli proliferated at 23 °C and 28 °C, these calli presented a darker color and stiffer morphology when cultured at 28 °C (Figure 3j). All of the shoot slices from the reinvigorated axillary shoots cultured in the SE induction media were able to produce a non-embryogenic callus. The tissue obtained in the S11 medium (Figure 3k) was brown, stiffer, and harder to disaggregate when compared to calli produced in the S10 induction medium (Figure 3i), which presented a lighter color, were softer, and had the easiest morphology to disaggregate. The calli induced in the S2 induction medium presented an intermediate aspect.

As described above, proliferation was consistent in all of the different initial explants and proliferation treatments. However, variations in the consistence, texture, and color of the developed tissue were observed between treatments. Also, during proliferation, tissue had similar characteristics to an embryogenic callus as it started growing, but with time it started losing those characteristics, becoming darker, stiffer, and developing non-embryogenic cells. In this sense, the initially developed cells were collected in an attempt at maturation but no somatic embryos could be obtained.

4. Discussion

4.1. Organogenic Process

The common criteria to identify reinvigoration or rejuvenation of explants are based on morphology, morphogenic and rooting capacity, and the ability to produce cones or flowers [31]. However, uncertainties remain over whether true rejuvenation can be reached by artificial methods, or whether these methods merely provide reinvigoration through the continuous in vitro subculture of the shoots. In the present study, for the first time, we were able to obtain reinvigorated axillary shoots through organogenesis, from the two BA treatments tested (22 and 44 µM), using apical shoot buds as explants. Then, successfully acclimatized plants were obtained.

Three different sterilization protocols were used, employing ethanol, commercial bleach, and silver nanoparticles, and the highest level of decontamination was obtained with bleach. The effective elimination of contamination could contribute to the better establishment of the shoot buds. This must be achieved with the least possible damage since a minor injury caused during sterilization can block the future growth and development of the explants. As reviewed in [32], ethanol and sodium hypochlorite are two of the most common chemicals used to decontaminate woody species. However, silver nanoparticles are quite effective at controlling physiochemical changes, preventing bacterial infections, and actively blocking ethylene through the release of silver ions, which could improve the induction of explants. Despite the fact that nanoparticle sterilization was not efficient in our case, changes to time or concentration could lead to better results and explant quality [33,34].

The evaluation of the organogenic response was made by considering the percentage of explants forming shoots (EFS) (%) and the number of shoots formed per explant (NS/E). The results showed that no statistically significant differences between treatments were obtained for EFS (%). When the mature zygotic embryos were used as initial explants in this species [25], a cytokinin alone, especially BA at the higher concentration tested (10 µM), also proved satisfactory for bud induction. Likewise, BA, either used alone or in combination with other cytokinins, was the most used growth regulator in organogenesis and its concentration played an important role in the explant response [18]. Several micropropagation protocols for the induction of axillary shoots in *Pinus* species supplement the culture media with different concentrations of BA ranging from 1 to 50 µM [20,21,25,35,36]. When the induction medium was supplemented with 44 µM BA, the NS/E obtained from the explants was more than double that of the explants induced at 22 µM BA. These results

agree with those obtained in *Pinus pinea* [37], where higher concentrations of BA (44.4 μM), as compared to regular doses of 4.4 and 10 μM, led to a significantly higher number of buds being formed per explant during the first 16 days of culture, and no differences between the three concentrations tested after 35 days in culture. In *Pinus roxburghii* [35], *Pinus pinaster* [18], and *Pinus radiata* [20], higher concentrations of BA promoted lower organogenic capacity or lower elongation rates. In *Pinus elliottii* [38], it was also shown that high levels of cytokinins may interfere with the normal development of axillary shoots and, despite the promotion of bud induction, they may compromise cell elongation and shoot elongation. It appears that the toxic environment created by the excess of BA seen in those species did not happen in our case and that the use of activated charcoal (AC) in our elongation medium proved efficient to detoxify the culture medium at both concentrations tested (22 and 44 μM BA). As has been reviewed previously [39], some of the positive effects of the use of AC in micropropagation can be attributed to the removal of inhibitory substances from the media itself and of toxic plant metabolites released from the tissue into the culture. Taking all the above-mentioned data into account, the induction of explants with 44 μM of BA and the addiction of AC to the elongation medium appears to be a good strategy for Aleppo pine organogenic induction.

It is widely recognized that different genotypes and genetic backgrounds may lead to differences in terms of the in vitro performance and cloning capacity in different conifer species [12,40]. Previously, studies developed by our group on the organogenesis of different *Pinus* species [18,20,41], and somatic embryogenesis in *Pinus halepensis* [26], also corroborated this effect. In this sense, no statistical analysis was performed concerning the genotype effect. Despite that fact, the mean results obtained for different genotypes presented, in general, a higher EFS (%) and NS/E for the explants cultured at 44 μM BA, and no differences were obtained regarding acclimatization between different genotypes.

The root induction treatment applied in this study, with long exposure to Indole-3-butyric acid (IBA), was not effective. Contrary, both pulses of IBA for five days in a solid medium and liquid pulses for four hours have previously proven efficient in the development of in vitro roots in this species [25]. For many years, IBA has been applied to different plant species to induce adventitious roots [42], however, the use of 1-Naphthaleneacetic acid (NAA), combined with IBA or alone, has proved successful for inducing root meristem differentiation in *Pinus* species [41,43]. Also, a study focused on the rooting of cuttings of mature *Pinus halepensis* [44], has shown that pulses for four hours of IBA alone, auxin combinations, and the use of the quick-dip method with IBA alone, are efficient for root development and further acclimatization of the cuttings. Our plants were able to produce ex vitro roots and acclimatize successfully. In this sense, further testing of different auxins, either alone or in different combinations, and of different induction times could improve this step of the Aleppo pine regeneration protocol. The application of short pulses or the quick-dip method could lead to a substantial reduction of time needed for the attainment of acclimatized true-to-type plants.

4.2. Attempts to Induce Embryogenic Tissue

In this work, we used various explants for the induction of embryogenic tissue, including apical shoot buds from both somatic and zygotic trees and reinvigorated axillary shoots developed during organogenesis. Woody species suffer a "phase change" or ontogenetic aging during their development, defined as a shift from the juvenile state to the adult state, which is usually characterized by a decrease in growth and the start of flowering [40,45]. Once this shift occurs, there is a significant loss of organogenic and embryogenic capacity. Also, initial explants had been considered the most important factor for mature SE accomplishment and it has been suggested that culture-derived material could be more responsive in tissue cultures [40,46].

In addition to explants, plant growth regulators, culture conditions and media composition also have a great influence on the embryogenic response [47]. Various induction media with different combinations of auxins (2,4-Dichlorophenoxyacetic acid and NAA)

along with lower concentrations of cytokinins (BA and kinetin) were tested for the induction of the explants. As mentioned above, the ratio between auxins and cytokinins can be determinant to the developmental fate of explants in vitro [16]. Cytokinins, or a higher ratio of cytokinin to auxin, are usually required for the induction of shoot organogenesis, while higher ratios of auxin to cytokinins typically favor SE [11]. Our basal media were based on one used in *Pinus contorta* [28] because they were able to induce embryogenic-like tissue from the shoot buds of mature trees, and one used for SE induction from immature megagametophytes of *P. halepensis*, as previously established in our laboratory [26,48].

All embryogenic-like tissue obtained initially produced non-embryogenic calli in the proliferation stage and no somatic embryos were produced. The primary objective of the induction phase is for the somatic cells of the explant to acquire embryogenic competence and, reprogramming the gene expression followed by polarized growth of the cells is required for that purpose [46]. The success of this step is essential for the entire process [49]. Nonetheless, the non-embryogenic tissue obtained had proliferation capacity, since it presented the ability to continuously originate new tissue throughout all subcultures performed in the proliferation media. Likewise, in *Pinus contorta* [28], they were able to develop calli with proliferation capacity and different morphologies, some of them with an embryogenic-like structure, that at the end were not able to produce somatic embryos. Non-embryogenic calli in other *Pinus* species have also been described as white-yellowish friable tissue containing spherical cells with prominent nuclei and without evidence of polarity that grow darker and necrotic with time [50,51]. Also, a study developed in our laboratory [52], tested different explants at different development stages with similar induction mediums.

Finally, we tested the influence of phytosulfokine, a small sulfated peptide involved in the initial step of cellular dedifferentiation, proliferation and re-differentiation, in the induction medium [53]. It did not lead to the ultimate formation of somatic embryos. However, it helped maintain the induced calli which were proliferating with a lighter color, and had the softer and easiest morphology to disaggregate. In *Daucus carota* [54], *Cryptomeria japonica* [53], and *Pinus elliottii* [55], phytosulfokine also significantly increased cell division, proliferation, and the number of somatic embryos developed.

5. Conclusions

This is the first report of successful in vitro regeneration of *Pinus halepensis* adult trees. The regeneration of *P. halepensis* through organogenesis using apical shoot buds as explants was achieved. Reinvigorated shoots from both juvenile somatic trees and adult trees were obtained, and a proliferative chain of microshoots coming from the buds of the adult trees was developed. In this sense, true-to-type plants from five different genotypes (H8, H32, 17.3, P1, and P8) were successfully obtained and plants acclimatized to ex vitro conditions were developed.

Despite our efforts to induce embryogenic tissue from mature trees, we were not able to produce somatic embryos. Nonetheless, we were able to produce proliferating embryogenic-like tissue and identify morphological differences with the application of different concentrations of phytosulfokine. Further experiments should be done, modifying the chemical and physical conditions of proliferation and the maturation of the embryogenic-like tissue.

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