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Efficient Procedure for Induction Somatic Embryogenesis in Holm Oak: Roles of Explant Type, Auxin Type, and Exposure Duration to Auxin

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Abstract: Holm oak is the dominant tree species in the Mediterranean climate. Currently, worrisome degradation of its ecosystems has been observed, produced, among other factors, by changes in land use, extreme weather events, forest fires, climate change, and especially the increasingly frequent episodes of high tree mortality caused by "oak decline", which has brought with it a social concern that transcends the productive interest. Breeding and conservation programs for this species are necessary to ensure the prevalence of these ecosystems for future generations. Biotechnological tools such as somatic embryogenesis (SE) have great potential value for tree improvement and have been shown to be highly efficient in the propagation and conservation of woody species. One challenge to this approach is that SE induction in holm oak has not yet been optimized. Here, we present a new reproducible procedure to induce SE in holm oak; we evaluated the responsiveness of different initial explants exposed to different types, concentrations, and durations of auxin. SE rates were significantly improved (37%) by culturing nodal segments for two weeks in induction medium. In addition, a significant auxin–genotype interaction was observed.

Keywords: auxin; indole-3-acetic acid; indole-3-butyric acid; leaves; 1-naphthaleneacetic acid; node explants; *Quercus ilex*; somatic embryogenesis induction; shoot apex

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1. Introduction

The evergreen oak *Quercus ilex* L. (holm oak) is one of the most important forest species in arid and semi-arid Mediterranean environments [1]. Approximately 90% of its worldwide distribution is in Morocco and the Iberian Peninsula [2]. In Spain, holm oak can form forest woodlands, and it is the predominant species in dehesas, which are the largest forest arrangements in the country and are equivalent to 27% of the Spanish forest area [3]. Dehesas are representative agroforestry systems of European agricultural systems and have high natural and cultural values [4,5]; they are models of compatibility between an efficient, diversified, extensive production system and the generation and conservation of high levels of biological diversity [6,7]. In addition to the productive role of dehesas (livestock, agriculture, forestry, hunting products), the traditional management of dehesas provides a wide variety of services, contributes to in the regulation of important natural cycles, and contributes to mitigating climate change and enhancing the conservation of biodiversity [4,5]. Finally, dehesas are an important part of the historical–cultural heritage of Spain and are increasingly exploited for tourism and recreational uses [8].

In recent years, the sustainability of these important ecosystems has been at high risk due to the lack of natural regeneration, extreme weather events, forest fires, climate change, and especially the presence of a severe disease named "oak decline syndrome" that has caused the loss of tens of thousands of hectares of dehesas and holm oak woodlands [9]. Oak decline is a complex syndrome, which causes the gradual and general deterioration of affected trees until their death and is produced by the joint action of silvicultural practices

and abiotic (episodes of drought or floods, and air or soil pollution) and biotic (pests and diseases) factors. *Phytophthora cinnamomi* is the biotic agent most related to oak decline, although other species such as *P. gonapodyides*, *P. quercina*, *P. psychrophila*, and *P. pseudocryptea* have also been identified as causative agents of this syndrome [10,11]. Despite the high economic and ecological importance of holm oak, currently there are no effective methods to control oak decline syndrome, and the vegetative propagation of tolerant genotypes of holm oak and their progenies may be one of the most realistic ways to address this problem.

Biotechnological tools such as somatic embryogenesis (SE) have great potential for tree improvement, and its high efficiency has been shown in many hardwood species [12]. Somatic embryogenesis in combination with genetic modification has enormous potential for improving forest species, but several bottlenecks must first be investigated and solved. Among these limitations, induction from adult tissues remains a challenge, as in many woody species, SE has only been reported from juvenile tissues. According to previous research on the topic, SE in oak species from very juvenile tissues (e.g., immature zygotic embryos) is relatively feasible, and induction rates of up to 100% have been achieved in some instances [13]. In contrast, the induction of somatic embryos from non-zygotic tissues, especially when derived from adult trees, remains problematic. To date, only three oak species, i.e., *Q. suber* [14], *Q. robur* [15,16], and *Q. alba* [17], have shown acceptable induction rates. In the case of holm oak, SE has been developed from zygotic embryos [18,19], floral tissues [20,21], and shoot and leaf explants [22–24], but induction frequencies were low, ranging from 0.2 to 11% [23–25]. Therefore, more efficient procedures for SE in holm oak need to be developed to apply this micropropagation technique for mass propagation of this species.

Substantial effort has been expended in recent decades to determine the factors that control SE. It is accepted that selection of the appropriate initial explant and the choice of plant growth regulators (PGRs) incorporated into the induction medium, as well as the exposure duration, are the most important factors for the successful induction of SE [12,26,27]. It is generally accepted that there are two stimuli that induce the reprogramming of differentiated plant cells to convert them into competent cells: (i) strong stress and (ii) changes in the internal and/or external cellular levels of PGRs [28,29]. There is also a consensus that among PGRs, auxins play a key role during SE induction, especially when the initial explants are non-zygotic tissues [12,30]. It is recognized that high doses of auxin with or without a cytokinin at low concentrations are crucial as an initial trigger in the acquisition of cellular competence, firstly promoting dedifferentiation followed by embryogenic differentiation [31]. The addition of exogenous auxins seems to act as a stressing agent and/or induces endogenous indole-3-acetic acid (IAA) production, which regulates the expression of a great number of transcription factors, several of them related to stress, and provokes changes in chromatin status [32,33]. Among the different types of auxin, the most used in SE induction, in order of frequency, are as follows: 2,4-dichlorophenoxyacetic (2,4-D) acid, 1-naphthaleneacetic acid (NAA), IAA, picloram, and dicamba [34]. Usually, NAA is applied when a strong auxin is not required to induce SE or because of its specificity for a given species. In the *Fagaceae* family, and specifically in the case of the *Quercus* species, to date NAA has been the most widely employed auxin to induce SE in non-zygotic tissues, whereas 2,4-D has been used to induce SE in zygotic tissues [13]. By contrast, IAA and indole-3-butyric acid (IBA) have hardly been used in these species [13,35]. Until now, most of the papers published on the effect of auxin on the induction of SE focus on the type and concentration of auxin used. However, less attention has been paid to the determination of the necessary exposure duration to trigger SE once the auxin type and concentration are set. Usually, a long exposure duration is routinely applied, whereas short periods and pulses have rarely been mentioned.

In addition to auxins, the type of explant and its well-defined developmental stage seem to be the most important factors that determine embryogenesis [12,36,37]. For SE induction in explants derived from adult trees, the general approach is to select explants that retain juvenile characteristics. Initially, maternal tissues (e.g., nucellus or inner teguments)

and floral tissues (e.g., immature inflorescences, petals, floral staminodes, pistils, stamens, or anther teguments) were the most used explants [12]. There was a general opinion that these tissues could contain dedifferentiated cells due to their proximity to the sites of fertilization and formation of zygotic embryos which facilitated the return to the embryogenic state [36]. However, in the last two decades, the use of other explant types such as shoot tips, nodes, internodes, and especially leaves has gained relevance in SE induction, mainly due to the fact that they are more abundant, which enables the evaluation of more factors, and, above all, they are easier to manage than flower tissues [25]. These explants can be isolated directly from a tree, although the strategy that has offered better results involves excision from shoots derived from forced flushing of branch segments or from axillary shoot cultures established from them [12,25,37]. In addition, the embryogenic ability of the explant type shows great variability in the function of the species. For instance, in *Eucalyptus globulus*, shoot apices presented a greater embryogenic response than leaves [38], while in *Q. alba*, the explants that best responded were the leaves [17]. In *Vitis vinifera*, nodes with a single axillary bud showed the highest rate of embryogenic induction [39].

In order to optimize the frequency of SE induction in holm oak, our objective was to identify the factors that can improve this step using axillary shoot cultures as the source of initial explants. In this study, we investigated the effects of (i) the explant type (leaves, nodes, and shoot tips), (ii) three auxins (NAA, IAA, and IBA), (iii) the auxin exposure duration, and (iv) the genotype role.

2. Materials and Methods

2.1. Plant Material

We used axillary shoot cultures of three holm oak genotypes, Q3-SE, Q10-SE, and E00, as the explant source. Genotype E00 was established from epicormic shoots of a 30-year-old tree as previously described [40]. Axillary shoot cultures of Q3-SE and Q10-SE were established by axillary budding from shoots derived from germinated somatic embryos induced from centenary trees as previously described [22]. Axillary shoot cultures of the three genotypes were maintained by subculture on Woody Plant Medium [41] (Duchefa Biochemie, The Netherlands) supplemented with sucrose (30 g/L), silver thiosulphate (20 µM), and Sigma agar (8 g/L), with a sequence of transfers performed every 2 weeks over a 6-week multiplication cycle as follows: 0.1 mg/L 6-benzyadenine (BA) for the first 2 weeks, 0.05 mg/L BA for the next 2 weeks, and 0.01 mg/L BA for the last 2 weeks. All culture media were brought to pH 5.6–5.7 before autoclaving at 115 °C for 20 min. Stock cultures were cultivated in a growth chamber with a 16 h photoperiod, provided by cool white fluorescent lamps (photon flux density of 50–60 µmol m^{-2} s^{-1}) at 25 °C light/20 °C dark (i.e., standard culture conditions).

2.2. Somatic Embryogenesis Initiation

To determine the optimal explant type for SE initiation, 3 types of explants were tested in this study: shoot apex (2 to 2.5 mm long, comprising the apical meristem and 2 to 3 pairs of leaf primordia), the most apical expanding leaf, and the node below the shoot apex of the three genotypes (Figure 1). Then, to determine the best combination of growth regulators, explants were cultured on a basal induction medium consisting of Murashige and Skoog medium (MS) [42] (Duchefa Biochemie, The Netherlands) added with casein hydrolysate (500 mg/L), sucrose (30 g/L), and Plant Propagation Agar (6 g/L; Pronadisa, Spain). This medium was added with three different PGR combinations: IAA (4 mg/L) plus BA (0.5 mg/L) (IAA treatment), NAA (4 mg/L) plus BA (0.5 mg/L) (NAA treatment), and IBA (3 mg/L) plus NAA (0.1 mg/L) (IBA treatment). The IAA and NAA treatments were chosen based on previous studies of SE induction in holm oak [22]. The IBA treatment was chosen following the appearance of somatic embryos when this treatment was used to induce adventitious root formation on E00 axillary shoots (Supplementary information S1). Nodal segments and shoot tips were cultured on auxin medium in the dark at 25 °C for 2, 4, and 8 weeks, whereas leaves were cultured only for 2 and 8 weeks. Then, explants

were transferred to basal induction medium without PGRs and cultured in light conditions (standard culture conditions) without transfers to fresh medium for at least 24 weeks. After this period, SE induction efficiency was estimated.

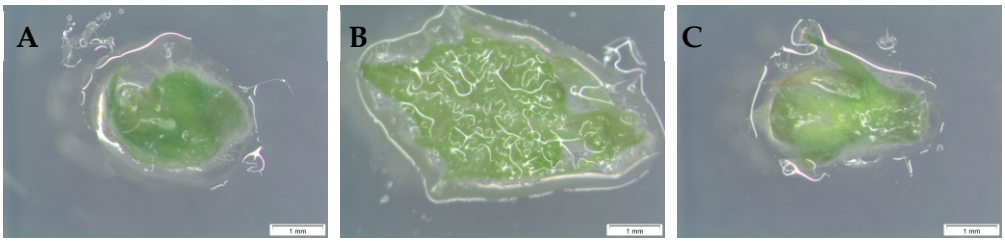

Figure 1. Initial explants used to induce somatic embryogenesis on holm oak at the excision day. (**A**) Shoot apex explant. (**B**) Leaf explant. (**C**) Node explant. Bar: 1 mm.

Ten leaves (abaxial side down) and ten shoot apices and nodal segments (horizontally orientated) (Figure 1) were cultured in 90 × 15 mm Petri dishes containing 25 mL of auxin treatment. For each genotype, explant type, auxin treatment, and auxin exposure duration, 50 explants were used, and each experiment was repeated twice.

At the end of the culture period, the following data were recorded: the percentage of explants that formed calli, the percentage of explants that formed roots, the percentage of explants that showed an embryogenic response, and the number of somatic embryos or nodular embryogenic structures per initial explant. An embryogenic response was described as the presence of nodular embryogenic structures and/or somatic embryos (torpedo/cotyledonary stage) on the initial explants. These parameters were determined by periodically examining the explants under a stereomicroscope (Olympus SZX9, Japan). Photographs were taken with an Olympus SC100 digital camera (Japan).

2.3. Statistical Analysis

The influence of the main experimental factors on the embryogenic response (measured as a percentage) and their interactions were statistically evaluated using analysis of variance (ANOVA). Prior to analysis, an arcsine square root transformation was applied to proportional data. A Levene test for normality and homogeneity of variance was performed prior to ANOVA. In the tables, non-transformed data are presented. SPSS for Windows (version 26.0, Chicago, IL, USA) was utilized to perform the statistical analysis.

3. Results

For the three genotypes evaluated, the first response observed was callus formation. The percentage, callus size, and its appearance mainly depended on the auxin treatment applied. The highest callus percentage (between 83 and 100%) was obtained when IBA treatment was used regardless of the genotype, exposure duration, and explant type (see Supplementary information S2). In this treatment, before and after the explants were transferred to media without PGRs, callus growth was disorderly, so the original explant became unrecognizable. With NAA treatment, the percentage of calli formed was also high, with the exception of the apex of genotype Q3-SE and the leaves of genotype E00. In this treatment, calli appeared only around wounded areas, and consequently, the initial explants were perfectly recognizable. Finally, IAA treatment produced only very small calli and usually in very low proportions. In addition to callus formation, after culture on induction media without PGRs, adventitious root formation was also observed. Among the three treatments, IBA treatment showed the highest percentage of roots, especially when the initial explants were leaves (e.g., about 95% in Q3-SE) (Supplementary information S3).

The embryogenic response was always indirect through callus formation on the original explant regardless of the type of explant, exposure duration, and auxin treatment; however, the size and percentage of the calli formed did not seem to be directly related to SE induction. In all genotypes, somatic embryos or nodular embryogenic structures arose

after the initial explants were transferred into PGR-free medium, becoming visible between 3 and 8 months after culture initiation (Figure 2A). Shoot tips and node explants produced the highest number of somatic embryos and/or nodular structures per explant, ranging mostly from one to three, while in foliar explants, the number of somatic embryos was always one per reactive explant (Figure 2B–D).

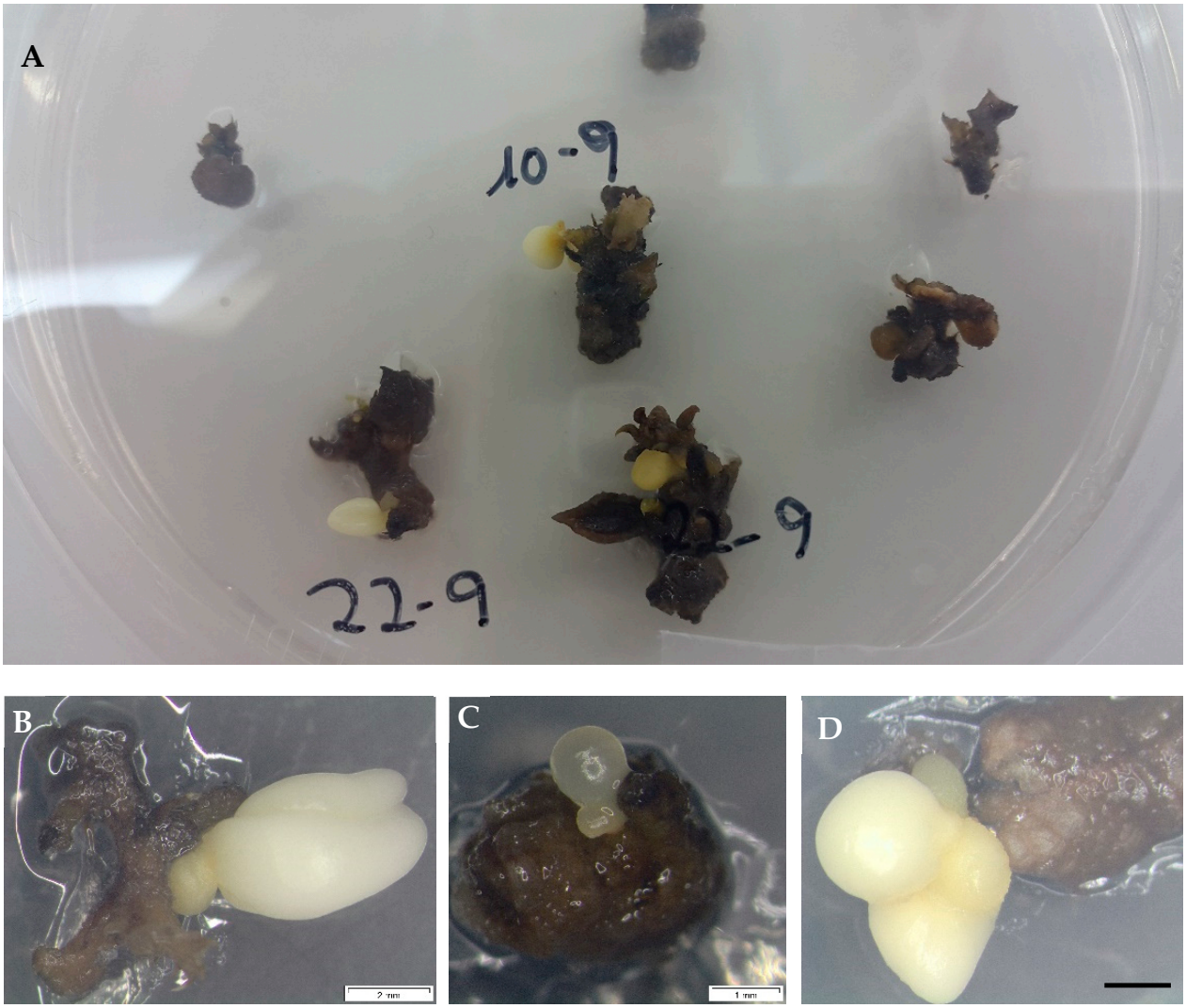

Figure 2. Somatic embryogenesis induction on different explants excised from axillary shoot cultures established from adult holm oak trees. (**A**) Somatic embryos and nodular embryogenic structures generated on different apex explants of genotype Q10-SE cultured on induction medium with IAA. (**B**–**D**) Embryogenic response on an apex (**B**), node (**C**), and leaf (**D**) explant of genotype Q3-SE. (**A**): diameter dish 90 mm. (**D**): bar 1 mm.

3.1. Embryogenic Response in Apex Explants

Somatic embryogenesis from apex explants was significantly affected by genotype ($p = 0.001$), auxin treatment ($p = 0.001$), and auxin exposure duration ($p = 0.049$), as well as by the genotype–auxin treatment interaction ($p = 0.001$) and the genotype–auxin treatment exposure-duration interaction ($p = 0.001$) (Table 1).

Table 1. Embryogenic response in apex tips excised from axillary shoot cultures of three adult genotypes of holm oak under the effects of different treatments and exposure duration to auxins.

Treatment (mg/L)	Somatic Embryogenesis (%)		
	Q10-SE	**Q3-SE**	**E00**
IAA 4 + BA 0.5			
2 wks	15.0 ± 2.9	2.0 ± 1.9	12.0 ± 3.1
4 wks	11.0 ± 3.6	7.0 ± 2.5	2.0 ± 1.3
8 wks	12.0 ± 2.8	15.0 ± 5.0	1.0 ± 0.9
NAA 4 + BA 0.5			
2 wks	3.0 ± 2.9	33.0 ± 4.3	0.0 ± 0.0
4 wks	0.0 ± 0.0	20.0 ± 7.2	0.0 ± 0.0
8 wks	0.0 ± 0.0	22.0 ± 4.2	0.0 ± 0.0
IBA 3 + NAA 0.1			
2 wks	0.0 ± 0.0	12.0 ± 4.2	0.0 ± 0.0
8 wks	0.0 ± 0.0	14.0 ± 2.9	0.0 ± 0.0
ANOVA			
Genotype (A)	$p = 0.001$ ***		
Treatment (B)	$p = 0.001$ ***		
Exposure duration (C)	$p = 0.049$ *		
A × B	$p = 0.001$ ***		
A × C	0.111 ns		
B × C	0.495 ns		
A × B × C	$p = 0.001$ ***		

BA: 6-Benzylaminopurine; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; NAA: 1-naphthaleneacetic acid; wks: weeks. Each value is the mean ± standard error of ten replicate dishes with ten explants per dish. ANOVA significance values are shown for each parameter. ns: not significant; * significant difference at 95.0% ($p \leq 0.05$); *** significant difference at 99.9% ($p \leq 0.001$).

The induction rates were higher with the shoot tips of Q3-SE cultured with NAA (33%) for two weeks. By contrast, in genotypes Q10-SE and E00, the best percentages were obtained with IAA also applied for 2 weeks (15% and 12%, respectively), but the values were lower than those in Q3-SE (Table 1). IAA treatment induced SE in all three genotypes, whereas NAA induced SE in genotypes Q10-SE and Q3-SE, although marked differences in the induction rates were observed between both genotypes (33% in Q3-SE versus 3% in Q10-SE). IBA induced SE only in genotype Q3-SE but without differences between the two auxin exposure periods evaluated (12% with two weeks versus 14% with eight weeks). Regarding the auxin application regime, a clear interaction between the genotype and the type of auxin was observed (Table 1). In the IAA treatment, 2 weeks of auxin exposure produced the best results in genotypes Q10-SE and E00, whereas in genotype Q3-SE, the highest induction percentages were obtained with an induction-medium culture period of 8 weeks. By contrast, in this genotype and in the NAA treatment, the best values were achieved with a 2-week culture period (Table 1).

3.2. Embryogenic Response in Node Explants

As occurred with the apex explants, SE from nodes was significantly influenced by genotype ($p = 0.001$), auxin treatment ($p = 0.001$), and auxin exposure duration ($p = 0.001$) (Table 2). Also similar to what was found with the shoot apex explants, three of the four possible interactions between treatments had significant effects on SE induction (i.e.,

genotype–auxin treatment, genotype–exposure duration, and genotype–auxin treatment–exposure duration).

Table 2. Embryogenic response in node explants excised from axillary shoot cultures of three adult genotypes of holm oak under the effects of different treatments and exposure duration to auxins.

Treatment (mg/L)	Somatic Embryogenesis (%)		
	Q10-SE	**Q3-SE**	**E00**
IAA 4 + BA 0.5			
2 wks	37.0 ± 5.3	2.0 ± 1.9	4.0 ± 2.1
4 wks	21.0 ± 5.6	2.0 ± 1.3	4.0 ± 2.1
8 wks	3.0 ± 2.0	4.0 ± 1.6	3.0 ± 2.0
NAA 4 + BA 0.5			
2 wks	0.0 ± 0.0	29.0 ± 5.0	1.0 ± 0.9
4 wks	0.0 ± 0.0	4.0 ± 2.1	0.0 ± 0.0
8 wks	0.0 ± 0.0	3.0 ± 1.5	0.0 ± 0.0
IBA 3 + NAA 0.1			
2 wks	4.0 ± 2.9	24.0 ± 3.2	1.0 ± 0.0
8 wks	0.0 ± 0.0	1.0 ± 0.95	0.0 ± 0.0
ANOVA			
Genotype (A)	$p = 0.001$ ***		
Treatment (B)	$p = 0.001$ ***		
Exposure duration (C)	$p = 0.001$ ***		
A × B	$p = 0.001$ ***		
A × C	$p = 0.001$ ***		
B × C	0.262 ns		
A × B × C	$p = 0.001$ ***		

BA: 6-benzylaminopurine; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; NAA: 1-naphthaleneacetic acid; wks: weeks. Each value is the mean ± standard error of ten replicate dishes with ten explants per dish. ANOVA significance values are shown for each parameter. ns: not significant; *** significant differences at 99.9% ($p \leq 0.001$).

Regarding auxin treatment, IAA induced SE in the three genotypes, but the best rate was observed in genotype Q10-SE (37%) (Table 2). Conversely, this treatment was less effective with the Q3-SE genotype (2%–4%), which responded with high SE induction frequencies when nodes were treated with NAA (29%) or IBA (24%) (Table 2). Treatment with IBA also produced an embryogenic response in the nodes of genotypes Q10-SE (4%) and E00 (1%), but the values were significantly lower than those obtained with IAA. With respect to the auxin exposure duration, the auxin treatment applied to the three genotypes for 2 weeks on induction medium produced the best results, with values of 4% for E00 in the IAA treatment, 29% for Q3-SE in the NAA treatment, and 37% for Q10-SE in the IAA treatment (Table 2). In addition, in genotypes Q10-SE and E00, for nodes cultured on NAA or IBA treatments, embryogenic responses were observed only when explants were cultured for 2 weeks.

3.3. Embryogenic Response in Leaf Explants

For the three genotypes, regardless of the auxin treatment and exposure duration, leaf explants were the least responsive explants, with values ranging between 1 and 9% (Table 3). Somatic embryogenesis in leaves was significantly influenced by genotype ($p = 0.003$) and auxin treatment ($p = 0.019$), and by the genotype–auxin treatment interaction ($p = 0.001$),

the auxin treatment–exposure duration interaction ($p = 0.019$), and the interaction among the three factors ($p = 0.001$) (Table 3). The highest embryogenic response (9%) was obtained for genotype Q3-SE with leaves cultured on medium with NAA for 2 weeks (Table 3). The results provide strong support for the argument that the leaves are not the best explant source by which to induce SE in holm oak if we continue to use these conditions. This low response is probably related to the high degree of necrosis shown by the leaves in comparison with the apex and node explants once they are excised from the shoots.

Table 3. Embryogenic response in leaf explants excised from axillary shoot cultures of three adult genotypes of holm oak under the effects of different treatments and exposure duration to auxins.

Treatment (mg/L)	Somatic Embryogenesis (%)		
	Q10-SE	**Q3-SE**	**E00**
IAA 4 + BA 0.5			
2 wks	1.0 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
8 wks	0.0 ± 0.0	2.0 ± 1.3	0.0 ± 0.0
NAA 4 + BA 0.5			
2 wks	0.0 ± 0.0	9.0 ± 3.0	0.0 ± 0.0
8 wks	0.0 ± 0.0	1.0 ± 0.9	1.0 ± 0.95
IBA 3 + NAA 0.1			
2 wks	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
8 wks	1.0 ± 0.9	1.0 ± 0.95	1.0 ± 0.9
ANOVA			
Genotype (A)		$p = 0.003$ **	
Treatment (B)		$p = 0.019$ *	
Exposure duration (C)		0.506 ns	
A × B		$p = 0.001$ ***	
A × C		0.071 ns	
B × C		$p = 0.019$ *	
A × B × C		$p = 0.001$ ***	

BA: 6-Benzylaminopurine; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; NAA: 1-naphthaleneacetic acid; wks: weeks. Each value is the mean ± standard error of ten replicate dishes with ten explants per dish. ANOVA significance values are shown for each parameter. ns: not significant; * significant differences at 95.0% ($p \leq 0.05$); ** significant differences at 99% ($p \leq 0.01$); *** significant differences at 99.9% ($p \leq 0.001$).

3.4. Overview of Results

To summarize, the results obtained indicate that all genotypes exhibited embryogenic response, but the induction rates varied substantially, with genotypes Q10-SE and Q3-SE being those with the highest embryogenic responses (37% and 33%, respectively). However, a very strong auxin–genotype interaction was observed regardless of the type of explant used and the auxin exposure duration; E00 and Q10-SE showed the highest induction rates when the IAA treatment was applied (12% and 15%, respectively, for apices and 4.0% and 37.0%, respectively, for nodal segments), while for genotype Q3-SE, the best response was obtained with the NAA and IBA treatments (33.0% and 14% for apex tips and 29% and 24% for nodes).

Among all three genotypes, regardless of the treatment and auxin exposure duration, leaves were the least reactive explants to induce SE. The shoot tip and node response rates were conditioned by the genotype, so that in Q3-SE (33%) and E00 (12%), the highest induction rates were recorded when the apices were used, and in Q10-SE (37%), the most reactive explants were the nodal segments.

The induction of embryos was possible for the three auxin exposure periods evaluated, although the results show that the percentage of explants with somatic embryos decreased when a 4-week exposure duration was applied. Regardless of the genotype, the best rates were recorded when the explants remained in the medium with auxin for 2 weeks.

In all three genotypes, embryogenic capacity was maintained by secondary embryogenesis subculturing proembryogenic masses according to the procedure described by [22] (see Supplementary Information S4). Likewise, plant regeneration was achieved by somatic embryo germination following the procedure developed by [22] (see Supplementary Information S4).

4. Discussion

The highly recalcitrant nature of holm oak represented by low rates of induction via somatic embryogenesis, together with the difficulty in obtaining the desired response in explants derived from mature trees, has slowed the application of this micropropagation pathway in breeding programs. One of the most important issues arising in SE induction of forest species, but also in the other two micropropagation pathways, is the difficulty in obtaining the desired response in explants derived from mature trees since as the tree age increases, the regeneration ability decreases [37,43,44]. In the present paper, regeneration through SE from adult tissues in the recalcitrant species holm oak was considerably improved by manipulating factors such as the explant type, auxin type, and auxin exposure duration for the three genotypes.

The initiation of the SE process may be conditioned by the explant source and by the condition of the donor plant [12,45]. A first key point in the present paper was the use of axillary shoot cultures as a source of initial explants. This type of explant source presents a great advantage over ex vitro explants, as it allows better control of the growing conditions of the stock material and avoids differences caused by the time of the collection of plant material from trees growing in the field. In addition, it enables the production of physiologically uniform explants while ensuring the supply of an unlimited number of explants throughout the year, which makes it possible to simultaneously evaluate multiple factors [12,37]. In particular, in holm oak, axillary shoot cultures have been shown be a good alternative by improving the embryogenic efficiency of previous attempts to induce SE from leaves or apex tips collected directly from selected field-growing trees [20,25]. In the same way, the induction of somatic embryos in *Arbutus unedo* was possible only when explants excised from axillary shoot cultures were cultured [46]. It is known that repetitive subcultures of shoots on medium supplemented with cytokinins exhibit certain rejuvenating effects in the shoots, which facilitates the induction of the embryogenic process [47]. Additionally, the embryogenic competence of genotypes Q3 and Q10 was enhanced in this research when axillary shoot cultures of Q3-SE and Q10-SE, established from somatic plants, were used as a source of explants. The induction rates obtained for Q3-SE and Q10-SE were significantly higher than the values previously published for the same genotypes when explants derived from axillary shoots established from forced shoots of the trees were used [22]. These results agree with those obtained by [48] for pedunculate oak; it was reported that for the same genotype, shoot multiplication and rooting rates were significantly higher in isolated shoots from cultures derived from germinated somatic embryos than those established from forced shoots of crown branches [48]. Both results confirm the idea that some rejuvenation occurred during the process of somatic embryogenesis, and axillary shoots derived from germinated embryos have more morphogenic ability than shoots derived directly from the tree.

The role of auxins in SE induction has been widely investigated on the basis of 'one-factor-at-a-time' and 'trial-and-error' assays, and in most of these tests, the evaluation of the auxin effect was insufficient due to the low availability of explants from which to study different experimental conditions. Moreover, the optimal PGR contents have been determined only for certain cultivars or genotypes. In the present study, the use of axillary shoot cultures as a source of initial explants allowed us to analyze three induction

treatments and three auxin exposure periods in three explant types collected from three different genotypes. Until now, NAA was the most widely used auxin to initiate somatic embryos in oak species when non-zygotic explants were employed [13,35]. However, in holm oak, IAA induced SE in the three genotypes, whereas NAA was effective only in genotype Q3-SE. There are some examples in which other less conventional auxins also had a successful effect on SE induction [12]. For example, IBA was applied in the SE of petioles of olive [49], leaves of *Camellia japonica* [50], and nodal segments of *C. sinensis* [51], whereas picloram was more effective in inducing somatic embryos in the apex of *Eucalyptus* [38]. We believe that this different embryogenic behavior of each genotype in the functioning of auxin types could be due to a different content of endogenous auxins. However, at present, very little is known about how exogenous auxin applied during the induction step interacts with the endogenous auxin of the initial explant used to induce SE.

While it has been suggested that a high concentration of auxin during a long period is required for the acquisition of embryogenic capacity, our results in holm oak show that the 2-week pulse treatment, together with the removal of auxin for the subsequent differentiation of somatic embryos, was sufficient to provide the necessary stimuli to induce somatic embryogenesis, although SE was also achieved when the period was extended to 8 weeks. Likewise, in *Cercis canadensis*, a greater number of somatic embryos was obtained using auxin pulse treatments compared to a long exposure to auxin [52]. A similar finding was also obtained for nucellar tissues of mango, where a short exposure time of 4-week culture was the optimal induction period [53]. In addition to added exogenous auxin, our results again emphasize the important role played by endogenous auxin levels, as well as the stress caused by the excision of the explant to generate an embryogenic response. Both factors combined with the 2-week pulse with a high auxin concentration seem sufficient to induce an embryogenic response for explants derived from adult trees of holm oak. It is well recognized that wounding is the first event that provides signals triggering the embryogenic process, as explant excision produces hormonal balance changes [54,55]. This is in accordance with our previous findings in holm oak, as we were able to induce somatic embryos in apex explants cultured on induction medium devoid of PGRs, concluding that in this species, wounding has a clear effect on the embryo induction process, but the addition of an auxin improves the induction rate [22,23]. Although two weeks of culture on induction medium increased the values, this approach did not reduce the time period required for the whole process of somatic embryo generation.

The novel achievement of this study is that the application of a two-step procedure, in which after culture on auxin medium explants are directly transferred to medium without PGRs, is effective. Our protocol makes it possible to induce somatic embryos or nodular embryogenic structures faster and at a lower cost than the three-step procedure previously published for holm oak [22] and other oak species such as cork oak [14], bicolor oak [56], or pedunculate oak [57], for which it has been observed that the embryogenic response occurs with a three-step procedure in which somatic embryos are induced in the presence of a high auxin concentration, following transfer to a second medium with a lower PGR concentration, and finally transfer to a third medium without PGRs. By contrast, as in the present work, in *Q. alba* [17], the application of a two-step culture induction process enhanced the embryogenic frequencies.

With regard to the choice of explant type, the aim is to identify those tissues that contain competent dedifferentiated cells, i.e., with the capacity to generate somatic embryos as a response to external or internal stimuli and/or signals [58]. To induce SE on adult genotypes of hardwoods, one of the most commonly used explants in recent decades is leaves [12]; however, in the case of holm oak, the morphogenetic ability of leaves was much lower than that of shoot tips and nodes, confirming previous published results for this species [22–24]. Holm oak leaves have the ability to form calli and roots, especially in induction medium supplemented with IBA, but their embryogenic competence is low. By contrast, the shoot apices of the three genotypes and node explants of Q3-SE and Q10-SE showed a strong ability to generate somatic embryos. Similar results have been reported by [59], who found

that shoot tips were more appropriate than petioles and entire leaves to obtain SE in two adult genotypes of olive. In the same way, the shoot apex of *Phoenix dactylifera* was the most reactive explant to generate somatic embryos in this species [60]. Equally, nodal segments taken from new sprouting branches of mature trees of *Santalum album* [61] or in vitro grown plantlets of *Vitis vinifera* [39] were able to generate somatic embryos. There is evidence in the literature supporting the hypothesis that the presence of meristematic tissues is strongly involved in the embryogenic response as they can be considered stem cell niches (i.e., pluripotent and totipotent cells) [37,39,62]. Moreover, it is important to highlight the effect of the timing of explant excision that determines the induction of SE; shoot apices should be collected with two or three primordial leaves and very young nodes (i.e., 2–3 days after formation).

In addition to auxin treatment and explant type and its developmental stage, the genotype of the mother tree used as the source of explants has a strong influence on embryogenic competence, and it is one of the main factors limiting SE induction [12,30,37]. There are many reports that have highlighted the influence of genotype on SE induction; however, there is little information regarding why the embryogenic response varies in different genotypes of the same species when the same induction treatments are used. In the present study, SE was achieved in the three genotypes evaluated, but large differences in embryogenic response were observed among them. Thus, for genotypes Q10-SE and E00, the best embryogenic responses were observed on induction medium supplemented with IAA, but very low or no somatic embryo formation was obtained in the medium with IBA or NAA. Conversely, in genotype Q3-SE, high embryogenic responsiveness was yielded with the latter two auxin types. These results are consistent with previous reports on SE induction in different oak species, which revealed a substantial participation of the genotype in the embryogenic response capacity. For example, for pedunculate oak, the authors of [57] obtained somatic embryos in three out of the five adult genotypes evaluated, with embryogenic rates ranging from 1.7 to 5.6%, while the authors of [63] obtained embryogenic lines in 12 out of 19 mature cork oak trees from four provenances with values ranging from 1.3 to 28.5%. The results obtained in the present research show the strong effect of the genotype–auxin interaction on SE induction but also indicate that the differences in embryogenic ability between genotypes can be significantly reduced by altering and optimizing culture conditions. Similar conclusions were drawn in the literature, demonstrating that SE induction in recalcitrant genotypes of coffee [64] or grapevine [45] was possible when the composition of the media was optimized.

5. Conclusions

The higher SE induction rates described in this work represent a considerable improvement with respect to what was previously published for this species. In addition to significantly increasing the induction percentages, the procedure was simplified by reducing the exposure duration to auxins to 6 weeks and eliminating the intermediate step to an expression medium with reduced PGRs (4 weeks), with consequent savings in time. The proposed experimental model might also be useful for developing studies on the anatomical, physiological, and epigenetic facets implicated in the embryogenic process of this species. This protocol has the potential to be used for mass-scale propagation of holm oak; however, several bottlenecks including scaled-up SE production, high-frequency germination and conversion, and improving somatic seedling quality should be overcome before SE could be employed for mass propagation or integration with breeding programs.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f14020430/s1, Supplementary information S1: Somatic embryo induced on the callus formed at the base of the axillary shoot subjected to rooting treatment consisting of IBA (3 mg/L) plus NAA (0.1 mg/L) for two weeks, Supplementary information S2: Callus response of three different explant types excised from axillary shoot cultures of three adult genotypes of holm oak and cultured on three different treatments, Supplementary information S3: Adventitious root formation in leaves cultured on induction medium with 3 mg/L IBA plus 0.1 mg/L NAA,

Supplementary information S4: A. Somatic embryos originated from proembryogenic masses after 6 weeks of culture on proliferation medium; B. Plant recovery after 8 weeks of culture of somatic embryos in germination medium; A: diameter dish 90 mm; B: diameter jar 90 cm.

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