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ARTICLE



# Secondary somatic embryogenesis in Cork oak: influence of plant growth regulators

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**ABSTRACT**

Cork oak (*Quercus suber* L.) is one of the most important Mediterranean forest tree species, and the Maâmora forest-Morocco is considered to be the world largest cork oak stand. Clonal propagation of *Quercus suber via* somatic embryogenesis is an alternative to conventional tree propagation methods. However, complete maturation of somatic embryos is considered the major bottleneck for mass propagation of *Quercus suber* during somatic embryogenesis. This study focuses on influences of cytokinins on secondary somatic embryogenesis. Cytokinins showed a negative effect on the induction of secondary somatic embryogenesis. For the various concentrations tested, the number of secondary somatic embryos was significantly reduced except in the case with low dose of 2iP, which showed a slight increase. Abscisic acid (ABA) may be beneficial at low concentrations between 0.3 and 0.5 mg/l. The best number of secondary embryos was given at 0.5 mg/l ABA with an average number of 5 embryos from each primary embryo, which represents a slight increase below 10% compared to control medium. Also, we found that the number of responsive embryos presenting secondary somatic embryos based on their cotyledons decreased once adding ABA. Phenylurea derivatives TDZ and DPU do not promoted the process of secondary somatic embryogenesis. DPU showed no significant effect with a similar response to that of the control medium and TDZ showed an inhibitory effect. Gibberellic acid ($GA_3$) partially blocked the process of somatic embryos multiplication, but promoted remarkably the germination of cotyledonary embryos without requesting a particular protocol. Our results provide a new insight into embryo development, establishing the basis for further research toward improvement of secondary somatic embryogenesis in Moroccan cork oak.

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Cork oak; somatic embryogenesis; in vitro culture; secondary somatic embryos; plant growth regulators; cytokinins

## Introduction

*Quercus suber* L. is a forest species playing a major role in many Mediterranean ecosystems because it has a high economical and ecological impact contributing to the rural development in its geographical distribution range (Pérez et al. 2015). Cork oak forest, as a carbon sink, is one of the mitigation strategies being considered to deal with climate change (Zribi 2016).

In Morocco, the current subericultural area is 377,482 ha, which represents 7% of the Moroccan forest. With this area, Morocco occupies the fourth place (15.2% of the world's subseries) and produce 5 to 6% of the cork world production. The average annual volume mobilized is around 129,700 st (about 15,000 tons), which represents an average realization rate of 73% and a productivity of 0.56 staves per hectare and per year (Hammoudi 2002). Most of the cork harvested is used for the manufacture of stoppers; the rest is destined for crushing and/or exported in a semi-processed state.

Cork oak is a long life cycle tree with irregular fructification, seasonality and difficulty of seed conservation. The restricted option for classical reproduction *via* breeding or vegetative reproduction of *Quercus suber* trees are serious drawbacks for its conservation (Gomez-Garay et al. 2014). Furthermore, vegetative propagation of mature individuals is not viable by classic methods (Vieitez et al. 2012). Thus, all these difficulties have impeded the development of genetic improvement programs for this species (Pérez et al. 2015). Among the alternatives to overcome the vegetative propagation problem, somatic embryogenesis (SE) has been the most successful and powerful technique used for large-scale propagation of selected material that is considered to be the most adequate tool for

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*in vitro* regeneration of woody species (Barra-Jiménez et al. 2014; Corredoira et al. 2014; Wang et al. 2020). However, there are several constraints when SE is applied to these species: in many cases, successful induction only occurs from juvenile tissues (limiting its use for the propagation of mature elite trees), and the quality of the somatic embryos obtained and their conversion rate into plantlets are dependent upon the genotype of the original explants (Stasolla et al. 2002). Several studies have been using SE with modified different experimental protocols. Scaling up and long-term maintenance of SE in different oak species depends on their capacity to produce embryos *via* repetitive or secondary embryogenesis. Isolation of somatic embryos induced in the original explants and cultured in an embryo proliferation medium can yield numerous secondary somatic embryos (SSE), thus giving rise to clonal embryogenic lines (Corredoira et al. 2014).

Despite the apparent feasibility of multiplying oak embryogenic lines by repetitive embryogenesis, the generation of SSE developed up to the torpedo/cotyledonary stage (bipolar structures) is not efficient in many oak embryogenic lines. Therefore, a bottleneck still remains associated with the production of well-developed embryos to be used in maturation and germination steps (Martínez et al. 2019). Factors affecting the embryo proliferation step, including plant growth regulators (PGRs), media composition, genotype, and embryogenic subculture, influence the character of the repetitive cycle (Merkle et al. 1995), but these factors have not been extensively investigated in Moroccan *Quercus suber*.

Phytohormones or "plant growth regulators" are organic molecules of average molecular weight produced by plants. They are endogenous (not supplied by the environment), oligodynamic (acting at doses in the micromole range), diffusible and vectors of a specific physiological process. Unlike animals, they are not formed in glands, each cell being potentially capable of producing them. PGRs affect seed growth, growth and senescence of stems, leaves, flowers, tissue growth orientation, flowering time, flower sex, fruit development and maturation, plant longevity and death, etc. The study of the influence of PGRs is an inevitable step for the establishment of optimal conditions for the successful secondary somatic embryogenesis of somatic embryos.

Currently, somatic embryogenesis is the basis of clonal forestry. Overall, with the aim of conserving and regenerating the Moroccan cork oak forest heritage, and improving the protection of the Maâmora forest, this study concerns primary somatic embryos of tree plus from Maâmora forest, and several PGRs were tested on the quality of induction of secondary embryogenesis: ABA, $GA_3$, BAP, Kinetin, Zeatin, 2iP, TDZ and DPU. These phytohormones were added separately to the culture medium at oligodynamic doses, and their effect was evaluated in terms of quantity and quality of expressed secondary embryos.

## Material and methods

### Plant material and culture medium

Embryogenic lines used in these experiences were obtained between 2016 and 2017 from explants of shoots resulting from adventitious shoots developed on branches segments. The starting explants have been isolated. They represent mature somatic embryos 8–10 mm length at the cotyledonary stage (Figure 1). They were taken from dicotyledonary embryogenic cultures, obtained from leaf cuttings from an elite tree located in the region of Maâmora (Morocco: GPS: N: 34°03'029, W: 006°38'207). The somatic embryos were kept during 1 year by recurrent embryogenesis on a series of subculture in a medium without growth regulators according to the protocols described by Ben Ali and Lamarti (2014), Toribio et al. (2005).

### Influence of PGRs on secondary somatic embryogenesis

Somatic embryos were placed into plastic Petri dishes (two embryos per Petri dishes) containing 40 ml of culture medium containing N30K macronutrients, MS (Murashige and Skoog 1962) micronutrients and vitamins, 0.7% agar (bacteriological agar type E) and 100 mg/l myo-inositol. Embryogenic lines were established from somatic embryos derived from cork oak leaves. Induction and expression of secondary somatic embryogenesis was achieved by maintaining cultures on proliferation medium supplemented with different concentrations of each PGR mentioned in each experiment (Tables 1–4).

In all experiments, pH was adjusted to 5.8 before autoclaving at 120 °C for 20 min. Primary embryos were placed in a proliferation medium consisting of N30K macroelements (Margara 1978), microelements (Murashige and Skoog 1962), Fe-EDTA and MS vitamins, 30 g/l glucose, 0.7% agar (bacteriological agar type E) and 100 mg/l meso-inositol. This medium without the addition of PGRs constituted the control medium. The tests were performed in sterile plastic Petri dishes of 90 mm diameter filled with 20 ml of culture medium closed with Parafilm®. For each treatment, at least 30 explants were used, and all experiments were repeated three times. Each culture period lasted eight weeks. Incubation took place at 25 ± 2 °C in the dark.

### Statistical analysis

Thirty explants of primary somatic embryos were cultured per experimental unit in each treatment. All the somatic embryos were homogeneously distributed between different treatments, and experiments were repeated three times. After 8 weeks culture, the recorded data included percentage of explants with secondary embryos, number of secondary somatic embryos directly formed on the primary embryos, number of viable clusters, and somatic embryos

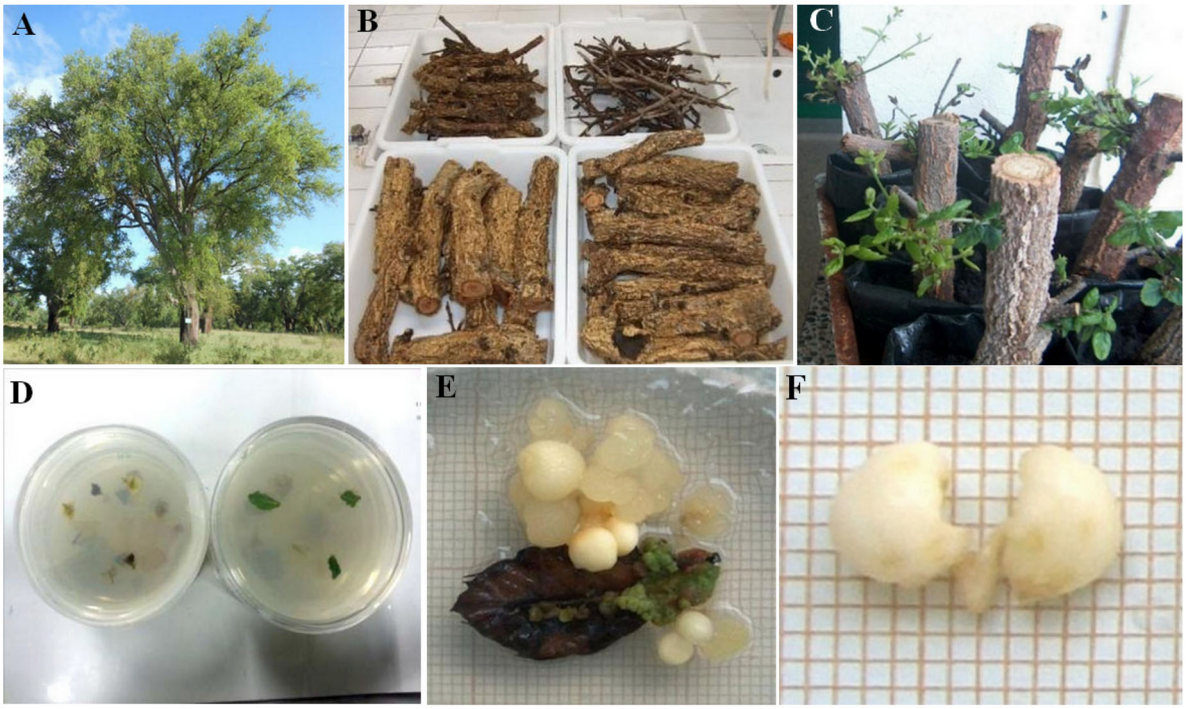


**Figure 1.** Budding of cuttings and realizatzion of primary somatic embryogenesis. (A) Tree used to obtain the first cuttings of *Q. suber*, located in Maâmora, Morocco (N: 34°03'029, W: 006°38'207); (B) Sterilization and classification of cuttings by diameter; (C) Disbudding of cuttings and formation of epicormic shoots; (D) Cultivation of young cork oak leaves; (E) Formation of primary somatic embryos; (F) Primary somatic embryo;.

**Table 1.** Effect of BAP on secondary somatic embryogenesis of cork oak after eight weeks of dark culture.

| BAP (mg/l) | Average number of secondary embryos | Average number of clusters | Average number of embryo clusters |
|---|---|---|---|
| **Control medium** | 4.04 ± 0.46 ab | 0.55 ± 0.11 abc | 1.44 ± 0.29 abc |
| **0.1** | 1.70 ± 0.30 bcd | 0.18 ± 0.07 d | 0.18 ± 0.07 c |
| **0.3** | 2.42 ± 0.40 bcd | 0.63 ± 0.11 abc | 1.71 ± 0.37 abc |
| **0.5** | 1.88 ± 0.30 bcd | 0.99 ± 0.21 a | 0.63 ± 0.10 bc |
| **0.7** | 1.98 ± 0.41 bcd | 0.27 ± 0.08 bc | 1.17 ± 0.36 abc |
| **1** | 2.34 ± 0.32 bcd | 0.18 ± 0.07 d | 0.36 ± 0.15 c |
| **1.5** | 2.16 ± 0.28 bcd | 0.36 ± 0.09 abc | 0.9 ± 0.23 bc |
| **2** | 2.60 ± 0.37 bcd | 0.18 ± 0.07 d | 0.54 ± 0.20 c |
| **4** | 1.44 ± 0.25 bcd | 0.36 ± 0.09 abc | 0.99 ± 0.29 bc |

**Table 2.** Effect of kinetin on secondary somatic embryogenesis of cork oak after eight weeks of dark culture.

| Kinetin (mg/l) | Average number of secondary embryos | Average number of clusters | Average number of embryo clusters |
|---|---|---|---|
| **Control medium** | 4.04 ± 0.46 ab | 0.55 ± 0.11 abc | 1.44 ± 0.29 abc |
| **0.1** | 2.20 ± 0.42 bcd | 0.99 ± 0.21 a | 2.70 ± 0.59 a |
| **0.3** | 3.14 ± 0.52 abcd | 0.72 ± 0.11 abc | 2.34 ± 0.38 ab |
| **0.5** | 3.50 ± 0.42 abc | 0.54 ± 0.10 abc | 1.35 ± 0.29 abc |
| **0.7** | 3.32 ± 0.40 abc | 0.63 ± 0.10 abc | 1.44 ± 0.27 abc |
| **1** | 2.34 ± 0.32 bcd | 0.27 ± 0.08 abc | 0.81 ± 0.15 bc |
| **1.5** | 3.06 ± 0.37 abcd | 0.45 ± 0.10 abc | 1.53 ± 0.36 abc |
| **2** | 3.6 ± 0.40 cd | 0.45 ± 0.10 abc | 1.26 ± 0.29 abc |
| **4** | 2.34 ± 0.49 bcd | 0.54 ± 0.13 abc | 1.08 ± 0.23 abc |

**Table 3.** Effect of zeatin on secondary somatic embryogenesis of cork oak after eight weeks of dark culture.

| Zeatine (mg/l) | Average number of secondary embryos | Average number of clusters | Average number of embryo clusters |
|---|---|---|---|
| **Control medium** | 4.04 ± 0.46 ab | 0.55 ± 0.11 abc | 1.44 ± 0.29 abc |
| **0.1** | 1.88 ± 0.43 bcd | 0.90 ± 0.21 ab | 0.90 ± 0.19 bc |
| **0.3** | 3.60 ± 0.40 abc | 0.45 ± 0.09 abc | 0.99 ± 0.23 bc |
| **0.5** | 3.96 ± 0.40 ab | 0.36 ± 0.10 abc | 0.90 ± 0.21 bc |
| **0.7** | 1.71 ± 0.31 bcd | 0.36 ± 0.10 abc | 0.72 ± 0.17 bc |
| **1** | 2.25 ± 0.30 bcd | 0.36 ± 0.10 abc | 0.81 ± 0.21 bc |
| **1.5** | 1.98 ± 0.37 bcd | 0.54 ± 0.10 abc | 1.53 ± 0.34 abc |
| **2** | 2.16 ± 0.37 bcd | 0.45 ± 0.09 abc | 0.99 ± 0.22 bc |
| **4** | 0.90 ± 0.25 d | 0.36 ± 0.09 abc | 0.63 ± 0.18 bc |

**Table 4.** Effect of 2iP on secondary somatic embryogenesis of cork oak after eight weeks of dark culture.

| 2iP (mg/l) | Average number of secondary embryos | Average number of clusters | Average number of embryo clusters |
|---|---|---|---|
| **Control medium** | 4.04 ± 0.46 ab | 0.55 ± 0.11 abc | 1.44 ± 0.29 abc |
| **0.1** | 5.22 ± 0.52 a | 0.63 ± 0.10 abc | 1.17 ± 0.22 abc |
| **0.3** | 3.50 ± 0.48 abc | 0.36 ± 0.09 abc | 0.90 ± 0.26 bc |
| **0.5** | 3.32 ± 0.43 bcd | 0.27 ± 0.08 bc | 0.36 ± 0.14 c |
| **0.7** | 2.60 ± 0.39 bcd | 0.54 ± 0.10 abc | 1.53 ± 0.33 abc |
| **1** | 2.60 ± 0.32 bcd | 0.09 ± 0.05 d | 0.18 ± 0.10 c |
| **1.5** | 2.06 ± 0.24 abc | 0.45 ± 0.09 abc | 0.99 ± 0.23 bc |
| **2** | 1.98 ± 0.23 bcd | 0.36 ± 0.09 abc | 1.08 ± 0.28 abc |
| **4** | 1.26 ± 0.37 cd | 0.27 ± 0.08 bc | 0.63 ± 0.19 bc |

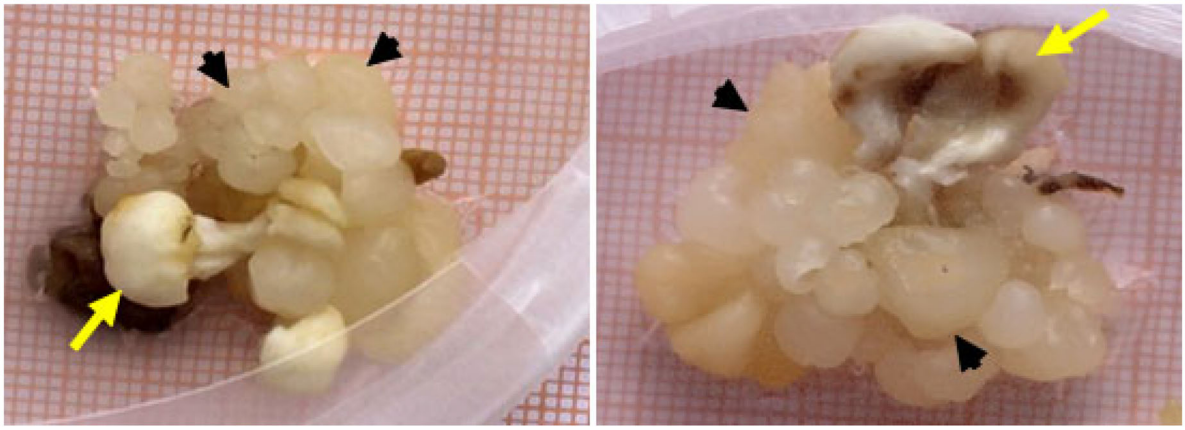

**Figure 2.** Formation of new secondary embryos (arrowheads) at the base of primary embryos (arrows).

formed on clusters per explants (primary somatic embryo). The data were uploaded to statistical software SPSS 17.0 (2019). One-way analysis of variance (ANOVA) was carried out to determine differences between the treatments that produced cotyledonary somatic embryos. Multiple comparisons were made using Duncan's post-hoc test ($p \leq 0.05$).

## Results and discussion

### *Effect of cytokinins*

The cytokinins concerned by this study are 6-benzylaminopurine (BAP), kinetin, zeatin and 2-isopentenyladenine (2iP), from 0.1 to 4 mg/l. The results obtained are represented in Tables 1–4.

In general, cytokinins were unfavorable for the induction of secondary somatic embryogenesis (SSEs), except for 2iP at 0.1 mg/l. The number of new secondary embryos formed at the base of primary embryos during the multiplication phase is influenced by cytokinins (Figure 2). At 0.1 mg/l, 2iP gives the best response that exceeds that of the control medium, which means that at this concentration, 2iP could stimulate the formation of new secondary embryos. The average number of secondary embryos, clusters and embryo clusters are respectively 5.22 ± 0.52, 0.63 ± 0.10 and 1.17 ± 0.22 (Table 4). Zeatin at 0.5 mg/l shows a response close to that of the control medium (Table 3).

The number of secondary embryos, clusters and embryo clusters are 3.96 ± 0.40, 0.36 ± 0.10 and 0.9 ± 0.21, respectively. The lowest response was recorded for BAP, where the average number of secondary embryos formed varies around 2.6 per primary embryo. However, in the case of BAP and kinetin, a very significant increase in the number of clusters and embryo clusters is noted. In the case of kinetin, the increase up to 80% at 0.3 mg/l. The comparison between our results and what the literature reveals cannot provide a satisfactory explanation of the effect of cytokinins on secondary somatic embryogenesis. The embryogenic response to PGRs in general and cytokinins in particular, depends strongly on the plant species, their genotypes and sometimes on other factors, such as the protocol adopted and the culture conditions.

One of the most influential factors in the embryogenic response associated with somatic embryo culture is the type and concentration of growth regulators (Jiménez 2005). Our results show that cytokinins can have a favorable embryogenic response only at low concentrations as seen in the case of 2iP. In other works, cytokinins have been used to stimulate somatic embryogenesis in several woody species, such as *Abies numidica*, *Prunus avium*, *Coffea arabica* and *Mangifera indica* (Fujimura and Komamine 1975; Sagare et al. 2000; Fernández-Da Silva and Menéndez-Yuffá 2003; Vooková et al. 2003; Gutièrrez Pesce and Rugini 2004; Xiao et al. 2004; Jiménez 2005). But all these studies mention that the cytokinins give a positive effect at low concentrations, which is confirmed in the case of 2iP at 0.1 mg/l. Takeno et al. (1983) reported that somatic embryo development can be regulated by cytokinins in several species such as *Vitis vinifera* and *V. rupestris*. However, in some cases, cytokinins can also inhibit embryos formation as in *Daucus carota* (Fujimura and Komamine 1975). Some authors also

Table 5. Influence of TDZ on the induction of secondary somatic embryogenesis in cork oak after eight weeks of *in vitro* culture.

| TDZ (mg/l) | Average number of secondary embryos | Average number of clusters | Average number of embryo clusters |
|---|---|---|---|
| **Control medium** | 3.89 ± 0.45 a | 0.50 ± 0.09 a | 1.58 ± 0.32 b |
| **0.2** | 0.07 ± 0.04 b | 0.07 ± 0.04 b | 0.14 ± 0.08 b |
| **0.4** | 0.21 ± 0.09 b | 0.07 ± 0.04 b | 0.14 ± 0.08 b |
| **0.6** | 0.36 ± 0.11 b | 0.00 ± 0.00 b | 0.00 ± 0.00 b |
| **0.8** | 0.43 ± 0.12 b | 0.00 ± 0.00 b | 0.00 ± 0.00 b |
| **1** | 0.50 ± 0.15 b | 0.14 ± 0.05 b | 0.28 ± 0.09 b |
| **1.5** | 0.28 ± 0.07 b | 0.00 ± 0.00 b | 0.00 ± 0.00 b |
| **2** | 0.36 ± 0.08 b | 0.07 ± 0.04 b | 0.14 ± 0.08 b |
| **5** | 1.08 ± 0.23 b | 0.21 ± 0.06 b | 0.28 ± 0.09 b |
| **10** | 1.22 ± 0.29 b | 0.23 ± 0.06 b | 0.50 ± 0.16 b |

Table 6. Influence of DPU on the induction of secondary somatic embryogenesis of cork oak after eight weeks of *in vitro* culture.

| DPU (mg/l) | Average number of secondary embryos | Average number of clusters | Average number of embryo clusters |
|---|---|---|---|
| **Control medium** | 3.89 ± 0.45 a | 0.50 ± 0.09 | 1.58 ± 0.32 ab |
| **0.2** | 2.43 ± 0.53 a | 0.27 ± 0.08 bc | 0.54 ± 0.18 b |
| **0.4** | 3.15 ± 0.63 a | 0.18 ± 0.07 bc | 0.36 ± 0.15 b |
| **0.6** | 2.07 ± 0.28 a | 0.45 ± 0.12 bc | 0.9 ± 0.25 b |
| **0.8** | 4.50 ± 0.66 a | 0.72 ± 0.18 ab | 1.17 ± 0.26 b |
| **1** | 3.42 ± 0.51 a | 0.27 ± 0.08 bc | 0.99 ± 0.30 b |
| **1.5** | 2.61 ± 0.38 a | 0.18 ± 0.07 bc | 0.54 ± 0.20 b |
| **2** | 3.60 ± 0.43 a | 0.09 ± 0.05 c | 0.18 ± 0.10 b |
| **5** | 4.59 ± 0.63 a | 1.08 ± 0.11 a | 2.52 ± 0.30 a |
| **10** | 2.79 ± 0.29 a | 0.45 ± 0.1 bc | 0.81 ± 0.20 b |

indicate that somatic embryogenesis can be effectively induced without growth regulators.

### Effect of TDZ and DPU

The tests conducted are designed to evaluate the primary reactive embryos potential of cork oak from somatic embryos regenerated in the presence of two phenylurea derivatives, diphenylurea (DPU) and thidiazuron (TDZ). They were tested separately with a range of concentrations from 0.2 to 10 mg/l. The results obtained after eight weeks are shown in Tables 5 and 6.

The first successful somatic embryos obtained after 15 days of culture. Two months later, the starting explants generate secondary embryos, clusters and embryo clusters offering the potential for further regeneration. Regarding the control medium, and where explants incubated in the absence of any phenylurea derivative, the rate of embryo multiplication by secondary somatic embryogenesis is higher compared to the media containing different concentrations of TDZ and DPU (Tables 5 and 6).

In the case of the DPU, Table 6 sometimes shows a slight increases in the number of secondary embryos, clusters and embryo clusters. The highest level was obtained at 5 mg/l DPU. At this concentration, the averages recorded for secondary embryos, cluster and embryo clusters are 4.59 ± 0.63, 1.08 ± 0.11 and 2.52 ± 0.30, respectively. Treatment with TDZ inhibits the whole process of secondary embryogenesis because the rates of embryo formation, clusters and embryo clusters are almost zero. The embryogenic potential of both TDZ and DPU remains lower than the embryogenic response of the previously tested cytokinins. However, according to Fiore et al. (2002), diphenylurea derivatives were able to positively induce somatic embryogenesis. For instance, 4-CPPU induced somatic embryogenesis of *Citrus* floral explants, and TDZ promoted somatic embryogenesis of rice caryopses and was used for regeneration of the best shoots, and the best results were obtained only with BAP (Gairi and Rashid 2004).

In our case, TDZ significantly inhibited the formation of secondary somatic embryos. In *Digitalis trojana*, TDZ at 1 mg/l was more effective than BAP, zeatin and kinetin in somatic embryos production (Verma et al. 2012). In *Cajanus cajan*, the formation of primary somatic embryos in the nodal parts of cotyledons was enhanced by the addition of 10–20 mg/l TDZ (Singh et al. 2003). Similar results were obtained in *Azadirachta* with 0.2 mg TDZ (Akula et al. 2003). Thus, it appears that the comparison between our results and what the literature reveals is difficult. Works concerning secondary somatic embryogenesis are rare. Similarly, the results cited in the literature concern in most cases the process of primary somatic embryogenesis, which may partially justify the differences with our results.

### Effect of abscisic acid

ABA was tested at concentrations between 0.1 and 4 mg/l (Table 7).

The variance analysis reveals that multiplication of primary somatic embryos was not significantly affected by ABA. A minimally significant optimal concentration was obtained at 0.5 mg/l. At this concentration the percentage of secondary embryos begins to decrease for higher concentrations (1–4 mg/l) (Table 7). In the presence of ABA, mature somatic embryos show inhibitory effects of cluster formation and embryo clusters. The best result was found using 0.5 mg/l of ABA, with 4.87 ± 0.5 secondary embryos, which represents an increase of 10%, but not significant compared to the control. Thus, the addition of ABA could be slightly beneficial at low concentrations, between 0.3 and 0.5 mg/l.

**Table 7.** Effect of ABA on secondary somatic embryogenesis of cork oak: N30K macroelements as culture medium, microelements (MS) 3% glucose and solidified with 0.7% agar.

| ABA (mg/l) | Average number of secondary embryos | Average number of clusters | Average number of embryo clusters |
|---|---|---|---|
| **Control medium** | 4.43 ± 0.49 a | 0.60 ± 0.09 a | 1.73 ± 0.36 a |
| **0.1** | 4.40 ± 0.61 a | 0.26 ± 0.08 bc | 1.06 ± 0.38 ab |
| **0.3** | 4.73 ± 0.54 a | 0.43 ± 0.09 abc | 1.33 ± 0.33 a |
| **0.5** | 4.87 ± 0.53 a | 0.50 ± 0.09 ab | 1.56 ± 0.32 a |
| **0.7** | 3.37 ± 0.65 ab | 0.47 ± 0.11 abc | 1.10 ± 0.29 ab |
| **1** | 2.53 ± 0.51 bc | 0.43 ± 0.10 abc | 1.00 ± 0.29 ab |
| **1.5** | 2.60 ± 0.57 bc | 0.40 ± 0.09 abc | 0.93 ± 0.25 ab |
| **2** | 2.10 ± 0.42 bc | 0.26 ± 0.11 bc | 0.86 ± 0.27 ab |
| **4** | 1.47 ± 0.35 c | 0.16 ± 0.07 c | 0.26 ± 0.69 b |

**Table 8.** Effect of ABA on the induction quality of secondary cork oak embryos.

| ABA (mg/l) | Number of reactive primary embryos | Reactive primary embryos (%) | Average size of secondary somatic embryos (mm) |
|---|---|---|---|
| **Control medium** | 28 | 93.3 | 6.62 |
| **0.1** | 20 | 66.6 | 5.82 |
| **0.3** | 26 | 86.6 | 6.55 |
| **0.5** | 27 | 90.0 | 6.09 |
| **0.7** | 20 | 66.6 | 5.70 |
| **1** | 20 | 66.6 | 5.65 |
| **1.5** | 19 | 63.3 | 5.40 |
| **2** | 19 | 63.3 | 3.86 |
| **4** | 18 | 60.0 | 3.78 |

Regarding the quality of the embryos, the average size of the secondary somatic embryos were examined (Table 8):

The number of reactive embryos (primary embryos showing secondary somatic embryos at the base of their cotyledons), decreases upon addition of 0.1 mg/l ABA, from 28 to 20 reactive embryos. However, between 0.3 and 0.5 mg/l, the results are slightly higher than those of the control medium.

The average size of secondary embryos generated at the base of primary embryos is less than 6.55 mm (6.62 mm for the control medium). This size is the length of the embryo from the root base to the top of the two cotyledonary part. It tends to decrease with high ABA concentrations. At 0.5 mg/l, the size of the secondary embryos remained close to that of the control embryos. At high doses, ABA appears to be an important inhibitor of secondary embryogenesis.

Generally, ABA can be considered as an agent that stimulates the maturation of somatic embryos in the primary embryogenesis phase (Kim and Moon 2007; Vahdati et al. 2008). ABA also plays a fundamental role in the regulation of the reserve protein synthesis and late embryogenesis proteins, also participates in the reduction of secondary embryogenesis and in the inhibition of early germination (Kernode 1995). It promotes the accumulation of storage material, i.e. lipids, protein bodies, and starch grains in somatic embryos. ABA is also known to promote the synthesis of a number of proteins in developing embryos (Skriver and Mundy 1990), including storage proteins (Sghaier-Hammami et al. 2010). At moderate concentrations, ABA permits normal embryonic growth and maturation, preventing the initiation and growth of new embryonic centers, aberrant forms and early germination of immature seeds (García-Martín et al. 2005). Thus, our results seem to be in agreement with the results already stated by other authors. In oaks, it has been shown that the addition of ABA to culture media significantly and undesirably reduces recurrent embryogenesis in *Q. ilex* (Mauri and Manzanera 2004) and significantly stimulates the maturation of somatic embryos in *Q. suber* (García-Martín et al. 2005). On the other hand, treatments in cork oak (Toribio et al. 2005) and pedunculate oak did not show significant effects on seedling germination and conversion.

### *Effect of gibberellic acid*

The effect of the phytohormone gibberellic acid ($GA_3$) was tested on the induction of secondary embryogenesis. Although a positive effect on secondary somatic embryogenesis seems unlikely, but these tests will also allow to test a possible effect on embryo germination. For this purpose, we added to the culture medium (N30K) a series of concentrations of $GA_3$ ranging from 0.3 to 4 mg/l (Table 9).

Analysis of these results shows that $GA_3$ has no significant effect on the induction of secondary embryogenesis. The maximum response is obtained at 1.5 mg/l. The average number of secondary embryos directly formed is 4.53 ± 0.66. In the absence of $GA_3$, the average recorded is 4.43 ± 0.49. This increase is not significant. For the other concentrations, a decrease in the number of secondary somatic embryos formed was observed.

From the literature, the situation is far from clear for $GA_3$, as the effect of exogenously applied $GA_3$ on somatic embryogenesis is highly variable between species or tissues (Jiménez 2005). Thus, $GA_3$ inhibits somatic embryogenesis of *Daucus carota* (Fujimura and Komamine 1975; Tokuji and Kuriyama 2003), *Citrus sinensis* (Kochba et al. 1978), and *Pelargonium* x *hortorum* (Hutchinson et al. 1997). In our case, $GA_3$ caused a decrease in the embryogenic response. However, in a few examples, exogenous $GA_3$ stimulates embryogenesis, such

**Table 9.** Effect of $GA_3$ on secondary somatic embryogenesis of cork oak.

| $GA_3$ (mg/l) | Average number of secondary embryos | Average number of clusters | Average number of embryo clusters |
|---|---|---|---|
| **Control medium** | 4.43 ± 0.49 ab | 0.60 ± 0.09 a | 1.73 ± 0.36 a |
| **0.1** | 3.43 ± 0.60 abc | 0.36 ± 0.08 ab | 1.20 ± 0.31 ab |
| **0.3** | 3.70 ± 0.57 abc | 0.37 ± 0.08 ab | 1.13 ± 0.29 a |
| **0.5** | 2.50 ± 0.40 c | 0.36 ± 0.08 ab | 0.86 ± 0.24 a |
| **0.7** | 2.73 ± 0.41 bc | 0.20 ± 0.07 b | 0.36 ± 0.15 ab |
| **1** | 2.03 ± 0.42 c | 0.20 ± 0.07 b | 0.50 ± 0.19 ab |
| **1.5** | 4.53 ± 0.66 a | 0.50 ± 0.10 a | 1.93 ± 0.49 ab |
| **2** | 3.40 ± 0.80 c | 0.16 ± 0.07 a | 1.80 ± 0.42 ab |
| 4 | 2.10 ± 0.43 c | 0.16 ± 0.07 b | 0.26 ± 0.12 b |

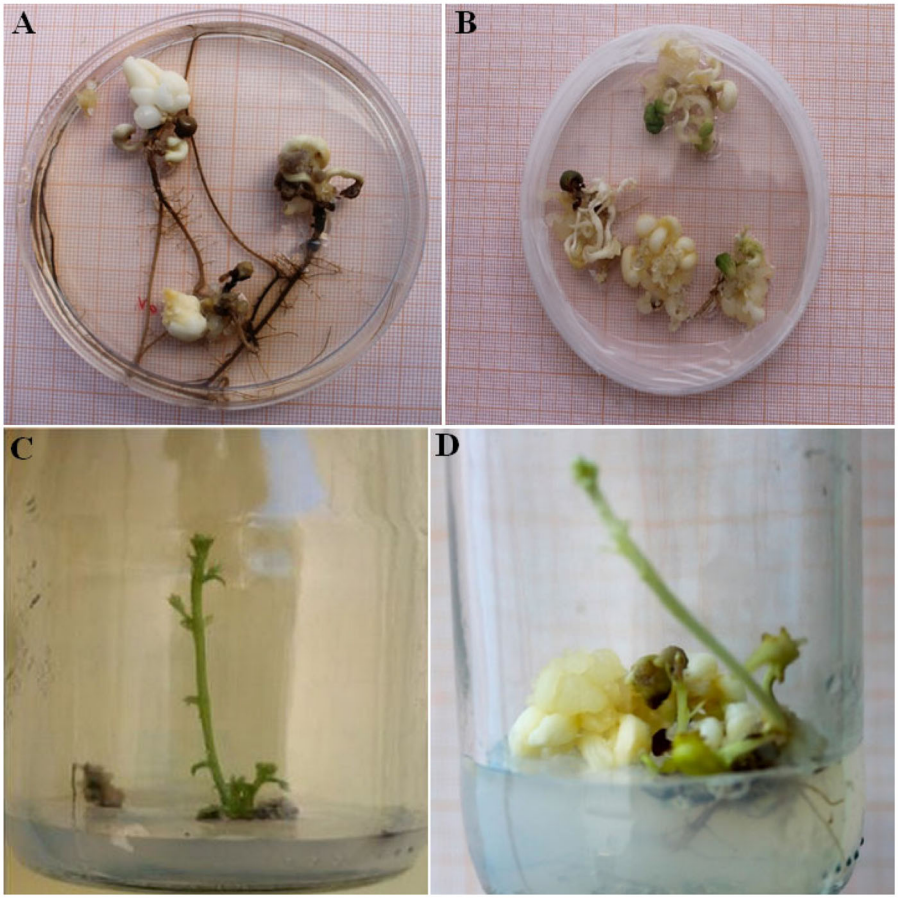


**Figure 3.** (A, B) Beginning of the germination phase in the presence of $GA_3$ after 60 days in the dark. The appearance of roots at the basal part of the embryos having reached the cotyledonary phase is noticed; (C, D) Conversion of a secondary somatic embryo of cork oak to a seedling in the presence of $GA_3$.

as in immature cotyledon cultures of *Cicer arietinum* (Hita et al. 1997) or petiole-derived tissue of *Medicago sativa* (Ruduś et al. 2002). It was also observed that the combination of L-glutamic acid and $GA_3$ in *Hardwickia binata* cultures greatly enhanced the frequency of normal embryonic differentiation (Das et al. 1995). The combination of BAP and $GA_3$ also accelerated primary regeneration of somatic embryos in *Cynodon dactylon* (Li and Qu 2002). Similar results were obtained in *Quercus alba* (Corredoira et al. 2012). Instead, we observed that induction of secondary somatic embryogenesis (SSEs) in culture medium containing $GA_3$ could promote germination of cotyledonary embryos without the use of a particular protocol. This spontaneous germination begins with the appearance of roots on the basal part of embryos that have reached the cotyledonary phase (Figure 3(A,B)).

However, only a portion of the embryos that initiated the germination phase was able to reach the conversion phase characterized by the appearance of a stem (Figure 3(C,D)). Table 10 shows the percentage of primary reactionary embryos, the percentage of embryos showing roots at their basal part, as well as the average size of the SSE. The percentage of reactive embryos is calculated from the total number launched at the beginning of the experiment (30 mature primary embryos, for each concentration of $GA_3$).

The germination rate depends significantly on the concentration of $GA_3$. The highest germination rate (36.3%) is recorded at 0.5 mg/l. From this value onwards, germination decreases as the $GA_3$ concentration increases.

These results show that $GA_3$ did not inhibit the SSEs process, but there was a decrease in the number of reactive primary somatic embryos compared to the

**Table 10.** Effect of $GA_3$ on the germination of cork oak embryos.

| $GA_3$ (mg/l) | Reactive primary embryos (%) | Rate of germination (%) (embryos showing rooting) | Average size of secondary somatic embryos (mm) |
|---|---|---|---|
| **Control medium** | 93.3 | 8.85 | 6.62 |
| **0.1** | 70.0 | 23.8 | 4.50 |
| **0.3** | 80.0 | 26.6 | 5.23 |
| **0.5** | 73.3 | 36.3 | 4.74 |
| **0.7** | 76.6 | 8.69 | 4.90 |
| **1** | 63.6 | 15.78 | 4.80 |
| **1.5** | 76.6 | 17.39 | 4.57 |
| **2** | 60.0 | 22.20 | 3.31 |
| **4** | 76.6 | 8.69 | 3,81 |

**Table 11.** Germination and seedling conversion rates of cork oak somatic embryos after 60 weeks in the dark in the presence of $GA_3$.

| $GA_3$ (mg/l) | Rate of germination (%) (embryos showing rooting) | Rate of de conversion (%) (embryos showing stems and roots) | Average stem size (mm) |
|---|---|---|---|
| **Control medium** | 8.85 | 0 | 0 |
| **0.1** | 23.8 | 14.28 | 33.0 |
| **0.3** | 26.6 | 0 | 0 |
| **0.5** | 36.3 | 9.9 | 37.5 |
| **0.7** | 8.69 | 0 | 0 |
| **1** | 15.78 | 0 | 0 |
| **1.5** | 17.39 | 0 | 0 |
| **2** | 22.2 | 5.55 | 12.0 |
| **4** | 8.69 | 4.34 | 39.0 |

control medium (93% in the absence of $GA_3$ and 75% in the presence of $GA_3$). The size of the embryos decreases at high concentrations of $GA_3$. It decreases from 6.62 mm in the absence of $GA_3$ to less than 4 mm at over 2 mg/l. $GA_3$ seems to partially block the secondary multiplication process in order to promote the germination process.

The second germination phase characterized by the appearance of stems was also observed with $GA_3$. Indeed, after eight weeks of *in vitro* culture, we observed that secondary embryos tending to transform into young plantlets with stems and roots. However, the conversion rate recorded (Table 11) is lower than that of the rooting phase. The highest rate was observed at 1 mg/l $GA_3$ (14.28%). Based on these results, it appears that there is no direct correlation between rooting and conversion rates. In the literature, several works mention the beneficial role of $GA_3$ in the process of germination and conversion to seedlings. Gaj (2004) and Jiménez (2005) showed that $GA_3$ stimulates both germination and conversion in *Arabidopsis thaliana*. In contrast, in *Cedrela odorata*, no conversion to seedling was observed in the presence of $GA_3$ (Peña-Ramírez et al. 2011).

Our results are in agreement with those obtained in *Quercus alba* (Corredoira et al. 2019) where the percentage of germination (embryos showing only roots) was 30% with the combination of 0.1 mg/l $GA_3$ and 0.05 mg/l ABA. In grapevine (*Vitis vinifera* x *V. rupestris*), the same germination protocol was adopted, the only difference being the treatment of the embryos, which underwent cold desiccation at 4 °C, which allowed a germination rate of these embryos exceeding 90% (Bueno et al. 1992).

In other studies, the size of *Persea americana* stems is between 5 and 10 mm (Litz and Litz 1999). In our case, the average stem size up to 37.5 mm at 0.5 mg/l (Table 11). In *Quercus rubra*, the germination rate exceeds 60% when combining 0.1 mg/l BAP and 0.1 mg/l $GA_3$ (Vengadesan and Pijut 2009). In contrast, the conversion rate was only 0.01% in *Persea americana* (Palomo-Ríos et al. 2012).

In the light of the results found in the literature and those obtained in this work, it can be seen that the reaction of somatic embryos to $GA_3$ depends on the concentration and combination of this phytohormone with other compounds, aimed primarily at enhancing the germination process, and on the other hand, on the cell tissue or genotype of the explant tested; however, this leaves open the question of the adequate dose of $GA_3$. Furthermore, we carried out tests concerning the acclimatization in these young seedlings, by adopting the experimental Protocol that we performed for the Moroccan genotype. The results show a great ability of resistance in these young seedlings to the natural climatic conditions, as shown in Figure 4. The next step of this work aims to transfer these young seedlings *in vivo* to the Moroccan Maâmora's forest, and accomplish a large greenhouse in order to regenerate and restore the Moroccan cork oak.

The objective of our work is part of a more general framework, which consists in developing a SSEs Protocol specific to cork oak. The present study was an unavoidable step to complete the protocol (among other studies already carried out: effect of the culture medium, carbon sources, amino acids, etc.). However, even if the results of the present study did not show significant effect of PGRs, it is considered as a precious information, since it lead to conclude that the secondary somatic embryogenesis process can be performed without PGRs. On the other hand, we demonstrate

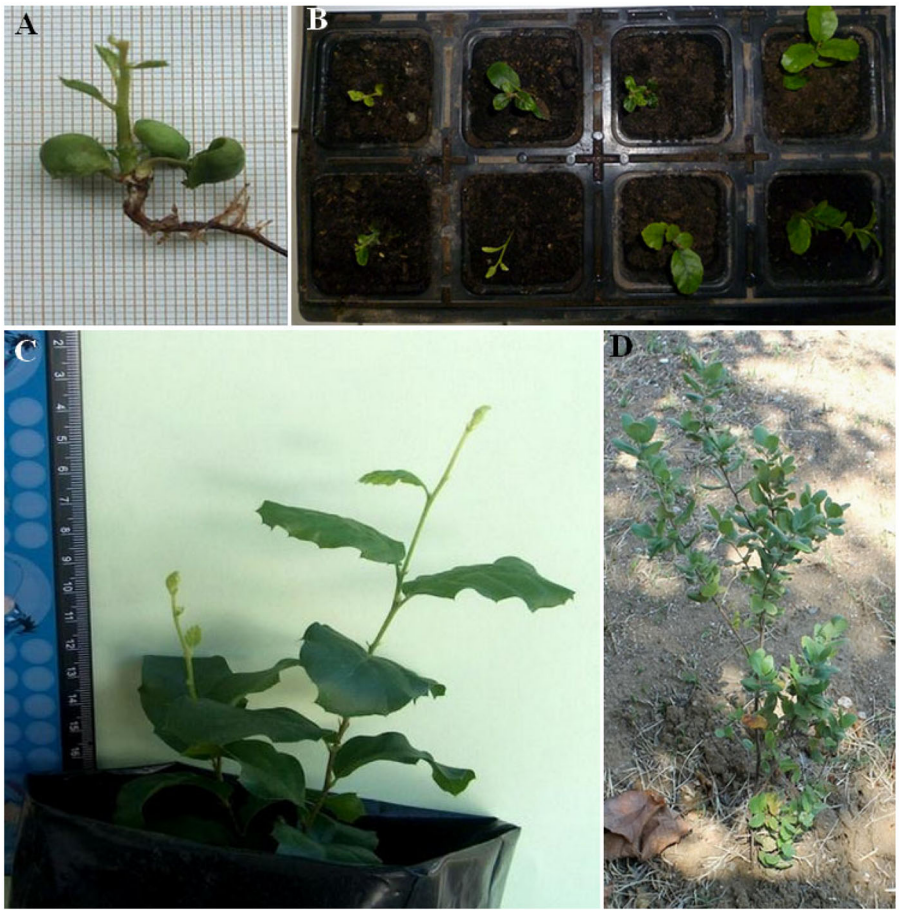


**Figure 4.** Acclimatization of young cork oak seedlings. (A) Development of a healthy plantlet after 8 weeks of culture prior to transferring to the acclimatization phase; (B) Transfer under greenhouse conditions (1000 lux, 16 h of light); (C) Ex vitro acclimatized plantlets after 8 weeks of transplanting; (D) Ex vitro acclimatized plantlets of transplanting; suitable for field planting.

that the presence of $GA_3$ can be used efficiently to enhance the germination process. These outcomes can be used to complete the SSEs Protocol already initiated, where the different constituents of the culture medium: micronutrients, macronutrients, the carbon sources, amino acids, and PGRs are specified properly.

## Conclusion

Somatic embryogenesis in cork oak is a technique for large-scale micropropagation from quality seedlings. It is a powerful tool to maintain the genetic stability of regenerated plants. Our work was devoted to the study of the process of primary and secondary somatic embryogenesis of Moroccan cork oak. The objective was to explore the embryogenic potential through the secondary process of somatic embryogenesis of cork oak. This process is considered as an efficient means for the massive multiplication and maintenance of somatic embryos, in view of its application in the micropropagation of Moroccan cork oak. Cytokinins showed a negative effect on the induction of secondary somatic embryogenesis. At the different concentrations tested, they significantly reduced the number of secondary somatic embryos, except for 2iP which showed a slight increase at low doses. Abscisic acid (ABA) can be slightly beneficial at low concentrations between 0.3 and 0.5 mg/l. The best result is obtained in the presence of 0.5 mg/l ABA with an average number of secondary embryos of 4.87 per primary embryo, which represents an increase of 10% compared to the control medium. However, we found that the number of reactive embryos, showing secondary somatic embryos at the base of their cotyledons, decreases with the addition of ABA.

Concerning phenylurea derivatives (DPU and TDZ), we found that they do not promote the secondary somatic embryogenesis process. DPU has no significant effect and the response remains close to that of the control medium. TDZ shows an inhibitory effect on the multiplication of secondary somatic embryos. Regarding gibberellic acid ($GA_3$), a slightly higher response than the control medium was observed for a concentration of 1.5 mg/l. $GA_3$ remarkably promotes the germination of cotyledonary embryos without the use of a particular protocol, which led us to focus on this behavior. Thus, the results obtained showed that the germination rate depends significantly on the concentration of $GA_3$. The latter does not inhibit the SSEs process but there is just a decrease in the number of primary somatic reactive embryos compared to the control medium. We attributed this behavior to the fact that $GA_3$ partially blocks the secondary multiplication process in order to favor the germination process.

## Disclosure statement

No potential conflict of interest relevant to this article was reported.

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