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Influence of exogenous polyamines on the secondary somatic embryogenesis of cork oak (*Quercus suber* L.)

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ABSTRACT

Quercus suber L. is the main woody tree species in the Mediterranean basin. The *in vitro* regeneration from adult material, through primary somatic embryogenesis, is a well-known process, but the use of secondary somatic embryos for plant regeneration remains a very sparsely studied process. The main objective of this work is to explore the cork oak regeneration potential by using the secondary somatic embryogenesis process. Mainly, in this work, we report the polyamine effect. Explants used consisted on primary mature embryos, derived from leaves rejuvenated by epicormic shoot of the Moroccan *Quercus suber*. Three different polyamines were added to the basal medium, which was composed by macronutrients of $N_{30}K$, 30 g/l glucose, and 7 g/l agar. Three polyamines, Putrescine, Spermine, and Spermidine, were added to the basal medium at 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/l. Explants were tested after 8 weeks. Morphological analysis showed that the medium with 0.4 mg/l Spermidine provided the best result for secondary embryos, which corresponds to a very significant ($p < 0.05$) increase of 375%. The number of secondary embryos directly formed was 2.70 ± 0.51. Similarly, the optimum concentrations for high number of clusters (0.50 ± 0.11) and embryo clusters (1.43 ± 0.35) were increased by 145% and 158%. The addition of the polyamine also acted on the quality of embryos formed. A very significant ($p < 0.05$) increase in the size of secondary embryos was observed compared to the medium without polyamines. Spermidine showed the greatest increase (about 38%).

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Introduction

Quercus suber L. has a wide geographic distribution in the Mediterranean ecosystem, spreading from the North African Atlantic coast to south-western Europe [1]. Consequently, of the phytosanitary state of oak tree populations, the need for reforestation is required in order to increase throughout the entire Europe and North Africa in the near future. The recent advance strategies and approach of forest tree breeding highlight on the use of vegetative propagation to quickly and successfully capture the possible potentials of selected individuals, which provide us the opportunity to establish them in plantations and performing the commonly named Multi-Varietal Forestry [2].

In the last few decades, *Quercus suber* gained lot of attention because of not only their health value but also for their applications in the biotechnology research area, there has been a steady flow of information on *Quercus* biotechnology, and now it is entering into the genomic era. Furthermore, *Quercus suber* is the most species of oaks in which somatic embryogenesis has reached the most advanced progress [3].

It is of great importance to use somatic embryogenesis to mass propagation and also to the improvement of genetic transformation protocols in forest trees [4]. Nonetheless, there are numerous limitations when somatic embryogenesis is applied: in numerous cases, successful induction only occurs from juvenile tissues, and the quality of the somatic embryos produced and the

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conversion rate of these somatic embryos into plantlets are dependent principally on the genotype of the original explants [5].

Long-term maintenance and scaling up of somatic embryogenesis in different species of *Quercus* depends on the ability of the explants for somatic embryogenesis. It is because of the low frequency of induction of somatic embryogenesis in the original explants [6–9]. In order to maintain embryogenic lines derived from mature and juvenile oak trees, a solid and semi-solid proliferation medium supplemented with low concentrations of auxins and/or cytokinins, has been used [9–12].

For *Q. suber*, numerous studies have been conducted by using somatic embryogenesis and with different modified experimental protocols, but few studies are carried out on the secondary somatic embryogenesis on *Q. suber*, if it presents some fairly large generative capacities in terms of embryo production. At the national scale, we tried to carry out studies on this process to improve the Moroccan genotype [13]. The protocol is based on the use of a range of phytohormones to develop the tissue structures, especially polyamines. As reported in various studies, polyamines are responsible for different plant developmental processes [14–16]. Including embryogenesis, polyamines have been considered as a new category of plant growth regulators and hormonal second messengers of cell differentiation and proliferation in many biological processes [17–21]. Against classical phytohormones, polyamines play a role structural and regulatory, which may explain their high cellular content [22]. These growth regulators include three different types of polyamines which are the most recognized in vitro culture of plant tissue: Spermine, Spermidine, and putrescine. Polyamines are small aliphatic amines with low molecular weight, organic polycations, and ubiquitous in all plant cells [23,24]. All of the three polyamines have been involved in the regulation of cell division and differentiation and in the morphogenesis [25,26]. These polycations have been shown to fluctuate as somatic embryogenesis proceeds, acting in root formation, cell division, floral initiation and development, secondary metabolism, fruit development, senescence, biotic and abiotic stress responses [11,26,27]. In addition, polyamines have been found to regulate the apoptosis [28]. During zygotic embryo development and somatic embryogenesis processes, polyamines have been used as developmental biomarkers [20,21,26,29], while few data describing variations in polyamines during the development of angiosperm seeds are available [24].

Treatments that modify polyamine levels, such as its exogenous application, are interesting ways for involved in many cellular processes, such as morphogenesis. It is generally known that exogenous polyamines can induce cell division and increase regeneration [18]. Various studies have revealed the positive impact of exogenous polyamines to improve in vitro processes, for instance somatic embryogenesis [14,30]. The influence of the polyamines on somatic embryogenesis is evident, while the precise mechanism through which they exert the effect is not yet noticeably clarified [31]. Despite the fact that much studies have been conducted on the effects of polyamines on tissue culture, but none of these studies have investigated their influence on secondary somatic embryogenesis in the cork oak.

In this work, we study the function of exogenous polyamines during the process of secondary somatic embryogenesis. It may provide interesting information about the behavior of embryos formed toward these polyamines. Thus, the aim of this work is to develop a protocol to induce somatic embryogenesis in adult explants from Moroccan cork oak genotype, by using the process of secondary somatic embryogenesis as a starting step for the multiplication and regeneration of somatic embryos. Therefore, the secondary somatic embryos generated can constitute the starting material for the next steps of the technique, such as germination and acclimatization. This will allow us to save time and effort expended during the early stages of somatic embryogenesis.

Materials and methods

Plant material

Experiments were carried out using a highly embryogenic line of *Quercus suber* obtained between 2018 and 2019 from explants of shoots resulting from adventitious shoots developed on branch segments as described by the protocol of

[31]. The starting explants have been isolated. They represent mature somatic embryos 8–10 mm length at the cotyledonary stage. They were taken from dicotyledonary embryogenic cultures, obtained from leaf cuttings from an elite tree located in the region of Maâmora (Morocco: GPS: N: 3403'029, W: 00638'207).

Culture conditions

Cotyledonary embryos were isolated from embryonal masses and placed in 9 cm Petri dishes (three embryos per dish). The primary embryos were maintained during 1 year by recurrent embryogenesis on a series of subculture on a multiplication medium every 4 weeks as described by [31]. The basal medium used was the $N_{30}K$ [32] supplemented with 30 g/l glucose and solidified with 7 g/l agar (Figure 1).

Influence of polyamines

Polyamines involved in this study were Putrescine, Spermine, and Spermidine. The mature somatic embryos were cultured in Petri dishes with 20 ml $N_{30}K$ medium [32], supplemented with vitamins and micronutrients of Murashige and Skoog [33], 100 mg/l myo-inositol and 3% glucose as reported by [13]. The medium was solidified using 7 g/l agar. For each polyamine tested, the concentrations were 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/l. Culture PGR-free medium without adding any polyamine was taken as control medium. Thirty Petri dishes containing three primary embryos, each were cultured for each treatment. After 60 days, the number of secondary embryos, the number of clusters, and the embryo clusters were recorded.

Another experiment was added to this study to mention the effect of culture medium on secondary somatic embryogenesis. The medium of Schenk and Hildebrandt [34] is commonly used in testing the polyamines. In order to confirm the advantage of the culture medium on the proliferation of secondary somatic embryos, a second series of tests was performed on a growth medium containing the SH macronutrients supplemented with the three polyamines, tested from 0.3 to 1 mg/l.

In all the experimental studies, the pH of mediums was adjusted to 5.8 ± 0.1 prior autoclaving. Cultures were carried out in 9 cm Petri dishes containing 20 ml of medium and sealed with

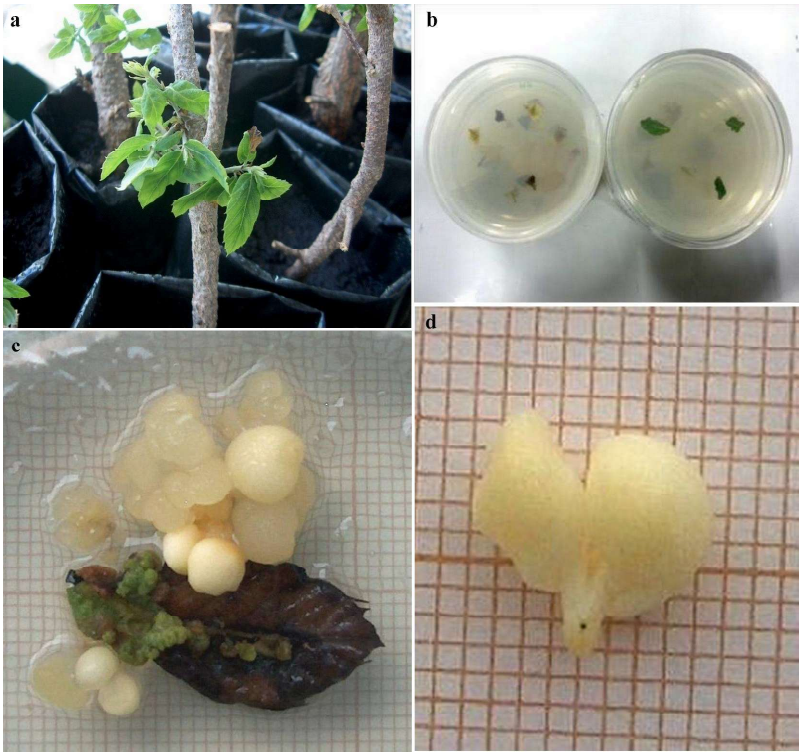

Figure 1. Primary somatic embryogenesis induction and plant material for secondary somatic embryogenesis. (a) Epicormic shoots sprouted in fragments of branches from elite cork oak tree, bearing expanding leaves. Scale bar: 20 mm; (b) Expanding leaves put into cultivation; (c) Induction of somatic embryogenesis in an expanding leaf collected from an epicormic shoot; (d) Matured somatic embryos.

Parafilm. Petri dishes were incubated at 25 ± 1°C in darkness.

Statistical analysis

Data were statistically analyzed using one-way ANOVA with post hoc Duncan test ($p \leq .05$). Each treatment (corresponding to a polyamine type and culture medium) had three replicates; each replicate included 10 Petri dishes containing three explants each. For all the treatments, three replicates were made to confirm the repeatability of the experiment.

All embryogenic responses in terms of number of secondary embryos, clusters, and embryo clusters were subsequently processed using SPSS 24.0 statistical software [35]. A one-way analysis of variance (ANOVA) was performed. Multiple comparisons were made using the post-hoc Duncan test ($p \leq .05$), at the 5% probability level. Values beyond $p \leq .05$ were considered as significant.

Results

The process of secondary somatic embryogenesis is characterized by a very high regeneration capacity of somatic embryos of cork oak, which helps to increase the regeneration performance of this species, while minimizing the cost and time of the somatic embryogenesis technique. Most of the studies that have been carried out to date have shown the capacity for somatic embryogenesis in regeneration of cork oak, but also time restrictions, which limit the application of this technique to a larger scale. To achieve this process and solve these constraints, we have focused our research on the process of secondary somatic embryogenesis, because our studies have shown that secondary somatic cork oak embryos have a remarkable regeneration potential, compared to the primary embryos where the successful induction only occurs directly in the leaves. Several parameters have been studied to test their influence on secondary somatic embryogenesis, including polyamines. Although much studies have been conducted on the effects of polyamines on tissue culture, none of these studies have investigated their influence on secondary somatic embryogenesis in the Moroccan cork oak. The results obtained showed a favorable response of exogenous polyamines to the induction of somatic embryos.

The results of various tests on the three polyamines are shown in Table (1) and Figures (2–4). There is a significant ($p < .05$) increase in the number of secondary embryos formed directly on primary embryos, clusters, and embryo clusters compared to the control medium for each polyamine. It is necessary to mention that the clusters correspond generally to a few structures in the process of somatic embryogenesis and in most cases remain without regenerative potential. But in this work, we have mentioned their formation, in which these clusters could serve as origin to viable somatic embryos. The presence of these embryos indicates that the starting embryos have the capacity of secondary somatic

Table 1. Influence of three polyamines at six concentrations on secondary embryogenesis of Moroccan cork oak. Primary embryos were cultured for 60 days in $N_{30}K$ medium.

Concentration of polyamines (mg/l)		Secondary embryos	Clusters	Embryo clusters
Control media		3.2 ± 0.52^{cd}	0.16 ± 0.07^{e}	0.53 ± 0.23^{c}
Put+Sp+Spd at 0.3 each		5.8 ± 0.54^{abc}	0.47 ± 0.09^{bcde}	1.06 ± 0.27^{c}
Putrescine	0.1	3.08 ± 0.43^{cd}	0.20 ± 0.07^{e}	0.47 ± 0.19^{c}
	0.2	5.12 ± 0.50^{abcd}	0.53 ± 0.10^{abcd}	1.20 ± 0.26^{bc}
	0.3	4.89 ± 0.57^{abcd}	0.43 ± 0.10^{bcde}	1.93 ± 0.37^{ab}
	0.4	6.97 ± 0.59^{ab}	0.60 ± 0.09^{ab}	0.77 ± 0.23^{c}
	0.5	4.96 ± 0.51^{ab}	0.51 ± 0.07^{bcde}	0.71 ± 0.32^{bc}
	0.6	4.78 ± 0.43^{abc}	0.46 ± 0.09^{bc}	0.56 ± 0.12^{bcd}
Spermine	0.1	4.20 ± 0.56^{cd}	0.30 ± 0.08^{cde}	0.60 ± 0.21^{c}
	0.2	5.64 ± 0.63^{abc}	0.30 ± 0.08^{e}	1.10 ± 0.25^{c}
	0.3	5.53 ± 0.41^{abc}	0.56 ± 0.09^{abc}	1.03 ± 0.24^{c}
	0.4	5.28 ± 0.38^{abc}	0.51 ± 0.07^{bcde}	1.10 ± 0.25^{c}
	0.5	4.81 ± 0.43^{ab}	0.46 ± 0.09^{bc}	1.23 ± 0.27^{c}
	0.6	4.00 ± 0.56^{abc}	0.3 ± 0.10^{de}	1.01 ± 0.32^{c}
Spermidine	0.1	4.00 ± 0.40^{cd}	0.40 ± 0.10^{bcde}	0.87 ± 0.24^{c}
	0.2	2.44 ± 0.37^{d}	0.23 ± 0.08^{e}	0.57 ± 0.21^{c}
	0.3	7.08 ± 0.70^{ab}	0.26 ± 0.08^{de}	0.67 ± 0.24^{c}
	0.4	7.84 ± 0.65^{a}	0.76 ± 0.2^{a}	1.37 ± 0.40^{a}
	0.5	5.28 ± 0.72^{ab}	0.60 ± 0.09^{ab}	0.53 ± 0.23^{c}
	0.6	4.43 ± 0.55^{ab}	0.50 ± 0.12^{ac}	1.06 ± 0.27^{c}

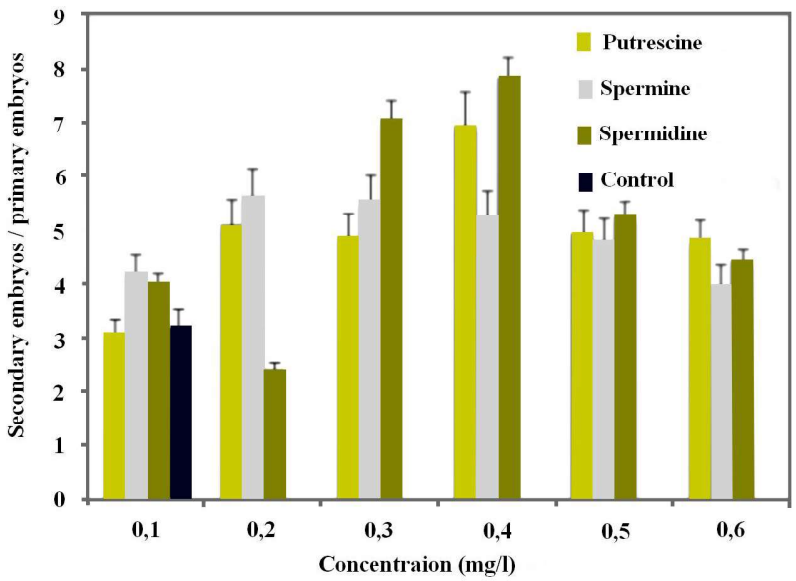

Figure 2. Influence of 3 polyamines on the number of secondary somatic embryos.

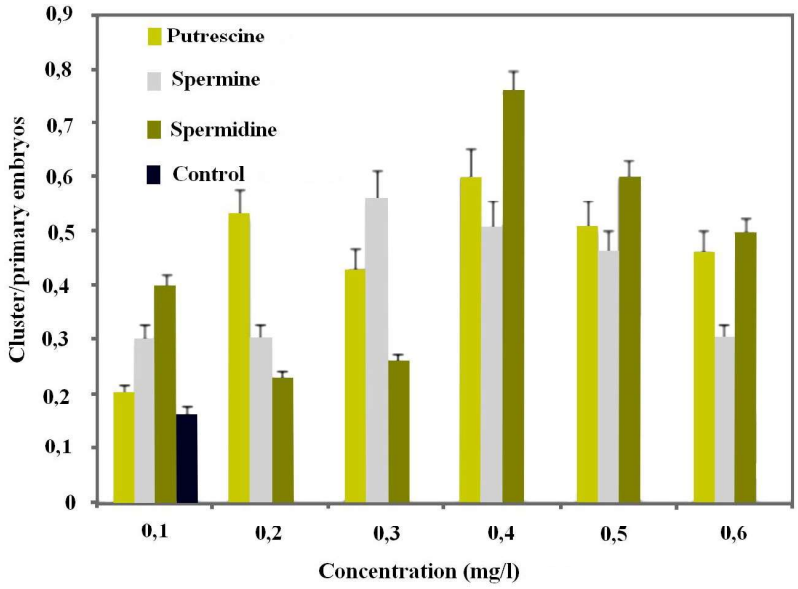

Figure 3. Influence of 3 polyamines on the number of clusters on primary somatic embryos.

embryogenesis. In this study, the embryogenic response was significantly influenced by the concentration and the type of polyamine tested.

For regeneration of the secondary embryos, Spermidine is the most favorable among the three polyamines tested. The highest number of secondary embryos formed was observed at a concentration of 0.4 mg/l. In this case, the averages of secondary somatic embryos, the number of clusters and the embryo clusters are 7.84 ± 0.65, 0.76 ± 0.2, and 1.37 ± 0.40, respectively, which correspond to a very significant ($p<0.05$) increase of 145%. Similarly, it is also noted that the optimum concentrations for the number of clusters and embryo clusters were increased by 145% and 158%. Putrescine also shows a very favorable response to induction of secondary somatic embryogenesis. Its effect reaches a maximum concentration at 0.4 mg/l, with

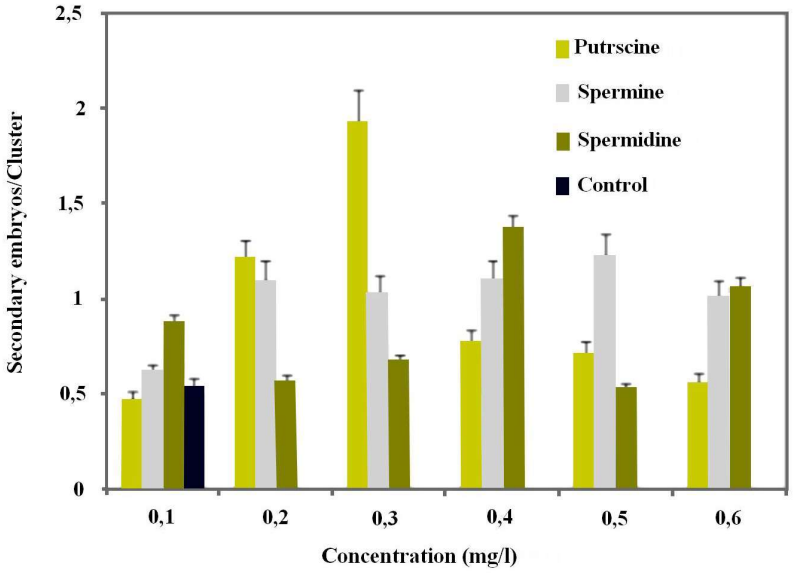

Figure 4. Influence of 3 polyamines on the number of secondary somatic embryos on clusters.

respective averages of 6.97 ± 0.59, 0.60 ± 0.09, and 0.77 ± 0.23, which corresponds to an increase of 108% compared to control medium.

Secondary somatic embryogenesis shows a lower response to Spermine compared to Spermidine and Putrescine. However, we found a marked increase of 76%, compared to the control medium, which also remains very interesting in the case of the obtained optimum concentration 0.3 mg/l. In general, the incorporation of the polyamines in the culture medium at concentrations varies between 0.1 and 0.6 mg/l, shows a very satisfactory uniform response regarded the secondary embryo process development.

As regards the morphological quality of embryos formed, Figure (5) represents our results after 2 months of culture. For each polyamine, they show some mature secondary embryos with regular morphology, a cotyledon appearance and a yellowish white color. The mature secondary embryos have a diameter that varies between 8 and 10 mm, globular stage embryos can also be observed with a diameter that varies between 4 and 5 mm.

Table (2) shows the average size of the secondary somatic embryos obtained from each polyamine, at a concentration of 0.3 mg/l. The results show that the addition of the polyamine also acts on the quality of embryos formed. Indeed, we can

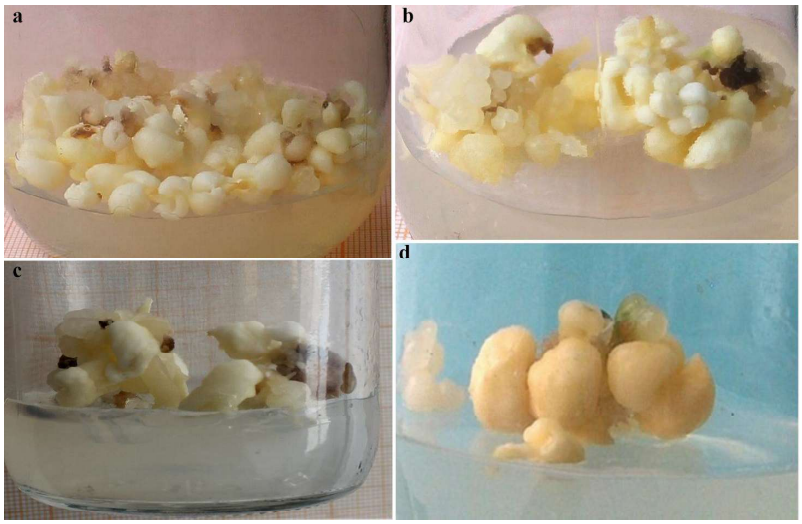

Figure 5. Morphology of secondary somatic embryos under treatment after 2 months of culture in medium containing polyamines: (a) Spermidine 0.4 mg/l, (b) Putrescine 0.4 mg/l, (c) Spermine 0.3 mg/l and (d) control medium.

Table 2. Influence of the three polyamines tested on the size of secondary somatic embryos.

Condition cultures	Control medium	Putrescine	Spermine	Spermidine
Average size of secondary embryos (mm) (mean ± standard error)	5.98 ± 0.36	7.84 ± 0.31	6.74 ± 2.04	8.30 ± 0.30

observe a very significant increase in the size of the secondary embryos, in comparison with the medium without polyamines. We found that the best stimulator is Spermidine, with an increase of 38%, followed by Putrescine and Spermine (Table 2). To confirm this result, a second series of tests was also performed with a concentration ranging from 0.3 to 1 mg/l, but this time in a growth medium containing macronutrients of Schenk and Hildebrandt [21], in order to confirm the advantage of Spermidine compared to Putrescine and Spermine in the presence of SH culture medium, that commonly used by other authors in testing polyamines. Furthermore, this experience allows us to check possible interference between the effect of each polyamine and that of $N_{30}K$ medium.

The results obtained clearly confirm the advantage of Spermidine compared to Putrescine and Spermine (Tables 3–5). It is also important to note that the absence of $N_{30}K$ medium decreases significantly the number of secondary somatic embryos formed directly on primary embryos. However, embryogenic behavior toward the concentration is maintained but with lower responses, while the optimum concentrations for each polyamine remain unchanged for Putrescine and Spermidine (0.4 mg/l) and Spermine (0.3 mg/l).

Finally, this result also confirms that N_{30} K medium actively stimulates the induction of secondary somatic embryogenesis without affecting or being affected by the existence of the polyamines.

Discussion

The addition of exogenous polyamines in the medium seems able to induce important effects in several *in vitro* organogenesis and somatic embryogenesis in many species [15,36]. However, very few studies have investigated the influence of this type of growth regulators on secondary somatic embryogenesis of woody species.

Our results show clearly the beneficial effect of polyamines in the secondary somatic embryogenesis process. Several studies have indicated the role of polyamines in the process of somatic embryogenesis [14–17,23,24,37–40]. However, no work has been devoted to the study of secondary embryogenesis in the Moroccan cork oak and very few to the study of secondary somatic embryogenesis in general. This lack of information makes comparisons between our results and other studies difficult and can be done only from an extrapolation of some specific primary somatic embryogenesis results. In our case, Spermidine at 0.4 mg/l showed a remarkable effect to both, the quality and quantity on the development and multiplication of secondary embryos, clusters, and embryo clusters. Our results seem consistent with those obtained for *Citrus clementina*, and *Panax ginseng* [18,28,41]. During the microsporogenesis of *Actinidia chinensis,* Biasi et al. [42] shown that Spermidine is present with significant concentration, and an exogenous supply of Spermidine induces an endogenous accumulation of this

Table 3. Influence of Spermidine (Spd) on secondary embryogenesis process of cork oak's embryos cultured on SH medium.

Concentration of Spd (mg/l)	Average number of secondary embryos	Average number of clusters	Average number of embryo clusters
Control media	1.97 ± 0.41^{a}	0.37 ± 0.09^{ab}	0.97 ± 0.28^{ab}
0.3	1.67 ± 0.36^{a}	0.43 ± 0.87^{a}	1.13 ± 0.36^{ab}
0.4	2.70 ± 0.51^{a}	0.50 ± 0.11^{a}	1.43 ± 0.35^{a}
0.5	2.17 ± 0.41^{a}	0.27 ± 0.08^{ab}	0.57 ± 0.21^{ab}
0.7	1.73 ± 0.37^{a}	0.33 ± 0.09^{ab}	0.90 ± 0.28^{ab}
1	2.17 ± 0.44^{a}	0.13 ± 0.06^{b}	0.50 ± 0.24^{b}

Table 4. Influence of Spermine (spm) on secondary embryogenesis process of cork oak's embryos cultured on SH medium.

Concentration of Spm (mg/l)	Average number of secondary embryos	Average number of clusters	Average number of embryo clusters
Control media	1.96 ± 0.41 a	0.36 ± 0.09 a	0.96 ± 0.28 a
0.3	2.10 ± 0.45 a	0.33 ± 0.10 a	0.73 ± 0.24 a
0.4	1.73 ± 0.47 a	0.23 ± 0.08 a	0.67 ± 0.25 a
0.5	1.20 ± 0.29 a	0.30 ± 0.10 a	0.70 ± 0.25 a
0.7	1.73 ± 0.30 a	0.27 ± 0.08 a	0.63 ± 0.20 a
1	2.07 ± 0.43 a	0.30 ± 0.08 a	0.73 ± 1.23 a

Table 5. Influence of Putrescine (Put) on secondary embryogenesis process of cork oak's embryos cultured on SH medium.

Concentration of Put (mg/l)	Average number of secondary embryos	Average number of clusters	Average number of embryo clusters
Control media	1.97 ± 0.41 ab	0.37 ± 0.09 a	0.97 ± 0.28 a
0.3	1.67 ± 0.29 ab	0.27 ± 0.08 ab	0.67 ± 0.21 a
0.4	2.40 ± 0.49 a	0.37 ± 0.09 a	1.00 ± 0.27 a
0.5	1.16 ± 0.25 b	0.10 ± 0.05 b	0.30 ± 0.17 a
0.7	1.30 ± 0.32 b	0.30 ± 0.08 ab	0.80 ± 0.25 a
1	1.10 ± 0.22 b	0.13 ± 0.06 b	0.43 ± 0.22 a

polyamine, which may explain the efficiency of the polyamines at low concentration.

In addition, it seems that at high doses, the exogenous amounts cause an excess in endogenous quantities of polyamines which may reduce its effectiveness on the secondary somatic embryogenesis process.

However, although Spermine has a beneficial effect, but it is less than that of Spermidine and Putrescine in all tests. The same results were also found by [43], which reported that Putrescine and Spermidine produced enhanced results compared with Spermine.

At high polyamine doses, no effect in the regeneration process was reported [44]. This result is confirmed in the present study, especially in the case of secondary somatic embryogenesis of cork oak. Also, several studies have tested the effect of endogenous polyamines. Domínguez et al. [24] reported that the addition of exogenous polyamines is strongly related to the increase of endogenous polyamine concentration in comparison with the control medium. From these results, we can explain the effectiveness of low doses performed in the culture medium, by the endogenous influence stimulated during treatment. In addition, the low effect of Spermine on secondary embryogenesis process may be due to the existence of a sufficient concentration of endogenous Spermine in primary somatic embryos; by consequence, the exogenous spermine remains less effective, which causes an overload of this polyamine. Similar results were observed in the embryogenic culture of *Bacopa monnieri* [25] and *Araucaria angustifolia* [41].

In *Araucaria angustifolia* [45], *Pinus taeda* [21], and *Ocotea catharinensis* [46], it was observed that the endogenous polyamine concentration is considerable during embryonic development. Their maximum level is detected in the primary stage of embryo development, then decreases during cotyledon stage, which indicated that the endogenous polyamine remains sufficient until a certain embryonic development stage. Also, it appears that an exogenous supply of these polyamines is necessary for completing the development of somatic embryos.

Furthermore, the increase in embryo size appears to be related to the tested polyamines in general, and the Spermidine in particular, as embryos passing from pre-cotyledonary to the cotyledonary stage, which agrees with the results obtained by [28,29] and which shows that Putrescine acts in the early embryo development by increasing its size at the globular stage, and Spermidine acts later in the somatic embryo formation, corresponding to the cotyledonal stage. This implies that the Spermidine promotes the maturation of secondary somatic embryos.

Conclusion

In this study, we demonstrated the efficacy of three different polyamines in the process of secondary somatic embryogenesis. Therefore, the results reported in this study may be the first for an oak species and especially the Moroccan cork oak. In general, polyamines seem to interfere with the growth and development of somatic embryos. Endogenous polyamines cause changes in the metabolism during somatic embryogenesis. However, until now, the role of endogenous and exogenous inputs is not completely well explained to understand the exact mechanism involved during the development of secondary embryos.

From a quantitative point of view, we found that the polyamines can increase in an exceptional way the number of secondary embryos formed directly on primary embryos. Embryogenic response depends on the concentration and the type of the tested polyamine with an optimal concentration, which differs from one polyamine to the other. Among the three tested polyamines, Spermidine has proved most effective with an increased number of secondary embryos of 145% at the optimal concentration of 0.4 mg/l, followed by Putrescine (108%) at a concentration 0.4 mg/l. Meanwhile, Spermine has shown the lower increase in the number of secondary somatic embryos of 76% at 0.3 mg/l. Furthermore, we have demonstrated that polyamines also affect the quality of the formed embryos. In the presence of polyamines, the size of the secondary embryos remarkably increases compared with medium without polyamines. The increase is about 38% for Spermidine.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Authors contributions

Naouar Ben Ali: acquisition of data, conception and design, methodology, formal analysis, writing, editing, and finalizing the manuscript; Rajae Benkaddour, Safaa Rahmouni, Ouafaa Hamdoun, Ibtissam Boussaoudi and Latifa Azaroual: Substantial contributions to the conception of this article, analysis and interpretation of data; Mustapha Hassoun: drafted the manuscript and critically revising it for important intellectual; Alain Badoc and Patrick Martin: critical review of important intellectual & editing the manuscript; Ahmed Lamarti: conception, design and editing the manuscript. All authors have read and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author [Naouar Ben Ali] upon reasonable request.

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References

[1] Lumaret R, Tryphon-Dionnet M, Michaud H, et al. Phylogeographical variation of chloroplast DNA in cork oak (*Quercus suber*). Ann Bot. 2005;96 (5):853–861. doi: 10.1093/aob/mci237

[2] Park Y-S, Beaulieu J, Bousquet J. Multi-varietal forestry integrating genomic selection and somatic embryogenesis. In: Park Y-S, Bonga J, and Moon H-K, editors Vegetative propagation of forest trees. Seoul, Korea: National Institute of Forest Science (NiFos); 2016. pp. 302–322.

[3] Ruiz-Galea M. Cork Oak *Quercus suber* L. Embryogenic Liquid Cultures. In: Jain S, Gupta P, et al., editors. Step wise protocols for somatic embryogenesis of important woody plants. DenmarkCham: Springer; 2018. pp. 243–254. doi: 10.1007/978-3-319-89483-6_18

[4] Loyola-Vargas VM, Ochoa-Alejo N. Somatic embryogenesis: fundamental aspects and application. 1st ed. Basel, Switzerland: Springer International Publishing; 2016.

[5] Stasolla C, Yeung EC. Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tissue Organ Cult. 2003;74 (1):15–35. doi: 10.1023/a:1023345803336

[6] Jangra A, Chaturvedi S, Kumar N, et al. Polyamines: the gleam of next-generation Plant growth regulators for growth, development, stress mitigation, and hormonal crosstalk in plants—A systematic review. *J Plant Growth Regul*. 2023;42(8):5167–5191. doi: 10.1007/s00344-022-10846-4

[7] Chambhare MR, Nikam TD. Influence of plant growth regulators on somatic embryogenesis in Niger (*Guizotia abyssinica* Cass.): an edible oilseed crop.

J Crop Sci Biotechnol. 2022;25(2):225–232. doi: 10.1007/s12892-021-00125-1

[8] Vengadesan G, Pijut PM. Somatic embryogenesis and plant regeneration of northern red oak (*Quercus rubra* L.). Plant Cell Tissue Organ Cult. 2009;97(2):141–149. doi: 10.1007/s11240-009-9508-z

[9] Ben Ali N, Benkaddour R, Rahmouni S, et al. Secondary somatic embryogenesis in cork oak: influence of plant growth regulators. Forest Sci Technol. 2023;19(1):78–88. doi: 10.1080/21580103.2023.2172462

[10] Chalupa V. Protocol of somatic embryogenesis: pedunculate oak (*Quercus robur* L.) and sessile oak (*Quercus petraea* Matt.Liebl.). In: Jain SM, and Gupta PK editors. Protocol for somatic embryogenesis in woody plants. Praha, Czech: Forestry Sciences; 2005. pp. 369–378. doi: 10.1007/1-4020-2985-3-29

[11] Valladares S, Sánchez C, Martínez MT, et al. Plant regeneration through somatic embryogenesis from tissues of mature oak trees: true-to-type conformity of plantlets by RAPD analysis. Plant Cell Rep. 2006;25(9):879–886. doi: 10.1007/s00299-005-0108-z

[12] Fernández-Guijarro B, Celestino C, Toribio M. Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber* L. Plant Cell Tissue *Organ Culture*. 1995;41(2):99–106. doi: 10.1007/bf00051578

[13] Ben Ali N, Lamarti A. Effect of carbon source on secondary somatic embryogenesis of Moroccan cork oak (*Quercus suber* L.). ScienceLib. 2013;5:1–14.

[14] Kapoor RT. Role of polyamines in plants under abiotic stresses: regulation of biochemical interactions. Plant Stress Mitigators. 2023;209–220. doi: 10.1016/B978-0-323-89871-3.00023-9.

[15] Sundararajan S, Sivakumar HP, Nayeem S, et al. Influence of exogenous polyamines on somatic embryogenesis and regeneration of fresh and long-term cultures of three elite indica rice cultivars. Cereal Res Commun. 2021;49(2):245–253. doi: 10.1007/s42976-020-00098-x

[16] De Moura LC, Xavier A, Cláudia A, et al. Effect of calcium, BAP and putrescine on somatic embryo induction in juvenile explants of eucalyptus grandis × E. urophylla hybrids. Aust J Crop Sci. 2019;13((04) 2019):513–519. doi: 10.21475/ajcs.19.13.04.p1345

[17] Mustafavi SH, Naghdi Badi H, Sekara A, et al. Polyamines and their possible mechanisms involved in plant physiological processes and elicitation of secondary metabolites. Acta Physiol Plant. 2018;40(6):102. doi: 10.1007/s11738-018-2671-2

[18] Rakesh B, Sudheer WN, Nagella P. Role of polyamines in plant tissue culture: an overview. Plant Cell Tiss Organ Cult. 2023;145(3):487–506. doi: 10.1007/s11240-021-02029-y

[19] Jimenez VM. Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. Plant Growth Regul. 2005;47(2):91–110. doi: 10.1007/s10725-005-3478-x

[20] Silveira V, Iochevet Segal Floh E, Handro W, et al. Effect of plant growth regulators on the cellular growth and levels of intracellular protein, starch and polyamines in embryogenic suspension cultures of *Pinus taeda*. Plant Cell Tissue Organ Cult. 2004;76(1):53–60. doi: 10.1023/a:1025847515435

[21] Silveira V, Balbuena TS, Santa-Catarina C, et al. Biochemical changes during seed development in *Pinus taeda* L. Plant Growth Regul. 2004b;44(2):147–156. doi: 10.1007/s10725-004-2601-8

[22] Klimaszewska K, Noceda C, Pelletier G, et al. Biological characterization of young and aged embryogenic cultures of *Pinus pinaster* (Ait.). In Vitro Cell Dev Biology-Plant. 2009;45(1):20–33. doi: 10.1007/s11627-008-9158-6

[23] Sathish D, Theboral J, Vasudevan V, et al. Exogenous polyamines enhance somatic embryogenesis and *Agrobacterium tumefaciens*-mediated transformation efficiency in sugarcane (*saccharum* spp. hybrid). Vitro Cell Dev Biol Plant. 2020;56(1):29–40. doi: 10.1007/s11627-019-10022-6

[24] Domínguez C, Martínez Ó, Nieto Ó, et al. Involvement of polyamines in the maturation of grapevine (*Vitis vinifera* L.'Mencía') somatic embryos over a semipermeable membrane. Sci Hortic. 2023;308:111537. doi: 10.1016/j.scienta.2022.111537

[25] Dey A, Hazra AK, Nongdam P, et al. Enhanced bacoside content in polyamine treated in-vitro raised *Bacopa monnieri* (L.) Wettst. S Afr J Bot. 2019;123:259–269. doi: 10.1016/j.sajb.2019.03.012

[26] Eren B, Türkoğlu A, Haliloğlu K, et al. Investigation of the Influence of polyamines on mature embryo culture and DNA methylation of wheat (*Triticum aestivum* L.) using the machine learning algorithm method. Plants. 2023;12(18):3261. doi: 10.3390/plants12183261

[27] Setia N. SETIA RC polyamines: an overview and prospects in crop improvement. Crop Improv Strateg. 2018;21:376–393.

[28] Niemi K, Sarjala T, Chen X, et al. Spermidine and methylglyoxal bis (guanylhydrazone) affect maturation and endogenous polyamine content of Scots pine embryogenic cultures. J Plant Physiol. 2002;159(10):1155–1158. doi: 10.1078/0176-1617-00634

[29] Ajithan C, Vasudevan V. Sathish D et al. The influential role of polyamines on the in vitro regeneration of pea (Pisum sativum L.) and genetic fidelity assessment by SCoT and RAPD markers. Plant Cell Tissue Organ Cult. 2019;39:245–252.

[30] Ebrahimzadeh H, Shariatpanahi ME, Ahmadi B, et al. Efficient parthenogenesis induction and in vitro haploid plant regeneration in cucumber (*Cucumis sativus* L.) using putrescine, spermidine, and cycocel. J. 2018;37(4):1127–1134. doi: 10.1007/s00344-018-9803-1

[31] Hernandez I, Celestino C, Toribio M. Vegetative propagation of *Quercus suber* L. by somatic embryogenesis: I. Factors affecting the induction in leaves from

mature cork oak tress. Plant Cell Rep. 2003;21(8):759–764. doi: 10.1007/s00299-003-0605-x

[32] Margara J. Bases de la multiplication végétative. Versailles, Paris: INRA; 1984.

[33] Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue culture. Physiol Plant. 1962;15(3):473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x

[34] Schenk RU, Hildebrandt AC. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can J Bot*. 1972;50(1):199–204. doi: 10.1139/b72-026

[35] IBM Corp. Released. IBM SPSS statistics for windows, version 24.0. Armonk, NY: IBM Corp; 2016.

[36] Chiancone B, Tassoni A, Bagni N, et al. Effect of polyamines on *in vitro* anther culture of *Citrus clementina* Hort. ex Tan. Plant Cell Tissue Organ Cult. 2006;87(2):145–153. doi: 10.1007/s11240-006-9149-4

[37] Kevers C, Gaspar T, Dommes J. The beneficial role of different auxins and polyamines at successive stages of somatic embryo formation and development of *Panax ginseng in vitro*. Plant Cell Tissue Organ Cult. 2002;70(2):181–188. doi: 10.1023/a:1016399905620

[38] Rajesh MK, Radha E, Karun A, et al. Plant regeneration from embryo-derived callus of oil palm - the effect of exogenous polyamines. Plant Cell Tissue Organ Cult. 2003;75(1):41–47. doi: 10.1023/A:1024679910085

[39] Bertoldi D, Tassoni A, Martinelli L, et al. Polyamines and somatic embryogenesis in two *Vitis vinifera* cultivars. Physiol Plant. 2004;120(4):657–666. doi: 10.1111/j.0031-9317.2004.0282.x

[40] Venkatachalam L, Bhagyalakshmi N. Spermine induced morphogenesis and effect of partial immersion system on the shoot cultures of banana. Appl Biochem Biotechnol. 2008;151(2–3):502–511. doi: 10.1007/s12010-008-8226-z

[41] Steiner N, Santa-Catarina C, Silveira V, et al. Polyamine effects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. Plant Cell Tissue Organ Cult. 2007;89(1):55–62. doi: 10.1007/s11240-007-9216-5

[42] Biasi R, Bagni N, Altamura MM. Identification of markers for sexual determination in *Actinidia deliciosa*. *Acta Hortic*. 1999;498(498):93–98. doi: 10.17660/ActaHortic.1999.498.10

[43] Cheruvathur MK, Abraham J, Mani B, et al. Adventitious shoot induction from cultured internodal explants of *malaxis acuminata* D. Don, a valuable terrestrial medicinal orchid. Plant Cell Tissue Organ Cult. 2010;101(2):163–170. doi: 10.1007/s11240-010-9673-0

[44] Petri C, Alburquerque N, Perez-Tornero O, et al. Auxin pulses and a synergistic interaction between polyamines and ethylene inhibitors improve adventitious regeneration from apricot leaves and Agrobacterium-mediated transformation of leaf tissues. Plant Cell Tissue Organ Cult. 2005;82(1):105–111. doi: 10.1007/s11240-004-7013-y

[45] Astarita LV, Handro W, Floh EIS. Changes in polyamines content associated with zygotic embryogenesis in the Brazilian pine, *Araucaria angustifolia* (Bert.) O. Ktze. Braz J Bot. 2003b;26(2):163–168. doi: 10.1590/S0100-84042003000200003

[46] Santa-Catarin AC, Silveira V, Balbuena TS, et al. IAA, ABA, polyamines and free amino acids associated with zygotic embryo development of *Ocotea catharinensis*. Plant Growth Regul. 2006;49(2–3):237–247. doi: 10.1007/s10725-006-9129-z