

# Macronutrients Effect on Secondary Somatic Embryogenesis of Moroccan Cork Oak (*Quercus suber* L.)

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Received 7 April 2014; revised 6 May 2014; accepted 17 May 2014

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## Abstract

To define the preliminary embryogenesis culture conditions of Moroccan Cork Oak (*Quercus suber* L.) in secondary propagation systems, secondary embryos formation from primary embryos were analyzed using seven macronutrient medias: (Chalupa) (BTM), Murashige and Skoog (MS), Schenk and Hildebrant (SH), Schenk and Hildebrant with half content macronutrients (SH ½), full Gamborg (G), Margara (N<sub>30</sub>K) and Woody Plant Media(WPM). Mature primary embryos at cotyledonal stage of 8 - 10 mm, were placed in each culture medium, and supplemented with 30 g/l of glucose and 7 g/l of agar without PGR. The experimental design consisted of a Petri dish containing three embryos explants. Each one of the seven treatments was composed of ten Petri dishes. Mean number of secondary somatic embryos, clusters and new embryogenic formation on clusters were recorded after 8 weeks, and evaluated by statistical analysis. There were no significant differences ( $p \leq 0.05$ ) in clusters and new embryos on clusters formation among evaluated media; but mean number of secondary embryos was significantly higher in N<sub>30</sub>K ( $4.37 \pm 0.48$ ) compared with control media ( $1.37 \pm 0.15$ ). The morphology of secondary embryos grown in the N<sub>30</sub>K medium exclusively showed the presence of three embryogenic stages: early cotyledonal with translucide aspect, white opaque, or green, and mature embryos. These results indicate that the medium do influence the morphogenic characteristics of produced embryos. Our finding revealed that secondary somatic embryos produced in N<sub>30</sub>K medium presented better morphogenic potential, with different stages of embryogenic formation.

## Keywords

*Quercus suber* L., Somatic Embryogenesis, Secondary Embryos, Mamora, Morocco

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## 1. Introduction

Cork oak (*Quercus suber* L.) is one of the most important species of the Mediterranean basin due to its ecological and socio-economical interests. In Morocco, around 350,000 hectares are covered with Cork oak trees [1]. Genetic variability of this specie is substantial embracing a range of ecotypes with different growth patterns.

These trees usually grow in agro forestry systems suffering intense pressures from wildlife, livestock and farmers activity. Under this situation, natural regeneration of this tree species becomes hampered. In addition, droughts and wildfires threaten seriously this species leading to an irreversible degradation of cork oak system [2].

The Mamora forest located at the northwest region of Morocco with a surface area around 60,000 ha, embraces 17% of the total area of cork oak forests, and 25% of the Atlantic forest area. It constitutes great importance in term of socio-economical and environmental values either at local and regional or national and international scales. The Mamora forest gives local communities an extra income, and benefits the country in term of cork exportation value [3]. However, the unprecedented pressure exerted by humans and livestock is among the main factors that had an adverse effect and led to degradation of the Mamora forest. Also, it has been found that the effect of several techniques and methods operating during development projects include gathering of firewood and some useful understory plants such as “doum”, “myrtle” and other medicinal and aromatic plants. In addition to illegal logging, clearing operations, wildfires, overgrazing had a serious impact on cork oak forest regeneration [4]. Currently, several sites occupied by cork oak suffer a really dramatic deterioration.

Nevertheless, the increasing demand for cork and the low natural regeneration of this species justifies intensive planting with improved material. Current tree improvement strategies in *Quercus* place great emphasis on breeding and cloning. Vegetative propagation of trees has been a useful tool in traditional tree improvement and holds important prospects for reforestation. It provides the possibility for multiplication of selected trees with favorable genetic combination and to produce genetically homogenous plant material that will grow predictably and uniformly. In addition, improved efficiency in management and finished product use may also be achieved [5].

Micropropagation techniques have been employed to overcome such problems, with studies being focused on the establishment of reliable protocols for somatic embryogenesis [6]. The main advantages of this system of regeneration include mass propagation of elite oak genotypes, high multiplication rates, scale-up for large scale production, genetic transformation, cryopreservation of embryos, and direct transfer to the field or greenhouse through artificial seeds. The combination of this technology could be very useful in an oak improvement program [7]. This system offers the capability to produce unlimited numbers of somatic embryos derived from plantlets [8] [9] or from artificial seeds [10]. Furthermore, somatic embryogenesis is an ideal system for genetic transformation because somatic embryos initiate from single cells [11], and have been already used in pines [12].

One of the main limitations of this protocol is associated to the late maturation, acclimation and establishment phase. Maturation has been hampered in many woody species by precocious conversion, spontaneous repetitive embryogenesis, embryo dormancy, and immaturity problems. Osmotic treatments have been used to promote somatic embryo conversion of cork oak [13] [14] and of several related species, such as *Q. robur* [15], and *Q. ilex* [16] [17]. Nevertheless, little attention has been devoted to late maturation and conversion [18].

Embryo production by recurrent or secondary embryogenesis is the step giving somatic embryogenesis a multiplicative potential for clonal mass propagation [19]. Secondary embryos arise from superficial single cells or by multicellular budding, usually at the hypocotyl of the mother embryo [20]. Embryo origin is especially relevant to the genetic uniformity of regenerated plants; as a multicellular origin may result in the formation of genetically variable plants, a uni-cellular origin is the desired pathway for practical applications of embryo cloning such as genetic transformation [21]. In the cork oak system, secondary embryos mainly originate by meristematic budding from a compact mass of proliferation [21].

With its high capacity for multiplication and susceptibility to automation, somatic embryogenesis becomes the clonal regeneration method of choice [22]. However, the production of mature embryos and the subsequent regeneration of plants are laborious, and the efficiencies are too low to enable economically competitive mass production of clonal material [23].

Multiplication of embryogenic lines via secondary embryogenesis was most frequently accomplished using culture media containing the cytokinin BAP, with auxin NAA or IBA (*Q. suber* [24]-[26] or 2,4-D (*Q. robur* [27]. More rarely BAP alone or in combination with GA<sub>3</sub> was used in *Q. petraea* [28] and *Q. robur* [27]. Zeatin

alone or in combination with NAA was also used successfully in *Q. robur* [29]. Secondary embryogenesis on culture media without growth regulators has been reported for a number of species including *Q. rubra* [31], *Q. suber* [25]-[32], *Q. acutissima* [33] and *Q. robur* [27]-[29].

The effect of culture medium is resulting from all interactions of various elements that compose it [34]. Mineral composition in appropriate culture medium [35] [36] and supplies of sucrose [37]-[39] are essential for the full extent balance of *in vitro* embryo development. Good absorption of water and metabolites nutrient medium is required for such development [37]. Fernández-Guijarro *et al.* [32] stated that on growth regulator free media, the secondary embryogenesis is influenced by macronutrient composition. Both high and low total nitrogen content decreased the percentage of somatic embryos that expressed secondary embryogenesis [30].

To confirm these hypotheses on the Moroccan genotypes, we try to test the effect of macronutrients on the secondary somatic embryogenesis regeneration capacity. Our study aims to evaluate the induction of secondary somatic embryogenesis, and plant regeneration of secondary somatic embryos. To do so, the suitable sources of macronutrients and culture conditions to induce proliferating embryogenic cultures of Moroccan *Quercus suber* L. were used in order to develop efficient *in vitro* regeneration methods to be applied in genetic transformation experiments of woody plants and in forest biotechnology. To achieve this aim, several factors affecting the proliferation and maturation of SSE, such as the basic formulation of culture medium, were evaluated.

## 2. Material and Methods

The initial explants which were isolated; are mature somatic embryos of 8 to 10 mm of length at cotyledonary stage. They are taken from embryogenic cultures originally obtained from the leaves of epicormic shoots extracted from selected tree in the Mamora region. The explants were maintained for more than 3 years by recurrent embryogenesis through a series of subculture on a medium without growth regulators according to the protocol referenced in [40].

### 2.1. Influence of Macronutrients on Secondary Somatic Embryogenesis

To study the influence of macronutrients composition on secondary somatic embryos formation, isolated embryos were grown for 60 days on a culture medium constituted of different macronutrients: MS (Murashige and Skoog, 1962) [41], SH (Schenk and Hildebrandt, 1972) [42], N<sub>30</sub>K (Margara, 1984) [43], BTM (Chalupa, 1981) [44], Woody Plant Medium (Lloyd and McCown; 1981) [45], SH ½ (including macronutrients were reduced to a half) and full Gamborg *et al.*, (Gamborg, 1968) [46], culture media were solidified with the agar (Bacteriological agar type E) at 0.8%.

### 2.2. Culture Conditions

The pH was adjusted to 5.8 before autoclaving at 120°C and 1 atmosphere during 20 min. primary embryos were placed on different media tested in sterile Petri dishes of 90 mm of diameter containing 20 ml of the culture medium and sealed with the Parafilm®. Incubation took place at 25°C ± 2°C under a photoperiod of 16 hours (50 pmol m<sup>-2</sup>·s<sup>-1</sup> cool white fluorescent tubes).

### 2.3. Statistical Analysis

For this experiment, 30 explants of primary somatic embryos were cultured per experimental unit in each of the 7 treatments. All the somatic embryos were homogeneously distributed between treatments. The experiment was repeated three times, thus a total of 630 embryos were cultured.

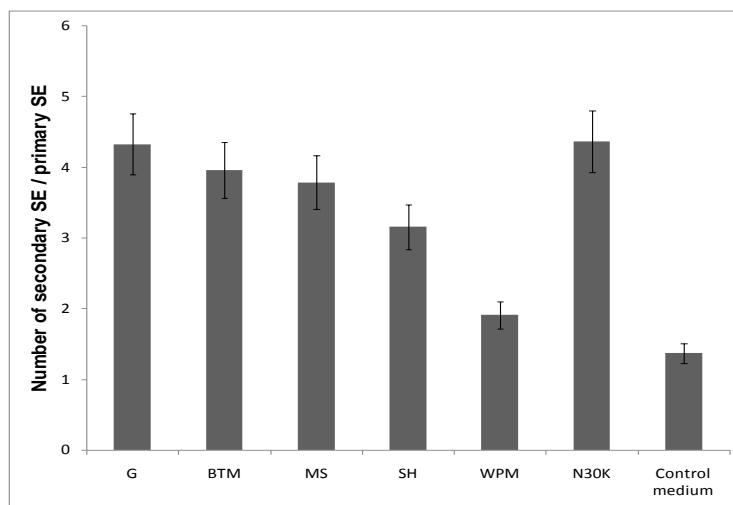
After 8 weeks of culture, the recorded data includes; percentage of explants with secondary SE, number of secondary somatic embryos directly formed on the primary embryos, number of viable clusters, and somatic embryos formed on clusters per explants (primary somatic embryo). The data uploaded to statistical software SPSS 17.0. (1999). 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 Duncans post-hoc test ( $p \leq 0.05$ ).

## 3. Results

The results showed that on all investigated media, cork oak primary somatic embryos produced a large number

of secondary embryos, which emerged all around the hypocotyl of the embryo axis but not on the cotyledons. The cotyledons were at first translucent (**Figure 2(A)**), then became white opaque (**Figure 2(B)**), and later, green (**Figure 2(C)**). The presence of more than two cotyledons per embryo was common, and cotyledons often exhibit a fuzzy morphology, but secondary embryo formation was not evident only after the first week of culture. However, in long-term culture, especially after 60 days, secondary embryos increase the size. On the surface of the cotyledons, the formation of embryonic outgrowths and/or occasionally the development of soft nodular masses (**Figure 2**) were observed.

Somatic embryos were grown in culture media formed by different composition of macronutrients. Significant differences were observed between different treatments in term of secondary embryos regeneration (**Table 1**). The highest rate of secondary embryogenesis ( $4.37 \pm 0.48$  of secondary embryos regenerated per primary explants) was recorded in the case of N<sub>30</sub>K medium, followed by G, BTM, MS and HS with a mean of ( $4.37 \pm 0.71$ ;  $3.96 \pm 0.36$ ;  $3.79 \pm 0.34$  and  $3.16 \pm 0.38$ ) respectively (**Table 1, Figure 1**). The lowest rate was observed



**Figure 1.** Influence of culture media on the number of secondary somatic embryos.



**Figure 2.** Somatic embryos at different developmental stages of (*Quercus Suber* L.) proliferated through secondary embryogenesis; (A) translucent aspect; (B) white opaque; (C) green mature embryo.

in a culture medium containing the WPM ( $1.91 \pm 0.21$ ) and the control medium SH  $\frac{1}{2}$  ( $1.37 \pm 0.15$ ), this last result is in agreement with the study realized by Mauri *et al.* [16].

Concerning the formation of clusters, among the seven solutions of macronutrients tested, BTM medium gives the best results ( $0.70 \pm 0.08$ ) (Table 1) and the WPM give low results ( $0.29 \pm 0.03$ ) in comparison with control medium ( $0.41 \pm 0.04$ ) (Figure 3).

Data were also recorded for small newly formed embryos on these clusters, they are newly formed cellular formations that tend to differentiate and proliferate into future embryos. The presence of these embryos in the culture medium indicates that the embryos have a starting regenerative capacity that will give even more secondary embryos through the process of secondary somatic embryogenesis. In our case we observed that the N<sub>30</sub>K environment has a significant influence on the formation of secondary somatic embryos on the clusters ( $1.62 \pm 0.19$ ) ( $0.58 \pm 0.07$  for the control medium). Figure 4 The analysis of the mineral composition of the 7 solutions tested showed that the mineral solution of N<sub>30</sub>K (Margara, 1984) [43] is nitrogen-rich (30 mEq/l) which consists of two-thirds in NO<sub>3</sub><sup>-</sup> and potassium (15 mEq/l) (Table 2 and Table 3). This medium is also characterized by a high content of SO<sub>4</sub><sup>2-</sup> and an average content of total nitrogen, where the third is supplied as NH<sub>4</sub><sup>+</sup>.

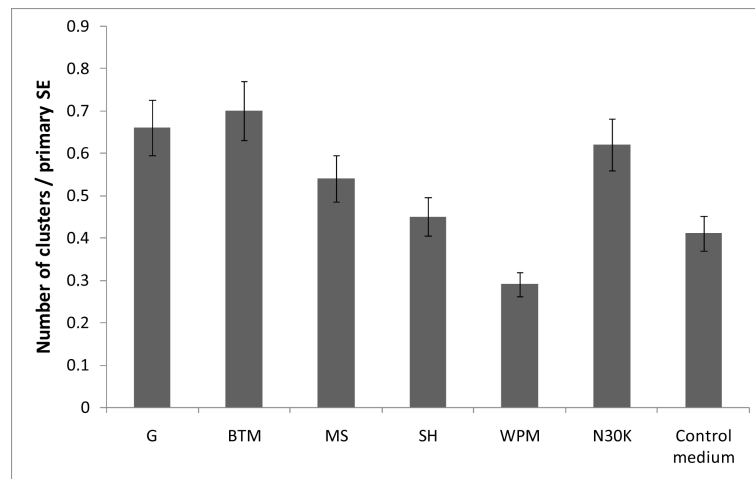


Figure 3. Influence of culture media on the number of clusters formed on primary embryos.

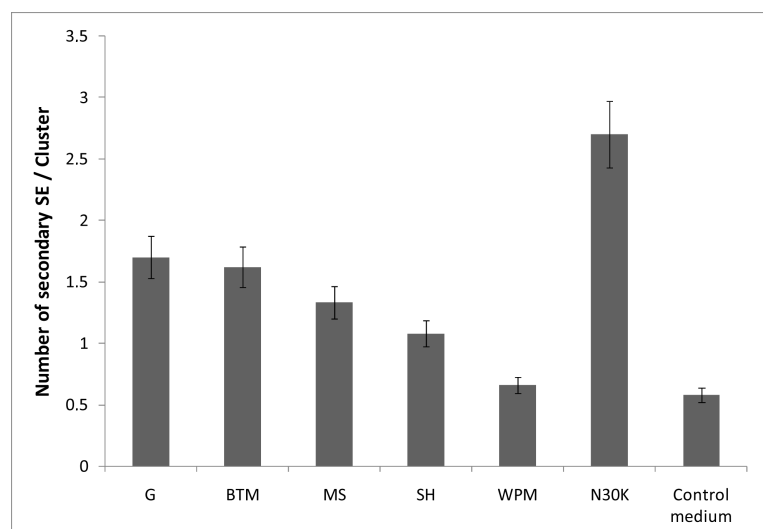


Figure 4. Influence of culture media on the number of secondary somatic embryos formed on clusters.

**Table 1.** Effect of macronutrients on the secondary somatic embryogenesis.

Macronutrients	Average number of secondary embryos per primary embryo	Average number of clusters	Average number of secondary embryos per cluster
G	4.33 ± 0.39 <sup>a</sup>	0.66 ± 0.08 <sup>ab</sup>	1.70 ± 0.19 <sup>ab</sup>
BTM	3.96 ± 0.36 <sup>a</sup>	0.70 ± 0.08 <sup>a</sup>	2.70 ± 0.30 <sup>a</sup>
MS	3.79 ± 0.34 <sup>a</sup>	0.54 ± 0.06 <sup>ab</sup>	1.33 ± 0.15 <sup>b</sup>
SH	3.16 ± 0.38 <sup>ab</sup>	0.45 ± 0.05 <sup>ab</sup>	1.08 ± 0.12 <sup>b</sup>
WPM	1.91 ± 0.21 <sup>bc</sup>	0.29 ± 0.03 <sup>b</sup>	0.66 ± 0.08 <sup>b</sup>
N <sub>30</sub> K	4.37 ± 0.48 <sup>a</sup>	0.62 ± 0.07 <sup>ab</sup>	1.62 ± 0.19 <sup>b</sup>
Control	1.37 ± 0.39 <sup>c</sup>	0.41 ± 0.04 <sup>ab</sup>	0.58 ± 0.07 <sup>b</sup>

**Table 2.** Composition in mg/l of macronutrients solutions used in the *in vitro* culture of somatic embryos of cork oak.

Medium	SH ½ (control medium)	N30K	MS	SH	BTM	WPM	G (1968)
NH <sub>4</sub> NO <sub>3</sub>	-	480	1650	-	165	400	-
KNO <sub>3</sub>	1570	1313	1900	2500	190	-	3000
MgSO <sub>4</sub> ·7H <sub>2</sub> O	199.62	246.0	370	399.24	370	370	500
KH <sub>2</sub> PO <sub>4</sub>	-	136.0	170	-	170	170	-
KCL	-	74.5	-	-	-	-	-
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	-	590	-	-	640	556	-
CaCl <sub>2</sub> ·2H <sub>2</sub> O	100.00	-	440	200.00	64	96	150
(NH <sub>4</sub> ) <sub>2</sub> H <sub>2</sub> PO <sub>4</sub>	150.00	-	-	300.00	-	-	134
K <sub>2</sub> SO <sub>4</sub>	-	-	-	-	860	990	-

**Table 3.** Ionic content (mEq) of the 7 macronutrients solutions used in the *in vitro* culture of cork oak.

Medium	SH ½ (control medium)	N <sub>30</sub> K	MS	SH	BTM	WPM	G (1968)
<b>Cations</b>							
Mg <sup>++</sup>	1.62	2.00	3	3.24	-	-	2
Ca <sup>++</sup>	1.36	5.00	6	2.72	3	3	2
NH <sub>4</sub> <sup>+</sup>	1.3	6.00	20.61	2.6	6.02	6	2
K <sup>+</sup>	12.5	15.00	20.04	25	5.69	5	25
K <sup>+</sup>	-	-	-	-	13	12.61	1.1
Na <sup>+</sup>							
<b>Anions</b>							
NO <sub>3</sub> <sup>-</sup>	12.5	24.00	3.00	25.00	16.5	14.36	25
SO <sub>4</sub> <sup>-</sup>	1.62	2.00	5.98	3.24	0.6	1.30	4
Cl <sup>-</sup>	1.30	1.00	39.4	2.60	9.36	9.70	2
H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	12.5	1.00	1.25	2.72	1.25	1.25	1.1
<b>Total Azote</b>		30.00	60.01		15.05	14.70	
<b>Totals Ions</b>		51.50	99.46		55.42	53.22	

Adapted from (Murashige and Skoog, 1962); (Schenk and Hildebrant, 1972); (Margara, 1984); (Chalupa, 1981); (Lloyd and McCown; 1981); SH ½ (including macronutrients were reduced by half) and (Gamborg, 1968). MS: (Murashige and Skoog, 1962), SH: (Schenk and Hildebrant, 1972), N<sub>30</sub>K: (Margara, 1984), BTM: (Chalupa, 1981), WPM: Woody Plant Medium (Lloyd and McCown; 1981); Control media: Schenk and Hildebrant with half content macronutrients (SH ½) (including macronutrients were reduced by half) and G: full Gamborg *et al.* (Gamborg, 1968).

#### 4. Discussion

The results showed very different reactions on the behavior of primary somatic embryos following the proliferation media used. These have significantly affected the embryogenesis of secondary embryos, clusters and newly formed embryos. Indeed, for the proliferation of secondary embryos, N<sub>30</sub>K medium proved generally to be more reactive with an average of 4.37 embryos per primary embryo. This is consistent with the result reported by Péjuiliaro *et al.*, [21] who showed that immature embryos of *Quercus suber* are longer and have swollen cotyledons when they are grown in a medium rich in nitrogen than those grown in nitrogen-free medium. It showed also that this contribution has a significant influence on the induction of secondary somatic embryogenesis. McCown & Sellmer [47] confirmed that the level of total nitrogen must be present in the culture medium. According to our results, the nitrogen level is satisfied in N<sub>30</sub>K medium for the embryogenic induction.

This is confirmed by Margara [43] who reported that the use of a solution enriched in Mn, Zn and Bo is generally promoting organogenesis.

However, BTM, MS, SH and G media provide acceptable results in terms of secondary embryogenesis induction but the efficiency remains generally not better than N<sub>30</sub>K medium. Furthermore, Pinto *et al.*, [18] reported that G medium give excellent results for the proliferation of globular secondary embryos but does not support the following stages of somatic embryogenesis process. Against MS showed a significant response for both the formation of embryos and maturation stage.

The use of other media tested as BTM, WPM and SH ½ can reduce the growth of secondary embryos, in some cases it can cause the necrosis (photo 1). This result confirms those obtained by Brhadda *et al.*, [34] concerning the growth of olive shoot (Figure 5 and Figure 6).

MS medium is particularly rich in ammonium nitrate. This excess in ammonium (compared with other media tested) can be detrimental for the induction of secondary somatic embryogenesis. Similarly, McCown *et al.* [48] found that *Salix babylonica* (Linnaeus) microcuttings cultured on MS medium offered a hypolignifications aspect. According to this author, this pathological aspect could be due to the high concentration of ammonium.

For SH medium, Mauri [16] showed that reducing by half the concentration of the medium was effective for both somatic embryo maturation and reducing the frequency of secondary embryogenesis, the latter result is well illustrated in our case.

N<sub>30</sub>K solution characterized by an average total nitrogen content, a predominance of Ca<sup>2+</sup> and low Mg<sup>2+</sup> and SO<sub>4</sub><sup>-</sup>, gave satisfactory results in terms of secondary embryogenesis regeneration. Based on these results, the N<sub>30</sub>K medium was more beneficial than the HS and MS medium for somatic embryogenesis of Moroccan cork oak. However, the literature show that our results cannot be generalized to the wholes genotypes of cork oak:



**Figure 5.** Secondary somatic embryos regenerated from mature primary embryos after 8 weeks of culture in WPM medium.



**Figure 6.** Secondary somatic embryos regenerated from mature primary embryos after 8 weeks of culture on N<sub>30</sub>K medium.

Valladares *et al.*, [7] adopted the MS medium for somatic embryogenesis of Spanish cork oak, Hernandez [41] and Fernández-Guijarro *et al.*, [32] showed that the SH medium allowed better growth of secondary embryos. Bueno *et al.*, [26] and Pintos *et al.*, [6] used the Sommer medium in their experiment. These results indicate that the genetic factor plays an important role in the choice of the culture medium to use, which requires adaptation of the medium mineral composition for cultivar multiplication. This difference may be related to the nutritional requirements which vary depending on the genotype.

## 5. Conclusions

This work has focused on the study of macronutrients effect on the process of secondary somatic embryogenesis of Moroccan cork oak (*Quercus suber* L.). Six macronutrients were tested through recognized Experimental protocols.

Results showed that the N30K medium is most suitable for the induction of secondary somatic embryogenesis of Moroccan cork oak (*Quercus suber* L.), followed respectively by the G, BTM, MS and HS mediums. For each medium tested, the averages of the number of secondary somatic embryos formed per primary embryo were respectively 4.37 (N30K), 4.33 (G), 3.96 (BTM), 3.79 (MS) and 3.16 (SH). WPM medium proved to be the least effective one.

Although secondary embryogenesis can be an efficient process of somatic embryo multiplication, it also seems to hamper embryos germination. Therefore, several studies should be carried out to succeed the germination and acclimatization phases which constitute the overall goal of cork oak cloning through the somatic embryogenesis.

## Acknowledgments

This work was partially funded by Hassan II Academy of Science and Technology (Morocco) through the project entitled “Study of the genomic variability of the cork oak (*Quercus suber* L.) and clonal multiplication by somatic embryogenesis”.

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