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Effect of Amino Acids on Secondary Somatic Embryogenesis of Moroccan Cork Oak (*Quercus suber* L.) Tree

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Abstract

In the present study, we tested the effect of amino acids on secondary somatic embryogenesis of Moroccan cork oak (*Quercus suber* L.). Secondary mature and immature somatic embryos were obtained from primary somatic embryos cultured in $N_{30}K$ medium supplemented with nineteen amino acids. Stimulation of embryogenesis was dependent on the type and concentration of amino acid in the medium. Thus, L-glutamine and L-asparagine at 3.42 mM have been proved to be the most favorable for the formation of functional somatic embryos and the induction of secondary somatic embryos.

Keywords

Cork Oak, *Quercus suber* L., Secondary Somatic Embryogenesis, Amino Acids

1. Introduction

Mediterranean forests have been extensively exploited for their different resources. These forests cover 85 million hectares, representing about two percent of the world's forest area (4033 million hectares) [1] and contain more than 25,000 plant species, especially conifers and hardwood species, like oaks [2].

Cork oak (*Quercus suber* L.), a species belonging to the Fagaceae family, is one of the most characteristic oak species of the Mediterranean ecosystem. It covers 2.2 million hectares of the world forest surface and plays important ecological, economic and social roles. *Quercus suber* is described for the first time

by Linnaeus [3] [4]. It belongs to the section *Suber* of the subgenus *Cerris* [5]. It is extremely polymorphic, distributed in the Western Mediterranean region, in the various European and African countries and territories: Portugal, Spain, Southern France, Morocco, Algeria, Tunisia, Corsica, Sardinia, Italy and Slovenia [6].

Moroccan cork oak forest covers a total area of 377,000 ha, in the central plateau (2%), the Atlantic plains (45%), the Western Rif (35%), the Taza-Taounate Region (18%) and that represents 14% of the total area on the Mediterranean scale [7]. It is an ecosystem of great ecological importance for the conservation of genetic resources and the protection of the environment [8] [9].

Quercus suber is traditionally propagated by acorns [10]. However, acorn production is irregular and highly dependent on climatic conditions [11] and cork oak seeds tend to lose their ability to germinate after a period of storage [10]. For this reason, new techniques of vegetative propagation (like micropropagation) have been used in order to preserve this ecological heritage and to improve some characteristics for agricultural and economic interests [12] [13].

Somatic embryogenesis, a technique of vegetative propagation applied successfully for several other forest species [14] [15], can be used for clonal propagation processes to produce a large number of somatic embryos, from selected trees and in a very short period of time. These embryos can be transformed into identical trees [16].

Secondary somatic embryogenesis allows the formation of new embryos from other somatic embryos. Secondary embryos have multicellular origin and are formed after a slight proliferation of cells in the external layers of the primary embryos cap. Meristematic primordia begin to appear on the periphery of the proliferated tissue and they soon develop into bipolar structures that form cotyledonary primordia. Then, new embryos emerge from the proliferation mass [17].

Composition of medium, culture system and genotype influence embryo multiplication rates and the quality of the newly developed somatic embryos [18]. The presence of cytokinin in the medium induces secondary somatic embryogenesis [19] [20] [21].

Studies on the induction of primary somatic embryogenesis in cork oak and other forest species are numerous [22] [23] [24] [25]. On the other hand, secondary somatic embryogenesis has not been sufficiently studied. Thereby, we established a protocol for secondary somatic embryogenesis of Moroccan cork oak (*Quercus suber* L.), by testing the effect of nineteen amino acids on the formation and multiplication of secondary embryos from primary somatic embryos.

2. Material and Methods

2.1. Plant Material and Culture Medium

We used mature somatic embryos that length is 8 - 12 mm, at cotyledonary stage

(Figure 1), proliferated from leaves of epicormic shoots; these latter were obtained by cuttings from segments (30 cm) of branches collected from one more cork oak tree (latitude N 35˚18'520 and longitude W 005˚40'622) of the Bouhachem-Chefchaouen region (Western Rif I2, Morocco) according to the procedure described by Hernandez [26] and Ben Ali [27]. Embryos were multiplied by transplanting every two months on Margara ($N_{30}K$) [28] medium using the method described by Ben Ali and Lamarti [29] [30].

2.2. Influence of Amino Acids on Secondary Somatic Embryogenesis

Somatic embryos were placed into flasks (two embryos per flask) containing 40 ml of culture medium containing N30K macronutrients, MS (Murashige and Skoog) [31] micronutrients and vitamins, 0.7% agar (Bacteriological agar type E) and 100 mg/l myo-inositol. Different concentrations of L-glutamine (Gln) and L-asparagine (Asn) (0.03; 0.05; 0.07; 0.1; 0.14; 0.2; 0.27; 3.42; 3.49 and 3.56 mM) were added to the culture medium. Also, nineteen amino acids were tested at 3.42 mM. A medium without amino acids was used as a control.

2.3. Culture Conditions

pH medium was adjusted between 5.6 and 5.8 before autoclaving at 121˚C for 20 min. However, all amino acids were sterilized by filtration (Filters Millex®—Merck Millipore 33 mm and pore size 0.45 µm) with Durapore membrane (PVDF, ultra-low protein adsorption). Embryos were incubated (without soaking) at 25˚C ± 2˚C in the dark for two months.

2.4. Statistical Analysis

For each condition, two replicates of 24 explants were carried out. After two months of culture, the percentage of functional embryos, the mean number of mature somatic embryos formed on the primary embryo, the mean number of immature somatic embryos formed on the primary embryo and the mean number of total embryos were counted. All results were tested using variance analysis (ANOVA 1) and means were compared using Duncan's multiple range test at $p < 0.05$.

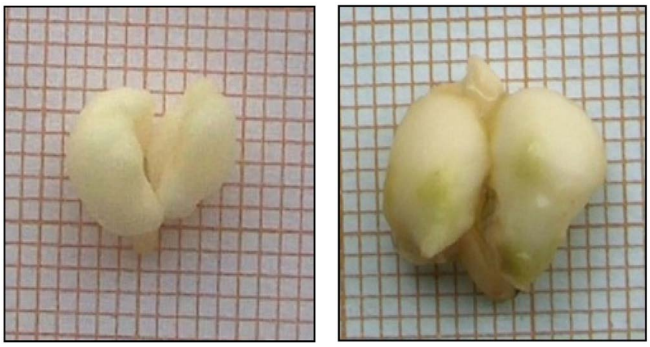


Figure 1. Mature somatic embryos of *Quercus suber* at cotyledonary stage.

3. Results

3.1. Effect of L-glutamine and L-asparagine

After two months of culture, the optimal percentage of functional somatic embryos is observed at 3.42, 3.49 and 3.56 mM of L-glutamine (79.97%, 77.37% and 76.17%, respectively), followed by L-asparagine at 3.42 and 3.49 mM (73.16% and 70.89%) (Figure 2 and Table 1). Also, the maximum mean number of mature somatic embryos is registered for L-glutamine at 3.42, 3.49 and 3.56 mM (3.90, 3.65 and 3.6 matures SE/primary SE respectively), followed by L-asparagine at 3.42 mM (3.35 matures SE/primary SE) (Figure 3, Figure 9 and Table 1). Moreover, the highest mean number of immature somatic embryos is recorded in the case of L-glutamine and L-asparagine at 3.42 mM (9.90 and 7.74 immature SE/primary SE, respectively), followed by L-glutamine at 3.49 mM (7.54 immature SE/primary SE respectively) and L-asparagine at 3.49 mM (7.16 immature SE/primary SE respectively) (Figure 4, Figure 9 and Table 1). In addition, the maximum mean number of total somatic embryos is obtained in the case of L-glutamine at 3.42 mM (11.5), 3.49 mM (10.1) and 3.56 mM (9.98 SE), followed by L-asparagine at 3.42 mM (9.83 SE) (Figure 5 and Table 1).

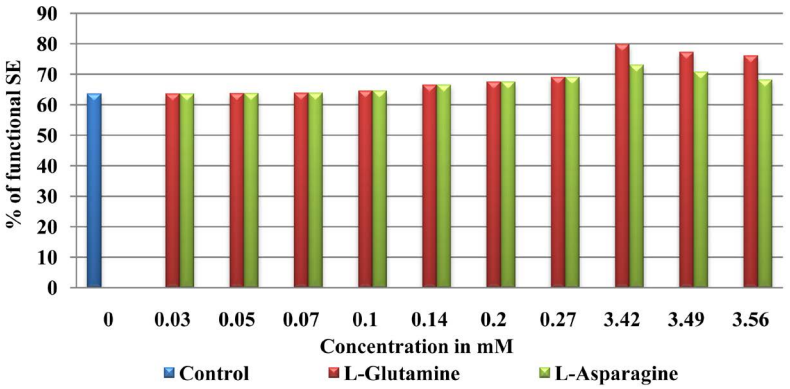


Figure 2. Effect of L-glutamine and L-asparagine at different concentrations on the percentage of functional embryos after 2-month culture in the dark.

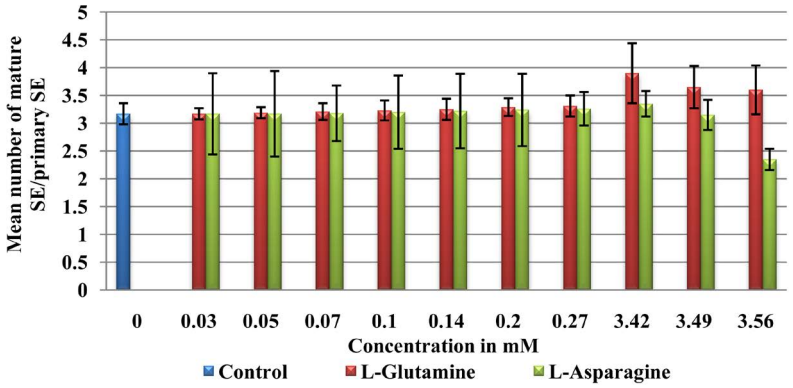


Figure 3. Effect of L-glutamine and L-asparagine at different concentrations on the mean number of mature embryos formed on the primary embryo after 2-month culture in the dark.

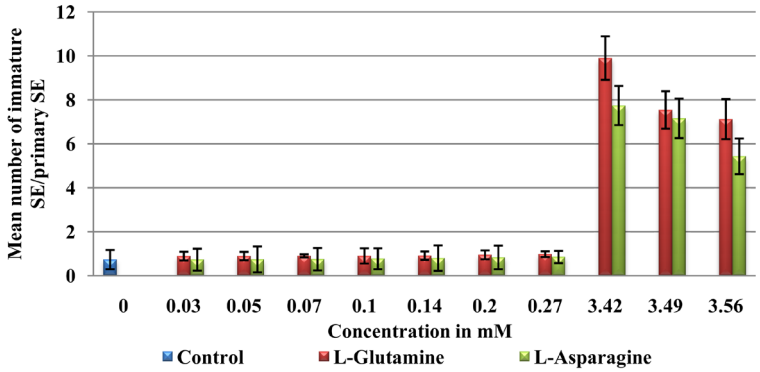


Figure 4. Effect of L-glutamine and L-asparagine at different concentrations on the mean number of immature embryos formed on the primary embryo after 2-month culture in the dark.

Table 1. Effect of L-glutamine and L-asparagine at different concentrations on the percentage of functional embryos, mean number of mature and immature embryos formed on primary somatic embryo and mean number of total embryos. Somatic embryo = SE.

Amino acids	Concentration in mM	% of functional SE	Mean number of mature SE/primary SE	Mean number of immature SE/primary SE	Mean number of total embryos
L-Glutamine	**0**	63.67	3.17 ± 0.19 gh	0.73 ± 0.34 i	3.19 ± 0.46 j
	0.03	63.70	3.17 ± 0.10 gh	0.89 ± 0.20 fgh	3.20 ± 0.33 ij
	0.05	63.82	3.19 ± 0.10 fgh	0.89 ± 0.19 fgh	3.23 ± 0.34 ij
	0.07	63.93	3.21 ± 0.15 efgh	0.90 ± 0.07 fgh	3.42 ± 0.25 gh
	0.10	64.67	3.23 ± 0.18 efgh	0.90 ± 0.35 fgh	3.44 ± 0.81 gh
	0.14	66.61	3.25 ± 0.19 defg	0.91 ± 0.19 fgh	3.67 ± 0.42 f
	0.20	67.63	3.29 ± 0.16 cde	0.95 ± 0.20 fg	3.70 ± 0.29 f
	0.27	69.15	3.31 ± 0.19 cd	0.98 ± 0.13 f	3.73 ± 0.68 f
	3.42	79.97	3.90 ± 0.54 a	9.90 ± 0.89 a	11.50 ± 1.01 a
	3.49	77.37	3.65 ± 0.38 b	7.54 ± 0.85 c	10.10 ± 0.97 b
	3.56	76.17	3.60 ± 0.44 b	7.12 ± 0.91 d	9.98 ± 0.90 b
L-Asparagine	**0.03**	63.70	3.17 ± 0.73 gh	0.73 ± 0.50 i	3.20 ± 0.27 j
	0.05	63.82	3.17 ± 0.77 gh	0.74 ± 0.59 i	3.20 ± 0.40 j
	0.07	63.93	3.18 ± 0.50 gh	0.75 ± 0.51 i	3.22 ± 0.88 ij
	0.10	64.67	3.20 ± 0.66 fgh	0.77 ± 0.48 hi	3.31 ± 0.86 hi
	0.14	66.61	3.22 ± 0.67 efgh	0.80 ± 0.58 hi	3.36 ± 0.91 ghi
	0.20	67.63	3.24 ± 0.65 defg	0.83 ± 0.54 ghi	3.41 ± 0.94 gh
	0.27	69.15	3.26 ± 0.30 def	0.85 ± 0.28 fghi	3.45 ± 0.31 g
	3.42	73.16	3.35 ± 0.23 c	7.74 ± 0.89 b	9.83 ± 1.00 c
	3.49	70.89	3.15 ± 0.27 h	7.16 ± 0.90 d	9.17 ± 1.01 d
	3.56	68.23	2.35 ± 0.19 i	5.43 ± 0.81 e	6.92 ± 0.99 e

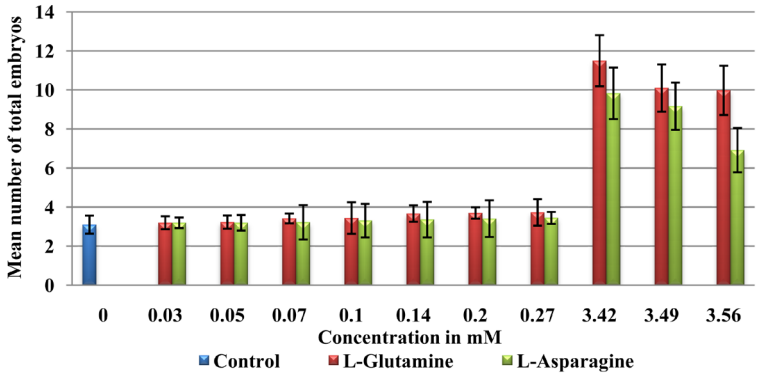


Figure 5. Effect of L-glutamine and L-asparagine at different concentrations on the mean number of total somatic embryos after 2-month culture in the dark.

3.2. Effect of Amino Acids

The percentage of functional somatic embryos is optimal in the presence of L-glutamine (79.97%), followed by L-asparagine (73.16%), L-ornithine (68.10%) and L-arginine (65.76%), on one hand. On the other hand, the minimum percentage (40.54%) was recorded in the case of L-leucine (**Figure 6** and **Table 2**). Also, the mean number of mature somatic embryos formed on the primary embryo is maximum for L-glutamine (3.9 mature SE/primary SE) and L-asparagine (3.35 mature SE/primary SE). On the other hand, the minimum value (0.59 mature SE/primary SE) is noted for L-cysteine (**Figure 7**, **Figure 9** and **Table 2**). In addition, a maximum mean number of immature somatic embryos is obtained in the case of L-glutamine (9.9 immature SE/primary SE). The lowest mean number is registered for the control (0.73 immature SE/primary SE) (**Figure 8**, **Figure 9** and **Table 2**). Moreover, the maximum mean number of total embryos is noticed for L-glutamine (11.5 SE), followed by L-asparagine (9.83 SE) and the minimum for L-cysteine (1.79 SE) (**Figure 10** and **Table 2**).

4. Discussion

The induction of secondary somatic embryogenesis from somatic embryos was reported on media containing plant growth regulators [21] [32] [33] and also on a free PGR (Plant Growth Regulators) media [33] [34] [35] [36] [37].

In our study, we tested the effect of amino acids on the process of secondary somatic embryogenesis in Moroccan cork oak primary embryos. Firstly, we tested L-glutamine and L-asparagine at different concentrations and then, nineteen other organic acids at 3.42 mM. Induction of embryogenesis and embryo development was strictly dependent on the type and concentration of amino acid in the medium. The best amino acid source was L-glutamine followed by L-asparagine at 3.42 mM.

In most cases, the addition of organic nitrogen form (from amino acids) has been mentioned as important factor for somatic embryogenesis, embryo proliferation and maturation and plant conversion [22] [23] [38] [39] [40] [41] [42]. The use of amino acids in herbaceous and conifer species improves embryo

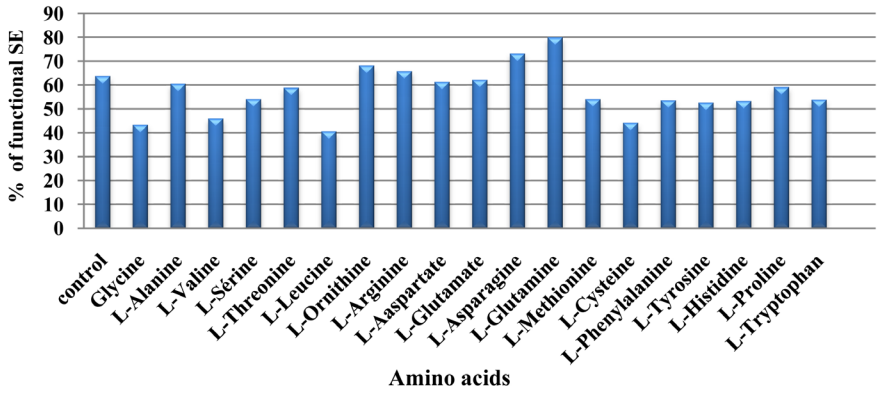


Figure 6. Effect of 19 amino acids at 3.42 mM on the percentage of functional embryos after 2-month culture in the dark.

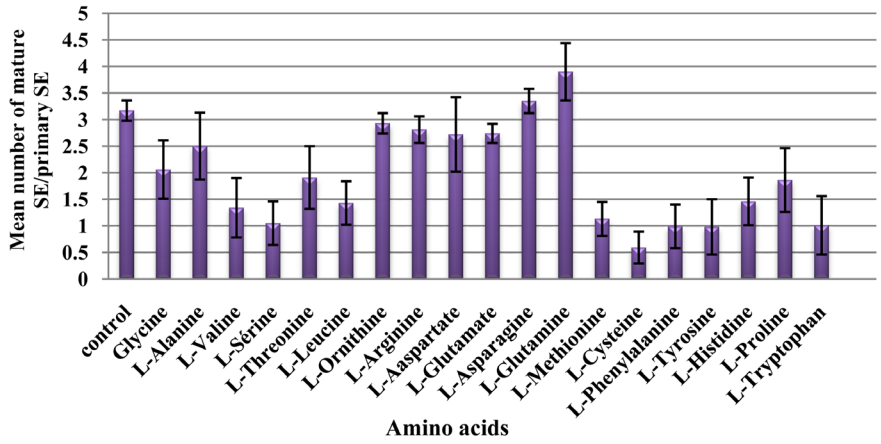


Figure 7. Effect of 19 amino acids at 3.42 mM on the mean number of mature embryos formed on the primary embryo after 2-month culture in the dark.

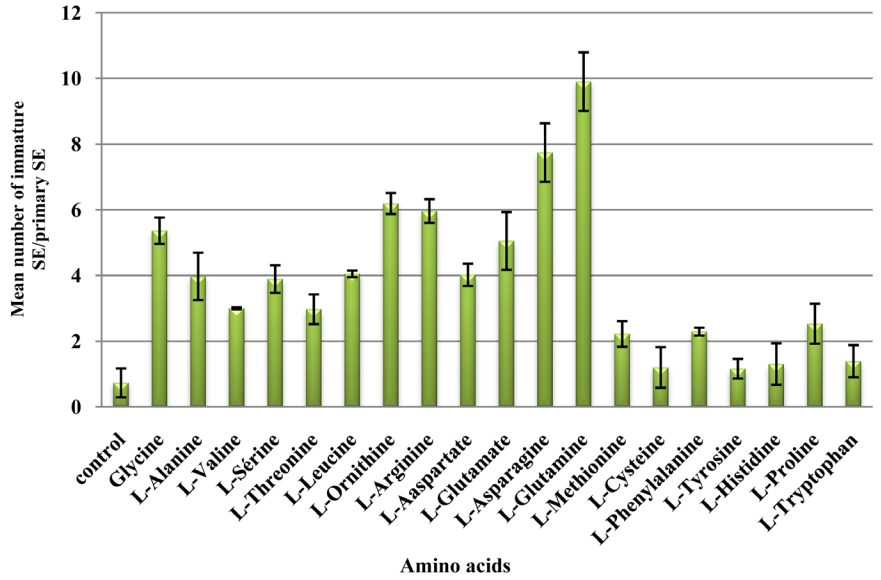
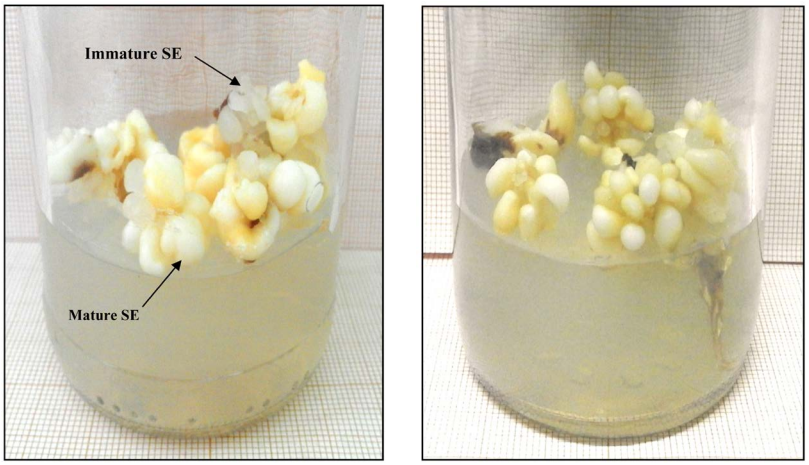


Figure 8. Effect of 19 amino acids at 3.42 mM on the mean number of immature embryos formed on the primary embryo after 2-month culture in the dark.



(a) (b)

Figure 9. Aspect of secondary somatic embryos in medium containing 3.42 mM of L-glutamine (a) and L-asparagine (b).

Table 2. Effect of nineteen amino acids at 3.42 mM on the percentage of functional embryos, mean number of mature and immature embryos formed on primary somatic embryo and mean number of total embryos. SE: Somatic embryo.

Amino acids	% of functional SE	Mean number of mature SE/primary SE	Mean number of immature SE/primary SE	Mean number of total embryos
Control	63.67	3.17 ± 0.19 c	0.73 ± 0.44 n	3.10 ± 0.46 i
Glycine	43.24	2.06 ± 0.55 g	5.36 ± 0.40 e	4.07 ± 0.60 f
L-Alanine	60.47	2.50 ± 0.63 f	3.97 ± 0.72 gh	6.47 ± 0.83 d
L-Valine	45.95	1.34 ± 0.56 i	3.00 ± 0.03 i	3.92 ± 0.64 g
L-Serine	54.05	1.05 ± 0.41 jk	3.89 ± 0.42 h	4.94 ± 0.62 e
L-Threonine	58.78	1.91 ± 0.59 h	2.97 ± 0.45 i	3.88 ± 0.76 g
L-Leucine	40.54	1.43 ± 0.41 i	4.05 ± 0.10 g	3.02 ± 0.47 i
L-Ornithine	68.10	2.93 ± 0.19 d	6.19 ± 0.32 c	7.33 ± 0.40 c
L-Arginine	65.76	2.81 ± 0.25 de	5.96 ± 0.36 d	7.13 ± 0.47 c
L-Aspartate	61.27	2.72 ± 0.82 e	4.02 ± 0.34 g	4.12 ± 0.84 f
L-Glutamate	62.16	2.74 ± 0.18 e	5.05 ± 0.88 f	6.35 ± 0.98 d
L-Asparagine	73.16	3.35 ± 0.23 b	7.74 ± 0.89 b	9.83 ± 0.9 b
L-Glutamine	79.97	3.90 ± 0.54 a	9.90 ± 0.89 a	11.50 ± 0.9 a
L-Methionine	54.05	1.13 ± 0.32 j	2.22 ± 0.39 k	2.92 ± 0.4 j
L-Cysteine	44.12	0.59 ± 0.30 m	1.20 ± 0.62 lm	1.79 ± 0.75 m
L-Phenylalanine	53.56	0.99 ± 0.41 kl	1.29 ± 0.12 lm	2.21 ± 0.57 l
L-Tyrosine	52.56	0.98 ± 0.62 l	1.16 ± 0.30 lm	2.20 ± 0.63 l
L-Histidine	53.27	1.46 ± 0.45 i	1.30 ± 0.63 lm	2.76 ± 0.85 j
L-Proline	59.12	1.86 ± 0.75 h	2.53 ± 0.61 j	3.39 ± 0.99 h
L-Tryptophane	53.85	1.01 ± 0.55 jkl	1.39 ± 0.49 l	2.53 ± 0.70 k

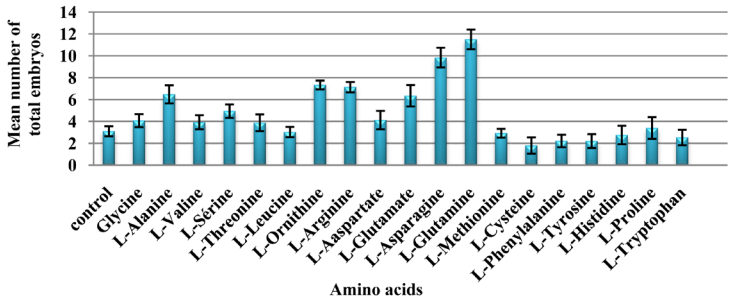


Figure 10. Effect of 19 amino acids at 3.42 mM on the mean number of total embryos after 2-month culture in the dark.

maturation. However, in hardwood species, systematic studies of the effect of organic nitrogen are rare [42].

Glutamine is effective to induce somatic embryogenesis in most studies. This can be due to direct absorption by cells, which leads to lower energy use [43]. Moreover, glutamine is involved in the synthesis of other amino acids and serves as a nitrogen transporter metabolite [42].

Similar to our results, Ara *et al.* [44] found that the addition of glutamine to the medium induces somatic embryogenesis in *Mangifera indica*. Also, it was demonstrated that glutamine had a positive effect on growth of *Picea abies* pro-embryogenic masses [45]. For *Sapindus trifoliatus*, addition of L-glutamine in the medium influenced influenced the frequency of embryogenesis, induction of nodular embryogenic structures from callus and differentiation of somatic embryos. Maximum results were observed on medium containing 200 mg/l glutamine [46]. Moreover, Vasanth and Vivier [47] used a medium containing glutamine for proliferation of *Vitis vinifera* embryo. In *Acca sellowiana*, the addition of L-glutamine, L-asparagine or arginine improves somatic embryo induction and development [48]. In *Cucumis sativus*, Ashok and Nurthy [49] studied the effect of amino acids (glutamine, glycine, arginine, asparagine and cysteine) on embryogenesis and plantlet regeneration. They found that the use of induction medium supplemented with a combination of amino acids enhanced both embryo induction and plantlet regeneration. Stuart and Strickland [50] noticed that in *Medicago sativa*, proline, alanine, glutamine, arginine, lysine, serine, asparagine and ornithine stimulated the number of somatic embryos formed, enhanced their structural quality, increased their size and stimulated embryo conversion.

Rathore *et al.* [51] reported that L-glutamine stimulated the maturation of *Acacia senegal* somatic embryos and reduced reduced embryo abnormalities. Rai *et al.* [52] found that 0.68 mM of glutamine increased maturation percentage of *Psidium guajava* somatic embryos. Moreover, L-glutamine increased plant conversion and reduced embryo abnormalities in *Abelmoschus esculentus* [41]. Smith [53] [54] supplemented the maturation medium of *Pinus radiata* with a solution of seven amino acids, to improve somatic embryos production and plantlet recovery. Khlifi and Tremblay [55] revealed that the maturation of so-

matic embryos of *Picea mariana* occurred in a medium supplemented with glutamine as the only source of nitrogen.

Gerdakaneh *et al.* [40] found that proline, glutamine and alanine were the best amino acids for strawberry embryo culture. Other researchers reported that proline stimulated growth of embryogenic cultures of larch (*Larix* x *leptoeuropaea*), sitka spruce (*Picea sitchensis*) and oak (*Quercus robur*) in stress conditions [56]. In our case, L-proline showed modest results compared to L-glutamine and L-asparagine on the induction of secondary somatic embryogenesis of Moroccan cork oak.

Also, L-glutamine was used separately or combined with other compounds to promote somatic embryos in *Pistacia vera*, *Phoenix dactylifera*, *Cullen corylifolium*, *Colocasia esculenta* and *Pelargonium sidoides* [57] [58] [59] [60] [61]. In *Abies alba*, embryogenic lines proliferated faster and matured better on a medium containing glutamine and casein hydrolysate [62].

George *et al.* [63] indicated that the addition of amino acids to media without ammonium ions was beneficial for growth or morphogenesis of cells and tissues. Müller and Grafe [64] cultured *Nicotiana tabacum* callus on a medium lacking of NO_3^- and NH_4^+ and supplemented with glycine, arginine, aspartic acid and glutamine. Also, Anderson [65] used glutamine as the only nitrogen source for the growth of wild carrot suspension.

However, Merkle *et al.* [43] reported that the addition of amino acids to the culture medium might promote or inhibit somatic embryos development and conversion. Pintos *et al.* [25] studied the effect of amino acids (glutamine, arginine and asparagine) on the growth of cork oak embryos. On the one hand, they showed that the combination of these three amino acids stimulated embryo growth. On the other hand, they noticed that amino acids did not show significant differences with the control. Also, Martinez *et al.* [66] found that glutamine decreased the number of nodular embryogenic structures and secondary somatic embryos of *Quercus ilex*. This is contradictory with our results in which we prove the positive effect of L-glutamine and L-asparagine.

Von Arnold [67] found a strong negative effect of L-glutamine, L-arginine, and L-asparagine on the induction of somatic embryogenesis in *Picea abies*. Changes in the somatic embryo phenotype could be induced by adding amino acid to the medium [68]. Pinto *et al.* [69] found that the addition of casein hydrolysate and glutamine increased the formation of abnormal embryo in *Eucalyptus globulus*. Moreover, in *Picea glauca*, organic nitrogen had a negative effect on somatic embryo maturation [70].

5. Conclusion

Secondary somatic embryogenesis is an effective tool for large-scale multiplication of cork oak in particular and for woody plants in general. Thus, the present study is the first one about the effect of amino acids on secondary somatic embryogenesis of cork oak (*Quercus suber*) from the north of Morocco.

First, we tested the effect of L-glutamine and L-asparagine at different concentrations and found that the addition of 3.42 mM to the culture medium increases the percentage of functional embryos, the mean number of mature and immature secondary somatic embryos and the mean number of total somatic embryos.

Then, we tested the effect of L-glutamine, L-asparagine and seventeen other amino acids at 3.42 mM and concluded that L-glutamine gives the highest results followed by L-asparagine.

Despite extensive work on somatic embryogenesis, the stage of secondary somatic embryogenesis has been less illustrated and very few studies have focused on optimization of secondary embryo production in oaks.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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