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# Micropropagation of Carob (*Ceratonia siliqua* L.) through Adventitious Buds of Immature Embryonic Cotyledons

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## Abstract

Adventitious budding from embryonic cotyledons of immature seeds of carob was obtained. The combination of BAP (4.44  $\mu$ M) and NAA (1.5  $\mu$ M) furthered the neoformation of adventitious buds. These latter were multiplied on MS medium added with BAP (2.22  $\mu$ M). Stems and leaves growing were improved by adding 2.02  $\mu$ M GA<sub>3</sub>. Elongation was favored by 0.5  $\mu$ M NAA. 70% of rooting was obtained with 10  $\mu$ M IBA.

## Keywords

*Ceratonia siliqua* L., Micropropagation, Adventitious Bud, Embryonic Cotyledons

## 1. Introduction

Carob tree (*Ceratonia siliqua* L., Fabaceae) is an agro-sylvo-pastoral species with socio-economic and ecological interests. It is very adapted to the various Mediterranean climates and different soil types [1] [2] [3].

Carob propagation is carried out in two main ways, by shoot and meristem culture for large-scale clonal propagation and by the development of cell and tissue culture techniques for the induction of calli *in vitro*.

Traditionally, the propagation of carob trees has been achieved by grafting young trees with selected female buds of productive trees [4]. This traditional

propagation method has failed to respond to market demand. The carob tree can also be propagated by seedlings, but the seeds do not show correct germination rates unless after scarification; Moreover, this multiplication does not guarantee the sex and the genetic characteristics of the cultivars. Cuttings multiplication is problematic because rooting is difficult [5]. In this context, micropropagation techniques offer an alternative to the propagation of carob trees in order to satisfy the increased demand for this tree [6].

Among the various techniques developed for the *in vitro* propagation of *Ceratonia siliqua*, we distinguish micropropagation by axillary budding from nodal explants of young stems, and by apex culture [6] [7] [8] or by adventive budding (direct organogenesis on leaf fragments, cotyledons or stems used as explants and indirect when buds appear without or after callus formation) [9], and somatic embryogenesis from immature cotyledons [10], mature cotyledons [11], zygotic embryos [12] or immature seeds [13].

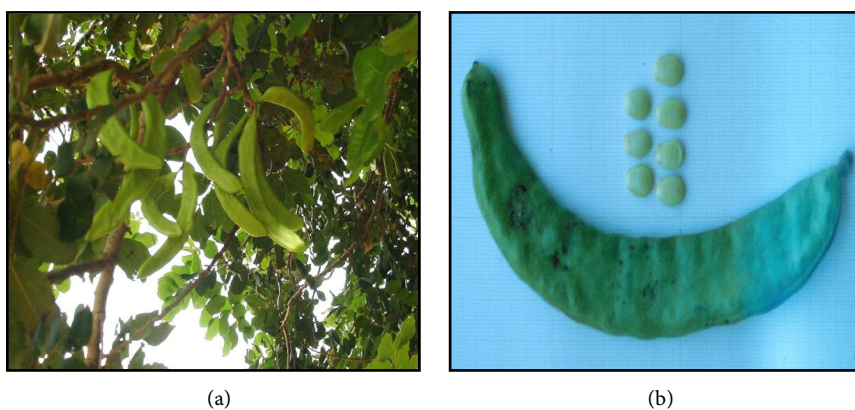
In the present study, embryonic cotyledons culture was established to evaluate organogenic capacity of these explants. Thereby, we tested the effect of different auxins combined with BAP on the development of embryonic cotyledons. Seedlings were regenerated from broken buds; also, several combinations of BAP with NAA were evaluated to improve their organogenicity. Shoot multiplication was studied on MS and WPM mediums added with different growth regulators (auxins, 6-Benzylaminopurine; BAP, and Gibberellic acid; GA<sub>3</sub>) often combined to each other.

## 2. Material and Methods

### 2.1. Explants Preparation

Immature carob fruits were harvested during June 2016, from a female tree on Tetouan-Chefchaouen road, Amtil region, Western Rif, Morocco (**Figure 1**).

After abundant washing with running water, seeds were placed for 20 minutes in a calcium hypochlorite (Ca(OCl)<sub>2</sub>) solution (7%) and then subjected to three



**Figure 1.** (a) Female tree with immature seed; (b) Immature pod and seeds harvested in June.

successive 10 minutes washes with sterilized distilled water. Then, the seeds were removed aseptically under a hood, immersed 3 minutes in mercuric chloride ( $\text{HgCl}_2$ ) solution 0.1% and afterward washed three times for 10 minutes with sterile distilled water. These seeds were shelled and separated from their albumen. The lower third of the embryonic cotyledons, with gemmule, plumule and radicle were removed and the upper part was implanted, either horizontally and vertically, in 200 ml flasks containing 50 ml of culture medium, solidified by 0.7% agar. In addition, the basic nutrient medium consists of Woody Plant Medium (WPM) macronutrients [14], supplemented with Murashige and Skoog (MS) [15] micronutrients and vitamins, as well as 3% sucrose and 0.1 g/l myo-inositol, the pH was adjusted at 5.8. Moreover, cultures were placed in a culture room with 16 hours photoperiod (4000 lux) at 23°C - 25°C during the day and 20°C at night. Results were evaluated after one month growth.

## 2.2. Characteristics of Immature Seeds

A sample of thirty seeds was selected for which the means of length, width and thickness were determined. The weights of fresh (FM) and dry (DM) matter of the seeds were also estimated after drying in an oven at 70°C for 15 days (Table 1).

## 2.3. Initiation of Embryonic Cotyledons Culture

### 2.3.1. Growth Regulators Effect

#### 1) Effect of Auxins Associated with BAP

Embryonic cotyledons were cultured vertically on MS medium supplemented with four auxins (Indole-3-acetic acid; IAA, Indole-3-butyric acid; IBA, 2-naphthalineacetic acid; NAA and 2,4-Dichlorophenoxyacetic acid; 2,4-D) in three concentrations (0.5; 1.5 and 2.5  $\mu\text{M}$ ) combined with BAP at 4.44  $\mu\text{M}$ . Results were evaluated after one month.

#### 2) Effect of different concentrations of BAP combined with NAA

1.5  $\mu\text{M}$  NAA combined with BAP was the best concentration for the development of adventitious buds from embryonic cotyledons. Then we tested other concentrations of BAP (0.44; 1.33; 2.22; 3.55; 4.44 and 6.66  $\mu\text{M}$ ).

### 2.3.2. Effect of the Position of Embryonic Cotyledons

To test the effect of the position, we tried on to put cotyledons vertically and horizontally in MS medium supplemented with three concentrations of BAP (1.33; 2.22 and 4.4  $\mu\text{M}$ ) combined with 1.5  $\mu\text{M}$  NAA. Results were reported after one month.

**Table 1.** Mean dimensions and weights of thirty immature carob seeds harvested in June 2016.

Length (mm)	Width (mm)	Thickness (mm)	FM (g)	DM (g)
10.40 ± 0.4	7.2 ± 0.4	3.95 ± 0.6	0.16 ± 0.03	0.05 ± 0.01

## 2.4. Multiplication of Shoots Obtained from Adventitious Buds

Shoots obtained after the break of adventitious buds from embryonic cotyledons, that presented three internodes of 5 - 7 mm, were transplanted into different mediums. Results were evaluated after one month growth.

### 2.4.1. Effect of Macronutrients MS and WPM

The effect of MS and WPM macronutrients, supplemented with BAP (2.22; 4.44 and 6.66  $\mu\text{M}$ ) was tested on the multiplication of shoots obtained after the break of adventitious buds on the embryonic cotyledons.

### 2.4.2. Effect of Growth Regulators

MS medium, more favorable than WPM for shoot multiplication and survival, was adopted to study the effect of other growth regulators.

Thereby, to study the effect of gibberellins on the multiplication and elongation of shoots obtained from embryonic cotyledons, different concentrations of  $\text{GA}_3$  (0.29; 0.58; 0.87; 1.44; 2.02 and 2.89  $\mu\text{M}$ ) were combined with 2.22  $\mu\text{M}$  BAP.

Furthermore, different auxins (IAA, IBA, NAA and 2,4-D) at 0.5  $\mu\text{M}$ , combined with 2.22  $\mu\text{M}$  BAP, were tested.

## 2.5. Shoot Elongation

Shoot elongation after multiplication was studied in the presence of three concentrations of BAP (1.33; 2.22 and 4.44  $\mu\text{M}$ ) and in the presence of  $\text{GA}_3$  (0.58; 1.44 and 2.02  $\mu\text{M}$ ) associated to BAP at 1.33  $\mu\text{M}$ .

## 2.6. Plantlets Rooting

IBA and IAA were adopted to induce shoot rooting after multiplication and elongation. These shoots were first cultured under darkness at  $\frac{1}{2}$  MS medium supplemented with IBA or IAA at 5 or 10  $\mu\text{M}$  during one week, then transferred to light in the same medium, but without growth regulators. Results were evaluated after one month.

## 2.7. Statistical Analysis

For each study, three replicates of thirty explants were carried out. All results were analyzed with a completely randomized design and tested using an analysis of variance (ANOVA) and means were compared using Duncan's multiple range test at  $p < 0.05$ .

## 3. Results

### 3.1. Embryonic Cotyledons Culture

#### 3.1.1. Growth Regulators Effect

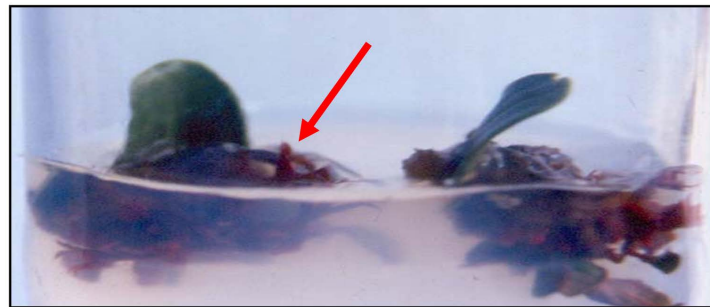
##### 1) Effect of different auxins associated with BAP

The reaction of embryonic cotyledons varies according to the conditions and the studied parameter. The percentage of only callogenic explants reaches 97% in the presence of 2,4-D at 2.5  $\mu\text{M}$ , followed by NAA at 2.5  $\mu\text{M}$  with a percentage

of 92%; moreover, the percentage of callogenic and caulogenic explants is generally low (Table 2, Figure 2). This latter reaches 27.86% in the presence of 1.5  $\mu\text{M}$  NAA, the number of buds per explants is 4.8. The concentration of 1.5  $\mu\text{M}$  seems to be the most appropriate for the neoformation of adventitious buds regardless of the studied auxin, a higher concentration favors callogenesis at the expense of caulogenesis.

## 2) Effect of different concentrations of BAP combined with NAA

The combination of BAP at different concentrations with NAA (1.5  $\mu\text{M}$ ) does not improve the percentage of caulogenic explants. Indeed, the best results are obtained, like before, with 4.44  $\mu\text{M}$  BAP. 3.55  $\mu\text{M}$  does not present significant difference with 4.44  $\mu\text{M}$  BAP (Table 3, Figure 3). In some cases with low concentrations of BAP, roots are formed on the embryonic cotyledons.

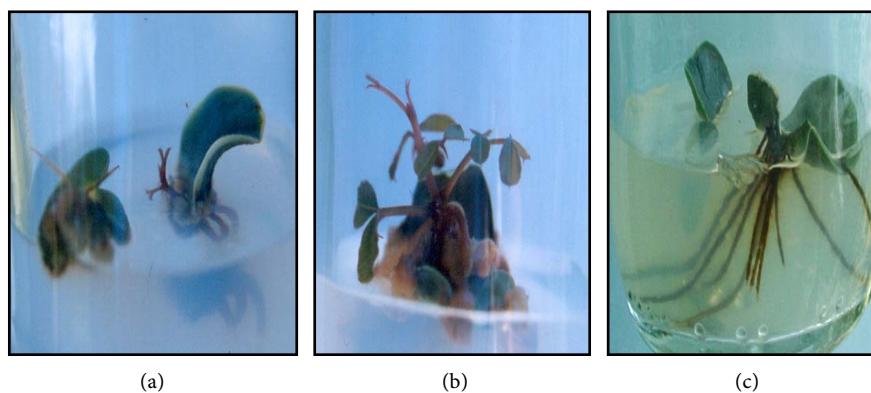


**Figure 2.** Adventitious buds obtained from embryonic cotyledons cultured in WPM medium added with NAA (1.5  $\mu\text{M}$ ) combined with BAP (4.44  $\mu\text{M}$ ), after one month growth.

**Table 2.** Effect of four auxins combined with BAP (4.44  $\mu\text{M}$ ) on the morphogenesis of carob embryonic cotyledons after one month culture.

Auxins	Concentrations ( $\mu\text{M}$ )	% callogenic explants	% callogenic and caulogenic explants	Number of buds by caulogenic explant	% of explants without reaction
IAA	0.5	74.20 g	6.45 c	1 c	19.35 a
	1.5	86.30 cd	6.80 c	1 c	6.90 e
	2.5	92.05 b	0 e	0 d	7.95 de
IBA	0.5	80.77 ef	0 e	0 d	19.23 a
	1.5	77.42 fg	6.45 c	1 c	16.13 b
	2.5	89.50 bc	0 e	0 d	10.50 c
NAA	0.5	82.35 e	8.55 b	2.50 $\pm$ 0.17 b	9.10 cd
	1.5	78.57 f	27.86 a	4.80 $\pm$ 0.39 a	3.57 gh
	2.5	92.12 b	2.70 d	1 c	5.18 fg
2,4-D	0.5	83.33 de	0 e	0 d	16.67 b
	1.5	86.28 cd	6.82 c	1 c	6.90 ef
	2.5	97.48 a	0 e	0 d	2.52 h

Means in the same column followed by the same letters are not significantly different from each other according to the Duncan's multiple range test ( $p < 0.05$ ).



**Figure 3.** Effect of different concentrations of BAP combined with NAA. (a) Adventitious buds obtained from embryonic cotyledons cultured in WPM medium, added with NAA (1.5  $\mu\text{M}$ ) and BAP (1.33  $\mu\text{M}$ ) after one month growth; (b) Adventitious buds obtained from embryonic cotyledons cultured in WPM medium added with 1.5  $\mu\text{M}$  NAA combined with BAP (4.44  $\mu\text{M}$ ), after one month growth; (c) Rooting of embryonic cotyledons cultured in WPM medium added with NAA (1.5  $\mu\text{M}$ ) and BAP (0.44  $\mu\text{M}$ ) after one month growth.

**Table 3.** Effect of BAP combined with NAA (1.5  $\mu\text{M}$ ) on the morphogenesis of carob embryonic cotyledons, after one month culture.

BAP ( $\mu\text{M}$ )	% of callogenic explants	% of callogenic and caulogenic explants	Number of buds by caulogenic explants	% of explants without reaction
0.44	86.67 a	0 e	0 e	13.33 b
1.33	76.22 c	7.92 c	1.87 $\pm$ 0.17 d	15.86 a
2.22	79.86 bc	10.04 c	2.50 $\pm$ 0.21 c	10.10 c
3.55	80.95 bc	14.28 b	4.32 $\pm$ 0.35 a	4.76 d
4.44	61.11 c	28.78 a	4.50 $\pm$ 0.43 a	11.11 c
6.66	85.31 ab	14.59 b	3.02 $\pm$ 0.28 bc	0 e

Means in the same column followed by the same letters are not significantly different from each other according to the Duncan's multiple range test ( $p < 0.05$ ).

### 3.1.2. Effect of Embryonic Cotyledons Position

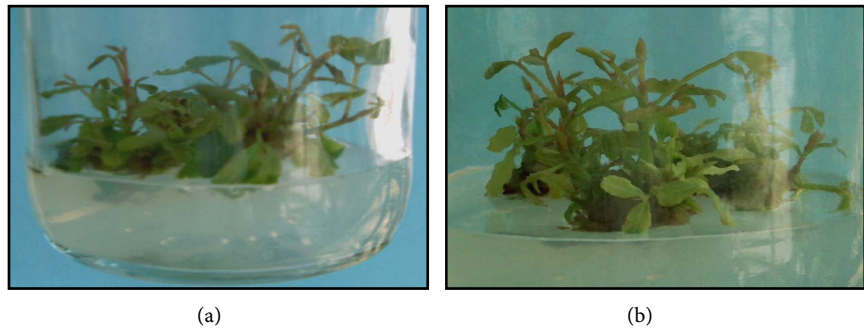
The position of embryonic cotyledons does not have a remarkable effect on their development (Table 4). Generally, the values obtained are close, with a slight preference for the vertical position, especially for the number of buds by explant, significantly higher at 4.44  $\mu\text{M}$ .

## 3.2. Multiplication of Shoots Obtained from Adventitious Buds

### 3.2.1. Effect of Macronutrients MS and WPM

The size of shoots varies according to the macronutrients used and the concentration of BAP (Table 5, Figure 4). In WPM medium, the elongation of shoots is maximal at 4.44 and 6.66  $\mu\text{M}$  (1.5 mm), and a similar value without significant difference is obtained at 2.22  $\mu\text{M}$  with MS medium.

The neoformation of shoots and leaves seems to be more favorable on MS medium, especially with BAP 2.22  $\mu\text{M}$ . Similar values are obtained on WPM



**Figure 4.** Effect of macronutrients MS and WPM. (a) Shoots of one month, obtained from adventitious buds deriving from embryonic cotyledons, on MS medium added with BAP (2.22  $\mu\text{M}$ ); (b) Shoots of one month, obtained from adventitious buds deriving from embryonic cotyledons, on WPM medium added with BAP (2.22  $\mu\text{M}$ ).

**Table 4.** Effect of two positions (vertical V and horizontal H) on the growth and development of embryonic cotyledons, cultured in WPM medium in presence of NAA (1.5  $\mu\text{M}$ ) and BAP.

BAP ( $\mu\text{M}$ )	Position	% of callogenic explants	% of callogenic and caulogenic explants	Number of buds by caulogenic explant	% of explants without reaction
1.33	V	73.58 b	8.80 bc	2.31 $\pm$ 0.21 cd	17.62 a
	H	73.35 b	7.78 c	1.65 $\pm$ 0.15 de	18.72 a
2.22	V	72.56 b	13.81 b	3.66 $\pm$ 0.35 b	13.64 b
	H	80.34 a	9.64 bc	2.70 $\pm$ 0.28 c	10.02 c
4.44	V	58.10 c	30.79 a	5.10 $\pm$ 0.43 a	11.10 c
	H	60.60 c	29.21 a	1.17 $\pm$ 0.10 e	10.20 c

Means in the same column followed by the same letters are not significantly different from each other according to the Duncan's multiple range test ( $p < 0.05$ ).

**Table 5.** Effect of two basic medium (MS and WPM) on carob shoot multiplication from adventitious buds after 30 day growth.

	BAP ( $\mu\text{M}$ )	Shoots size (mm)	Shoots number	Number of leaves by shoot	% of poorly developed shoots (<5 mm)
WPM	2.22	12.2 $\pm$ 1.1 c	3.25 $\pm$ 0.31 c	2.46 $\pm$ 0.24 c	0
	4.44	14.5 $\pm$ 1.2 ab	3.34 $\pm$ 0.34 c	3.83 $\pm$ 0.32 a	0
	6.66	15.1 $\pm$ 1.3 a	4.00 $\pm$ 0.38 ab	3.90 $\pm$ 0.35 a	0
MS	2.22	15.7 $\pm$ 1.3 a	4.80 $\pm$ 0.45 a	3.30 $\pm$ 0.34 b	0
	4.44	13.5 $\pm$ 1.1 bc	4.52 $\pm$ 0.44 a	3.01 $\pm$ 0.31 b	0
	6.66	12.1 $\pm$ 1.1 c	3.39 $\pm$ 0.40 ab	3.75 $\pm$ 0.32 a	0

Means in the same column followed by the same letters are not significantly different from each other according to the Duncan's multiple range test ( $p < 0.05$ ).



medium at a high concentration of BAP, however, some physiological disorders were observed, such as the formation of lenticels or the vitrification of explants.

### 3.2.2. Effect of Growth Regulators

#### 1) Effect of GA<sub>3</sub>

The combination of GA<sub>3</sub> with BAP does not influence strongly shoot growth. Values registered are generally lower than those obtained with BAP alone. Nevertheless, neoformation of shoots and leaves is improved and the best results (6.13 and 3.94, respectively) are obtained with 2.02 μM of GA<sub>3</sub> (Table 6, Figure 5).

#### 2) Effect of auxins

The combination of auxins (IAA, IBA and NAA) does not improve significantly shoot elongation. Furthermore, the neoformation of shoots seems to be globally inhibited by the addition of auxins, especially in the case of NAA and 2,4-D, while leaf neoformation increases slightly (Table 7).



**Figure 5.** Shoots of one month obtained from adventitious buds deriving from embryonic cotyledons, on MS medium added with GA<sub>3</sub> (2.02 μM) and BAP (2.22 μM).

**Table 6.** Effect of GA<sub>3</sub> combined with BAP (2.22 μM) on carob shoot proliferation from adventitious buds after 30 day growth on MS medium.

GA <sub>3</sub> (μM)	Shoots size (mm)	Shoots number	Number of leaves by shoot	% of shoots poorly developed
0	15.1 ± 1.4 a	4.80 ± 0.50 b	3.29 ± 0.30 c	0
0.29	11.7 ± 1.1 c	4.68 ± 0.47 b	4.16 ± 0.38 a	0
0.58	12.1 ± 1.1 bc	4.84 ± 0.45 b	3.65 ± 0.33 b	0
1.44	13.2 ± 1.2 bc	4.99 ± 0.52 b	3.53 ± 0.34 b	0
2.02	14.5 ± 1.3 ab	6.13 ± 0.58 a	3.94 ± 0.35 a	0
2.89	13.0 ± 1.2 bc	5.08 ± 0.46 b	3.62 ± 0.34 b	0

Means in the same column followed by the same letters are not significantly different from each other according to the Duncan's multiple range test ( $p < 0.05$ ).

**Table 7.** Effect of four auxins at 0.5  $\mu\text{M}$  combined with BAP (2.22  $\mu\text{M}$ ) on carob shoot multiplication from adventitious buds after 30 day growth on MS medium.

Auxin	Shoots size (mm)	Shoots number	Number of leaves by shoot	% of shoots poorly developed
0	15.2 $\pm$ 1.3 ab	4.76 $\pm$ 0.47 a	3.36 $\pm$ 0.29 b	0
IAA	15.3 $\pm$ 1.5 ab	3.67 $\pm$ 0.29 bc	3.70 $\pm$ 0.35 a	0
IBA	15.4 $\pm$ 1.4 ab	4.21 $\pm$ 0.38 ab	2.91 $\pm$ 0.28 c	0
NAA	16.3 $\pm$ 1.5 a	3.00 $\pm$ 0.26 c	3.71 $\pm$ 0.36 a	0
2,4-D	13.3 $\pm$ 1.2 b	1.35 $\pm$ 0.32 d	3.65 $\pm$ 0.55 a	0

Means in the same column followed by the same letters are not significantly different from each other according to the Duncan's multiple range test ( $p < 0.05$ ).

### 3.3. Shoot Elongation

Shoot elongation is mostly promoted by BAP at 1.33  $\mu\text{M}$ , but increasing the concentration has a negative effect on their growth. Besides, the addition of  $\text{GA}_3$  does not improve shoot size. Moreover, an acceleration of growth is observed between the second and the third week, what is translated by a steep slope, which tends to dampen after this period (**Figure 6** and **Figure 7**).

### 3.4. Shoot Rooting and Acclimatization of Plantlets

#### 3.4.1. Shoot Rooting

Induction of rooting in the presence of IAA and IBA shows that this latter is the most favorable, especially in 10  $\mu\text{M}$ , with a maximum percentage of 70%. However, the maximum size of roots is obtained at 5  $\mu\text{M}$  of IBA (**Table 8**, **Figure 8**).

#### 3.4.2. Acclimatization of Plantlets

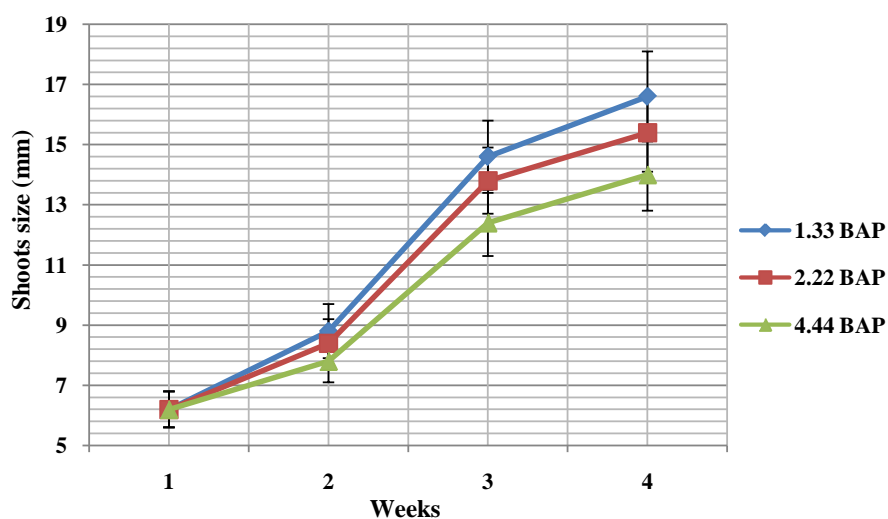
The acclimatization of the resulted plantlets in the peat is difficult; they often wither during the first week. The success rate did not exceed 40% (**Figure 9**).

## 4. Discussion

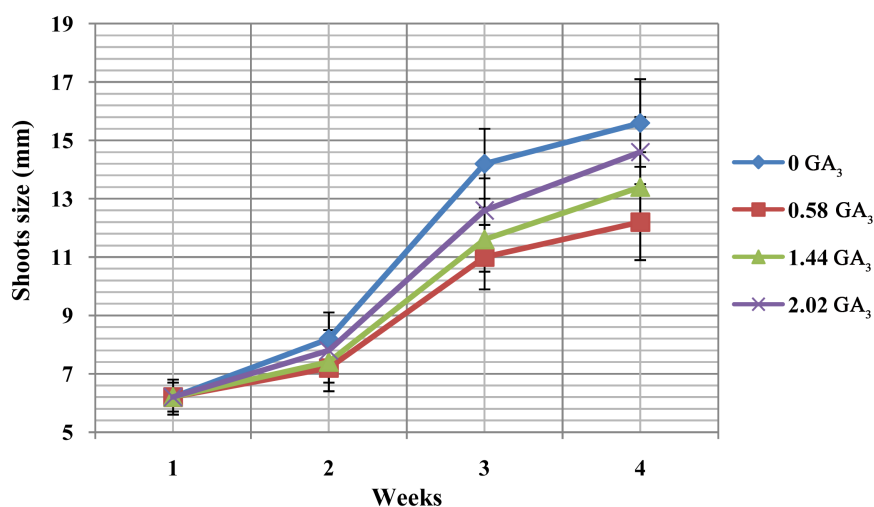
The culture of embryonic cotyledons of carob tree, established only recently [9], offers a new way of micropropagation. Shoots obtained after multiplication phase are very similar to those obtained by apex culture or by cotyledonary buds culture.

After culturing, embryonic cotyledons, with reduced size (about 7 mm), undergo a considerable increase (about 20 mm). Generally, a callus develops in the contact of explants with culture medium. The development of adventitious buds is made anarchically within the culture medium. In rare cases, a neoformation of roots is noticed. The percentage of callogenic explants is often high (74.2% - 97.48%), while that of callogenic and caulogenic explants is globally low (0% - 27.86%).

Among the studied auxins, in different concentrations and combined with



**Figure 6.** Effect of the concentration of BAP on the elongation of shoots obtained from adventitious buds.

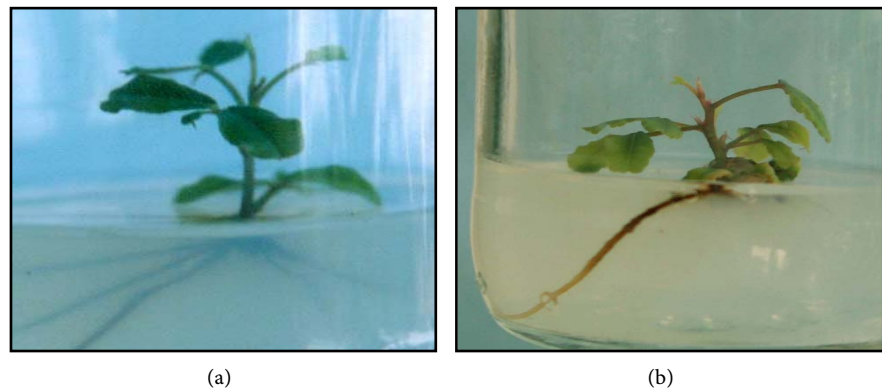


**Figure 7.** Effect of GA<sub>3</sub> combined with BAP (1.33 μM) on the elongation of shoots obtained from adventitious buds.

**Table 8.** Effect of IAA and IBA on carob shoot rooting after one week under dark on ½ MS medium, added with auxins (IAA and IBA at 5 and 10 μM) and their transfer to ½ MS without auxins, under light.

Auxins (μM)	Shoots size (mm)	Number of stems by shoot	Number of leaves by shoot	% of rooting	Number of roots by plantlet	Length of roots (mm)	
IAA	5	10.8 ± 1.1 b	1.00 ± 0.0 b	4.58 ± 0.36 b	32.45 c	1.97 ± 0.20 b	28.34 ± 2.33 c
	10	10.4 ± 0.9 b	1.00 ± 0.0 b	3.20 ± 0.29 c	25.80 d	1.50 ± 0.45 b	25.62 ± 2.54 c
IBA	5	12.8 ± 1.1 a	1.00 ± 0.0 b	4.35 ± 0.37 b	56.20 b	3.22 ± 0.27 a	43.54 ± 3.87 a
	10	13.9 ± 1.2 a	2.45 ± 0.2 a	6.50 ± 0.54 a	70.37 a	4.33 ± 0.36 a	35.85 ± 2.65 b

Means in the same column followed by the same letters are not significantly different from each other according to the Duncan's multiple range test ( $p < 0.05$ ).



**Figure 8.** Rooting phase. (a) Rooted plantlet obtained one month after rooting induction in darkness, on  $\frac{1}{2}$  MS added with  $10 \mu\text{M}$  IBA; (b) Rooted plantlet obtained one month after rooting induction in darkness, on  $\frac{1}{2}$  MS added with  $5 \mu\text{M}$  IBA.



**Figure 9.** Plantlet obtained after three month acclimatization in peat substrate.

BAP ( $4.44 \mu\text{M}$ ), the neoformation of adventitious buds is mostly favored by NAA at  $1.5 \mu\text{M}$ , contrary to 2,4-D, that favors callogenesis (reaching 97.48% at  $2.5 \mu\text{M}$ ). Indeed, among various types of auxins, 2,4-D is employed in callus induction [16] [17]. Custódio *et al.* (2004) [18] obtained the best result in terms of calli induction from anthers, with the higher tested concentration ( $2.26 \mu\text{M}$ ). Also, Carimi *et al.* (1997) [19] demonstrated that concentrations ranging from  $0.45$  to  $4.52 \mu\text{M}$  promote a good development of callus on cultured ova. Therefore, 2,4-D could induce callogenesis, while NAA could be used to induce both callogenesis and caulogenesis. We also want to point out that the effectiveness of BAP in cotyledonary nodes culture was well indicated and produced better results in multiplication of shoots. However, shoot tips, hypocotyls, cotyledonary leaves and roots produced only callus which were non-regenerative [9].

The combination of NAA ( $1.5 \mu\text{M}$ ) with other concentrations of BAP does not improve the percentage of caulogenic explants. The obtained results are less

important than those found with 4.44  $\mu\text{M}$ ; beyond this concentration, BAP seems to have an inhibitory effect on the neoformation of adventitious buds. The inhibitory effect of high concentration of BAP was observed in other studies: Belaizi *et al.* (1995) [20] noticed that during the study of carob regeneration from lateral buds, concentrations higher than 4.44  $\mu\text{M}$  does not increase the number of broken buds. As well, Romano *et al.* (2002) [6] affirm that the best multiple shoot response was obtained in MS medium supplemented with 4.44  $\mu\text{M}$  BAP, for in vitro propagation protocol based on axillary bud proliferation.

Also, survival of meristems reaches the maximum with 8.88  $\mu\text{M}$  BAP and decreases in a higher concentration [21]. However, Radi *et al.* (2013) [22] found that MS medium added with 8.88  $\mu\text{M}$  BAP affects significantly the size of shoots obtained from lateral buds and gives the best results among lower concentrations.

The position of embryonic cotyledons on culture medium does not have a considerable effect on their development. The area in contact with the medium reacts by initiating small clusters of callus that will end by forming a big calli accompanied or not by adventitious buds.

Concerning the multiplication and elongation of shoots deriving from adventitious buds, WPM and MS mediums give close results, even if the hormonal balance used is different. The maximum of shoots and leaves is obtained in WPM added with 6.66  $\mu\text{M}$  BAP (4 and 3.9, respectively), while close values are obtained in MS medium added with 2.22  $\mu\text{M}$  BAP (4.8 and 3.3, respectively). Gharnit and Ennabili (2009) [23] showed that WPM, in comparison with MS and Gresshoff and Doy (GD) [24], added with 0.44  $\mu\text{M}$  BAP is the most favorable for the development of explants from shoot tip cultures, with 100% production of buds, a low rate of apical necrosis and callogenesis.

The addition of  $\text{GA}_3$  at 2.02  $\mu\text{M}$  to BAP (2.22  $\mu\text{M}$ ) increases slightly the number of shoots and leaves. Thus,  $\text{GA}_3$  could be added to BAP during shoot multiplication. In fact, Sebastian and McComb (1986) [25] affirmed that addition of  $\text{GA}_3$  (2.5  $\mu\text{M}$ ) in the shoot multiplication medium inhibited subsequent rooting. This inhibition was partially overcome by a passage in a medium added with zeatin (5  $\mu\text{M}$ ) and without  $\text{GA}_3$ . As well, multiple shoots, developed using axillary buds, were regenerated when explants were cultured in MS medium, supplemented with 6.66  $\mu\text{M}$  BAP and 1.44  $\mu\text{M}$   $\text{GA}_3$  [26]. Furthermore, bud break via lateral budding, was ensured with BAP and improved with 2.22  $\mu\text{M}$  BAP combined with 1.44  $\mu\text{M}$   $\text{GA}_3$  [27]. Belaizi *et al.* (1995) [20] also obtained good results when combining BAP and  $\text{GA}_3$ , but high concentrations of BAP causes some physiological disorders, like shoot vitrification, apical necrosis, appearance of lenticels and reduction in leaf size.

The combination of auxins with BAP does not improve shoot multiplication. However, auxins like IAA and IBA, in low concentration (0.57 and 0.49  $\mu\text{M}$ , respectively), have been already used, combined with several concentrations of BAP, and sometimes with  $\text{GA}_3$ , with good results when culturing microshoots

from a tree [28] and using micropropagation by shoot tip cultures [29]. Other studies proved also that high concentrations of auxins combined with BAP and GA<sub>3</sub> give good results in the culture of young buds [26], as well as for apical buds break from a mature tree [21]. Radi *et al.* (2013) [22] observed that the best effect of the combination of auxins and BAP was obtained with 2.22 BAP and 2.46 μM IBA, 2.68 NAA, and 2.58 IAA, but the combination of 2.22 μM BAP, 2.46 μM IBA and 1.44 μM GA<sub>3</sub> induced shoot multiplication and also provided rooting of shoots.

Actually, an elongation phase of shoots was added to favor the rooting phase, as in vitro propagation of some other tree species [30] [31]. According to Belaizi *et al.* (1989) [32], this step is optional and is only required for very small shoots in order to obtain elongated leafy stems of *Pyros malus* which are then used for rooting. Moreover, after the multiplication phase, small shoots can be directly placed on a rooting medium, but it was proved that the success of this phase depended on the size of the explant. Thus, very short stems (2 to 3 mm), placed on a rooting medium containing IBA, showed necrosis very frequently, also, the growth of those who developed roots was blocked subsequently [33]. The same observations were also made by Boxus and Quoirin (1974) [34], IBA applied too early to buds can irreversibly inhibit their elongation. However, after the multiplication phase, the length of new shoots is very variable. In some clones, some buds eventually lengthen after several subcultures and can then be rooted. However, this elongation seems to occur randomly and in most cases, the young shoots do not exceed 5 mm in length. It is therefore necessary to find a medium allowing the elongation of shoots obtained during the propagation phase, so that they can react to the rhizogenic treatments and then have a normal development [33].

Initiation of shoot rooting after multiplication phase is mostly favored by IBA (10 μM) in half strength MS medium, which is in agreement with several other studies [6] [35]. Actually, in many woody plants, IBA is commonly used to promote root initiation [36] [37].

Nevertheless, several authors have shown that auxins are only required during the initiation phase, and become inhibitory for root growth [38] [39] [40]. Also, the effect of mother tree age on rooting of carob shoots was demonstrated: rooting capacity of micro-shoots originated from juvenile parts of mature trees is the best [41]. In some other references, it was reported that carob shoots obtained from shoot tips cultures can root without auxins [23] [29] [42]. Shahzad *et al.* (2017) [9] reported that IBA promoted rhizogenic response in shoots.

## 5. Conclusion

The results of the present study demonstrate that carob can be cultured in vitro, starting from embryonic cotyledons and going through adventitious buds that develop stems, leaves and roots, to be finally acclimatized with a survival rate of 40%.

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