

Studies on Carob (*Ceratonia siliqua* L.) Propagation

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Abstract: Carob (*Ceratonia siliqua* L.) is an environmentally and economically important tree and is among the most difficult to propagate fruit species. In this study, efforts were made to propagate the plant by using three different methods; seeds germination, cuttings and micropropagation. Seeds and cuttings were scarified and disinfected under aseptic conditions to improve the germination percentage and the percentage of success of cuttings in April and September through two successive seasons (2011 and 2012). Results showed that the highest values of seed germination percentage, the fastest germination, the greatest plant length, number of leaves/plant, root length and dry weight were obtained by soaking seeds in 60% H₂SO₄ in April and September through the two seasons, but there was no significant difference between the first and the second date in both studied seasons for most of the studied parameters. April was found the best time for propagation of Carob by cuttings, for all treatments. Besides, carob cuttings treated with indole-3-butyric acid (IBA) at 8000 mg/l + β -naphthalene acetic acid (NAA) at 200 mg/l gave the highest values of parameters (shoot length, no. of lateral shoot, no. of leaves/plant, root length, no. of roots/plant and root dry weight) followed by IBA at 6000 mg/l + NAA at 200 mg/l, in both seasons. While, all cuttings failed to root for all treatments in September, except when they were treated with IBA at 6000 mg/l + NAA at 200 mg/l and IBA at 8000 mg/l + NAA at 200 mg/l, in the two studied seasons. Micropropagation of carob was developed by using shoot tips and stem node segments, of *ex vitro* grown seedlings, as explants. Hundred % of growth induction was obtained, for both explants, with the highest number of shoots on Murashige and Skoog (MS) medium containing 0.4 mg/l NAA + 2 mg/l 6-benzylaminopurine (BAP). Shoots were best multiplied on MS medium containing BAP (1.0 mg/l), while the media containing N⁶-(2-isopentenyl) adenine (2iP) gave the highest lengths of shoots (2iP at 1.0 mg/l). For further elongation and multiplication of shoots, the combination of both BAP and 2iP gave promising results at concentrations of 0.5 mg/l BAP + 0.5 mg/l 2iP or 1.0 mg/l BAP + 0.5 mg/l 2iP. Attempts to achieve rooting were unsuccessful, since only shoot development was observed.

Key Words: Carob, propagation, germination, cuttings, micropropagation.

Abbreviations: BAP, 6-benzylaminopurine; GA₃, gibberellic acid; IBA, indole-3-butyric acid; 2iP, N⁶-(2-isopentenyl) adenine; MS, Murashige and Skoog; NAA, β -naphthalene acetic acid; TDZ, Thidiazuron; PGRs, plant growth regulators

I. Introduction

Carob tree (*Ceratonia siliqua* L.) belongs to family Fabaceae. It is a long-lived evergreen and thermophilous fruit and forms an important part of Mediterranean vegetation (Battle and Tous, 1997). Carob is native to the eastern Mediterranean, probably the Middle East, where it has been in cultivation for at least 4000 years. It grows well in the Mediterranean basin, and some parts of Western Asia and the Middle East. The tree is well adapted to mild and dry areas with poor soils. It is often interplant with olives, grapes, almonds, and barley in low intensive farming system. It grows well in warm temperate and subtropical areas. It tolerates adverse conditions, drought, and alkali soils and is extremely tolerant to heat and dry atmosphere. They are used naturally against soil erosion and land reclamation in most Mediterranean areas (Janick and Paull, 2008).

Carob is well known in the Mediterranean countries for its ornamental, nutritional and medicinal value (Battle and Tous, 1997). Carob pods were also used in ancient Egypt, where the pulp of the pods was mixed in porridge, with a little honey, and wax as a treatment for diarrhea and some other diseases. The trees are also useful as ornamentals and for landscaping, windbreaks and a forestation. Cattle can browse on leaves and the wood is suitable for fuel (Shereen et al., 2011). Annual world production of Carob pods mainly from Mediterranean countries including Spain, Italy and Portugal reaches 180.000 tons. As an origin country of Carob, Turkey produces around 14.000 tons Carob annually (Anonymous, 2009).

Carob trees may be male, female and hermaphrodite or play gummous inflorescences, showing high plasticity in inflorescences and flowering characteristics. Female cultivars are the most important trees commercial groves of Mediterranean countries (Laubscher and Ndakidemi, 2008).

Carob may be propagated by seeds, budding, grafting or cutting (Romano et al., 2002), and has been described by Lee et al. (1977) and Hartman and Kester (1983) as one of the most difficult species to root. Carob seeds are difficult to germinate. The seed coat is extremely hard and not ready to absorb water (Gubbuk et al., 2011). Alorda et al. (1987), Cabrita et al. (1988), Martins-Louçao et al. (1996) and Piotto and Di Noi (2003) showed that the best pre-sowing treatments for enhancing Carob seeds germination were chemical scarification with acids, treatment with warm water and scarification with the appropriate machines (mechanical scarification). Esma et al. (2009) study evaluated the effect of different H₂SO₄ concentrations on seed germination. Seeds, obtained from wild types of Carob were used as the experimental material. In this respect, scarified Carob seeds were treated with different H₂SO₄ concentrations (control, 10, 20, 40, 60, 80 and 98%) for 30 minutes in petri dishes at 26°C and placed in the plant growth chamber. The results of this study indicated that the lowest germination rate was 22.92% in the control treatment followed by 23.61% by soaking the seeds in 60% H₂SO₄. The highest germination rate (93.06%) was obtained with the treatment of 98% H₂SO₄ for 30 minutes.

The art of propagation by cuttings is a very old technique popular in the field of horticulture. However, there are many species, which are difficult to root. In recent years, many such difficult to root species are made to root easily by use of root inducing chemicals and modifying the surrounding environment. Such attempts were successful to get higher rooting percentage and early rooting. The rooting generally affected by internal and external factors; i.e. type, date of taking and growth regulators application (Hartman et al., 2002, Manan et al., 2002; Gerakakis and Ozkaya, 2005; Laubscher and Ndakidemi, 2008; Mohy, 2009 and Sayed et al., 2010). Plastic tunnels with water mist gave the best rooting and vegetative growth followed by the cutting under plastic tunnels without mist in *Hamelia patens* (Elgimabi, 2009).

The use of micropropagation technique can be the solution to solve propagation problems in Carob and to faster its propagation (Gubbuk et al., 2011). It is also considered a technique for the preservation of plant genetic material, the development of plant breeding programs and the fast production of large quantities of propagation material (George, 1993). Efforts were made previously to micropropagate Carob tree from various explants; for example from seeds (Sebastian and McComb, 1986), explants from suckers of female trees (El-Shafey et al., 1998), explants of mature trees of Galhosa and Mulata cultivars (Romano et al., 2002) or explants from *in vitro* germinated seedlings (Hakim et al., 2010 and Radi et al., 2013). However, little data are reported about micropropagation of carob in Egypt. Romano et al. (2002) reported that the composition of medium changed with the cultivar studied, which required an optimization in the composition of the culture medium used for the micropropagation of Carob coming from different geographical areas.

Therefore, the aim of this study was to optimize easy and reliable methods for rapid propagation of the Egyptian Carob (*Ceratonia siliqua* L.) by seeds, cuttings and by micropropagation. Also, to study the effect of date of Carob propagation by seeds or cuttings and some soaking or dipping treatments to overcome the difficulties of its propagation. This study reports, also, a trial for micropropagating Carob from seedling's explants, in order to fulfill the increasing demand for propagating this tree in Egypt.

II. Materials and Methods

This investigation was conducted through two seasons of 2011 and 2012 on Carob. The mature Carob seeds and cuttings were collected from a female tree grown at the Experimental Orchard Station of Horticulture Research Institute at Giza, Egypt. Seeds and cuttings were scarified and disinfected under aseptic conditions with some treatments to improve the germination percentage and the percentage of success of cuttings.

This investigation included three main experiments:

1. Seeds Germination

In a split plot design, the effect of two different dates of culture (1st of April and 1st of September), as a first factor, and some soaking and dipping treatments, as a second factor, on Carob seeds germination, were studied. The following treatments were applied to the seeds:

- 1- Control (untreated seeds).
- 2- Soaking in the water at 30°C for 72 h.
- 3- Soaking in hot water for 10 min, then in tap water at room temperature for 48 h.
- 4- Dipping in 60% H₂SO₄ for 30 min. The seeds were then washed in running water for 1 h to remove any traces of acid, before being tested for germination.
- 5- Dipping in GA₃ solution (25 mg/l) for 24 h.
- 6- Dipping in 80% acetone.

All seeds were tested for germination by using four replicates (each contained 20 seeds).

Measurements

The germination percentage (%), germination time (day), plant length (cm), number of leaves/plant and root length (cm) were recorded and roots after drying at 80°C were weighed.

2. Vegetative propagation by cuttings

In a split plot design, the effect of two different dates of culture (1st of April and 1st of September), as a first factor, and some growth regulators, as a second factor, on vegetative propagation by cuttings were studied. The following treatments were applied to the cuttings:

- 1- Control (dipping in water only).
- 2- Dipping in IBA at 6000 mg/l.
- 3- Dipping in IBA at 8000 mg/l.
- 4- Dipping in IBA at 6000 mg/l + NAA at 100 mg/l.
- 5- Dipping in IBA at 8000 mg/l + NAA at 100 mg/l.
- 6- Dipping in radecant contains 80% NAA.

After dipping in the previous treatments, cuttings were then cultivated in pots under a tunnel. Four replicates were prepared per treatment (ten stem cuttings for each replicate).

Measurements

Shoot length (cm), number of lateral shoots, number of leaves/plant, root length (cm) and number of roots/plant were recorded after 3 months of planting and roots after drying at 80°C were weighed.

3. Micropropagation

Plant material and sterilization

Shoot tips and stem node segments of Carob were collected from two months old seedlings grown in the greenhouse (from the first experiment). The explants were washed under running tap water followed by a detergent for 5 min. Explants were immersed in an antioxidant solution containing 200 mg/l of both citric and ascorbic acid and kept in a refrigerator for 1 h, to overcome the phenolic exudates. Surface sterilization was done by dipping both shoot tips and stem node segments for 20 min in 20 and 30% (v/v) commercial bleach solution (Clorox) (containing 3% sodium hypochlorite), respectively. Finally explants were rinsed thoroughly with six changes of sterile distilled water. Explants were cultured vertically on sterile nutrient medium.

Culture medium and conditions

Explants were cultured on Murashige and Skoog (MS) basal medium (Duchefa, Haarlem, the Netherlands) (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and gelled with 2.5 g/l phytigel (Duchefa, Haarlem, the Netherlands). Different plant growth regulators (PGRs) – cytokinins (BAP, 2iP and TDZ) and auxins (NAA and IBA) (Sigma Cell Culture, min. 90%, St. Louis, USA), at different concentrations (mg/l) – either individually or in combination, were added to the medium to optimize hormonal requirements for bud sprouting, multiple shoot induction and rooting, in addition to the control medium without PGRs. The pH of the medium was adjusted to 5.7-5.8 before autoclaving at a pressure of 1.06 Kg cm⁻² and 121°C for 15 min. All cultures were incubated in a culture room at 25 ± 2°C with a 16-h photoperiod under cool white fluorescent tubes (F140t9d/38, Toshiba). At least 10 cultures were raised for each treatment and data were scored after 8 weeks of culture.

Culture establishment, multiplication, elongation and rooting

Various concentrations of BAP, 2iP or TDZ (0.5, 1.0 and 2.0 mg/l), in addition to 0.25 mg/l TDZ, in combination with NAA (0.4 mg/l) were added to the MS medium for the establishment and bud break. For multiplication of shoots, MS medium was supplemented with BAP or 2iP (0.5, 1.0, 2.0 and 3.0 mg/l), or in combination to enhance both multiplication and elongation.

The percentage of explants initiating growth, the mean number of shoots/explant and mean shoot length (cm) were measured after 8 weeks of culture. The micropropagation cycle consisted of the regular subculture of produced shoots onto fresh medium.

Shoots regenerated from the multiplication stage were excised and transferred to MS medium supplemented with different concentrations of IBA (0.5, 1.0, 2.0 and 4.0 mg/l), either individually or in combination with NAA at a concentration of 0.5 mg/l, for *in vitro* root induction.

Statistical analysis of data

Analysis of variance (ANOVA) and Duncan's multiple range test (Duncan, 1955), as modified by Snedecor and Cochran (1998), were performed to analyze the obtained data. The differences among means of

the recorded parameters for all treatments were tested for significance at 5% level. Means followed by the same letter are not significantly different at $P \leq 0.05$.

III. Results and Discussion

1. Seeds germination

The Carob seeds like those of the majority of legumes have germination difficulty. This is mainly due to an inhibition of seeds coat, because it is extremely hard and not ready to absorb water and consequently affects the germination. Seed germination percentage was significantly affected by all pre-sowing treatments in April as a first date and September as a second date in both studied seasons, 2011 and 2012 (Figures 1 and 2). The highest values of seed germination percentage were obtained with soaking seeds in 60% H_2SO_4 in April and September in both studied seasons, their values was recorded as 91.22 and 91.63%, respectively, followed by the seeds that were treated with 25 mg/l GA_3 (78.50 and 78.55%) and hot water treatment (76.03 and 77.21%), as compared with the control (25.69 and 25.43%), in the first and second seasons, respectively.

On the other hand, the relationship between time of germination on Carob seeds and soaking treatments was shown in Figures 3 and 4. Seed germination time was significantly affected by all pre-sowing treatments (cold water, hot water, 60% H_2SO_4 , 25 mg/l GA_3 and acetone) in the first (April) and second (September) dates of germination, in both studied seasons. In this respect, the fastest germination was recorded with 60% H_2SO_4 (germination occurred after 7.24 and 7.20 days), followed by 25 mg/l GA_3 (germination occurred after 8.07 and 7.92 days) and then the hot water (germination occurred after 8.50 and 8.77 days) in the first and second seasons, respectively. All these treatments were markedly higher than the control and other treatments. The longest germination time was 11 days for the control treatment (without any additives) in both studied seasons.

Results in Table 1 showed that all treatments were significantly different in plant length, number of leaves/plant, root length and root dry weight from the control, in both studied seasons. Besides, it is clear that there was no significant difference between first and second dates of germination (April and September) with respect to all studied parameters except for the number of leaves/plant in 2012 and the plant length in both seasons (2011 and 2012). Moreover, the interaction between soaking treatments and culture date showed significant differences in plant length, number of leaves/plant, root length and root dry weight, as compared with the control, in both the first and second seasons.

The greatest number of leaves/plant and plant length were found with 60% H_2SO_4 treatment in both 2011 and 2012 seasons (8.12, 8.50 and 18.00, 20.50 cm, respectively), followed by 25 mg/l GA_3 (4.87, 4.75 and 16.25 cm, respectively). However, the maximum root dry weight and length, in the two seasons, were obtained with the same treatments (7.29, 7.18 and 3.79, 3.77 cm, respectively), followed by 25 mg/l GA_3 (6.33, 6.31 and 3.29, 3.38 cm, respectively), as compared with the control and other treatments of the seeds (Table 1).

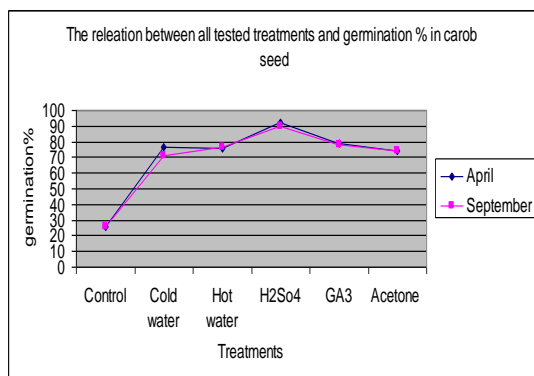


Figure 1. The effect of different pre-sowing treatments on the germination percentage of Carob seeds in 2011 seasons.

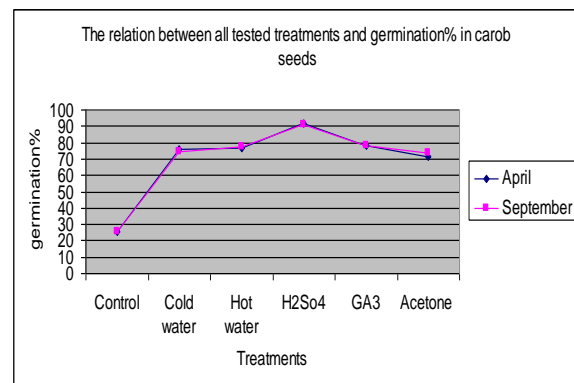


Figure 2. The effect of different pre-sowing treatments on the germination percentage of Carob seeds in 2012 seasons.

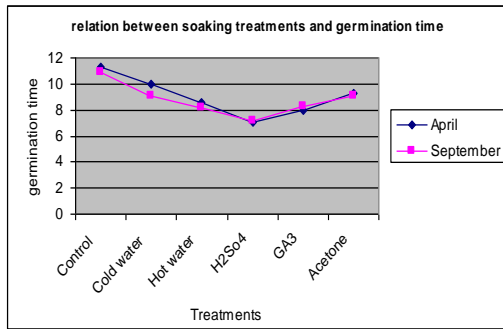


Figure 3. The effect of different pre-sowing treatments on the germination time of Carob seeds in 2011 seasons.

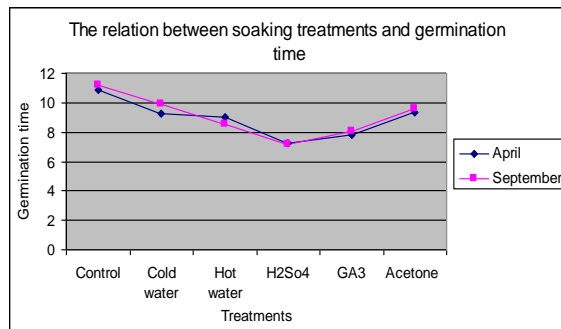


Figure 4. The effect of different pre-sowing treatments on the germination time of Carob seeds in 2012 seasons.

Table 1. Effect of some pre-sowing treatments and time of propagation by seeds on number of leaves/plant, plant length, root length and root dry weight of carob, during 2011 and 2012 seasons.

Seasons \ Treatments	2011			2012			2011			2012		
	April	Sep.	Mean	April	Sep.	Mean	April	Sep	Mean	April	Sep.	Mean
No. of leaves/plant												
Control	3.75 ^d	3.50 ^d	3.62 ^c	4.75 ^c	4.50 ^c	4.62 ^b	14.25 ^c	15.25 ^{bc}	14.75 ^C	14.50 ^d	14.50 ^d	14.50 ^c
Cold water	4.25 ^c	4.50 ^c	4.37 ^b	4.75 ^c	5.25 ^b	5.00 ^b	13.00 ^d	13.50 ^d	13.25 ^d	15.75 ^d	14.00 ^d	13.37 ^d
Hot water	5.25 ^b	4.00 ^c	4.62 ^b	4.00 ^c	4.75 ^c	4.37 ^b	12.00 ^c	12.75 ^{de}	12.37 ^d	12.25 ^f	11.25 ^f	11.75 ^e
H ₂ SO ₄	7.50 ^a	8.75 ^a	8.12 ^a	8.75 ^a	8.25 ^a	8.50 ^a	15.75 ^{bc}	20.25 ^a	18.00 ^a	18.50 ^b	22.50 ^a	20.50 ^a
GA ₃	4.00 ^c	5.75 ^b	4.87 ^b	3.50 ^d	6.00 ^b	4.75 ^b	15.75 ^{bc}	16.75 ^b	16.25 ^b	16.25 ^c	16.25 ^c	16.25 ^b
Acetone	4.25 ^c	4.25 ^c	4.25 ^b	4.00 ^b	4.50 ^c	4.25 ^b	13.50 ^d	15.75 ^{bc}	14.62 ^C	15.00 ^d	16.00 ^c	15.50 ^b
Mean	4.83 ^a	5.12 ^a		4.96 ^b	5.54 ^a		14.04 ^b	15.71 ^a		15.37 ^b	15.75 ^a	
Root dry weight (g)												
Control	3.45 ^e	3.27 ^e	3.36 ^f	3.33 ^j	3.55 ⁱ	3.43 ^e	2.52 ^g	2.60 ^g	2.56 ^e	2.47 ^g	2.70 ^f	2.58 ^e
Cold water	4.61 ^e	4.22 ^f	4.42 ^e	4.25 ^g	4.04 ^h	4.14 ^d	3.30 ^c	2.95 ^d	3.12 ^c	3.12 ^d	2.80 ^f	2.96 ^c
Hot water	4.63 ^e	5.42 ^d	5.03 ^d	4.66 ^f	5.77 ^d	5.22 ^c	3.25 ^c	3.50 ^b	3.57 ^b	3.40 ^e	3.25 ^c	3.25 ^b
H ₂ SO ₄	7.06 ^b	7.41 ^a	7.29 ^a	7.49 ^a	6.88 ^b	7.18 ^a	3.85 ^a	3.75 ^a	3.79 ^a	3.75 ^a	3.82 ^a	3.77 ^a
GA ₃	6.43 ^c	6.21 ^c	6.33 ^b	6.33 ^c	6.29 ^c	6.31 ^b	3.10 ^d	3.47 ^b	3.29 ^b	3.25 ^c	3.52 ^b	3.38 ^b
Acetone	5.35 ^d	5.36 ^d	5.35 ^c	5.23 ^e	5.30 ^e	5.27 ^c	2.77 ^e	2.75 ^f	2.75 ^d	2.70 ^f	2.87 ^e	2.78 ^d
Mean	5.26 ^a	5.32 ^a		5.21 ^a	5.30 ^a		3.13 ^a	3.16 ^a		3.11 ^a	3.16 ^a	
Root length (cm)												
Control	3.45 ^e	3.27 ^e	3.36 ^f	3.33 ^j	3.55 ⁱ	3.43 ^e	2.52 ^g	2.60 ^g	2.56 ^e	2.47 ^g	2.70 ^f	2.58 ^e
Cold water	4.61 ^e	4.22 ^f	4.42 ^e	4.25 ^g	4.04 ^h	4.14 ^d	3.30 ^c	2.95 ^d	3.12 ^c	3.12 ^d	2.80 ^f	2.96 ^c
Hot water	4.63 ^e	5.42 ^d	5.03 ^d	4.66 ^f	5.77 ^d	5.22 ^c	3.25 ^c	3.50 ^b	3.57 ^b	3.40 ^e	3.25 ^c	3.25 ^b
H ₂ SO ₄	7.06 ^b	7.41 ^a	7.29 ^a	7.49 ^a	6.88 ^b	7.18 ^a	3.85 ^a	3.75 ^a	3.79 ^a	3.75 ^a	3.82 ^a	3.77 ^a
GA ₃	6.43 ^c	6.21 ^c	6.33 ^b	6.33 ^c	6.29 ^c	6.31 ^b	3.10 ^d	3.47 ^b	3.29 ^b	3.25 ^c	3.52 ^b	3.38 ^b
Acetone	5.35 ^d	5.36 ^d	5.35 ^c	5.23 ^e	5.30 ^e	5.27 ^c	2.77 ^e	2.75 ^f	2.75 ^d	2.70 ^f	2.87 ^e	2.78 ^d
Mean	5.26 ^a	5.32 ^a		5.21 ^a	5.30 ^a		3.13 ^a	3.16 ^a		3.11 ^a	3.16 ^a	

Physical dormancy is an adaptive trait, allowing seed germination over time and space, thus increasing the probability of enhancing the propagation of a species (Baskin and Baskin, 2000). Similar seed dormancy characteristics were reported in Carob by Pérez-García (2009). Seed dormancy can be broken by mechanical scarification and certain pretreatments such as H₂SO₄, cold water, hot water GA₃ and acetone treatments (El-Shatnawi et al., 2001; Karaguzel et al., 2002; Pérez-García, 2009; Piotto and Di Noi, 2003). All pretreatments enhanced the seed resulting in higher germination and good growth in Carob producing seedlings. Consequently, H₂SO₄, GA₃ and hot water beside other treatment was found to be superior in terms of germination percentage, average germination time, plant length, number of leaves/plant, root length and root dry weight. However, Pérez-García (2009) used various treatments to enhance seed germination in Carob and found the lowest germination rate in the control seeds and highest (99%) in scarified seeds. Similarly, Tsakalimi and Ganatsas (2001) found that the highest germination rate (87%) was found on seeds dipped in concentrated acid for 15 minutes but that the shortest average germination time (8 days) was observed with hot water soaking for 5 minutes. In this study, the highest germination percentage and fastest germination time was obtained with 60% H₂SO₄ treatment, but the least mean germination percentage and longest germination time was found using tap water as a control treatment. Piotto and Di Noi (2003) have demonstrated that if Carob seeds had been either soaked in hot water, or dipped in concentrated H₂SO₄ and then washed in water, or mechanically scarified, then there is no subsequent problem in terms of seed germination.

2. Vegetative propagation by cuttings

The effects of two dates (April and September) of culture, as a first factor, and concentration of PGRs, as a second factor, on vegetative propagation of Carob by using stem cuttings, under tunnel, were studied. Data illustrated in Table 2 showed great significant differences between treatments for all growth parameters (shoot length, number of lateral shoot, number of leaves/plant, root length, number of roots/plant and root dry weight) of Carob cuttings.

Table 2. Effect of some growth regulators and time of vegetative propagation by stem cuttings, on shoot length, no. of lateral shoot, no. of leaves/plant, root length, no. of roots/plant and root dry weight, of carob during 2011 and 2012 seasons.

Treatments	2011			2012			2011			2012		
	April	Sep.	Mean	April	Sep.	Mean	April	Sep.	Mean	April	Sep.	Mean
	Shoot length (cm)						No. of lateral shoots					
Control	0.00 [#]	0.00 [#]	0.00 ^c	0.00 [#]	0.00 [#]	0.00 ^c	0.00 [#]	0.00 [#]	0.00 ^c	0.00 [#]	0.00 [#]	0.00 ^d
6000 mg/l IBA	11.00 ^f	0.00 [#]	5.50 ^b	11.75 ^d	0.00 [#]	5.87 ^b	1.50 ^d	0.00 [#]	0.75 ^c	1.75 ^d	0.00 [#]	0.87 ^c
8000 mg/l IBA	13.00 [#]	0.00 [#]	6.50 ^b	13.00 [#]	0.00 [#]	6.50 ^b	1.75 ^d	0.00 [#]	0.78 ^c	2.00 ^d	0.00 [#]	1.00 ^c
6000 mg/l IBA + 200 mg/l NAA	17.25 ^e	16.25 ^b	16.75 ^a	15.75 ^b	16.75 ^b	16.25 ^a	3.50 ^b	5.00 ^a	4.25 ^a	4.00 ^c	4.75 ^b	4.37 ^b
8000 mg/l IBA + 200mg/l NAA	18.25 ^b	19.25 ^a	18.75 ^a	19.00 ^a	18.75 ^a	18.87 ^a	5.25 ^a	4.75 ^a	5.00 ^a	5.50 ^a	5.50 ^a	5.50 ^a
Radecant 80%	10.75 ^f	0.00 [#]	5.37 ^b	12.75 ^d	0.00 [#]	6.37 ^b	2.50 ^c	0.00 [#]	1.25 ^b	2.25 ^d	0.00 [#]	1.12 ^c
Mean	11.71 ^a	5.91 ^b		12.04 ^a	5.91 ^b		2.41 ^a	1.62 ^b		2.58 ^a		1.70 ^b
	No. of leaves plant						Root length (cm)					
Control	0.00 [#]	0.00 [#]	0.00 ^c	0.00 ^d	0.00 ^d	0.00 ^c	0.00 [#]	0.00 [#]	0.00 ^d	0.00 [#]	0.00 [#]	0.00 ^c
6000 mg/l IBA	3.50 ^d	0.00 [#]	1.75 ^b	4.00 ^c	0.00 ^d	2.00 ^b	5.55 ^d	0.00 [#]	2.77 ^c	5.70 ^c	0.00 [#]	2.85 ^b
8000 mg/l IBA	4.00 ^c	0.00 [#]	2.00 ^b	3.50 ^c	0.00 ^d	1.75 ^b	5.35 ^d	0.00 [#]	2.67 ^c	5.05 ^d	0.00 [#]	2.52 ^b
6000 mg/l IBA + 200 mg/l NAA	4.25 ^c	4.75 ^b	4.50 ^a	4.00 ^c	5.00 ^b	4.50 ^a	5.80 ^c	6.07 ^b	5.93 ^b	5.80 ^c	6.57 ^b	6.18 ^a
8000 mg/l IBA + 200 mg/l NAA	5.50 ^a	5.75 ^a	5.62 ^a	5.50 ^a	5.75 ^a	5.62 ^a	7.57 ^a	7.50 ^a	7.53 ^a	7.55 ^a	7.37 ^a	7.46 ^a
Radecant 80%	3.75 ^c	0.00 [#]	1.87 ^b	4.25 ^b	0.00 ^d	2.12 ^b	5.45 ^d	0.00 [#]	2.72 ^c	5.57 ^c	0.00 [#]	2.78 ^b
Mean	3.50 ^a	1.75 ^b		3.54 ^a	1.79 ^b		4.95 ^a	2.26 ^b		4.94 ^a		2.32 ^b
	No. of roots plant						Root dry weight (g)					
Control	0.00 [#]	0.00 [#]	0.00 ^d	0.00 ^f	0.00 ^f	0.00 ^d	0.00 [#]	0.00 [#]	0.00 ^d	0.00 [#]	0.00 [#]	0.00 ^d
6000 mg/l IBA	4.34 ^f	0.00 [#]	2.17 ^c	4.51 ^e	0.00 ^f	2.25 ^c	3.50 ^f	0.00 [#]	1.65 ^d	3.57 ^e	0.00 [#]	1.78 ^c
8000 mg/l IBA	5.39 ^e	0.00 [#]	2.69 ^c	5.32 ^d	0.00 ^f	2.66 ^c	4.39 ^e	0.00 [#]	2.19 ^c	4.24 ^f	0.00 [#]	2.12 ^c
6000 mg/l IBA + 200 mg/l NAA	6.44 ^d	7.34 ^c	6.89 ^b	6.28 ^c	7.42 ^b	6.85 ^b	4.66 ^d	4.62 ^d	4.64 ^b	4.66 ^d	4.46 ^d	4.56 ^d
8000 mg/l IBA + 200mg/l NAA	8.16 ^b	9.45 ^a	8.80 ^a	8.92 ^a	8.35 ^a	8.64 ^a	7.08 ^b	7.62 ^a	7.37 ^a	7.49 ^a	6.83 ^b	7.16 ^a
Radecant 80%	6.20 ^d	0.00 [#]	3.10 ^c	6.63 ^c	0.00 ^f	3.31 ^c	6.51 ^c	0.00 [#]	3.26 ^c	6.36 ^c	0.00 [#]	3.18 ^c
Mean	5.30 ^a	2.58 ^b		5.28 ^a	2.63 ^b		4.32 ^a	2.04 ^b		4.38 ^a		1.88 ^b

Regarding the effect of different PGRs treatments on all the studied parameters of cuttings, it is obvious that all treatments positively affect these parameters, compared with the control treatment, in both seasons. It can be clearly noticed in Tables 2 that the best treatments, which gave the highest values of shoot length, no. of lateral shoot, no. of leaves/plant, root length, no. of roots/plant and root dry weight, were IBA at 8000 mg/l + NAA at 200 mg/l (18.75 and 18.87 cm, 5.00 and 5.50, 5.62, 7.53 and 7.46 cm, 8.80 and 8.64, 7.37 and 7.16 g, respectively), compared with the control and other treatments in both 2011 and 2012 seasons.

On the other hand, April was found the best time for the treated Carob cuttings for all treatments compared with September, in both 2011 and 2012 seasons. It was clear that treated cuttings showed, in general, the significantly best and highest values of all tested parameters in April. While, in September, treated cuttings failed to root with all treatments, except when cuttings were treated with IBA at 6000 mg/l + NAA at 200 mg/l and IBA at 8000 mg/l + NAA at 200 mg/l, in 2011 and 2012 seasons.

Regarding, the interaction, data clearly showed that Carob cuttings treated with IBA at 8000 mg/l + NAA at 200 mg/l, followed by cuttings treated with IBA at 6000 mg/l + NAA at 200 mg/l gave the highest values of parameters, in April and September, in both seasons. While, in September, treated cutting failed to root for all treatments, except when cuttings were treated with IBA at 6000 mg/l + NAA at 200 mg/l and IBA at 8000 mg/l + NAA at 200 mg/l, in the two studied seasons.

These results were in agreement with the findings of Çelik et al. (1994) and Ibrahim et al. (2009) on olive. Plastic tunnels with water mist gave the best rooting and vegetative growth, followed by the cuttings under plastic tunnel without mist in *Hamelia patens* (Elgimabi, 2009). Loach (1997) studied the leaf water potential and the rooting of cuttings of *Rhododendron* under mist and polythene. He stated that propagation under polythene gave better results than mist in the lower radiation conditions. He studied also the variation in water potential in cuttings under mist and polythene and found that cuttings in mist depended on three variables namely; current days radiation, number of days from insertion of cuttings and the previous days leaf water potential, which was relatively unimportant, probably because cuttings were able to take up water over night from condensation on the under surface of the polythene.

3. Micropropagation

Culture establishment

MS basal medium supplemented with various cytokinins in addition to NAA showed that shoots could initiate from shoot tips and stem node segments of Carob on all the tested media. The percentage of explants initiating growth reached 100% (Tables 3 and 4, Figures 5A and B). Concerning the *in vitro* establishment of shoot tips (Table 3), data obtained after 8 weeks of culture revealed that shoots could be induced on all tested media with 100% growth percentage. MS medium supplemented with 0.4 mg/l NAA + 2.0 mg/l BAP gave the significantly highest number of shoots (3.0 shoots/explant), but with a length of only 0.6 cm (Figure 5A). Followed by the medium containing 0.4 mg/l NAA + 0.5 mg/l 2iP, which gave 2.0 shoots/explant, with the significantly highest shoot length (1.18 cm).

Table 3. Effect of different cytokinins in combination with NAA on the *in vitro* establishment of shoot tips of Carob.

Medium composition	Growth %	Mean no. of shoots/ explant	Mean length of shoots (cm)
MS control medium	100	1.00 ^c	0.42 ^{de}
MS medium+0.4 mg/l NAA+0.50 mg/l BAP	100	1.33 ^c	0.70 ^{bc}
MS medium+0.4 mg/l NAA+1.00 mg/l BAP	100	1.33 ^c	0.88 ^b
MS medium+0.4 mg/l NAA+2.00 mg/l BAP	100	3.00 ^a	0.60 ^{cd}
MS medium+0.4 mg/l NAA+0.50 mg/l 2iP	100	2.00 ^b	1.18 ^a
MS medium+0.4 mg/l NAA+1.00 mg/l 2iP	100	1.00 ^c	0.78 ^{bc}
MS medium+0.4 mg/l NAA+2.00 mg/l 2iP	100	1.00 ^c	0.60 ^{cd}
MS medium+0.4 mg/l NAA+0.25 mg/l TDZ	100	1.00 ^c	0.26 ^{ef}
MS medium+0.4 mg/l NAA+0.50 mg/l TDZ	100	1.67 ^{bc}	1.10 ^a
MS medium+0.4 mg/l NAA+1.00 mg/l TDZ	100	1.33 ^c	0.58 ^{cd}
MS medium+0.4 mg/l NAA+2.00 mg/l TDZ	100	1.00 ^c	0.20 ^f

Decreasing concentration of BAP brought about a significant decrease in the number of shoots. Although 0.5 mg/l 2iP produced the highest length of shoots, increasing 2iP concentration significantly decreased the shoot length and also the number of shoots. TDZ gave the least response in terms of the number of shoots, compared to the other used cytokinins (BAP and 2iP), however, MS medium containing 0.4 mg/l NAA + 0.5 mg/l TDZ gave significantly high shoot length (1.1 cm). The control MS medium without PGRs gave only one shoot with a length of 0.42 cm.

Regarding the *in vitro* establishment of stem node segments of Carob (Table 4), the maximum shoot number (2.6 shoots/explant) was produced on MS medium containing 0.4 mg/l NAA + 2.0 mg/l BAP (Figure 5B), followed by the other concentrations of BAP (0.5 and 1 mg/l), which produced 1.8 shoots/explant. The other used cytokinins (2iP and TDZ) gave the least response regarding to the number of shoots, even when compared to the control. This result is in harmony with that obtained by Hakim et al. (2010) who found that multiple shoots of Carob were regenerated when explants were cultured on MS medium fortified with BAP with an auxin. By taking into consideration the stem length, it was found that among the concentrations tested, the control MS medium produced the significantly highest length of shoots (1.5 cm), followed by lengths of shoots of 1.4 and 1.2 cm on MS medium containing 0.4 mg/l NAA + 2.0 and 1.0 mg/l 2iP, respectively. TDZ didn't enhance the elongation of shoots at all tested concentrations compared to the control MS medium or the application of 2iP. Romano et al. (2002) found that shoot culture initiation of Carob is greatly influenced by explanting season. In conclusion, the best *in vitro* establishment of both tested explants of Carob was induced on MS medium contained the combination of 0.4 mg/l NAA + 2.0 mg/l BAP. This result agrees with Radi et al. (2013) who reported that the concentration of auxin and the balance between the concentration of auxin and cytokinin affected the organogenic potentiality of Carob.

Table 4. Effect of different cytokinins in combination with NAA on the *in vitro* establishment of stem segments of Carob.

Medium composition	Growth %	Mean no. of shoots/ explant	Mean length of shoots (cm)
MS control medium	100	1.8 ^b	1.50 ^a
MS medium + 0.4 mg/l NAA + 0.50 mg/l BAP	100	1.8 ^b	0.88 ^{cd}
MS medium + 0.4 mg/l NAA + 1.00 mg/l BAP	100	1.8 ^b	0.52 ^d
MS medium + 0.4 mg/l NAA + 2.00 mg/l BAP	100	2.6 ^a	0.84 ^{cd}
MS medium + 0.4 mg/l NAA + 0.50 mg/l 2iP	100	1.0 ^c	0.84 ^{cd}
MS medium + 0.4 mg/l NAA + 1.00 mg/l 2iP	100	1.2 ^{bc}	1.20 ^{abc}
MS medium + 0.4 mg/l NAA + 2.00 mg/l 2iP	100	1.4 ^{bc}	1.40 ^{ab}
MS medium + 0.4 mg/l NAA + 0.25 mg/l TDZ	100	1.0 ^c	0.88 ^{cd}
MS medium + 0.4 mg/l NAA + 0.50 mg/l TDZ	100	1.0 ^c	0.68 ^d
MS medium + 0.4 mg/l NAA + 1.00 mg/l TDZ	100	1.0 ^c	0.56 ^d
MS medium + 0.4 mg/l NAA + 2.00 mg/l TDZ	100	1.2 ^{bc}	0.94 ^{bcd}

Shoot multiplication and elongation

Shoot multiplication rates were not significantly affected by the concentration of BAP or 2iP, as up to 3.8 shoots/explant were recorded using 1.0 mg/l BAP, which was the highest value. This result agrees with that of Romano et al. (2002) who obtained the best multiple-shoot response with Mulata Carob cultivar on MS medium supplemented with BAP. The longest shoots of 1.4 and 1.28 cm were obtained on MS medium containing 1.0 and 0.5 mg/l 2iP, respectively, followed by the higher 2iP concentration (2.0 mg/l) and the control treatment (Table 5).

Table 5. Influence of BAP or 2iP individually on the multiplication of Carob shoots

Medium composition	Growth %	Mean no. of shoots/ explant	Mean length of shoots (cm)
MS control medium	100	1.4 ^c	0.84 ^b
MS medium + 0.5 mg/l BAP	100	3.2 ^{ab}	0.70 ^{bc}
MS medium + 1.0 mg/l BAP	100	3.8 ^a	0.58 ^{bc}
MS medium + 2.0 mg/l BAP	100	2.6 ^{abc}	0.46 ^c
MS medium + 3.0 mg/l BAP	100	2.6 ^{abc}	0.38 ^c
MS medium + 0.5 mg/l 2iP	100	2.6 ^{abc}	1.28 ^a
MS medium + 1.0 mg/l 2iP	100	2.4 ^{abc}	1.40 ^a
MS medium + 2.0 mg/l 2iP	100	2.2 ^{abc}	0.92 ^b
MS medium + 3.0 mg/l 2iP	100	2.0 ^{bc}	0.64 ^{bc}

Effect of the combination of BAP and 2iP on the shoots multiplication and elongation was studied and represented in Table 6. It was found that the combination of the two cytokinins enhanced both the multiplication and elongation of *in vitro* produced Carob shoots. The highest mean number of shoots (8.6 and 7.2 shoots/explant) was observed on MS medium containing 1.0 mg/l BAP + 0.5 mg/l 2iP (Figure 5C and D) and 0.5 mg/l BAP + 0.5 mg/l 2iP, with also the significantly highest mean shoots length of 2.3 and 2.54 cm, respectively. Increasing the concentration of 2iP to 1.0 mg/l in addition to BAP (at 0.5 or 1.0 mg/l) decreased both number and length of shoots. Romano et al. (2002) reported that for Carob cultivar, cytokinin type and concentration are the most important factors affecting shoots multiplication.

Table 6. Influence of BAP in combination with 2iP on multiplication and elongation of Carob shoots

Medium composition	Growth %	Mean no. of shoots/explant	Mean length of shoots (cm)
MS control medium	100	1.0 ^c	0.70 ^c
MS medium + 0.5 mg/l BAP + 0.5 mg/l 2iP	100	7.2 ^a	2.54 ^a
MS medium + 1.0 mg/l BAP + 0.5 mg/l 2iP	100	8.6 ^a	2.30 ^{ab}
MS medium + 0.5 mg/l BAP + 1.0 mg/l 2iP	100	3.2 ^b	2.00 ^{abc}
MS medium + 1.0 mg/l BAP + 1.0 mg/l 2iP	100	2.2 ^b	1.60 ^{bc}

The rooting of Carob shoots was very difficult, as all the tested treatments gave negative results, although, the reported superior effect of IBA over other auxins, which may be due to its slow movement and slow degradation. Previous studies demonstrated that the hormonal concentration in the shoot culture medium might have a carryover effect on rooting (McComb, 1995). Also, the explants may contain endogenous auxin in addition to the added concentration, which could be supra-optimum for them to induce rooting. It has been repeatedly confirmed that auxin is required for initiation of adventitious roots on stems and the development of the first initial root cells is dependent upon either the added or the endogenous auxins (Pignatti and Crobeddu, 2005 and Radi et al., 2013). Carob has been described as one of the most difficult species to root (Lee et al., 1977; Hartman and Kester, 1983 and Gubbuk et al., 2011). Since the Carob traditional propagation methods failed to meet the market request, the use of micropropagation seems to be appropriate in order to fulfill the increasing demand of this valuable species (Romano et al., 2002).

In conclusion, this study describes three successful methods for the propagation of Carob, as one of the environmentally and economically important species. Although, the *in vitro* establishment and multiple shoots production gave positive results, the micropropagation of this plant needs further studies to overcome the difficulties in the root induction.

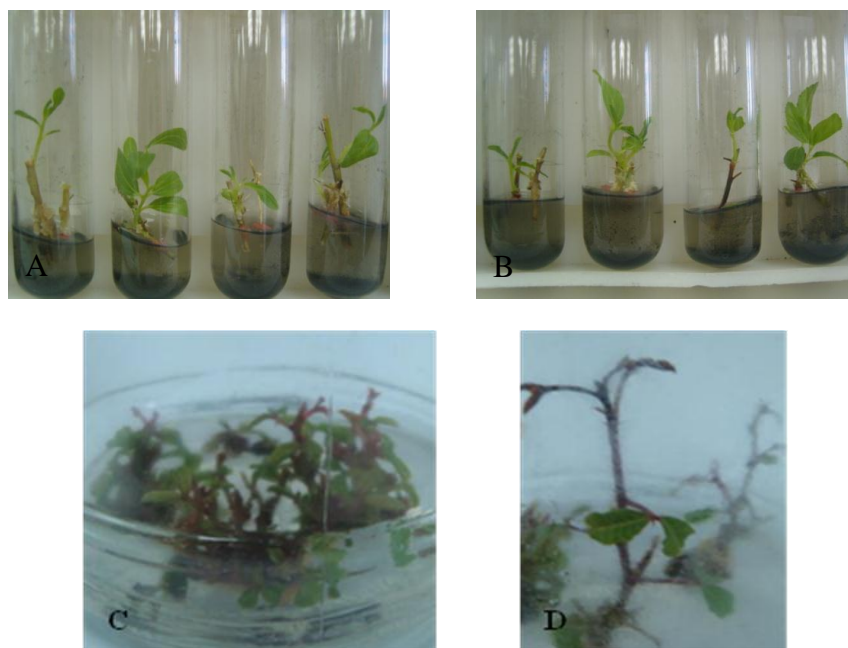


Figure 5. Micropropagation of Carob. *In vitro* establishment of (A) shoot tips and (B) stem node sections on MS medium containing 0.4 mg/l NAA + 2.0 mg/l BAP. (C) Multiplication and (D) elongation of shoots on MS medium containing 1.0 mg/l BAP + 0.5 mg/l 2iP.

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