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The effects of different explants, basal media and growth regulators on regeneration of carob (*Ceratonia siliqua L.*)

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Abstract
Ceratonia siliqua L. is a slow growing evergreen tree of the family Fabaceae used for the rehabilitation of marginal and submarginal dry areas of the Mediterranean basin due to it's resistant to drought and salt tolerance. In this study, the effects of different basal media (Woody Plant Medium and Murashige and Skoog medium), explant types (cotyledon and hypocotyl) and growth regulators (BA, Kinetin and NAA) on *in vitro* callus formation, differentiation of callus to shoot and root formation were investigated. High frequencies of caullogenesis were obtained and the best medium for callus induction was WPM supplemented with 1.0 mg L^{-1} BA + 0.5 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BA + 1.0 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA for hypocotyl explants and 0.5 mg L^{-1} BA + 1.0 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA; 0.5 mg L^{-1} BA + 0.5 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA and 1 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA for cotyledon explants. Callus induction was more readily obtained from hypocotyl explants than cotyledon explants. It was determined that explant types as significant on shoot formation, statistically. The shoot ratio was obtained from cotyledon explants in WPM as 10%. The best regeneration was obtained from cotyledon explants placed on WPM (30%) instead of MS medium.
Keywords: Callus, *in vitro*, plant hormone, tissue culture, woody plants

INTRODUCTION

Carob (*Ceratonia siliqua* L.), a perennial leguminous tree, which is widely distributed around the Mediterranean basin (North Africa, Southern Europe and Western Asia) and in other zones having Mediterranean-type climates, usually in mild, dry areas on poor soils [4, 22, 29, 48]. The carob tree is an important part of the Mediterranean vegetation and it is often interplanted with olives, grapes, almonds, and barley in low-intensive farming systems. The trees are also useful as ornamentals and for landscaping, windbreaks, and forestation [17, 63] According to recent data, carob pod production worldwide nearly 400000 tons per year from 200000 ha [33].

Turkey is one of the countries the carob tree grown naturally. Carob tree grow in, about 1750 km of shoreline from İzmir (Urla) to Hatay (Samandağ). It also grows in low-altitude areas 600-700 m above sea level [17, 43, 55]. There are different names used for carob tree such as "Harnup", "Harup", "Boynuz", "Buynuz" and "Kerti"in different districts in Turkey. Carob plants show either a tree form or bushy habitat. Although there is no commercial scale carob plantations in Turkey, recent long term rental policy of state lands had made popular to establish large carob plantations. Besides pods and seeds, the tree itself is a popular landscape tree due to its distinguished evergreen characteristic [17].

The carobs have been propagated from seeds while cultivated varieties are easily propagated vegetatively by grafting in Turkey. Three carob types determined in Turkey by Vardar et al. (1980): Wild type (pods are thin, long and flat, sometimes curled with a brown color), Fleshy type (pods are thick, long, and flat with a dark brown color) and Sisam type (pods are thick, short and flat with a light brown color) In another study, researchers selected five superior cultivars and seven wild genotypes from the eastern part of the Mediterranean region. They also have selected four grafted and ten wild genotypes from the west part of the Mediterranean and the Aegean regions of Turkey [42, 43, 61].

Carob pods with their sugary pulp have been a staple in the diet of farm animals and were eaten by children as snacks or by people in times of famine. Seed production is the main interest of carob growers. The endosperm of carob seeds produces a gum (carob bean gum) rich in galactomannans, used as a food additive [3]. In addition, the seed coat and pod pulp are used in the food industry to produce locust bean gum (LBG) and natural antioxidants [11, 12, 40]. Carob pods are also used in the pharmaceutical and cosmetic industries [2, 32, 40] and could provide an economical source of bioethanol in the future, particularly in arid regions [40, 51, 65]. This gum is used as a thickener, stabilizer, binder and gelling agent in the food industry and in several technical applications, including the manufacture of chemicals, paper and cosmetics and the pharmaceutical industry [33]. Certainly, carob trees are candidates for the reforestation of arid and degraded areas in the Mediterranean basin [4, 40, 50]. Propagation by cuttings has difficulties because of rooting problem [28]. In this context, *in vitro* techniques offer an alternative method for carob propagation [5, 16, 21, 47]. Micropropagation of carob through axillary meristem and somatic embryogenesis [6, 7, 9, 10, 13, 27, 38, 41, 47. 54, 64], from protoplast [21], with adequate nutrient medium [6] have been reported by several researchers and plants were established in to the field. Also, a micrografting technique was developed for carob using *in vitro* germinated

seedlings as rootstocks and apex or shoot cultures of pistillate cultivars as microscions [23].

From the previous studies, low frequencies of embryogenesis induction from fertilized ovules were obtained in Murashige and Skoog (MS) medium supplemented with 0.45 µM 2,4-D (2,4-Dichlorophenoxyacetic acid) using the somatic embryogenesis technique as (4%) [7]. Micropropagation of carob was possible from seedlings and mature trees using MS medium with 5 µM Zeatin for shoot multiplication and 10 µM Indole-butyric acid for root induction [54]. Gibberellic acid (2.5 µM) in shoot multiplication medium inhibited subsequent rooting. This inhibition was partially overcome by submersion in a medium with Zeatin (5 µM) without Gibberellic acid and was completely reversed by submersion in a medium with Zeatin (5 µM) and Ancymidol (5 µM). The success of *in vitro* tissue culture was strongly dependent on the chemical composition of the culture medium [1, 5, 16, 18, 37, 49, 52, 53, 60]. The macronutrient concentrations were optimized for *in vitro* rooting of *Ceratonia siliqua* micropropagated shoots [16]. MS and ½MS were tested. The frequency of *in vitro* rooting was enhanced when ½MS was used (50% rooted shoots). In another research, somatic embryos of carob were induced from cotyledon segments excised from immature seeds when cultured on MS media supplemented with several combinations of BA and IBA [6]. The best frequencies of induction were obtained from the medium with 4.4 µM BA and 0.5 µM IBA as (33.8%).

Micropropagation of *Ceratonia siliqua* L. (carob) from the apex of seedlings was attempted on different growth media (based on WPM, Durzan 'modified or not', SH, B5, MS, 1/2MS, modified Heller, BTM and GD) supplemented with microelements and MS vitamins. Four different auxins (IAA, IBA, NAA and 2,4-D) were tested in conjunction with BAP in WPM for the stimulation of bud development [52]. BAP at 0.5 mg L^{-1} as well as cytokinine favored bud development mainly in WPM and MS media. Combination of BA (0.5 mg L^{-1}) with IBA or IAA (0.1 mg L^{-1}) has also given good results. Multiplication was stimulated by IBA (0.1 mg L^{-1}) concentrations and direct rooting of obtained plantlets was better in the medium without any auxin pretreatment in ½ MS than the others.

The effect of Woody Plant Medium (WPM) and Olive Medium (OM) with various concentrations of Zeatin and BA alone or in combinations with each other was investigated in *in vitro* shoot proliferation of the olive cultivar 'Moraiolo' [1]. Olive medium was proved to be the most effective one, resulting in better and morphologically superior microshoots as compared to WPM. Zeatin (3 mg L^{-1}) in combination with 0.5 mg L^{-1} BA resulted in the highest number of microshoots

Table 1. Effects of plant growth regulators, explant types and media on callus formation

PGRs (mg L^{-1}) Concentration	Callus				Mean of PGRs
	MS Medium		WP Medium		
	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	
Control	0.00 k (0.00)	0.00 k (0.00)	0.00 k (0.00)	0.00 k (0.00)	0.00 G (0.00)
0.0BA, 0.5Kin, 0.0NAA	0.00 k (0.00)	10.00 jk (13.28)	0.00 k (0.00)	25.00 hij (26.25)	8.75 FG (9.88)
0.0BA, 1.0Kin, 0.0NAA	90.00 abc (80.19)	0.00 k (0.00)	25.00 ijk (22.50)	0.00 k (0.00)	28.75 DE (25.67)
0.5BA, 0.0Kin, 0.0NAA	0.00 k (0.00)	90.00 abc (80.19)	0.00 k (0.00)	60.00 defg (54.80)	37.50 D (33.75)
1.0BA, 0.0Kin, 0.0NAA	40.00 ghij (35.19)	100.00 a (90.00)	0.00 k (0.00)	100.00 a (90.00)	60.00 C (53.79)
0.5BA, 0.5Kin, 0.0NAA	15.00 jk (12.69)	65.00 defg (54.21)	0.00 k (0.00)	0.00 k (0.00)	20.00 EF (16.72)
0.5BA, 1.0Kin, 0.0NAA	65.00 cdefg (57.69)	65.00 cdefg (57.69)	0.00 k (0.00)	15.00 jk (12.69)	36.25 D (31.15)
1.0BA, 0.5Kin, 0.0NAA	60.00 efg (51.05)	25.00 ijk (22.50)	0.00 k (0.00)	0.00 k (0.00)	21.25 EF (18.38)
0.5BA, 0.0Kin, 0.5NAA	75.00 bcdef (63.74)	95.00 ab (83.35)	95.00 ab (83.35)	90.00 abcd (76.71)	88.75 A (76.79)
1.0BA, 0.0Kin, 0.5NAA	90.00 abcd (76.71)	90.00 abcd (76.71)	95.00 ab (83.35)	100.00 a (90.00)	93.75 A (81.69)
0.0BA, 0.5Kin, 0.5NAA	95.00 ab (83.35)	55.00 fgh (48.16)	95.00 ab (83.35)	85.00 abcd (77.30)	82.50 AB (73.04)
0.0BA, 1.0Kin, 0.5NAA	70.00 cdefg (57.10)	90.00 abc (80.19)	85.00 abcde (73.55)	100.00 a (90.00)	86.25 A (75.21)
0.5BA, 0.5Kin, 0.5NAA	40.00 ghi (38.94)	85.00 abcde (73.55)	95.00 ab (83.35)	100.00 a (90.00)	80.00 AB (71.46)
0.5BA, 1.0Kin, 0.5NAA	70.00 cdefg (57.10)	95.00 ab (83.35)	100.00 a (90.00)	100.00 a (90.00)	91.25 A (80.11)
1.0BA, 0.5Kin, 0.5NAA	90.00 abcd (76.71)	50.00 fghi (45.00)	100.00 a (90.00)	45.00 ghi (38.35)	71.25 DC (62.51)
1.0BA, 1.0Kin, 0.5NAA	45.00 ghi (38.35)	45.00 ghi (38.35)	95.00 ab (83.35)	85.00 abcde (73.55)	67.50 C (58.40)
Mean of Media	56.40 A (49.12)		52.81 A (46.95)		

LSD_{medium}=not significant, $LSD_{explant}$=4.30***, LSD_{PGRs}=12.17***, $LSD_{medium*explant*PGRs}$=24.34**, $LSD_{medium*explant}$=not significant, $LSD_{medium*PGRs}$=17.25***, $LSD_{explant*PGRs}$=16.95***,

P<0.05*, P<0.01**, P<0.001***

The numbers in parenthesis represent the angle value of percentages. Different letters within a column indicate significantly different means (LSD test). Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total media and mean of PGRs values

Table 2. Effects of plant growth regulators, explant types and media on differentiation to shoot buds

PGRs (mg L^{-1}) Concentration	Regeneration of Shoot Buds				Mean of PGRs
	MS Medium		WP Medium		
	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	
Control	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	0.00 B (0.00)
0.0BA, 0.5Kin, 0.0NAA	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	5.00 bc (6.64)	1.25 B (1.66)
1.0BA, 0.0Kin, 0.0NAA	0.00 c (0.00)	5.00 bc (6.64)	0.00 c (0.00)	0.00 c (0.00)	1.25 B (1.66)
0.5BA, 1.0Kin, 0.0NAA	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	30.00 a (29.14)	7.50 A (7.28)
0.5BA, 1.0Kin, 0.5NAA	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	10.00 b (13.28)	2.50 B (3.32)
1.0BA, 0.5Kin, 0.5NAA	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	5.00 bc (6.64)	1.25 B (1.66)
1.0BA, 1.0Kin, 0.5NAA	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	10.00 b (9.80)	2.50 B (2.45)
Mean of Media	0.156 B (0.207)		1.875 A (2.047)		

LSD_{medium}=1.24**, $LSD_{explant}$=1.24***, LSD_{PGRs}=3.51**, $LSD_{medium*explant*PGRs}$=7.02***, $LSD_{medium*explant}$=1.75**, $LSD_{medium*PGRs}$=4.96***, $LSD_{explant*PGRs}$=4.96**,

P<0.05*, P<0.01**, P<0.001***

The numbers in parenthesis represent the angle value of percentages. Different letters within a column indicate significantly different means (LSD test). Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total media and mean of PGRs values.

Table 3. Effects of plant growth regulators, explant types and media on shoot formation

PGRs (mg L^{-1}) Concentration	Shoot formation				Mean of PGRs
	MS Medium		WP Medium		
	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	
Control	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	0.00 B (0.00)
0.0BA, 1.0Kin, 0.0NAA	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	10.00 a (13.28)	2.50 A (3.32)
1.0BA, 0.0Kin, 0.0NAA	0.00 c (0.00)	5.00 b (6.64)	0.00 c (0.00)	5.00 b (6.64)	2.50 A (3.32)
0.5BA, 0.5Kin, 0.0NAA	0.00 c (0.00)	5.00 b (6.64)	0.00 c (0.00)	0.00 c (0.00)	1.25 AB (1.66)
1.0BA, 0.5Kin, 0.0NAA	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	5.00 b (6.64)	1.25 AB (1.66)
0.0BA, 0.5Kin, 0.5NAA	0.00 c (0.00)	0.00 c (0.00)	0.25 bc (1.43)	0.00 c (0.00)	0.06 B (0.35)
1.0BA, 0.5Kin, 0.5NAA	0.00 c (0.00)	0.00 c (0.00)	0.00 c (0.00)	5.00 b (6.64)	1.25 AB (1.66)
Mean of Media	0.31 A (0.41)		0.78 A (1.08)		

LSD_{medium}=not significant, $LSD_{explant}$=1.03**, LSD_{PGRs}= not significant, $LSD_{medium*explant*PGRs}$= not significant, $LSD_{medium*explant}$=not significant, $LSD_{medium*PGRs}$= not significant, $LSD_{explant*PGRs}$= not significant,

P<0.05*, P<0.01**, P<0.001***

The numbers in parenthesis represent the angle value of percentages. Different letters within a column indicate significantly different means (LSD test). Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total media and mean of PGRs values

Table 4. Effects of plant growth regulators, explant types and media on root formation

PGRs (mg L^{-1}) Concentration	Root				Mean of PGRs
	MS Medium		WP Medium		
	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	
Control	0.00 f (0.00)	0.00 f (0.00)	0.00 f (0.00)	0.00 f (0.00)	0.00 C (0.00)
0.5BA, 0.0Kin, 0.5NAA	0.00 f (0.00)	20.00 d (23.09)	0.00 f (0.00)	20.00 d (23.09)	10.00 B (11.54)
1.0BA, 0.0Kin, 0.5NAA	0.00 f (0.00)	10.00 ef (9.80)	0.00 f (0.00)	5.00 f (6.64)	3.75 C (4.11)
0.0BA, 0.5Kin, 0.5NAA	0.00 f (0.00)	80.00 a (66.91)	0.00 f (0.00)	35.00 bc (35.78)	28.75 A (25.67)
0.0BA, 1.0Kin, 0.5NAA	0.00 f (0.00)	25.00 cd (26.25)	0.00 f (0.00)	15.00 de (19.92)	10.00 B (11.54)
0.5BA, 0.5Kin, 0.5NAA	0.00 f (0.00)	45.00 b (38.35)	0.00 f (0.00)	5.00 f (6.64)	12.50 B (11.25)
0.5BA, 1.0Kin, 0.5NAA	0.00 f (0.00)	15.00 de (19.92)	0.00 f (0.00)	50.00 b (45.28)	16.25 B (16.30)
1.0BA, 0.5Kin, 0.5NAA	0.00 f (0.00)	5.00 f (6.64)	0.00 f (0.00)	0.00 f (0.00)	1.25 C (1.66)
Mean of Media	6.25 A (5.96)		4.06 A (4.29)		

LSD_{medium}= not significant, $LSD_{explant}$=1.81***, LSD_{PGRs}=5.13***, $LSD_{medium*explant*PGRs}$=10.26***,
$LSD_{medium*explant}$= not significant, $LSD_{medium*PGRs}$=7.25***, $LSD_{explant*PGRs}$=7.25***
P<0.05*, P<0.01**, P<0.001***

The numbers in parenthesis represent the angle value of percentages. Different letters within a column indicate significantly different means (LSD test). Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total media and mean of PGRs values

per explant, with shoot length and number of nodes on OM as compared to its single use of Zeatin. The best interaction of both media with cytokinins occurred when 3 mg L^{-1} Zeatin was used in combination with 0.5 mg L^{-1} BA. In another research, used four different culture media (Modified MS, MS, WPM and NN) for culturing two types of leaf explant, (leaves and nodes) of Anthurium [60]. All media were supplemented with 3% sucrose, 0.5 mg L^{-1} TDZ and 0.5 mg L^{-1} BA. Modified MS medium gave the highest callus formation from both leaf (86.6%) and node (100%). Callus grown in NN and modified MS was meristematic nodular callus while WPM created embryogenic like callus.

The aim of the present study was to determine the effects of different explants, basal media and plant growth regulators on *in vitro* possibility of regeneration for *Ceratonia cilica* L.

MATERIALS and METHODS

Plant material

The three types of *Ceratonia* have been determined in Turkey (Fleshy type, wild type and sisam type). In this study "Wild Type" carob type were used (The fruits are thin, long and flat, sometimes curled with a light brown color) [66] (Fig. 1 A). The mature "Wild Type" carob seeds were collected from a female tree grown at the **Çukurova University Campus Area, Adana, Turkey** (Fig. 1 B).

Surface sterilization

The carob seeds like those of the majority of legumes have difficulties on germination. 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. Therefore**,** the seeds of carob were immersed for two hours in diluted sulfuric acid solution (50%) and soaked in sterile water for 3 days at 4ºC. Afterward, carob seeds were sterilized by immersion in a 70% ethanol solution for 30 s, followed by immersion in a 30% sodium hypochlorite solution (commercial bleach solution with 4.5% active chlorine, v/v, NaOCl; Domestos©) in water with two drop of Tween 20 per 500 ml for 20 min, and rinsing three times with sterile distilled water. The seed coats were removed by surgical blade in the laminar air flow cabinet.

Seed germination and medium preparation

Seeds were germinated in hormone free MS basal medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar (Phyto Technology Laboratories©) and cultured for 16 days in darkness in a growth chamber at 25±2ºC. After 16 days of culture, hypocotyl (approximately, 5 mm) and cotyledon (approx. 4 x 5 mm) sections were excised from sterile etiolated seedlings and cultured on a MS basal medium [36] and Woody Plant Medium (WPM) [30] supplemented with 30 g L^{-1} sucrose, and 3.5 g L^{-1} gelrite (Duchefa). Hypocotyl and cotyledon explants were used on MS and WPM containing different concentration of BA (0, 0.5 and 1 mg L^{-1}), NAA (0 and 0.5 mg L^{-1}) and Kinetin (0, 0.5 and 1 mg L^{-1}) for regeneration ratio, callus, shoot and root formation. The pH level was adjusted to 5.7 prior to autoclaving at 121ºC for 15 min. Cultures were incubated in a growth chamber at 25±2ºC with a 16 h photoperiod under 75 $Mmolm^{-2}s^{-1}$ cool white fluorescent light. Each experiment consisted of six explants per petri dishes (90 x 15 mm) and five petri dishes per plant growth regulator treatments. For determination of the optimal duration of exposure of the explants to the medium containing BA, Kinetin and NAA of fifteen combination, 5 plates of each treatment were subcultured onto MS and WPM after 3, 6, 9, and 12 days of culture initiation. All cultures were incubated in a growth chamber with a 16 h photoperiod under cool-white light (Model F40/CW/RS/EW-II Philips Canada, Scarborough,

Ont.; 40–60 mmol 7 m^{-2} 7 s^{-1}). Regeneration and callus formation were quantified after 18 and 23 days of culture.

Experiment design and statistical analysis

All experiments were set up in a completely randomized factorial design and repeated twice with five replicates per treatment. Callus percentage (callus formed/total number of explants) and the number of shoot/explant (the number of shoots/cotyledon and hypocotyl explants) were calculated. All quantitative data expressed as percentages were subjected to arcsine transformation. Means were separated by analysis of variance and the least significant difference (LSD) test was performed to examine differences in callus formation and shoot production among different explants in Carob. All data analysis was examined using the JMP® program (SAS Institute, Cary, NC) ver. 5.00 and significance was considered at $p<0.01$.

RESULTS and DISCUSSION

In this research, the effects of different explant types (cotyledon and hypocotyl from etiolated seedlings) (Fig. 1 C, D, E), basal media (WPM and MS) and growth regulators (BA, Kinetin and NAA) were investigated on *in vitro* callus formation and differentiation of callus to shoot and root. Effects of plant growth regulators on callus formation, shoot buds, shoot formation and root growth were shown in Table 1, 2, 3 and 4, respectively.

After 3 weeks of culture, explants began to differentiate callus structures depending on the type of explant and PGRs used. In the meantime, different media induced light brown compact callus from hypocotyl (Fig. 1) or yellow brown compact callus (Fig. 3) from cotyledon explant. There were no significant differences statistically between the plant media and explant types interaction on callus formation (Table 1). But it was found that PGRs concentration was significant. It was found that different explant types also have a statistically significant effect on shoot buds and callus formation (Table 1 and 2). The best callus was obtained from WPM containing 0.5 mg L^{-1} BA + 0.5 mg L^{-1} NAA, 1 mg L^{-1} BA + 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA and 1 mg L^{-1} BA + 1 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA as 95%, 0.5 mg L^{-1} BA + 1 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA and 1 mg L^{-1} BA + 0.5 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA as 100% for hypocotyl explant. This was followed by WPM containing 1 mg L^{-1} BA, 1 mg L^{-1} BA + 0.5 mg L^{-1} NAA, 1 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} BA + 0.5 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} BA + 1 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA as 100% also 0.5 mg L^{-1} BA + 0.5 mg L^{-1} NAA as 90% for cotyledon explant. (Table 1) (Fig. 2A, B). After four weeks of culture, among 16 combination of PGRs, the best results of caullogenesis were provided with MS medium containing BA, Kinetin or NAA it was found that 90-95% (Table 1).

In our study, the effect of the different cytokinins on differentiation of explants depended strongly on their concentration in the culture medium. *In vitro* explants response was positively correlated with the cytokinin concentrations. Higher cytokinin levels increased shoot formation (Table 2 and 3).

It was determined that explant types were statistically important on shoot buds. The number of shoot buds obtained from cotyledon explants (30%) was higher than the shoots obtained from hypocotyl explants (10%) on WPM (Table 2 and Figure 1, 2). A strong interaction of cytokinin-auxin in inducing shoot bud and callus formation was observed. It was found that combination of BA, Kin or NAA were low effective in inducing shoot bud formation. When NAA was substituted for BA or Kin shoot bud development was completely suppressed; instead, the explants produced inadequate yellowish callus. Consistent shoot bud differentiation was observed only on WPM containing BA or Kin. Kinetin proved totally effective in inducing bud differentiation.

Te-chato et al. (2006), determined that explant type (leaf, internode, node) has statistically important effect on callus formation within the same anthurium cultivar "Plew Thien Phuket" (45.3%; 50.3% and 41.2%, respectively) and also within different anthurium cultivars "Plew Thien Phuket, Sonat and Valantino" such as in carob in our study.

Different explant types such as stem nodal segments [5, 16, 18, 38, 47], meristem tip culture [39], hormone concentrations [18, 35, 38, 39, 45, 47, 49, 52, 53, 62] and basal media [1, 5, 16, 37, 60] have statistically important effect on regeneration and callus formation.

Researchers investigated that micropropagated carob (*Ceratonia siliqua* L.) shoots were rooted on ½ MS medium, supplemented with different types and concentrations of sugars, in order to determine the effects of sugar composition and concentration on *in vitro* rooting and *in vivo* establishment of the plantlets [9]. The best rooting response was obtained from the medium with 145 mM sucrose. The usage of filter-sterilized fructose increased root number and root length than autoclaved one. Plantlet acclimatization was affected positively by reduction of glucose concentration during *in vitro* rooting. In another study, investigated *in vitro* micropropagation of carob (*Ceratonia siliqua* L.) by cotyledonary buds attempted on WPM supplemented with microelements and MS vitamins [53]. Seedling growth was stimulated by Zeatin (1 mg L^{-1}), has better under BAP or Zeatin at 1 mg L^{-1}. Stem formation and growth were stimulated by NAA (0.1 mg L^{-1}) with BAP (0.5 mg L^{-1}). Shoot multiplication was stimulated with BAP (0.5 mg L^{-1}) alone or with GA_3 (0.2 mg L^{-1}). IBA (2 mg L^{-1}) was the most efficient auxin for root formation. The best shoot formation was obtained from cotyledon explants (10%) grown on WPM supplemented with and 1 mg L^{-1} Kinetin than on MS medium (Table 3).

The most important differences between WPM and MS medium in terms of elemental composition were lower concentrations of N, P and Ca in WPM compared to the MS medium. It was previously shown that carob was sensitive to high concentrations of N in the medium [8, 34, 35]. In another experiment, MS medium was also compared with ½ MS medium for carob rooting [16]. They obtained a higher rooting ratio in ½ MS compared to full strength MS medium because of lower P and higher Ca and Mg concentrations. Also Brugaletta et al. (2009) obtained more regenerated shoots in ½ MS than full strength MS medium. Chemical composition of the culture medium has been reported on the effects of all types of morphogenic responses, including axillary bud proliferation [56], caullogenesis [37], plant regeneration [46] and embryogenesis [6, 10, 26, 31]. In this research, WPM containing 0.5 mg L^{-1} BA and 1.0 mg L^{-1} Kinetin showed the best regeneration ratio as 30% (Table 1). This was followed with the WPM containing 0.5 mg L^{-1} BA + 1.0 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BA + 1.0 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA as 10%. But the explant types and hormone concentrations were important on root formation (Figure 3). The highest frequency of root

Fig 1. A) The wild type carob tree form University of Çukurova campus area; **B)** Wild type pod and seeds; **C)** Removing seed coat; **D, E)** Germination of seeds in *in vitro*

formation was obtained from cotyledon explants as 80% in MS medium with 0.5 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA and as 50% in WPM with 0.5 mg L^{-1} BA + 1 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA hormone concentrations (Table 3). The plant growth regulators play a very critical role in the induction of any morphogenic structure [52, 57]. BA has been widely accepted as the preferred cytokinin capable of inducing satisfactory explant regeneration [5, 18]. However, the combined use of BA and Kinetin has also been considered to have a synergistic effect on the morphogenesis of carob in this study. Tefera and Wannakrairoj (2006), reported that using 0.5 mg L^{-1} thidiazuron (TDZ) in combination with 3 mg L^{-1} paclobutrazol (PBZ) gave about 26 shoots/explant (about 12.6-fold than the control) and shoot multiplication was also enhanced when TDZ (0.5 mg L^{-1}) was simultaneously used with either 2 mg L^{-1} imazalil (IMA) or 3 mg L^{-1} N 6 -benzyladenine (BA) in the culture medium (MS medium added with 5% coconut water).

Various reports indicate that the exogenous supply of growth regulators may affect the uptake and utilization of mineral nutrients [24, 25, 44, 59]. Mobilization of nutrients by cytokinins has also been reported [58]. They demonstrated that nutrients were preferentially transported and accumulated in cytokinin treated tissues [20]. It has been postulated that the plant growth regulators caused nutrient mobilization by creating a new source sink relationship [14]. Cytokinins are derived from adenine and accelerate the process of morphogenesis by producing two immediate effects on undifferentiated cells: the stimulation of DNA synthesis and increased cell division [62].

The WPM stimulated callogenesis more than MS, QL or DKW media did [57]. This might be associated with it's low nitrogen level and low salt concentration. The stimulatory effect of medium with low nitrogen level and low salt was demonstrated in *Malus*, where the N_6 medium was more effective than MS on shoot regeneration. In *Prunus avium*, it was also reported that shoot regeneration was more efficient when using N_6 basal medium than MS or other media [15, 67]. Regeneration of black cherry (*Prunus serotina*) occurred more frequently when leaves were cultured on WPM rather than DKW [19]. and poorer micropropagation of common ash (*Fraxinus excelsior*) on WPM compared to DKW could be overcome by supplementing the WPM with ammonium nitrate [19, 57].

From the results presented in this study, it can be concluded that the explant types, culture media composition and hormone concentration are the main factors controlling regeneration in carob. It was found that WPM was more effective than MS medium for woody plants. In addition to media composition, cotyledon explants showed better regeneration and callus formation than hypocotyl explants. Combination of BA with Kinetin in WPM also gave better result than BA or Kinetin alone.

CONCLUSION

In this paper, the effects of different PGR concentrations (BA, Kinetin, and NAA), different basal media (WPM and MS) and different explants (cotyledon, hypocotyl) on regeneration, caullogenesis, shoot and root formation in carob were investigated. It was determined that the WPM containing 0.5 mg L^{-1} BA and 1 mg L^{-1} Kinetin showed the best regeneration capacity for carob cotyledon explants.

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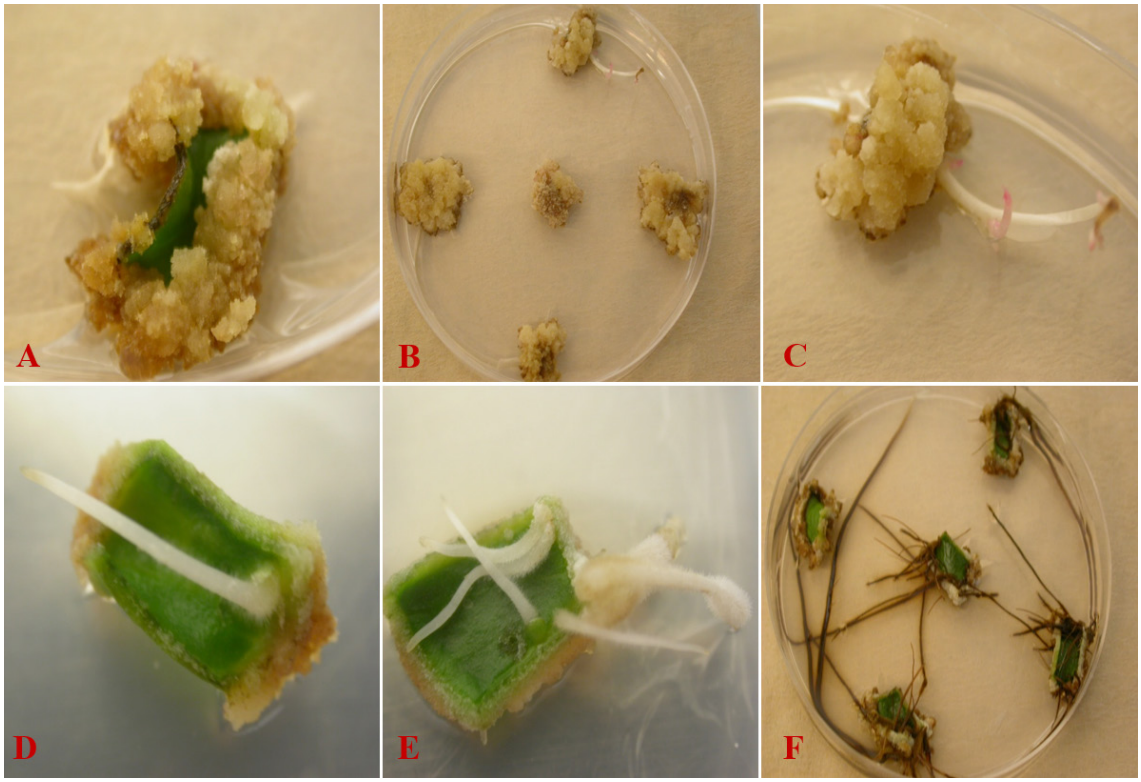

Fig 2. Shoot and root formation from cotyledon explants cultured on WPM. A, B and C) Callus and shoot formation from cotyledon on WPM containing 1 mg L^{-1} Kinetin; D, E and F) Root formation from cotyledon explant cultured on WPM containing 0.5 mg l^{-1} Kinetin and 0.5 mg l^{-1} NAA

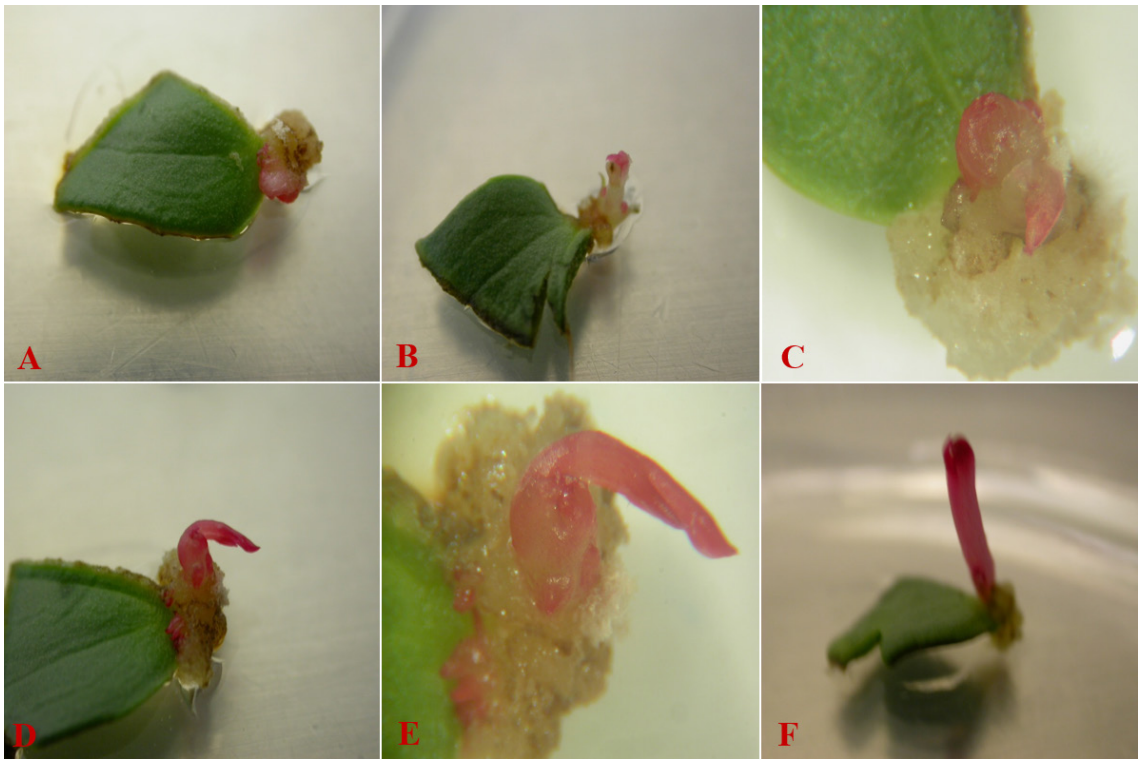

Fig 3. Early shoot formation from cotyledon explants. A, B and C) Early developmental stage of shoot formation from cotyledon explant; D, E and F) Shoot formation from cotyledon explants cultured on WPM containing 1 mg L^{-1} Kinetin

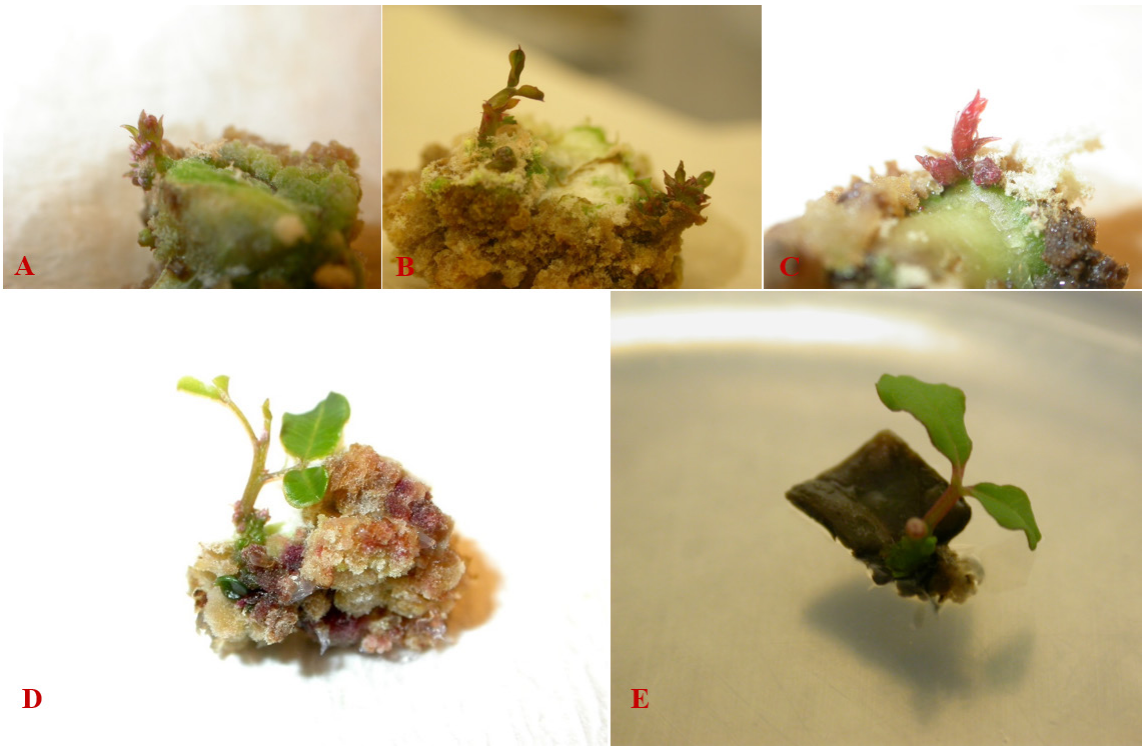

Fig 4. Development stages of shoot formation. **A, B** and **C)** After 4 weeks of culture, first shoot structure occurred from cotyledon explant on WPM containing 1 mg L^{-1} Kinetin; **D** and **E)** Shoots regenerated from cotyledon explant of carob after 6 weeks of culture on WPM

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