Production of *Ceratonia siliqua* Female Plantlets through Tissue Culture Technique

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Y.H. El-Shafey*; O.M. Elshihy*; E.M. Youssef**
and Mervat M. Gad**
* Fac. of Agric., Cairo University.
** Hort. Res. Inst., Agric. Res. Centre

ABSTRACT

Ceratonia siliqua is a multipurpose, economical tree that stands drought and is considered as a threatened tree. Therefore, a tissue culture technique was adopted to produce female plantlets vegetatively in a short period of time. Phenolic excretion problem of explants was overcome by several consequent steps, i.e., soaking in antioxidants (Ascorbic acid, 150 mg/l + Citric acid, 100 mg/l) overnight, dark treatment and in situ explant transfer. Explants were transferred into MS-solidified medium supplemented with 25 plant growth regulator treatments comprising; N^6-benzyl-aminopurine (BAP), 6-furfuryl aminopurine (Kinetin), α-naphthaleneacetic acid (NAA) and indole buteric acid (IBA) at concentrations (0.0, 0.5 and 1.0 mg/l). Three types of explants, i.e., shoot tips, nodal cuttings and lateral buds were taken from recent suckers of a productive female tree and were subjected to this trial. Nodal cutting explants achieved the highest survival percentage (80%) and optimal shootlets formation (100%), when cultured on the most adequate supplements (BAP, 0.5 + IBA, 0.5) mg/l or (Kin, 1.0 + IBA, 1.0) mg/l.

Key words: *Ceratonia siliqua, carob tree, phenolic excretion, tissue culture, suckers, female plantlets.*

INTRODUCTION

Ceratonia siliqua, Linn (Carob) is the only species now widely distributed in warm countries, being grown for shade, edible pods and its hard wood. Imam (1971) and Lo Gullo *et al.,* (1986) stated that carob is of much importance as a farm crop throughout the Mediterranean basin and other hot and semi-arid regions of hot, dry summer and cooler, but frostless winter. Thomas (1981) suggested that carob stands drought and brilliant sun, and grows well in ordinary well drained soil. The best kinds are dioecious, and a sufficient number of staminate trees, therefore, must be planted to pollinate the female trees.

Bailey (1933) reported that carob grew from seeds after soaking for 2-3 days before sowing and afterwards seedlings budded to the best varieties. It can be also raised from cuttings, but requires bottom heat and careful treatment, however, the large pods contain protein (5.05-

7.8%), sugar (40-46.9%) and fiber 25.85%. In addition, the pulp contains 0.51% fat, 3.01% ash, 18.5% tanins, 4.7% pectins and 2.7% amino acids (Ezdakova and Panaiotu, 1984; Saura-Calexto, 1987). *Ceratonia siliqua* is a very important forage crop and is also used for the manufacture of syrups and different fermented drinks. It was mentioned that it has a high digestive coefficient.

Despite the economical importance of this tree and the need for female trees production, it received little attention and only limited success has been reported. *Ceratonia siliqua* has hardly been studied *in vitro* with only a single micropropagation, with success on working with seeds as a source of explants though difficulties have been arisen for obtaining shootlets from mature trees because of contamination and phenolic compounds excretion problems from explants in the medium (Sebastian and McComb, 1986). This work describes experiments where nodal cuttings, from juvenile grown suckers resulted in promising female shootlets production after a successful trial for overcoming phenolic excretion of explants. These experiments led to a micropropagation protocol that can be used in further studies for mass production of shootlets.

MATERIALS AND METHODS

Surface sterilization of plant material

This work was carried out during the three successive years 1994-1996. The plant source was taken from young suckers of a fruitful female carob tree exhibiting characteristics (e.g. high fruit production, sweaty pods). This tree was located at the research garden of the Horticultural Research Institute, Agricultural Research Center (A.R.C.), Giza, Egypt. Cuttings of 2-3 nodes (~15 cm) were collected in February, directly washed with running tap water and the following steps were performed: Cuttings were continuously agitated for 40 minutes in a disinfecting solution containing commercial liquid soap (2%), and savlon disinfectant (3%), (v/v) each. Continuous rinsing with running tap water for one hour was applied. Plant material was soaked overnight under cooling in an antioxidant solution containing 100 mg/l citric acid and 150 mg/l ascorbic acid, in order to circumvent problems with phenol oxidation and its diffusion into the medium. Surface sterilization under aseptic conditions was adopted by cuttings transfer into laminar air flow cabinet after discarding antioxidant solution. Cuttings were agitated, first in 70% ethanol for 5 minutes followed by 20% (v/v) Chlorox (sodium hypochlorite, 5.25% active material) with few drops of Tween-20 (as a wetting agent), agitation was performed for 10 minutes. Finally, cuttings were agitated for 7 minutes in mercury chloride, $HgCl_2$ 0.2% (w/v), and a few drops of Tween-20 were added. Each sterilizing treatment was followed by three rinses with sterile distilled water. The explants were held in sterile distilled water till inoculation.

Establishment of plant material

Surface sterilized cuttings were separated into three types of explants in order to obtain sterile shoot proliferation. The first type of explant was shoot tip(s) of suckers, (~0.5 cm length); the second type of explant was nodal cuttings (~1 cm long), those close to the shoot tip having soft wood involving one bud per node. While, the third explant was axillary buds (~1 cm long) joined with a piece of the bark (b), its position was just beneath the soft area of explant (n). These explants were inoculated into medium in glass jars (Ca. 250 ml), covered with transparent polypropylene lids containing 25 ml (MS) solidified basal medium (Murashige and Skoog,

1962). This medium was solidified with 0.7% agar (BDH). Medium pH was adjusted to 5.70 ± 0.05 before solidification. The medium was then autoclaved at 121°C and 1.2 kg/cm^2 for 15 min. Four portions of each type of explants was embedded with the cut basal end towards the medium. Each jar was considered as a replicate and 10 replicates were used for each type of explant. Cultures were incubated in air conditioned growth chamber at 25°C ± 1°C under dark conditions for one week. Explants were *in situ* transferred from one position into another inside the same jar in order to avoid any phenolic compounds secretion, as mentioned by Danielle (1990). Cultures were then transferred into photo period of 16 hours (Philips day light fluorescent tubes) and light intensity of (40 µE. cm^{-2}.sec.$^{-1}$) for two more weeks and transferring explants *in situ* was performed as previously mentioned till explant secretion was over.

Three weeks of dis-colouration treatment were conducted till recovery, then explants were transferred into establishing medium. Twenty five different treatments using MS-basal medium supplemented with combinations of two types of auxins (NAA and IBA) and two types of cytokinins (BAP and Kin) in concentrations of 0.0, 0.5 and 1.0 mg/l were adopted in order to obtain aseptic shoot proliferation.

All 25 treatments were supplemented with 100 mg/l casein hydrolysate; 80 mg/l adenine hemisulphate; 1 mg/l Ca-pantothenate; 1 mg/l GA_3 and solidified with 0.7% agar. Ten glass jars were used as replicates per treatment and per type of explant. Four portions of each explant were partially embedded in each jar as mentioned before. Cultures were incubated in a growth chamber with the aforementioned conditions. Three subcultures were adopted at 4 weeks intervals. This work was repeated three times and data of survival percentage and shoot formation percentage were recorded after three months. Data were subjected to analysis of variance according to Snedecor and Cochran (1982) assuming completely randomized design with two factors. Mean separation was made using least significant difference (L.S.D.) at 5% level of significance. Shoots formation and the result of browning recovery were photographed.

RESULTS AND DISCUSSION

Data represented in Table (1) revealed that the highest survival percentage of explants (53.18%) was recorded by the application of (IBA, 0.5 + BAP, 1.0) mg/l. However, no significant difference was detected with the application of IBA, 0.5 + BAP, 0.5 mg/l which resulted in 51.52% survival of explants. The cytokinin BAP exhibited more promotive effect on explant survival percentage 53.18%, when applied at 1.0 mg/l to 0.5 mg/l IBA than kinetine which showed a maximum survival potentiality of 43.15% when applied at 1.0 mg/l to IBA 1.0 mg/l. On the other hand, the auxin IBA surpassed NAA in promoting explant survival potentiality, specially when IBA was supplemented at 0.5 mg/l to 1.0 mg/l BAP which brought about 53.18% of explant survival. The highest survival percentage due to the application of NAA at 1.0 mg/l to BAP 1.0 mg/l resulted in survival percentage of 48.88%. The maximume increase in survival percentage, as affected by type of explant was clearly observed by using nodal cutting explant (55.28%) surpassing the effects of lateral bud (25.8%) and shoot tip (13.45%) explants.

In general, from Fig. (1), it is obvious that nodal cutting explant brought about the highest magnitude of survival percentage (80%) when cultured on MS-medium supplemented with either (BAP, 1.0 mg/l) or (IBA, 0.5+BAP, 0.5) mg/l or

(IBA, 1.0+Kin, 1.0) mg/l. As represented in Table (2), the highest increase shoot formation (69.44%), took place by supplementing MS-medium with (IBA, 0.5+BAP, 1.0) mg/l. However, this was no significantly different the percentage of shoot formation obtained by the application of (IBA, 0.5+BAP, 0.5) mg/l. The cytokinin BAP surpassed Kin in promoting shootlet formation when applied either, individually or combined with auxins. While, the auxin IBA was superior to NAA, when combined with cytokinins, particularly at lower concentration of IBA with BAP at both concentrations (0.5, 1.0 mg/l). Nodal cutting explant induced the highest increase in percentage of shoots formation (67.53%), exceeding the lateral bud (33.57%) and the shoot tip (20.68%) explants in this respect.

Table (1): Effect of various plant growth regulators and various explant types on survival (%) of Ceratonia siliqua explants cultured in vitro.

Explant type	Shoot tip		Nodal cutting		Lateral Bud		Mean value of plant growth regulators	
Plant growth regulators	Mean %	Log**	Mean	Log**	Mean %	Log**	Mean %	Log**
1-Control	15.67	1.2472 b-d	23.33	1.4036 b-j	34.08	1.5573 b	24.36	1.4210 a-i
2-BAP (0.5mg/l)	12.00	1.1461 c-j	51.11	1.7252 a-j	24.33	1.4205 a-h	29.15	1.4934 a-i
3- BAP (1.0mg/l)	13.33	1.1856 c-j	80.00	1.9138 a	21.33	1.3680 a-h	38.22	1.6045 a-d
4- Kin (0.5mg/l)	3.00	0.6990 fp	19.11	1.3245 e-j	19.33	1.3291 a-h	13.81	1.1990e-i
5-Kin (1.0mg/l)	0.00	0.3010 k-p	53.00	1.7404 a-j	2.00	0.6021 v	19.33	1.3291 d-i
6- NAA (0.5mg/l)	0.00	0.3010 k-p	60.01	1.7925 a-h	15.24	1.2366 b-h	25.08	1.4327 a-i
7- NAA(0.5 mg/l) + BAP (0.5mg/l)	0.00	0.3010 k-p	61.67	1.8039 a-h	27.33	1.4674 a-h	29.67	1.5006 a-i
8- NAA(0.5 mg/l) +BAP (1.0mg/l)	0.00	0.3010 k-p	40.00	1.6233 a-j	28.89	1.4898 a-h	2.96	1.3973 a-i
9- NAA(0.5 mg/l) +Kin (0.5mg/l)	0.00	0.3010 k-p	49.63	1.7129 a-j	20.00	1.3424 a-h	23.21	1.4016 a-i
10- NAA (0.5 mg/l) +Kin (1.0mg/l)	0.00	0.3010 k-p	48.33	1.7018 a-j	27.50	1.4698 a-h	25.28	1.4358 a-i
11- NAA(1.0mg/l)	13.33	1.1856 c-j	59.89	1.7916 a-j	23.33	1.4037 a-h	35.19	1.5464 a-i
12- NAA(1.0mg/l) +BAP (0.5mg/l)	18.33	1.3082 a-d	78.58	1.9061 a-d	33.06	1.5448 ab	44.32	1.6657 a-d
13- NAA(1.0mg/l) +BAP (1.0mg/l)	53.33	1.7430 ab	56.67	1.7684 a-j	33.64	1.5519 ab	48.88	1.7066 abc
14- NAA (1.0mg/l) + Kin (5.0mg/l)	32.22	1.5343 bc	73.33	1.8770 a-d	22.61	1.3911 a-h	45.72	1.6601 a-d
15- NAA (1.0mg/l) + Kin (1.0mg/l)	30.000	1.5052 bc	56.66	1.7684 a-j	23.97	1.4144 a-h	37.88	1.6007 a-d
16- IBA (0.5mg/l)	0.00	0.3010 k-p	17.83	1.2974 I-j	24.28	1.4197 a	13.04	1.2314 e-i
17- IBA(0.5 mg/l) +BAP (0.5mg/l)	9.90	1.0756 c-j	80.00	1.9138 a	61.67	1.8039 a-h	51.52	1.7285 a
18- IBA (0.5 mg/L) +BAP (1.0mg/l)	61.67	1.8039 a	70.33	1.8593 a-d	24.54	1.4240 a-h	53.18	1.7418 a
19- IBA(0.5 mg/l) +Kin (0.5mg/l)	18.33	1.3082 a-d	40.00	1.6233 a-j	18.00	1.3010 a-h	25.44	1.4385 a-i
20- IBA(0.5 mg/l) +Kin (1.0mg/l)	18.33	1.3082 a-d	64.22	1.8210 a-h	8.74	1.0310 c-h	30.43	1.5110 a-i
21- IBA(1.0mg/l)	0.00	0.3010 k-p	39.44	1.6174 a-j	32.83	1.5420 ab	24.09	1.4165 a-i
22- IBA(1.0mg/l) +BAP (0.5mg/l)	23.33	1.4037 abc	71.07	1.8637 a-d	27.50	1.4698 a-h	40.63	1.6298 a-d
23- IBA(1.0mg/l) +BAP (1.0mg/l)	0.00	0.3010 k-p	64.50	1.8228 a-d	33.75	1.5533 ab	32.75	1.5410 a-i
24- IBA(1.0mg/l) +Kin (0.5mg/l)	6.67	0.9379 d-j	43.24	1.6555 a-j	14.61	1.2205 b-h	21.51	1.3712 b-i
25- IBA(1.0mg/l) +Kin (1.0mg/l)	6.86	0.9474 d-j	80.00	1.9138 a	42.59	1.6492 ab	43.150	1.6547 a-d
Mean valu eof explant type	13.45	1.1890 c	55.28	1.7580 a	25.81	1.4442 b		

L.S.D. (5%)

Plant growth regulators (G.R.) 0.353 **

Explant type (E.) 0.101 **

G.R.xE. 0.5052 **

* Means value followed by the same letter are not significanlty different.

** Corresponding log values were calculated in order to identify the catagories(e.g. a, b,c..).

Fig. (1): Shootlets formation of Ceratonia siliqua on nodal explants:

1- ***Browning recovery of explants.***
2- ***Shootlets formation on MS medium supplemented with IBA (0.5 mg/l) + (0.5 mg/l).***
3- ***Shootlets formation on MS medium supplemented with IBA (1.0 mg/l) + Kin (1.0 mg/l).***

Table (2): Effect of various plant growth regulators and different explant types on shoot formation (%) from Ceratonia siliqua cultured In vitro.

Explant type	Shoot tip		Nodal cutting		Lateral Bud		Mean value of plant growth regulators	
Plant growth regulators	Mean%	Log**	Mean %	Log**	Mean %	Log**	Mean %	Log**
1-Control	23.400	1.4048 a-c	33.22	1.5468 a-j	43.75	1.6604 ab	33.46	1.5497 b-o
2-BAP (0.5mg/l)	40.00	1.6232 a-c	61.11	1.8001 a-j	34.62	1.5637 ab	45.24	1.6743 a-h
3- BAP (1.0mg/l)	20.00	1.3424 bc	90.00	1.9638 a	31.55	1.5257 ab	47.18	1.6918 a-h
4- Kin (0.5mg/L)	34.00	1.5563 a-c	6.67	0.9379 k-u	8.19	1.3051 b-u	19.62	1.3348 i-o
5-Kin (1.0mg/l)	0.00	0.3010 d-g	62.23	1.8077 a-j	6.67	0.9379 c-u	22.96	1.3973 d-o
6- NAA (0.5mg/l)	0.00	0.3010 d-g	77.00	1.8976 a	22.92	1.3965 b-u	33.31	1.5479 b-o
7- NAA (0.5mg/l) + BAP (0.5mg/l)	0.00	0.3010 d-g	72.00	1.8692 a	37.50	1.5966 ab	36.50	1.5855 a-o
8- NAA (0.5mg/l) +BAP (1.0mg/l)	0.00	0.3010 d-g	50.00	1.7160 a-j	38.89	1.6116 ab	29.63	1.5001 c-o
9- NAA (0.5mg/l) +Kin (0.5mg/l)	0.00	0.3010 d-g	60.11	1.7931 a-j	30.00	1.5051 ab	30.04	1.5056 c-o
10- NAA (0.5mg/l) +Kin (1.0mg/l)	0.00	0.3010 d-g	58.33	1.7805 a-j	37.50	1.5966 ab	31.94	1.5308 b-o
11- NAA (1.0 mg/L)	20.00	1.3424 bc	73.33	1.8770 a	33.33	1.5482 ab	42.22	1.6456 a-h
12- NAA (1.0 mg/L) +BAP (0.5mg/l)	25.00	1.4314 a-c	93.33	1.9792 a	43.06	1.6538 ab	53.80	1.7466 a-c
13- NAA (1.0 mg/L) +BAP (1.0mg/l)	66.6	1.8367 a	66.89	1.8254 a-j	37.88	1.6008 ab	57.15	1.7719 a-c
14- NAA (1.0 mg/L) +Kin (5.0mg/l)	40.00	1.6232 a-c	76.67	1.8958 a	29.17	1.4937 ab	48.61	1.7043 a-c
15- NAA (1.0 mg/L) +Kin (1.0mg/l)	40.00	1.6232 a-c	66.66	1.8367 a-j	27.27	1.4665 ab	44.65	1.6688 a-h
16- IBA (0.5mg/l)	0.00	0.3010 d-g	20.83	1.3586 b-u	30.95	1.5179 ab	17.26	1.2847 n-o
17- IBA (0.5mg/L) +BAP (0.5mg/l)	16.67	1.2711 bc	100.00	2.0086 a	75.00	1.8865 a	63.89	1.8188 ab
18- IBA (0.5mg/L) +BAP (1.0mg/l)	75.00	1.8865 a	100.00	2.0086 a	33.00	1.5441 ab	69.44	1.8540 a
19- IBA (0.5mg/L)+Kin (0.5mg/l)	25.00	1.4314 a-c	50.00	1.7160 a-j	25.00	1.4314 ab	33.33	1.5482 b-o
20- IBA (0.5mg/L) +Kin (1.0mg/l)	25.00	1.4314 a-c	77.77	1.9019 a	17.22	1.2838 b-u	40.00	1.6232a-m
21- IBA (1.0mg/l)	0.00	0.3010 d-g	52.77	1.6606 a-j	43.53	1.6583 ab	32.10	1.5328b-o
22- IBA (1.0 mg/L) +BAP (0.5mg/l)	33.11	1.5454 a-c	86.66	1.9477 a	37.74	1.5992 ab	52.50	1.7364 a-c
23- IBA (1.0 mg/L) +BAP (1.0mg/l)	0.00	0.3010 d-g	100.00	2.0086 a	43.77	1.6606 ab	47.92	1.6983 a-h
24- IBA (1.0 mg/L) +Kin (0.5mg/l)	16.67	1.2711 bc	52.77	1.7385 a-j	18.75	1.3170 b-u	29.39	1.4969 c-o
25- IBA(1.0 mg/L) +Kin (1.0mg/l)	16.56	1.2685 bc	100.00	2.0086 a	41.56	1.6391 ab	52.70	1.7380 a-c
Mean value of explant type	20.68	1.3557 c	67.53	1.8422 a	33.57	1.5510 b		

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L.S.D.	(5%)	Plant growth regulators (G.R.)	0.3010 **
Explant type (E.)	0.0965 **	G.R. xE.	0.4817 **

* Means value followed by the same letter are not significantly different.

**Corresponding log values were calculated in order to identify the catogrories (e.g. a,b,c..).

In general, as shown in Figs. (2&3), the maximum increase in shoot formation (100% of survived explants) was induced by nodal cuttings when cultured on either of the combinations (IBA, 0.5+BAP, 0.5 mg/l), or (IBA, 0.5+BAP, 1.0 mg/l), or (IBA, 1.0+BAP, 1.0 mg/l), or (IBA, 1.0+Kin, 1.0 mg/l).

These results pointed out that nodal cutting explant fulfilled the optimal values concerning explant survival potentiality (80%) and shoots formation (100%), particularly by supplementing MS-medium with (IBA, 0.5+BAP, 0.5) mg/l or (IBA, 1.0+Kin, 1.0) mg/l, with no significant difference.

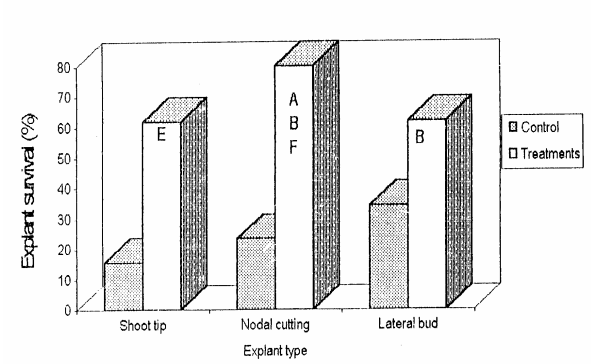

Fig. (2): Effect of explant type and plant growth regulators on explant survival (%) of Ceratonia siliqua relative to control.

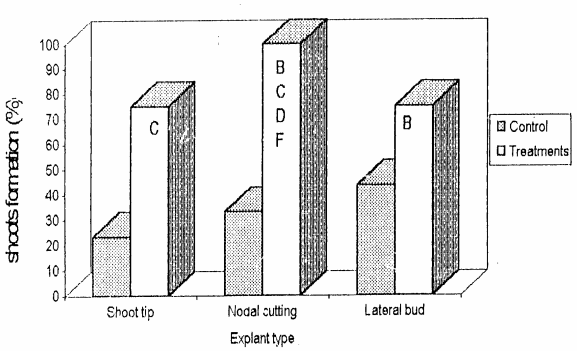

Fig. (3): Effect of explant type and plant growth regulators on shoots formation (%) of Ceratonia siliqua relative to control.

TREATMENTS

Co-Ms-basal medium (control) (Fig. 2 and 3)

A- BAP, 1.0 mg / l.
B- (IBA, 0.5 + BAP, 0.5 mg/l).
C- (IBA, 0.5 + BAP, 1.0 mg/l).
D- (IBA, 1.0 + BAP, 1.0 mg/l).
E- (IBA, 0.5 + Kin, 0.5 mg/l).
F- (IBA, 1.0 + Kin, 1.0 mg/l).

In this experiment, explant survival of *Ceratonia siliqua* was mainly affected by two factors, plant growth regulators applied and the type of explant used. The type of both cytokinin and auxin played a significant role affecting the explant survival potentiality. This may be ascribed the possible percentage of some of the plant tissue exudates which would suppress its survival potentiality. Hence, cytokinins, specially BAP, and auxins, particularly IBA seemed to play a role in reducing the endogenous exudates, such as phenolic compounds. Similar reports were cited by Williamson (1950); Belkengren and Miller

(1962); Von Arnold and Erikson (1988) and Bosila *et al.* (1994). Moreover, Danielle (1990) stated that antioxidants do not prevent phenolic oxidation, but prevent the polymerization of quinons, reducing their chances to react with protein. In addition, the explant type plays a critical role on explant survival. These results may be explained on the basis that the position of explant played an important role on its capability for survival, as the larger and more mature explant in the middle part of the shoot, the more vascular tissues were formed to transfer the necessary nutrients for survival. In addition, shoot apices may produce smaller amounts of cytokinins, therefore, cytokinins synthesized in shoot apices were insufficient to sustain their prolonged growth *in vitro,* so exogenous application of cytokinins is frequently required. These results were supported by those reported by Letham (1967); Koda and Okazawa (1980); George and Sherrington (1984) and Arafa (1994).

Shoot formation was influenced by the same two factors. Cytokinins have been shown to stimulate cell division as well as cell elongation and to activate RNA synthesis and to stimulate protein synthesis and enzyme activity as well (Kshankia and Niranjini, 1995). Moreover, auxin is thought to be mainly responsible for the initial differentiation of phloem and xylem elements in tissue culture. A balance between auxin and cytokinin is often required for different aspects of cellular differentiation and organogenesis in tissue and organ cultures. Relatively high levels of cytokinin are presumed to encourage the growth of axillary buds and reduce apical dominance of the main shoot. This agreed with Thorpe and Meier (1972); Kulaeva (1980), Aloni (1980), Vieitez and Vieitez (1980b) and Vieitez *et al.* (1993). The explant type and its position play a significant role, on the potentiality of inducing shootlets. The central buds were observed to be more vigorous, having mature vascular tissues to transfer various nutrients and hormonal components essential for different physiological processes, giving rise to shootlets formation in a pronounced higher potentiality than other explants. Analogous conclusions were stated by Arafa (1992) and Kshanika and Niranjini (1995).

Thus, it could be concluded that each type of explant under investigation subjected to the influence of specific exogenous convenient balance between auxin and cytokinin as morphogenic regulant necessary for controlling the internal hormonal balance for preserving the highest explant survival and optimal shootlets formation percentage which varied from one type of explant to another, which could be due to exogenous plant growth regulators according to the physiological state of the explant under investigation. Actually, no reports on direct shoot formation from organ culture of *Ceratonia siliqua* have been published, therefore, this finding is of much economical importance due to the fulfilment of obtaining female plantlets out of *Ceratonia siliqua* female trees through *in vitro* vegetative propagation which is not considered an easy task by traditional methods. Moreover, this research, needs to be continued to realize mass clonal production which would save hard currency spent on importing carob fruits.

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