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Effect of growth regulators and explant origin on *in vitro* propagation of *Ceratonia siliqua* L. via cuttings

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The present work was undertaken to develop a basic and simple protocol for micropropagation of *Ceratonia siliqua*. Axillaries bud sprouting and shoot development were stimulated on MS supplemented with BAP (0.5 mg·l^{-1}), IBA (0.1 mg·l^{-1}) and GA_3 (0.5 mg·l^{-1}), shoot multiplication was obtained on MS supplemented with BAP (2 mg·l^{-1}) and rooting of microshoots was achieved on MS supplemented with IBA (2 mg·l^{-1}) and charcoal (2 mg·l^{-1}). The best results were obtained with herbaceous explants taken from juvenile trees. Significant differences in proliferation, multiplication and rooting due to the type and origin of explant and to the concentration of growth regulators were found.
Keywords. *Ceratonia siliqua*, *in vitro*, bud sprouting, multiplication, rooting.

Effet des régulateurs de croissance et de l'origine de l'explant sur la culture *in vitro* du caroubier (*Ceratonia siliqua* L.) par le microbouturage. Une méthode de micropropagation du caroubier (*Ceratonia siliqua* L.) à partir de microboutures comportant un ou deux bourgeons axillaires est décrite. Le débourrement et le développement des pousses sont obtenus sur le milieu MS additionné de BAP (0,5 mg·l^{-1}), d'AIB (0,1 mg·l^{-1}) et de GA_3 (0,5 mg·l^{-1}). Les pousses feuillées sont multipliées sur le milieu de MS avec de la BAP à raison de 2 mg·l^{-1} puis enracinées sur le milieu de MS dont les macro-éléments et les micro-éléments ont été dilués de moitié et additionnés de charbon actif (2 mg·l^{-1}) et d'AIB (2mg·l^{-1}). Les meilleurs résultats sont obtenus avec les explants herbacés, issus d'arbres jeunes. Des différences significatives de résultats sont obtenus aux stades d'initiation, de multiplication et d'enracinement. Elles sont dues à l'origine des explants et aux différentes concentrations hormonales.
Mots-clés. *Ceratonia siliqua*, *in vitro*, débourrement, multiplication, enracinement.

1. INTRODUCTION

The carob tree (*Ceratonia siliqua* L.) belonging to the family Cesalpiniaceae, is widely used in the Mediterranean regions (Rejeb, 1989; Tous et al., 1990) and cultivated for ornamental and industrial purposes (Girolamo et al., 2002). The carob is mainly used in pharmaceutical and in liquor industries (Van Uden, 1981; Maria et al., 1997; Haslberg, 2000).

There is a great intraspecific variability with a large number of cultivars of carob (Mitrakos, 1987). The high phenotypic variation between cultivars has important implications for selection, cultivation practices and establishment of new plantations to improve productivity of this crop (Battle et al., 1997) which is hampered by its high morphological variability (Naghmouchi et al., 2004) and its high long reproductive cycle.

To optimise the productivity of plantations, it is essential to plant a maximum number of female plants. This could be achieved through *in vitro* culture method for cloning superior carob plants of each sex.

Most studies on carob tree have focused on applied aspects, such as agricultural, industrial and commercial. Nevertheless, there are some aspects on carob flower phenology, fruiting and pollination (Tucker, 1992; Retana et al., 1994; Bosch et al., 1996; Ortiz et al., 1996; Arista et al., 1999).

Organogenesis in carob has been reported in the past, Martins-Loucao et al. (1981) obtained calli and

shoot regeneration from cotyledon cultures. Sebastian et al. (1986) and Romano et al. (2002) reported a micropropagation protocol from seedlings and mature trees, using Murashige & Skoog (1962) medium (MS) supplemented with zeatin for shoot multiplication and MS medium supplemented with indole-3-butyric acid (IBA) for root induction. Bhalerao et al. (1992) tested young male inflorescences on MS medium supplemented with 6-benzylaminopurine (BAP) and casein hydrolysate. On this medium, the floral buds were transformed into shoot buds, and developed into various types of shoots. After transferring to MS medium supplemented with BAP and kinetin, the shoots elongated and formed 2-3 leaf pairs. Belazi et al. (1994) cultured nodal segments of seedlings in Quoirin & Lepoivre (1977) medium added with IBA, GA_3 and IBA at different concentrations.

Adventitious root formation is essential for successful vegetative propagation of many woody plants in a cutting. However, in several tree species, rooting is still a major problem. Furthermore, rooting ability declines with age (Dimitris et al., 2002). Some progress have been made in rooting of different species using different chemical or natural compounds in the rooting media such as auxins combined with phenolic compounds (Onay et al., 2003; Fotso et al., 2004).

Root formation of ligneous species is regulated by a great number of factors, and to a great extent by auxins (Shwab et al., 1988; Baksha et al., 2003) and by addition of charcoal in the media which is indispensable to reduce the effect of polyphenols secreted by the microcuttings.

Our work aimed at developing an *in vitro* vegetative propagation method of selected trees of *C. siliqua* and founding optimal conditions for shoot proliferation and root formation.

2. MATERIAL AND METHODS

2.1. Explant origin, sterilization protocol and cultivation

Nodal segments of carob were harvested from trees growing in the Botanical Garden of the Institute of Research in Rural Engineering, Water and Forestry in Tunisia.

The material collected from February to April, from herbaceous and semi-ligneous parts of the stem of mother-trees, was used as initial explant for *in vitro* propagation of carob trees of different ages (5, 12 and 25 years).

These explants were washed with water and detergent (Teepol). They were shaken successively for 5 minutes in 70% ethanol, 20 minutes in a NaClO solution and 20 minutes in a 0.1% $HgCl_2$ solution. Finally, explants were rinsed three times with sterile distilled water and cultured on agar nutrient medium.

2.2. Media and culture conditions

Cuttings of 20 mm long (one node with a single or two axillary buds) were excised and individually placed in 20x150 mm pyrex test tubes containing 15 ml of MS basal medium (Murashige & Skoog, 1962) supplemented with 30 $g \cdot l^{-1}$ sucrose and solidified with agar (8 $g \cdot l^{-1}$). The pH of medium was adjusted to 5.6 with HCl 0.1N or NaOH 0.1N before sterilization by autoclaving at 121°C for 105 minutes.

Growth regulators: 6-Benzylaminopurine (BAP), Indol-3-butyric acid (IBA) and Gibberelic acid (GA_3) were added in media according to the experimental stage. After inoculation, cultures were incubated under a 16:8 h photoperiod of cool-white light at 1250 Lux.

2.3. Shoot culture

In vitro regenerated shoots, longer than 20 mm, were excised from the microcuttings and maintained by subculturing every five weeks on a shoot multiplication medium: MS salts supplemented with IBA (0, 0.1 and 0.5 $mg \cdot l^{-1}$) and BAP (0, 0.5, 1 and 2 $mg \cdot l^{-1}$).

Observations were made after five weeks including percentage of axillary bud sprouting, shoot lengh, shoot numbers and multiplication rate.

2.4. Rooting induction

Multiplying microshoots were cultured on rooting medium. The rooting process was separated into two treatments:

– Shoots were cultured on darkness on the rooting medium that contained macro and micronutrients in half-strength with 30 $g \cdot l^{-1}$ sucrose and 8 $g \cdot l^{-1}$ agar, supplemented with IBA (0, 1 and 2 $mg \cdot l^{-1}$)
– Shoots were transferred to the basal medium and incubated under the same light and temperature regime as shoot multiplication cultures.

Data on rooting percent were recorded after five weeks of transfer in rooting medium.

2.5. Statistical design

Forty-eight microcuttings were tested on initiation medium and 24 shoots were tested on each multiplication and rooting medium.

Data from each experimental stage were analysed separately by an analysis of variance and the means compared with Duncan's multiple-range test at $P < 0.05$.

3. RESULTS

3.1. Effect of plant growth regulators and explant origin on axillary bud sprouting and shoots development

Nodal segments placed on MS agar medium started to form shoots within two to three weeks. Fast axillary bud sprouting was achieved on MS medium supplemented with different concentrations of BAP and IBA.

All tested BAP concentrations stimulated the fast developpment of new shoots from axillary buds of nodal segments.

Experiments showed that the major treatment was significantly different from the control. MS medium supplemented with BAP (0.5 mg·l^{-1}) plus IBA (0.1 mg·l^{-1}) and GA_3 (0.5 mg·l^{-1}) gives a higher rate of bud sprouting and promoted effectively formation of longer shoots in nodal segments (**Figure 1**).

The MS medium supplemented with higher concentration of BAP (1 and 2 mg·l^{-1}) stimulated formation of shoots. However, the shoots were shorter than those obtained with MS added with BAP (0.5 mg·l^{-1}), IBA (0.1 mg·l^{-1}) and GA_3 (0.5 mg·l^{-1}) (**Figure 1**).

Nodal segments, taken from herbaceous and semi-ligneous parts of trees at different ages, and cultured on MS medium supplemented with BAP and IBA produced new shoots within two to three weeks.

Results showed that bud sprouting decreased with increasing the age of mother tree and with lignification of the explant.

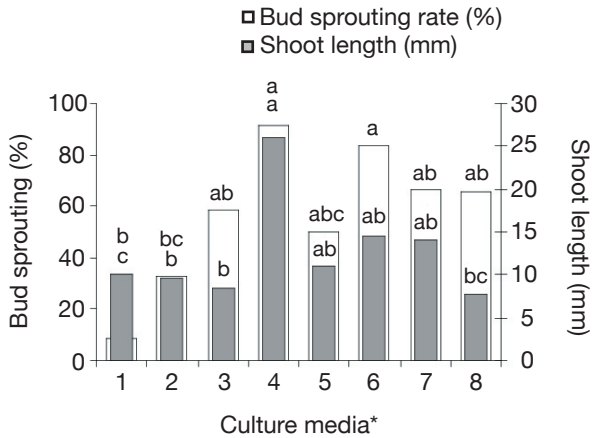

Figure 1. Hormonal effect on axillary bud sprouting and shoot development of herbaceous carob microcuttings taken from 5 years age trees — *Effet des hormones de croissance sur le débourrement axillaire et le développement des micropousses chez les microboutures herbacées prélevées d'arbres de 5 ans d'âge.*

Values marked with the same letter are not significantly different at 5% — *Les valeurs suivies de la même lettre ne sont pas significativement différentes à 5 %.*

* see **Table 1**

Table 1. Code of culture medium and concentrations of growth regulators used in the carob tree's bud sprouting stage — *Code et concentrations des régulateurs de croissance utilisés durant le stade d'initiation des microboutures de caroubier.*

Culture media		1	2	3	4	5	6	7	8
PGRs (mg·l^{-1})	BAP	0	0.5	1	0.5	1	1	2	2
	IBA	0	0	0	0.1	0	0.5	0	0.5
	GA_3	0	0	0	0.5	0	0	0	0

There is a significant difference in response of the different explants inoculated on the better selected medium: MS plus BAP (0.5 mg·l^{-1}), IBA (0.1 mg·l^{-1}) and GA_3 (0.5 mg·l^{-1}) (**Figure 2**).

Bud sprouting rates and shoot length of herbaceous microcuttings were higher than that of semi-ligneous microcuttings (**Figures 2** and **3**).

3.2. Effect of plant growth regulators and explant origin on shoot multiplication of *Ceratonia siliqua*

The highest multiplication rate and the high number of shoots regenerated by microshoot were obtained on MS medium supplemented with 2 mg·l^{-1} BAP (**Figure 4**).

In general, the number of shoots produced on media supplemented with BAP increased with increasing BAP concentration.

Our experiments with explants taken from trees of different ages and different parts of mature trees of *C. siliqua* showed that age of explants and lignification

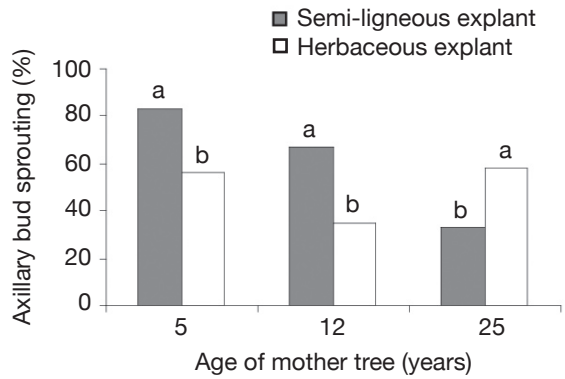

Figure 2. Effect of explant origin on axillary bud sprouting of *Ceratonia siliqua* L. cultured on MS medium supplemented with BAP (0.5 mg·l^{-1}), IBA (0.1 mg·l^{-1}) and GA_3 (0.5 mg·l^{-1}) — *Effet de l'origine de l'explant sur le débourrement axillaire de* Ceratonia siliqua *L. cultivée sur le milieu MS additionné de BAP (0,5 mg·l^{-1}), AIB (0,1 mg·l^{-1}) et GA_3 (0,5 mg·l^{-1}).*

Percentages marked with the same letter are not significantly different at 5% — *Les pourcentages suivis de la même lettre ne sont pas significativement différents à 5 %.*

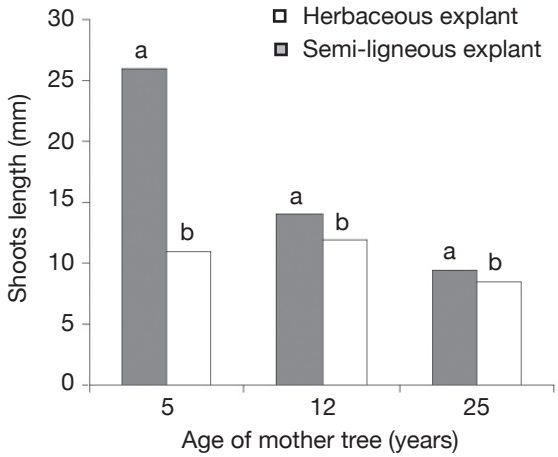

Figure 3. Effect of explant origin on shoot development of *Ceratonia siliqua* L. cultured on MS medium supplemented with BAP (0.5 mg·l^{-1}), IBA (0.1 mg·l^{-1}) and GA_3 (0.5 mg·l^{-1}) — *Effet de l'origine de l'explant sur le développement des feuilles de* Ceratonia siliqua *L. cultivée sur le milieu MS additionné de BAP (0,5 mg·l^{-1}), AIB (0,1 mg·l^{-1}) et GA_3 (0,5 mg·l^{-1}).*
Values marked with the same letter are not significantly different at 5% — *Les valeurs suivies de la même lettre ne sont pas significativement différentes à 5 %.*

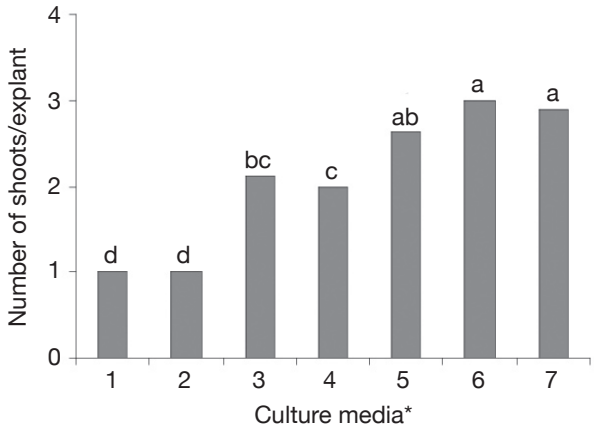

Figure 4. Hormonal effect on shoot multiplication of *Ceratonia siliqua — Effet des hormones de croissance sur la multiplication des vitropousses de* Ceratonia siliqua.
Values marked with the same letter are not significantly different at 5% — *Les valeurs suivies de la même lettre ne sont pas significativement différentes à 5 %.* * See **Table 2.**

Table 2. Code of culture medium and concentrations of growth regulators used in the carob tree's multiplication stage — *Code et concentrations des régulateurs de croissance utilisés durant le stade de multiplication des microboutures de caroubier.*

Culture media		**1**	**2**	**3**	**4**	**5**	**6**	**7**
PGRs (mg·l^{-1})	BAP	0	0.5	0.5	1	1	2	2
	IBA	0	0	0.5	0	0.5	0	0.5

of material are two important factors influencing shoot multiplication (**Figure 5**).

Shoots culture was produced from all tested explants. However, multiplication rates of explants issued from herbaceous parts were higher than those from semi-ligneous parts. Also microshoots produced from nodal segments taken from young trees (five years) exhibited higher multiplication rates and produced numerous and longer shoots than the other experimented materials.

3.3. Effect of explant origin, darkness exposure time, growth regulators and charcoal on rooting

Shoots excised from multiplying cultures and transferred on rooting medium started to form adventitious roots within three weeks after darkness exposure.

Figure 6 shows results on root formation with different concentrations of IBA.

When no IBA was supplemented in the medium, no roots were formed.

In order to promote maximum rooting, an exposure time for one week was required for explants of carob with 2 mg·l^{-1} IBA (**Figure 7**). The percentage of rooted shoots and the number of roots increased with increasing time of exposure, whereas the long time of exposure (more than 7 days) decreased rooting and the explants became chloritic.

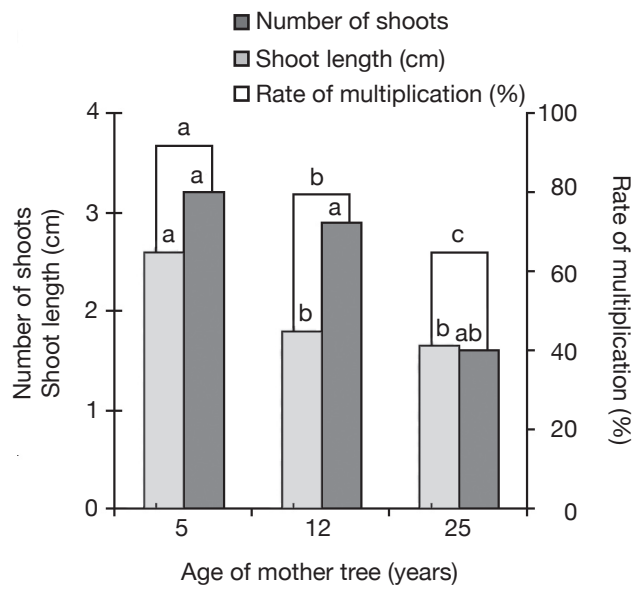

Figure 5. Effect of explants origin on shoot multiplication of *Ceratonia siliqua* L. cultured on MS medium supplemented with BAP (2 mg·l^{-1}) — *Effet de l'origine de l'explant sur la multiplication de* Ceratonia siliqua *L. cultivée sur le milieu MS additionné de BAP (2 mg·l^{-1}).*
Values marked with the same letter are not significantly different at 5% — *Les valeurs suivies de la même lettre ne sont pas significativement différentes à 5 %.*

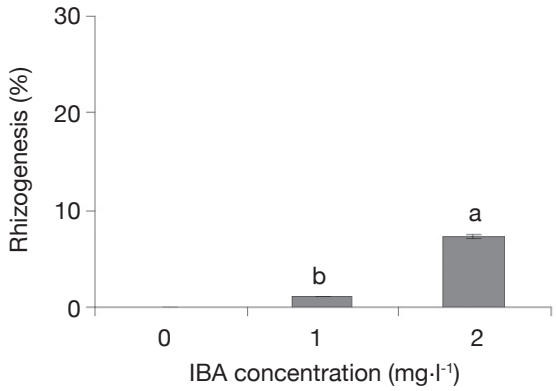

Figure 6. Effect of IBA concentration on rooting of shoots of *Ceratonia siliqua* L. — *Effet des concentrations de l'AIB sur l'enracinement des vitropousses de* Ceratonia siliqua *L.*
Percentages marked with the same letter are not significantly different at 5% — *Les pourcentages suivis de la même lettre ne sont pas significativement différents à 5 %.*

Figure 8. Effect of mother tree age on rooting of shoots of *Ceratonia siliqua* L. — *Effet de l'âge de l'arbre-mère sur l'enracinement des vitropousses de* Ceratonia siliqua *L.*
Percentages marked with the same letter are not significantly different at 5% — *Les pourcentages suivis de la même lettre ne sont pas significativement différents à 5 %.*

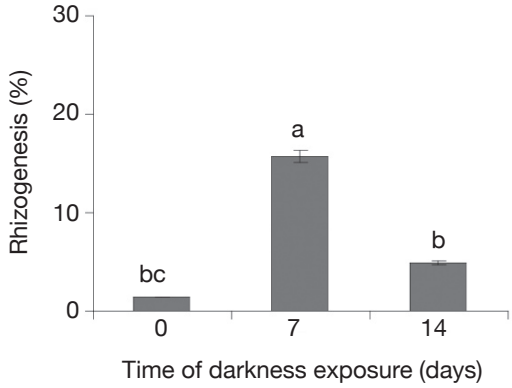

Figure 7. Effect of darkness time exposure on rooting of shoots of *Ceratonia siliqua* L. — *Effet du temps d'induction à l'obscurité sur l'enracinement des vitropousses de* Ceratonia siliqua *L.*
Percentages marked with the same letter are not significantly different at 5% — *Les pourcentages suivis de la même lettre ne sont pas significativement différents à 5 %.*

Shoots issued from nodal segments taken from young trees gave highest percentage of rhizogenesis (**Figure 8**). Shoots derived from juvenile parts of mature trees exhibited also good rooting response. Twenty-five percents of shoots formed adventitious roots within three to five weeks.

The best rate of rhizogenesis was observed with charcoal (2 mg·l^{-1}). The addition of charcoal ameliorates the development of roots and callus.

4. DISCUSSION

Recent advances in micropropagation of forest trees have opened great opportunities for mass propagation of selected valuable genotypes (Chalupa, 1993).

Selection of explants, composition of nutrient media, concentration of phytohormones and methods used for micropropagation had significant effects on shoot multiplication and rooting rates of carob explants.

Shoot elongation depends on the medium used. The addition of GA_3, IBA and BAP ameliorates bud sprouting and shoot elongation. The best elongation was shown on media with BAP. Shoots obtained on free BAP medium presented short internodes and quickly lost their ability to elongate. At 0.5 mg·l^{-1} BAP, the leaves were curled. Those formed on media with BAP were normal and no apparent difference was observed between shoots.

The development of microshoots had a similar tendency as bud sprouting. The best results were obtained with 2 mg·l^{-1} for herbaceous material collected from juvenile trees.

On media without growth regulators, nodal segments produced only one shoot.

The best shoot multiplication was obtained on media supplemented with 2 mg·l^{-1} BAP or with BAP (2 mg·l^{-1}) supplemented with IBA (0.5 mg·l^{-1}).

Shoot multiplication and elongation were also greatly affected with juvenility of explants.

It was demonstrated that technique of *in vitro* culture is used for rejuvenation of mature trees (Vieitez, 1994; Chalupa, 2000, 2002). The capacity of trees to be propagated vegetatively decreases with age; mature trees have usually a low capacity to be vegetative propagation (Brhadda et al., 2003).

Bud sprouting ability has been found to be closely related to cuttings origin in a number of other tree species. For example, *Olea europea* bud sprouting was the same in juvenile and adult material (Abousalim et al., 2005).

Our experiments with different explants origin of *C. siliqua* indicate that explants taken from juvenile parts from mature trees and those taken from juvenile trees, exhibit juvenile characteristics. Cultures initiated from these explants showed a high capacity for shoot formation and fast shoot proliferation.

A difference between herbaceous and semi-ligneous explants was found. Herbaceous explants are at a younger stage of development than semi-ligneous ones. A young developmental stage has often been found to be more optimal for shoot regeneration than older stages, which may be explained by differences in anatomical and physiological properties.

The potential of cuttings of *C. siliqua* to form adventitious roots decreased with increasing plant age. Furthermore mature trees are characterized by decreasing capacity for vegetative propagation. However, different parts of mature tree are often in different degree of maturity.

Despite the recalcitrance to vegetative propagation of *C. siliqua*, our results indicate that the species can be rooted by addition of IBA on the induction medium. Auxins are involved in the process of adventitious root formation. In many woody plants, IBA is commonly used to promote root initiation (Onay et al., 2003; Bhatt, 2004). In our study, the absence of IBA in the rooting medium did not lead to root formation. Several authors have shown that auxin is only required during the initiation phase, and becomes inhibitory for root out growth (Shwab et al., 1988; Elhamdouni et al., 2000; Chalupa, 2002). This inhibition of rooting is often accompanied with callus formation. The presence of callus on the shoots increased time for rooting as well as the number of roots formed. Studies on *in vitro* rooting of explants of *Eucalyptus* (Fazal et al., 2003) also showed an increased callus formation with increased IBA concentrations.

Charcoal is indispensable to induce good development of roots, clamping the reaction of tannins. In fact the tannin constitutes a physiological inhibitor (Souayah et al., 2002; Custódio et al., 2005).

The low rates of rhizogenesis were found with other species (Wallali et al., 1993). The difficulty to root *in vitro* can be overcome by rejuvenation of materials by different methods (Franclet et al., 1982; Howard et al., 1989). With carob, Sebastian et al. (1986) demonstrate the importance of multiplication medium on rhizogenesis and the presence of GA_3 in the shoot multiplication medium suppressed rooting.

Rooting experiments demonstrated the good rooting capacity of microshoots originated from juvenile parts of mature trees.

This study induces plantlets regeneration from explants of *C. siliqua*. These regenerated plantlets could be further transferred to the natural conditions.

5. CONCLUSION

Plant growth regulators, age of mother tree and type of explant clearly affected shoot development from nodal explants cultivated *in vitro* and shoot multiplication.

In vitro propagation of mature or juvenil carob trees can be achieved by the use of MS medium with BAP, IBA and GA_3 on the initiated medium and BAP, or BAP supplemented with IBA on the multiplication medium.

It can be concluded that the presence of IBA and charcoal in the medium is indispensable to induce a good development of roots, clamping the reaction of tannins. Thus, this medicinal plant has a considerable potential morphogenic capacity. This potential can be optimized by searching the performing factors in each stage of the breeding technique: age of the mother tree and type and concentration of plant growth regulators, and by optimizing the different stages.

The problems associated with the great variability of carob (*C. siliqua* L.) and the long stage of juvenility can be now overcome, using *in vitro* microcutting or other techniques like meristem culture and direct and indirect organogenesis.

List of abbreviations

BAP: 6- Benzylaminopurine
GA_3: Gibberelic acid
$HgCl_2$: Bichlorure mercure
IBA: Indol-3-butyric acid
MS: Murashige and Skoog (1962)
NaClO: Hypochlorite sodium
GRs: Growth Regulators

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