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Micropropagation of the Mediterranean tree *Ceratonia siliqua*

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Abstract

An *in vitro* propagation protocol based on axillary bud proliferation has been developed for mature female trees of *Ceratonia siliqua* L. 'Galhosa' and 'Mulata'. Browning and contaminants were the major obstacles for culture establishment. Shoot culture initiation was greatly influenced by explanting season, with the highest survival percentage observed in spring. The cultivar, cytokinin type and concentration were the most important factors affecting shoot multiplication. The best multiple-shoot response was obtained with 'Mulata' on Murashige and Skoog medium supplemented with 4.44 μM 6-benzyladenine or 4.56 μM zeatin. Rooting was achieved on growth-regulator-free medium after basal dipping of shoots in indole-3-butyric acid (4.9 mM). Plantlets were successfully acclimatized (80–85%) under high relative humidity and then moved to the glasshouse. A field trial was established to follow their agronomic behaviour.

Abbreviations: 2iP – 2-isopentenyladenine; BA – 6-benzyladenine; GD – Gresshoff and Doy (1972) medium; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; MS – Murashige and Skoog (1962) medium; 1/2MS – half-strength MS medium; NAA – 2-naphthaleneacetic acid; PG – phloroglucinol

Introduction

Carob (*Ceratonia siliqua* L.) is a tree belonging to the *Leguminosae*, and is well adapted to the Mediterranean climate. It is an evergreen sclerophylous tree suitable for the revegetation of marginal and submarginal dry areas of the Mediterranean basin (Batlle and Tous, 1997). The demand for carob products has steadily increased due to the growth of food industries using locust bean gum. Portugal is one of the most important exporters of locust bean gum (1330 tons in 1999). This gum, obtained from carob seed endosperm, is rich in galactomannans and improves the water retention properties. Its use as a food additive is the most important outlet for locust bean gum. It is employed in a wide range of products, among the most important of which are ice cream, baby foods and pet foods.

The presence of wild trees growing adjacent to established orchards, as well as the great variation in sexuality of different carob varieties (male, female, hermaphrodite and polygamous inflorescences) causes great intraspecific variability and a large number of cultivars (Mitrakos, 1987). The high phenotypic variability within and between cultivars has important implications for selection, cultivation practices, and establishment of new plantations and productivity optimization of this crop (Batlle and Tous, 1997).

Traditional carob propagation has been achieved by grafting saplings with female buds of chosen productive trees (Batlle and Tous, 1997). This traditional method of propagation has failed to meet the market demand for new, selected plant material. Thus, the use of micropropagation techniques seems to be appropriate, in order to fulfill the increased demand for propagating this tree.

Of the few reports published on micropropagation of *C. siliqua* (Thomas and Metha, 1983; Alorda and Medrano, 1986; Sebastian and McComb, 1986), only

Table 1. Seasonal influence of explant collection on *in vitro* culture initiation: percentage of surface sterile explants, percentage of viable explants and percentage of browning. Cultivars: 'Mulata' and 'Galhosa

Explanting season	% surface sterile	% viable	% browning[1]
'Mulata'			
March–April	75.3 a	24.3 a	62.3 bc
July–August	12.1 b	12.4 b	70.4 ab
November–December	0 c	0 c	100 a
'Galhosa'			
March–April	80.6 a	30.2 a	72.4 a
July–August	20.4 b	15.7 b	75.6 ab
November–December	0 c	0 c	100 a

Results are the mean of two collections during the first week of each mentioned month. Values represent means±SE of three replicates with 30 shoots. Values followed by the same letter are not significantly different at $p \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test).
[1]Browning of explant and/or medium.

Table 2. Effect of the basal media (MS and GD) on the mean number of shoots per culture and elongation (mm) of the longest shoot of cultivars 'Mulata' and 'Galhosa'

	Mean number of shoots	Longest shoot length (mm)
'Mulata'		
MS	1.3±0.1 a	13.5±0.6 a
GD	1.1±0.1 a	8.8±0.5 b
'Galhosa'		
MS	1.0±0.0 b	9.2±0.4 b
GD	1.4±0.1 a	10.9±0.5 a
Significance of two-way ANOVA		
Cultivar (A)	ns	ns
Medium (B)	ns	*
A×B	***	***

Media were supplemented with 2.22 μM BA.
Values represent means±SE of three replicates with 30 cultures. For each cultivar and variable, a single factor analysis of variance was performed between media. Values followed by the same letter are not significantly different at $p \leq 0.05$. ns, *, **, ***: non significant, significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively (two-way ANOVA, Ducan's Multiple Range Test).

one describes procedures for propagation of mature trees (Sebastian and McComb, 1986). The Portuguese cultivars 'Galhosa' and 'Mulata' are economical and ecologically interesting (Martins-Loução and Brito de Carvalho, 1989; Batlle and Tous, 1997) and the present paper describes a protocol for micropropagating these selections through *in vitro* culture of nodal explants from mature female trees.

Materials and methods

Plant material

Stem cuttings, 20–30 cm length, were collected from the crown of 12 year-old adult female trees 'Galhosa' and 'Mulata' growing in a germplasm repository in Tavira (Algarve, Portugal). Cuttings were randomly collected from five trees of each cultivar which have been propagated by grafting from two original trees. The cuttings were defoliated just after collection and surface disinfected using the system reported previously (Romano et al., 1992). Nodal segments, 1–2 cm length, with one unopened bud were used as explants.

Alternatively, the buds were forced to grow under controlled conditions as described previously (Romano et al., 1992). The developed sprouts, 4–5 cm in length, were collected for establishment *in vitro*. These sprouts were surface disinfected in commercial bleach (20% v/v, plus a few drops of Tween 20) for 15 min and then washed three times in sterile distilled water.

Establishment

In order to control contaminants, different sterilizing agents were tried in the spring of 1998, including NaOCl, $Ca(ClO)_2$ and $HgCl_2$ at various concentrations for different times. Thirty explants were tested per sterilization method and per cultivar. After sterilization, the explants were rinsed four times with sterile water and established in MS (Murashige and Skoog, 1962) medium supplemented with 2.22 μM 6-benzyladenine (BA). Sucrose (20 g l^{-1}) was used as carbon source and media were solidified with 0.7%

Table 3. Effect of cytokinin (BA, kinetin, zeatin and 2iP), and concentration (0.5 mg $l^{-1,a}$ and 1 mg $l^{-1,b}$) on the mean number of shoots per culture and elongation (mm) of the longest shoot of cultivars 'Mulata' and 'Galhosa'

	Mean number of shoots	Longest shoot length (mm)
'Mulata'		
BA	1.4±0.1 a	13.8±0.5 a
kinetin	1.1±0.0 b	8.8±0.3 c
zeatin	1.5±0.1 a	12.3±0.5 b
2iP	1.1±0.0 b	8.0±0.3 c
'Galhosa'		
BA	1.1±0.0 ab	9.2±0.2 a
kinetin	1.1±0.1 ab	8.2±0.3 b
zeatin	1.3±0.1 a	9.5±0.4 a
2iP	1.0±0.0 b	8.0±0.4 b
'Mulata'		
0.5 mg l^{-1}	1.2±0.0 b	11.1±0.4 a
1 mg l^{-1}	1.5±0.1 a	11.9±0.5 a
'Galhosa'		
0.5 mg l^{-1}	1.1±0.0 a	9.0±0.3 a
1 mg l^{-1}	1.2±0.0 a	8.6±0.2 a
Significance of three-way ANOVA		
Cultivar (A)	***	***
Cytokinin (B)	***	***
Concentration (C)	***	ns
A×B	**	***
A×C	**	*
B×C	ns	ns
A×B×C	*	ns

Basal medium: MS.
Values represent means±SE of three replicates with 30 cultures. For each cultivar and variable, a single factor analysis of variance was performed between treatments (cytokinin and concentration). Values followed by the same letter are not significantly different at $p \leq 0.05$. ns, *, **, ***: non significant, significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively (three-way ANOVA, Ducan's Multiple Range Test).
[a] Equivalent to 2.22 μM BA, 2.32 μM kinetin, and 2.28 μM zeatin 2.46 μM 2iP.
[b] Equivalent to 44 μM BA, 4.64 μM kinetin, 4.56 μM zeatin and 4.92 μM 2iP.

(w/v) agar (Iberagar). pH was adjusted to 5.8 before autoclaving at 121 °C and 1.1 kg cm^{-1} for 20 min.

In order to prevent browning of the medium and tissue, three strategies were tried. After sterilization, the explants were washed in an antioxidant solution (ascorbic acid 100 mg l^{-1}) for 5 min; the cultures were incubated in darkness during the first 48 h; and MS salts were reduced to half strength.

Primary explants were grown in test tubes (25×160 mm) containing 10 ml of medium, and closed with transparent polyethylene caps. Cultures were incubated for 6 weeks at 25±2 °C under a 16-h photoperiod at 60 μmol m^{-2} s^{-1} provided by cool-white fluorescent lights.

Shoot multiplication

After establishment, shoots ≥1 cm were separated from their basal callus and transferred onto multiplication media. During the multiplication phase, MS and GD (Gresshoff and Doy, 1972) media were compared. The cytokinin BA (2.22 and 4.44 μM), kinetin (2.32 and 4.6 μM), 2iP (2-isopentenyladenine) (2.46 and 4.92 μM), and zeatin (2.28 and 4.56 μM) were assayed. The auxins NAA (2-naphthaleneacetic acid) at 0.54 μM, IAA (indole-3-acetic acid) at 0.57 μM, and IBA (indole-3-butyric acid) at 0.49 μM were tested in combination with BA at 2.22 or 4.4 μM. The effect of 162 mg l^{-1} phloroglucinol (PG) in the multiplication medium was also examined (Thomas and Mehta, 1983).

After 6 weeks, the newly developed shoots were separated and subcultured for further multiplication. Shoot multiplication was determined as the total number of shoots produced from each culture, and the length of the longest shoot per culture, during three multiplication cycles of 6 weeks.

Rooting

For root induction, two methods were tested: inclusion of auxins in the rooting medium and dipping the base of shoots in a concentrated auxin solution. In the first method, individual shoots 3 cm in length, harvested at the end of the multiplication stage on MS medium supplemented with 2.22 μM BA, were cultured on half-strength MS medium (1/2MS) containing 11.42 μM of IAA, 9.8 μM of IBA or 10.74 μM of NAA. Shoots were grown for 1 week in darkness and then transferred to a 16-h photoperiod, 60 μmol m^{-2} s^{-1}, produced from cool-white fluorescent lamps for 3 weeks.

Alternatively, the basal ends of the shoots were dipped in 4.9 mM IBA for 2 or 3 min, followed by culture on 1/2MS growth-regulator-free-medium. Shoots were grown in the dark for 1 week and then under normal light conditions for 3 weeks.

For both methods, shoots were grown in test tubes (32×200 mm) containing 20 ml of medium, capped with aluminium foil. Rooting was evaluated 1 month

after induction and was expressed in terms of rooting percentage, root number, the longest root length per plantlet, the shoot length and the mean number of leaves.

Acclimatization

For acclimatization, plantlets with at least three roots were soaked in a dilute fungicide solution (Benomyl®, 1 g l^{-1}) for 1 min, and placed in 350 ml plastic pots containing a mixture of peat and vermiculite (3:1, v/v). The potted plantlets were maintained inside a plant growth chamber (Aralab 500 E) at 90–95% relative humidity, 25±2 °C, with a 16-h photoperiod (100 μmol m^{-2} s^{-1}), for 4 weeks. The relative humidity was gradually decreased to 65% for the next 4 weeks and plantlets were finally transferred to the glasshouse. Plantlets were watered bi-weekly with a diluted solution of MS macronutrients (1:10).

The results presented are the mean±SE of 30 explants per experiment. All the experiments were carried out three times. The results were compared through analysis of variance (ANOVA) and Duncan's Multiple Range Test. To analyze the data on establishment, rooting and survival percentages, arcsin square root transformation was used.

Results and discussion

The establishment of cultures was very difficult due to high incidence of contamination and phenolic compound exudation. Most of the disinfection methods were unsuccessful (∼100% contamination). Only 0.1% $HgCl_2$ for 5 min eradicated fungi and bacteria with 75% surface sterile explants and 25% of the explants giving rise to single shoots (5–10 mm) during the first 2 weeks of establishment. This disinfection method was used during the following establishment assays.

Contamination was more severe in November–December and the best results were observed in March–April for both cultivars (Table 1). Unexpectedly (Romano et al., 1992), the use of shoots flushed under controlled conditions did not decrease the incidence of contamination (∼100% of contamination).

The severe browning of explants and medium caused by phenolic exudate oxidation, and the lack of response to *in vitro* culture conditions of the non-contaminated explants inhibited establishment. None of the anti-browning methods assayed were successful. This problem could be circumvented by transfer of explants onto fresh medium whenever exudation occurred. Usually one transfer was enough to stop exudation. The inefficiency of antioxidant treatments has been previously reported for other woody plants (Romano and Martins-Loução, 1992; Sharma and Ramamurthy, 2000).

For both cultivars, the incidence of browning was maximum in November–December, although exudation was observed throughout the year (Table 1). A seasonal fluctuation of phenolic exudation has been reported for other woody species (Romano and Martins-Loução, 1992; Dhar and Upreti, 1999; Sharma and Ramamurthy, 2000).

Establishment of cultures was not possible year-round since significant seasonal fluctuation responses affected the explants viability. March–April was the best season for culture initiation (Table 1). The seasonal influence of explant collection on establishment has already been reported for other species (Hohtola, 1988; Pattnaik and Chand, 1997; Sharma and Ramamurthy, 2000).

During the first 2 weeks of establishment, axillary bud development was followed by callus initiation at the base of the explant. Four weeks later, the callus enlarged and produced adventitious shoots that failed to develop further. The axillary buds were separated from the callus after 6 weeks in culture. Basal callus grown on fresh medium was a source of adventitious shoot production (results not shown). This high callus formation and organogenic potential was already observed for this species (Thomas and Metha, 1983; Sebastian and McComb, 1986).

Multiplication phase was significantly affected ($p<0.001$) by the interaction of cultivar and basal medium and no statistically significant differences were observed with either basal media or cultivars (Table 2). However, shoot multiplication rate and shoot elongation were significantly higher on GD medium for 'Galhosa' as compared with MS. With 'Mulata', although no statistical significance was observed for the mean number of shoots, the shoot length was significantly higher on MS medium (Table 2).

Mean number of shoots per culture was strongly dependent on cultivar ($p<0.001$), cytokinin type ($p<0.001$), and concentration ($p<0.001$) (Table 3). 'Mulata' presented higher multiplication rates, with zeatin and BA the most effective cytokinins (Table 3). Although no statistical differences were observed between these two cytokinins in terms of multiplication rate, shoot length was statistically higher with BA (Figure 1a). Furthermore, the mean number of shoots

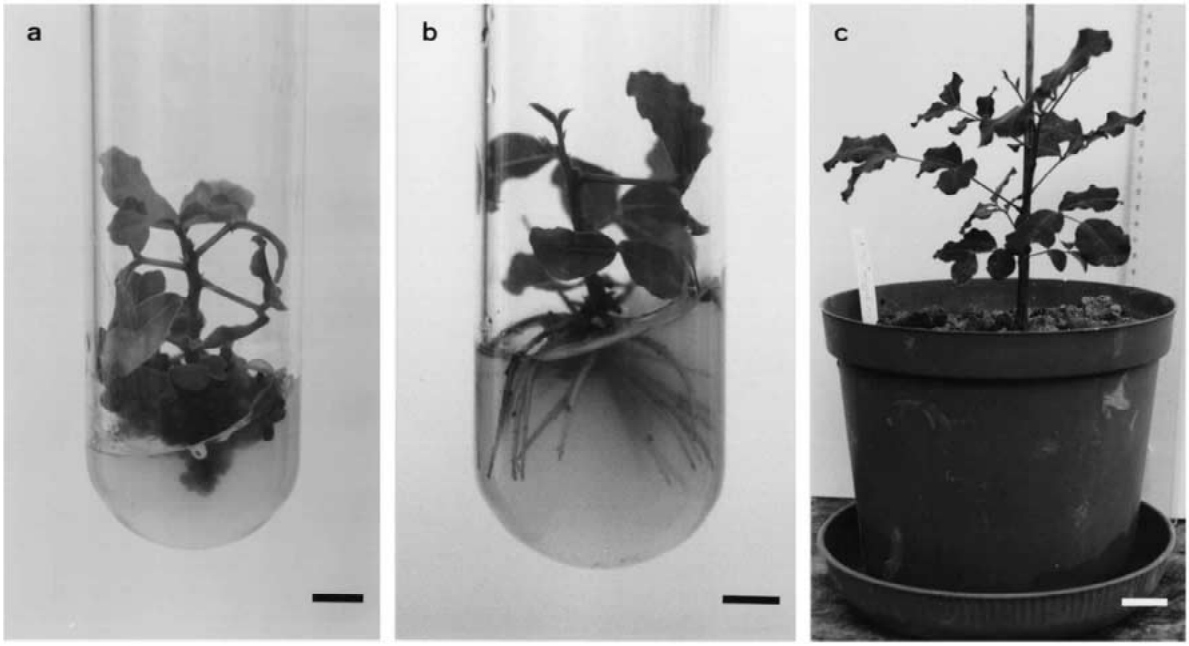

Figure 1. *(a)* Shoot-culture ('Galhosa') at the end of multiplication phase in BA (4.9 μM) containing medium (bar=2 cm). *(b)* Rooted shoot ('Mulata') 20 days after rooting induction by basal immersion in a 4.9 mM IBA solution for 3 min (bar=2 cm). *(c)* Plantlet ('Galhosa') 3 months after acclimatization growing in the glasshouse (bar=0.5 cm).

per culture increased with increasing concentrations of cytokinin (Table 3). For 'Galhosa', only shoot length was statistically higher for zeatin and BA.

No statistically significant interaction was found between cytokinin type and concentration on shoot number and shoot length. With respect to the shoot length, the most important factors were cultivar ($p<0.001$), cytokinin type ($p<0.001$), and their interaction ($p<0.001$) (Table 3).

In contrast to other woody plants (Romano et al., 1992; Ajithkumar and Seeni, 1998) the combination of BA (2.22 and 4.44 μM) and various auxins (0.57 μM IAA, 0.49 μM IBA or 0.54 μM NAA) did not improve the multiplication rate or elongation of the shoots. However, Alorda and Medrano (1986) and Sebastian and McComb (1986) observed that 4.44 μM BA and 4.56 μM zeatin, respectively, induced the best shoot growth and axillary bud development of carob. In contrast, Thomas and Mehta (1983) observed the best results with MS medium supplemented with 8.9 μM BA, 0.49 μM IBA and 5.77 μM GA_3. Furthermore, addition of 162 mg l^{-1} of PG enhanced general growth and leaf expansion (Thomas and Mehta, 1983).

The use of PG during multiplication has improved shoot multiplication in several species (Hammatt, 1993a; Ibanez and Amo-Marco, 1998) but can also be deleterious (Hammatt, 1993b; Ramirez-Malagon et al., 1997). Addition of 162 mg l^{-1} PG to the multiplication media was ineffective. No growth was observed and the cultures turned brown and dead.

During establishment and proliferation lenticel hypertrophy was observed. This appeared first at the basal shoot internode and gradually spread acropetally. Vinterhalter et al. (1992) reported development of lenticel callus in shoot cultures obtained from epicotyls of carob immature embryos.

Adventitious rooting of woody plants is usually induced by auxin. The effect of different auxins (IAA, IBA and NAA) was tested with 'Galhosa', with the best results observed with IBA (Table 4). These plantlets developed a high number of roots, but only a 70% of rooting frequency was attained.

Previous results with juvenile carob material indicated that IAA is the most adequate auxin (36–40% of rooting) (Alorda and Medrano, 1986). Thomas and Mehta (1983), also working with juvenile material, reported that neither NAA nor IBA induced rooting, and that the addition of PG was essential. In contrast, Sebastian and McComb (1986) reported a high rooting frequency (92%) with 9.8 μM IBA.

PG was completely ineffective for rooting. PG can improve rooting of several species e.g. fraser photinia

Table 4. Effect of auxins NAA, IAA and IBA added to 1/2MS medium on the percentage of rooting (%R), the mean number of roots, the length of the longest root (mm), total shoot length (mm) and number of leaves per rooted plantlet

Auxin (μM)[1]	%R	Mean root number	Longest root length (mm)	Shoot length (mm)	Mean leaf number
10.74 NAA	5	*nd*[2]	*nd*	*nd*	*nd*
11.42 IAA	10 b	3±2 b	36.5±6.5 a	27.0±5.0 a	6±1 a
9.8 IBA	70 a	10±1 a	31.1±4.6 a	35.1±2.6 a	7±1 a

Values represent means±SE of three replicates with 30 shoots. Values followed by the same letter are not significantly different at $p \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test).
[1]Equivalent to 2 mg l^{-1} of each auxin.
[2]*nd* – not determined.

Table 5. Influence of the duration of dipping (2 or 3 min) in 4.9 mM IBA, of the basal medium (1/2MS or GD) and cultivar ('Mulata' and 'Galhosa'), on the percentage of rooting (%R), the mean number of roots per plantlet, the length of the longest root (mm), and total shoot length (mm) and number of leaves per rooted plantlet

Dipping time (min)	Basal medium	Cultivar	%R	Mean root number	Longest root length (mm)	Shoot length (mm)	Mean leaf number
2	1/2MS	'Galhosa'	70b	9±1 ab	50.5±7.3 a	39.6±2.9 a	7±1 ab
	1/2MS	'Mulata'	67 b	7±2 bc	44.8±12.8 ab	*nd*[1]	*nd*
	GD	'Galhosa'	80 ab	5±1 c	25.8±2.7 c	23.1±1.5 b	6±1 bc
3	1/2MS	'Galhosa'	85 a	12±1 a	34.8±4.1 b	38.2±3.0 a	8±1 a
	1/2MS	'Mulata'	59 c	4±1 c	30.2±9.4 bc	*nd*	*nd*
	GD	'Galhosa'	85 a	10±1 ab	21.8±2.8 c	27.0±1.9 b	5±1c

Values represent means±SE of three replicates with 30 shoots. Values followed by the same letter are not significantly different at $p \leq 0.05$ (one-way ANOVA, Duncan's Multiple Range Test).
[1]*nd* – not determined.

(Ramirez-Malagon et al., 1997), wild cherry (Hammatt, 1993b) and apple 'Tydeman's Early Worcester' (Modgil et al., 1999), but is ineffective for *Pyrus* species (Berardi, 1993; Al-Maarri et al., 1994) and *Azadirachta excelsa* (Kooi et al., 1999).

The rooting percentages were increased to 85% by immersion of shoots in a 4.9 mM IBA solution for 3 min (Table 5). Increasing dipping time from 2 to 3 min increased rooting of 'Galhosa' shoots, while for 'Mulata' the opposite was observed. The same rooting frequency and mean number of roots were observed with 1/2MS and GD with 'Galhosa' shoots for 3 min of dipping time. However, better results were observed for root and shoot length, and mean number of leaves with 1/2MS (Table 5). For 3 min of dipping and 1/2MS medium, 'Galhosa' shoots had higher rooting potential than 'Mulata' (Table 5).

A large number of long and vigorous roots developed after IBA treatment (Table 5, Figure 1b). These roots gradually turned brown and only the terminal part of the root remained white. This rooting protocol is a simple and efficient method for root induction of carob-tree.

Regenerated plantlets were successfully acclimatized *ex vitro* in an acclimatization chamber with high relative humidity. After 2 months, plants were transferred to glasshouse conditions and the percentage of surviving plantlets was 85% and 80% for 'Gathosa' and 'Mulata', respectively (Figure 1c). A field trial has been established with 95% success. Plants showed good growth and uniformity 8 months after transfer to the field.

The results show that Portuguese carob 'Mulata' and 'Galhosa', which are difficult to propagate asexually, can be cultured *in vitro* in a zeatin or BA enriched media using nodal segments from adult trees of 'Gaihosa' and 'Mulata' growing in the field. The positive response of the explants during the establishment phase is however, seasonally dependent.

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