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BRIEF COMMUNICATION

Influence of sugars on *in vitro* rooting and acclimatization of carob tree

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Abstract

Carob tree (*Ceratonia siliqua* L.) micropropagated shoots were rooted on half-strength Murashige and Skoog 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. Among the various sugars tested, the best rooting response was obtained with 145 mM sucrose, both in terms of rooting frequency and index of rooting. The use of filter-sterilized rather that autoclaved fructose increased root number and root length. Sugar treatment during rooting slightly influenced plantlet survival and growth during acclimatization. A reduction in the glucose concentration during rooting was beneficial for plantlet acclimatization.

Additional key words: *Ceratonia siliqua* L., fructose, glucose, mannitol, sorbitol, sucrose.

Adventitious root formation is a difficult step in tissue culture propagation for many woody plants. Root initiation and growth are high energy requiring processes that can occur only at the expense of available metabolic substrates, mainly sugars. Quality of tissue-cultured plantlets is an important factor for success during the transition to *ex vitro* conditions. Preconditioning and rooting shoots with different concentrations and types of sugars influences the quality of plants produced *in vitro* (Wainwright and Scrace 1989, Van Telgen *et al.* 1992, Romano *et al.* 1995, Morini and Melai 2003/4). By amending the sugar content in the culture medium prior to *in vivo* transfer it may be possible to improve the quality of established plantlets derived from aseptic cultures (Wainwright and Scrace 1989, Moncousin *et al.* 1992, Van Telgen *et al*. 1992, Marino *et al.* 1993).

Carob (*Ceratonia siliqua* L.) is a slow growing evergreen tree of the family *Fabaceae* suitable for the revegetation of marginal and submarginal dry areas of the Mediterranean basin. Portuguese cultivar Galhosa is of great economic importance (Battle and Tous 1997) due to the locust bean gum obtained from its pods. This gum is reach in galactomannans and is used in the food industry as viscosofier, stabilizer and gelling agent, in products such as juices, dietetic beverages, desserts, baby foods and pet foods (Battle and Tous 1997).

A micropropagation system was successfully developed for carob tree (Romano *et al*. 2002). The present investigation was undertaken to examine the effects of several sugars on *in vitro* rooting and subsequent *ex vitro* growth of carob, in order to improve rooting and acclimatization for large-scale propagation of this species.

Shoots (3 cm long) were obtained from stock cultures of *Ceratonia siliqua* cv. Galhosa (Romano *et al*. 2002). For root induction the basal ends of the shoots were dipped in 4.9 mM indole-3-butyric acid (IBA) for 3 min, followed by culture on half-strength Murashige and Skoog medium (1962; MS). Sucrose (58, 87, 116 or 145 mM), glucose (58, 116 or 145 mM), 116 mM fructose, 116 mM sorbitol, or filter-sterilized 116 mM fructose, were added. Two combinations of sugars were also used: 58 mM glucose + 58 mM fructose and 58 mM glucose + 58 mM mannitol, corresponding to 116 mM

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Abbreviations: IBA - indole-3-butyric acid; MS medium - Murashige and Skoog medium.
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of total sugars. Except for filter-sterilized fructose, all sugars were added to the medium before autoclaving. The pH was adjusted to 5.8 before autoclaving at 121 ºC and 1.1 kg cm^{-2} for 20 min. Shoots were grown individually in test tubes (25 × 160 mm) containing 10 cm^3 culture medium and closed with transparent polyethylene caps. Shoots were grown in the dark for one week and then placed under a 16-h photoperiod (irradiance of 60 µmol m^{-2} s^{-1}, provided by cool-white fluorescent lamps) at a temperature of 25 ± 2 ºC for 3 weeks. Thirty shoots were used per treatment, and the experiment was repeated three times. Rooting was evaluated one month after root induction and expressed in terms of rooting percentage, root number and the longest root length per plantlet. Date of first root emergence was recorded for each treatment. Index of rooting (IR) was evaluated as: (total number of roots formed × number of shoots with roots)/(total number of shoots)^2.

For acclimatization, plantlets with at least three roots were soaked in a fungicide solution (*Benomyl*®, 1 g dm^{-3}) for 1 min, and placed in 350 cm^3 plastic pots containing a mixture of peat and vermiculite (3:1, v/v). The potted plantlets were maintained inside a plant growth chamber (*Aralab 500E*, Lisboa, Portugal) set at relative humidity 90 - 95 %, temperature of 25 ± 2 ºC, 16-h photoperiod (irradiance of 100 µmol m^{-2} s^{-1}), for four weeks. The relative humidity was gradually decreased to 65 % for the next four weeks and finally plants were transferred to the glasshouse. Plants were watered bi-weekly with a solution of MS macronutrients (1:10). Success of acclimatization was expressed in terms of survival percentage, and plant growth was assessed weekly by measuring plant height and counting leaves and leaflets.

Data (except IR and root emergence) were subjected to analysis of variance (*ANOVA*) to assess treatment differences using the *SPSS* statistical package for *Windows* (release *11.0*, *SPSS Inc*.). Significance between means was tested by Duncan's New Multiple Range Test ($P = 0.05$). To analyse the data on rooting and survival percentages, arcsin square root transformation was used.

In this work the 145 mM sucrose treatment induced the highest IR (Table 1). The use of this concentration also resulted in the highest rooting frequency (Table 1). Those observations do not confirm the concept that the reduction in sugar content improves rooting (Kooi *et al*. 1999). Beneficial effect of high sucrose concentration on rooting has been reported for cork oak and apple (Romano *et al*. 1995, Calamar and De Klerk 2002). The mean root number was significantly higher ($P \geq 0.05$) on 87 mM sucrose, except for 145 mM sucrose, 145 mM glucose and filter-sterilized fructose (Table 1).

With glucose, a significantly ($P \geq 0.05$) higher mean root number was observed in the highest concentration tested (Table 1). Similar results were obtained with equimolar concentrations of glucose and sucrose, except for 145 mM glucose, where roots emerged later, IR was lower and rooting frequency was significantly lower ($P \geq 0.05$) (Table 1). Nevertheless, glucose proved to be an efficient carbon source for tissue culture in other species, namely in *Ficus lyrata* (Wainwright and Scrace 1989) and *Quercus suber* (Romano *et al*. 1995).

Root elongation was greater on filter-sterilized fructose, excluding 87 and 145 mM sucrose and 116 mM glucose (Table 1). Autoclaved and filter-sterilized fructose proved to be similarly effective in terms of rooting frequency (Table 1). However, roots emerged earlier and IR, root number and elongation were higher when 116 mM fructose was filter-sterilized rather than incorporated into the medium before autoclaving (Table 1). There seems to be differences in sensitivity of

Table 1. Effect of sugar composition and concentration in medium on *in vitro* rooting of microcuttings of *Ceratonia siliqua* cv. Galhosa. IR - index of rooting. Means of three replicates with 30 shoots. Values followed by the same letter are not significantly different at $P \geq 0.05$.

Sugar	Concentration [mM]	Root emergence [d]	Rooting [%]	IR	Mean root number	Longest root length [cm]
Sucrose	58	12-14	60 b	4.3	12 b	1.8 de
	87	12-17	70 b	8.7	18 a	4.5 abc
	116	10-21	65 b	5.1	12 b	2.6 cd
	145	13-14	90 a	11.2	14 ab	5.0 ab
Glucose	58	14-21	60 b	3.8	11 b	2.4 cd
	116	11-13	60 b	4.6	13 b	3.8 abcd
	145	14-21	40 bc	2.4	15 a	3.5 bcd
Fructose	116	18-22	60 b	3.8	11 c	2.9 bcd
Filtered fructose	116	13-14	60 b	5.7	16 a	5.7 a
Glucose + fructose	116	14-21	35 bc	0.5	4 c	2.1 de
Glucose + mannitol	116	12-14	55 bc	1.8	6 c	2.6 cd
Sorbitol	116	20-21	15 c	0.05	2 c	0.3 e

Table 2. Effect of sugar composition and concentration in medium during *in vitro* root initiation on *Ceratonia siliqua* cv. Galhosa survival and growth after five months of *ex vitro* acclimatization. Values represent means of three replicates with 10 plantlets. Values followed by the same letter are not significantly different at $P \geq 0.05$.

Sugar	Concentration [mM]	Survival [%]	Increase in shoot length [cm]	Mean leaf number	Mean leaflet number
Sucrose	58	88 a	2.3 abc	3 ab	7 a
	87	63 ab	2.7 abc	4 ab	9 a
	116	90 a	2.6 abc	4 ab	9 a
	145	81 a	3.3 ab	4 ab	10 a
Glucose	58	90 a	1.6 bc	5 ab	10 a
	116	94 a	2.0 abc	3 b	8 a
	145	42 b	1.8 bc	3 b	7 a
Fructose	116	90 a	2.9 abc	4 ab	9 a
Filtered fructose	116	92 a	3.7 a	4 ab	9 a
Glucose + fructose	116	50 b	1.4 c	6 a	12 a
Glucose + mannitol	116	100 a	1.5 c	3 ab	7 a

plant species, to the degradation products formed as a result of fructose autoclaving, namely furfural and hydroxymethylfurfural, which may explain the contradictory results of fructose as a carbon source (Moncousin *et al*. 1992, Romano *et al*. 1995, Uosukainen and Vasara 1995, Peterson *et al*. 1999).

Sorbitol, an effective carbon source for apple and related species (Pua and Chong 1985, Marino *et al*. 1993, Kadota *et al*. 2001), was the least effective sugar in terms of rooting frequency (Table 1). The mean number of roots and root length were also very low (Table 1). Similar results were observed for cork oak (Romano *et al*. 1995). This negative effect could not be caused by osmotic effects, since the carbon sources were added in equimolar concentrations to ensure similar osmotic potentials. To check whether osmoticum played any role in rooting response, mannitol was added to glucose-containing medium, which resulted in a significant decrease in root number (Table 1).

Sucrose is usually added as the carbon source on *in vitro* culture, since it is the most common sugar in the phloem sap of the angiosperms (Zimmermann and Ziegler 1975). Nevertheless, the explants are usually exposed to a mixture of sucrose, glucose and fructose, due to the activity of the invertase released by the explants into the medium (George 1993). So, we could expect similar results in medium containing fructose + glucose as compared to those obtained with equimolar concentrations of sucrose. Instead, mean number of roots was significantly lower ($P \geq 0.05$), resulting in a lower IR (Table 1). The same was observed when comparing this combination of sugars with equimolar concentration of glucose. Vinterhalter *et al*. (2001) have previously reported the superiority of sucrose as compared to glucose and fructose during *in vitro* culture of carob tree. Similar results were observed by Jain and Babbar (2003/4) in black plum.

Percentage of survival at the end of acclimatization was superior to 80 % for all the sugars tested, except for 87 mM sucrose, 145 mM glucose and glucose + fructose (Table 2). For glucose we observed a significant decrease in the survival percentage when concentration increased from 116 to 145 mM (Table 2), which is in agreement with other studies where a reduction in the sugar concentration during rooting was beneficial for plantlet acclimatization, by increasing plantlets photosynthetic ability (Langford and Wainwright 1987, Serret *et al*. 1997). Plants rooted on medium with filter-sterilized fructose presented higher growth compared to glucose 58 and 145 mM, glucose + fructose and glucose + mannitol (Table 2). Rooting medium with glucose + fructose induced significantly higher leaf number than glucose alone at the same (116 mM) or higher concentrations (145 mM) (Table 2).

Preconditioning and rooting *in vitro* with different types and concentrations of sugars has shown to influence quality of plantlets and their response during acclimatization (Van Telgen *et al*. 1992). Photoautotrophic cultivation of plantlets on medium without sugars has been considered to have positive effects on plantlet growth and survival (Kozai *et al*. 1997, Pospíšilová *et al*. 1999). The sugars used during rooting did not remarkably influence plantlet survival and growth at the end of acclimatization period, except for glucose (Table 2). This is the first report of the effect of carbon source on rooting of carob tree and provides a basis for the improvement of carob tree micropropagation.

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