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PLANT TISSUE CULTURE

Development of a new culture medium and efficient protocol for *in vitro* micropropagation of *Ceratonia siliqua* L.

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Abstract
A new basal culture medium was developed and tested using a rapid and efficient protocol of *in vitro* axillary shoot bud proliferation of *Ceratonia siliqua* L., an important Mediterranean Fabaceae plant species. In a first experiment, the new formulated 'LA' mineral composition significantly improved shoot growth and proliferation as compared with Murashige and Skoog medium (MS, 1962) in both solid and liquid culture media. However, the liquid culture system proved to be the most suitable for shoot induction, shoot length (about fourfold higher), and multiplication rate (about two-fold higher), the difference being significant. The measured growth and proliferation parameters were further improved when LA mineral composition was optimized, in a second experiment. The highest multiplication rate (6.3) was achieved during the second subculture using the optimized 'LAC' medium. Noticeably, hyperhydricity and shoot-tip necrosis symptoms were absent in both formulated LA and LAC compositions when using the liquid culture system. *In vitro* rooting in solid medium showed 41.7 to 46.3% response on a solid medium which was more suitable than the liquid culture system, the difference being significant. In contrast, pretreated microcuttings with 3 μM IBA (indole-3-butyric acid) were successfully rooted *ex vitro*, showing significantly higher response (91.7%), average root number (8.3), and root length (31.5 mm). The plantlets were successfully acclimatized showing more than 90% survivability and normal morphology. The present study is a first cost-effective protocol for carob micropropagation combining the use of the newly formulated LAC basal medium, a liquid culture system, and *ex vitro* rooting.

Keywords *Ceratonia siliqua* · *Ex vitro* rooting · Micropropagation · Liquid medium · New basal medium

Introduction

The carob tree (*Ceratonia siliqua* L.), is an important Fabaceae plant species in the Mediterranean area. The world production of carob pods is estimated at about 158,609 t per year and the main producers are Portugal (25.46%), Italy (18.24%), Spain (16.51%), Morocco (13.89%), and Turkey (8.45%) (FAOSTAT 2016). Seeds and pod pulp are used to produce a wide range of products. The most economically important use of carob seeds is the production of locust bean gum, a valuable stabilizing and thickening additive (E 410) used in food and pharmaceutical industries (Karababa and Coşkuner 2013). Carob pulp is rich in total sugar content (31–50%) that can easily be water extracted to be used for syrups production (El Batal et al. 2011). It can also be exploited for the production of bioethanol (Mazaheri et al. 2012) and natural antioxidants (Roseiro et al. 2013; Benchikh et al. 2014; Amessis-Ouchemoukh et al. 2017). Due to these economical and ecological benefits, many countries have promoted the cultivation and exploitation of this valuable genetic resource (Battle and Tous 1997; Lozzi et al. 2015).

Establishing a rapid and large-scale propagation system is urgently needed to make successful the large planned world-wide modern plantation projects. Conventional methods of plant multiplication are, however, unable to fulfill the important and increasing local and regional demands for *C. siliqua*-selected plant material. In this context, micropropagation techniques provide an alternative for rapid and large-scale carob propagation. Axillary buds proliferation is the most widely

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used technique in clonal commercial micropropagation (George and Debergh 2008; Gamborg and Phillips 2013). In the last years, several reports have been published on carob regeneration through axillary buds using juvenile (Alorda and Medrano 1986; Sebastian and McComb 1986; Radi et al. 2013) and adult material (Romano and Barros 2002; Naghmouchi et al. 2008) but with a limited success in growth and development of regenerated shoots.

Mineral composition of culture media is one of the most important factors influencing *in vitro* growth and morphogenic responses (George and De Klerk 2008). Each nutrient has its individual role within the plant growth process and each deficiency may disrupt normal plant metabolism that could be manifested as physiological, morphological, and biochemical changes (Monteiro et al. 2000). Literature highlights several culture media formulations differing in mineral composition being selected for *in vitro* plant propagation. Murashige and Skoog basal medium (MS, 1962), initially developed for optimal growth of tobacco calli, remains the most used composition in the case of many species (George and De Klerk 2008) including *C. siliqua* (Naghmouchi et al. 2008; Osório et al. 2012; Radi et al. 2013; Shahzad et al. 2017). However, this medium is not necessarily optimal for all plant species (Pierik 1997; Niedz and Evens 2007). George and De Klerk (2008) already reported that the nitrogen concentration of MS is too high and the balance between nitrogen and ammonia is not optimal. In our previous study (Lozzi et al. 2019), this medium was also found not optimal for carob embryonic callus growth. Other basal media were tested for carob micropropagation through auxiliary buds such as Gamborg (Gamborg et al. 1968), Gresshoff and Doy (Gresshoff and Doy 1972), and Woody Plant Medium (Lloyd and McCown 1980) but with limited improvement as argued in different works (Sebastian and McComb 1986; Romano and Barros 2002; Saidi et al. 2007).

Several authors emphasized the need to optimize the mineral composition for each plant species to elaborate efficient micropropagation protocols (George and De Klerk 2008; Aranda-Peres et al. 2009). Indeed, every species has its own mineral requirements that could be considered during medium formulation (George and De Klerk 2008). In this context, a number of attempts for estimating the correct balance of mineral nutrients were reported for a number of species such as *Passiflora edulis* (Monteiro et al. 2000), *Corylus avellana* (Nas and Read 2004), and *Vriesea duvaliana* (Aranda-Peres et al. 2009). In *C. siliqua*, an attempt to optimize the mineral component of the culture medium concerned *in vitro* rooting of shoots initially propagated on MS medium (Gonçalves et al. 2005).

A number of published studies also noted that agar is the most expensive ingredient of plant culture media and constitutes about 70–80% of the medium cost (Martin 2003; Rathore et al. 2015). In the case of *C. siliqua* micropropagation, agar or phytagel as the gelled agents was used to support explants. Eliminating this component would, therefore, lead to a significant cost reduction. Nowadays, many researchers underline the importance of using liquid culture systems not only in the establishment of a low-cost protocol for micropropagation but also because it offers many potential advantages, including the following: (i) better growth of plants as a consequence of uniform dispersal and better availability of nutrients and growth regulators; (ii) lack of impurities from the solidifying agent; (iii) dilution of exudates from the explants; and (iv) greater efficiency in transferring plantlets to *ex vitro* environment (Ascough et al. 2004; Savio et al. 2012; Shekhawat et al. 2015). Liquid culture system was successfully used for micropropagation of several woody plants such as *Prunus avium* (Godoy et al. 2017) and *Juglans nigra* (Stevens and Pijut 2018). However, several reports indicated that it may promote hyperhydricity (Ascough et al. 2004; Thorpe et al. 2008; Heikrujam et al. 2014). Various supporting materials including cotton wool, filter paper, and glass seeds were suggested to overcome this disorder (Farahani and Majd 2012; Grzegorczyk-Karolak et al. 2017).

Rooting and acclimatization are also two challenging problems in *C. siliqua* micropropagation (Radi et al. 2013). *In vitro* rooting step may account for 30–75% of the total cost of micropropagated plants (Ranaweera et al. 2013). The *ex vitro* rooting offers an alternative tool which would help reducing cost and duration of *in vitro* rooting and acclimatization (Ranaweera et al. 2013). In addition, it allows for better root development and high plant survival rates (Phulwaria et al. 2013; Rathore et al. 2015). *Ex vitro* root induction was successfully proved in many woody plant species such as *Psoralea corylifolia* (Fabaceae) (Baskaran and Jayabalan 2009), *Acacia nilotica* (Fabaceae) (Rathore et al. 2015) and *Populus* (Arencibia et al. 2017). To the best of our knowledge, no similar studies were conducted on *C. siliqua*. The objective of the present study was, therefore, to develop an improved and a low-cost *C. siliqua* micropropagation protocol, through combining the use of the newly formulated basal medium, a liquid culture system and *ex vitro* rooting of micropropagated shoots.

Material and Methods

Experiment 1: Formulation of a new carob basal medium (LA)
Determination of mineralsIn this study, we hypothesized that a medium formulated based on mineral proportions in cotyledons would favor the growth and proliferation of carob shoots. For this reason, the mineral contents of carob mature cotyledons were first determined. Mature carob pods (about 50 kg per tree) were collected from 25 trees growing in the area zone

of the rural commune of El Ksiba (32° 33′ 54″ N latitude, 6° 01′ 59″ W longitude) located in the Middle-Atlas mountains of Morocco. Extracted seeds of each tree (1 kg per tree) were mixed and randomly distributed and stored in 1-kg batches. Approximately 300 g of seeds were randomly sampled in three replicates and used in the analysis. Cotyledons were extracted from scarified seeds using concentrated sulfuric acid (98%, 60 min), dried in vacuum at 50°C to constant weight, and ground to a powder that could pass through a 0.50-mm stainless steel sieve. The total nitrogen content was measured by the Kjehdahl method (Bradford 1976). The mineral constituents (K, Ca, Na, Fe, Mg, Zn, and Cu) were analyzed using an atomic absorption spectrophotometer (AA-7000, Labindia Analytical Instruments Pvt. Ltd., Mumbai, India), and P content was determined spectrophotometrically according to AOAC 970.39. Data were expressed on a dry weight basis.

Medium formulation The mineral proportions in the cotyledons were used to formulate a new basal culture medium for carob (LA) using N concentration as a reference. The nitrogen level was fixed at 44 mM to overcome the adverse effects of high N concentrations as was widely reported. Preliminary experiments we conducted also confirmed that the high N concentration in MS medium (60 mM) remarkably reduced carob shoots growth (data not shown). The calcium and iron levels were, however, increased. Carob is known to be highly dependent on Ca and this was also confirmed under hydroponic culture conditions (Correia et al. 2003). The modification of this macronutrient was achieved using CaCl2·2H2O and Ca(NO3)2·4H2O, not normally present in MS medium. In the case of Fe, it was increased to double the MS level considering the fact that carob cotyledons we analyzed showed remarkably high Fe concentration. The importance of this micronutrient in carob shoot growth was also proved in hydroponic culture system (Correia et al. 2003). The other MS micronutrients were maintained unchanged.

Plant material and culture conditions Scarified carob seeds using concentrated sulfuric acid (98%, 60 min), were washed with sterile water 3 times and then soaked in sterile distilled water for 24 h. The seeds were germinated in 125-ml Erlenmeyer flask containing 50 ml of LA basal medium supplemented with MS vitamins, 3% (*w*/*v*) sucrose, 0.7% (*w*/*v*) agar, and 0.1% (*w*/*v*) activated charcoal. The pH was adjusted to 5.6 with 1 M NaOH before autoclaving at 120°C for 20 min. After 1 wk, the germinated seeds were transferred to test tubes (250 × 25 mm) containing 20 ml of fresh medium. Both tubes and flask were sealed with cotton plugs. The cultures were incubated under a constant temperature of 26 ± 2°C with a 16-h photoperiod (60 μmol m^{-2} s^{-1}). The nodal segments (10 mm) obtained from 2 mo-old seedlings were used for axillary shoot bud induction and proliferation.

Comparative response of shoot induction and proliferation in MS and LA media using solid and liquid culture systems. A comparative evaluation of shoot induction and proliferation in both LA and MS basal media was made using solid (0.7% (*w*/*v*) agar) and static liquid culture system. All media were supplemented with 4.44 μM BA (6-Benzyladenine) and 0.1% (*w*/*v*) activated charcoal. Shoots were grown in 150 × 25 mm test tubes closed with cotton plugs. Observations carried out after 4 wk of culture allowed to produce percentages of shoot induction and shoot-tip necrosis as well as shoot length and multiplication rate. The latter parameter was calculated as the ratio of shoot number at the end of a subculture to the initial shoot number.

Table 1. Total levels of macro and micronutrients in *C. siliqua* cotyledons (*n* = 3)

Elements	*C. siliqua* cotyledons
	g/kg
N	42.9 ± 3.3
P	7.7 ± 1.5
K	15.7 ± 3.9
Ca	7.1 ± 0.6
Mg	1.5 ± 0.2
Na	0.3 ± 0.1
	mg/kg
Fe	115.0 ± 8.9
Mn	102.4 ± 5.9
Zn	55.2 ± 8.5
Cu	14.2 ± 0.8

Experiment 2: Optimization of LA and formulation of LAC medium In a second experiment, LA basal medium was

Table 2. Macro- and micronutrients composition of culture media tested during *C. siliqua* micropropagation. Concentrations are given in milligram per liter

	MS	LA	LAC
KNO_3	1900	770	1700
NH_4NO_3	1650	1350	970
$CaCl_2·2H_2O$	440	440	440
$MgSO_4·7H_2O$	370	217	217
$Ca(NO_3)_2·4H_2O$	–	350	350
KH_2P0_4	170	486	486
KI	0.83	0.83	0.83
$MnSO_4·4H_2O$	22.3	22.3	22.3
$ZnSO_4·7H_2O$	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2
$Na_2MoO_4·2H_2O$	0.25	0.25	0.25
$CuSO_4·H_2O$	0.025	0.025	0.025
$CoCl_2·6H_2O$	0.025	0.025	0.025
Na_2-EDTA	37.25	74.5	74.5
$FeSO_4·7H_2O$	27.8	55.6	55.6
Total N (mM)	60.01	44.33	44.03
NO_3^-/NH_4^+ ratio	1.91	1.63	2.6

Table 3. Effect of MS and LA media and type of culture media (semisolid and liquid media) on shoots induction and proliferation of *C. siliqua* after 4 wk

Mineral composition	Type of culture medium	Shoot induction (%)	Length of shoots (mm) ± S.E.	Multiplication rate ± S.E.	Shoot-tip necrosis (%)
MS	Semisolid	40.0[c]	6.1 ± 0.9[d]	1.3 ± 0.1[c]	66.7[a]
	Liquid	76.7[b]	25.8 ± 2.2[b]	2.5 ± 0.6[b]	17.4[b]
LA	Semisolid	66.7[b]	10.1 ± 0.3[c]	1.9 ± 0.1[b]	15.0[b]
	Liquid	93.3[a]	38.1 ± 0.3[a]	4.0 ± 0.7[a]	0.0[c]
Significance of two-way ANOVA					
Mineral composition (A)		**	***	***	**
Type of culture medium (B)		***	***	***	*
A × B		NS	**	***	NS

Mean values within a *column* followed by the same *letter* are not significantly different by Duncan's multiple range test (5%)

NS nonsignificant

*Significant at $P \leq 0.05$ (two-way ANOVA)

**Significant at $P \leq 0.01$ (two-way ANOVA)

***Significant at $P \leq 0.001$ (two-way ANOVA)

taken up for optimization of its constituents. The ratio of NO_3^-/NH_4^+ was increased in LAC medium using KNO_3 and $Ca(NO_3)_2 \cdot 4H_2O$. In addition to increasing nitrate concentration, this approach permitted a further increase of Ca and K. Cultures were grown in test tubes containing liquid media supplemented with 4.44 μM BA and 0.1% (*w*/*v*) activated charcoal. The newly formed shoots were excised and microcuttings were subcultured every 4 wk in a 125-ml Erlenmeyer flask containing 50 ml of a fresh liquid medium using sterile perlite as a support.

Experiment 3: In vitro and ex vitro rooting of shoots Regenerated shoots of 3 cm in length were excised at the end of the multiplication stage and rooted under *in vitro* conditions. The basal ends of the micropropagated shoots were first dipped, for 3 min, in 4.8 mM IBA (indole-3-butyric acid) before they were cultured in ½LAC and ½MS solid and liquid media. All media were supplemented with 0.1% (*w*/*v*) activated charcoal and 3% (*w*/*v*) sucrose. Cultures were kept at 26 ± 2°C in the dark for 1 wk and then exposed to 16-h photoperiod (60 μmol m^{-2} s^{-1}).

To induce *ex vitro* rooting, the shoot's basal ends were dipped, for 5 min, in various concentrations (0, 4.8, 9.6, and 14.4 μM) of IBA or NAA (naphthalene acetic acid). Both *ex vitro* rhizogenesis and acclimatization of *in vitro* rooted shoot were carried out in plastic pots containing autoclaved sand and peat (1:1, *v*/*v*) and covered to maintain high relative humidity. The plastic covers were gradually opened over a period of 2 wk before they were removed. Cultures were maintained at 26 ± 2°C under light conditions and moistened twice a week

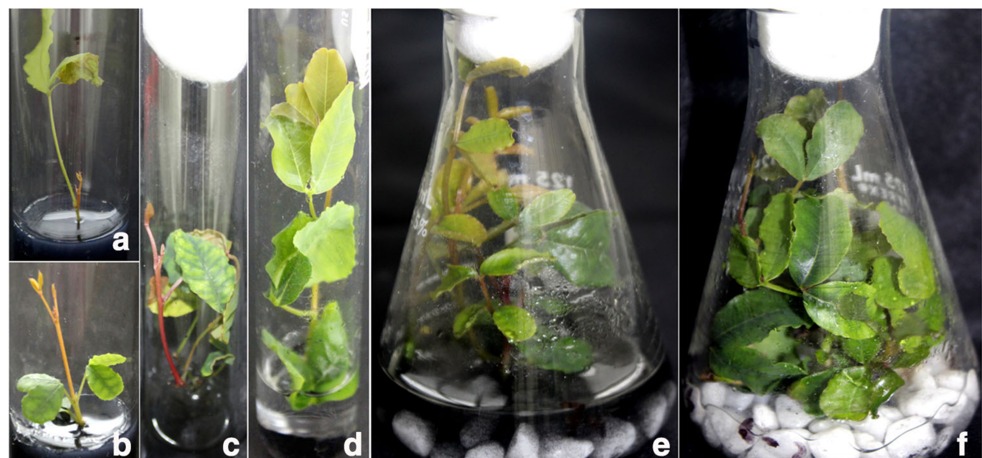

Figure 1. Shoot proliferation of *C. siliqua* in (**a**) MS semisolid medium, (**b**) LA semisolid medium, (**c**) MS liquid medium, (**d**) LA liquid medium, (**e**) third subculture in LA liquid medium, and (**f**) Third subculture in LAC liquid medium.

with one-sixth strength of LAC basal salts. After 4 wk, *in vitro* and *ex vitro* rooted shoots were shifted to the greenhouse.

Observations were carried out after 4 wk of root initiation to calculate the percentage of root induction and the number and length of roots. The rate of survivability was calculated after 3 mo of culture under greenhouse conditions.

Statistical analysisAll experiments were repeated three times with a minimum of 12 replicates per treatment. The results are expressed as mean ± SD of the three experiments and data were processed with the analysis of variance (ANOVA) method using the IBM SPSS Statistics 21 tool (IBM Corporation, Armonk, NY). The significance of differences among the mean values was examined using Duncan's multiple range test (DMRT) at 5%.

Results and Discussion

Experiment 1: Formulation of LA basal medium and shoot bud multiplication using solid and liquid culture systems Mineral optimization of plant tissue culture media usually involves testing a small range of ready-made media or reducing the strength of MS macronutients. In the absence of a universal approach to formulate a tissue culture medium, suggestions to modify some minerals among others were made years ago (George 1993; Bouman and Tiekstra 2005; Gonçalves et al. 2005) but have generally been ignored. The difficulty of optimizing a mineral composition of a culture medium arises from the interaction between its different components that requires numerous time-consuming and factorial experiments (Nas and Read 2004). In the present study, we instead formulated a new basal nutrient medium on the basis of mineral proportions in mature cotyledons with a view to improve carob micropropagation. A similar approach based on plant leaves and seeds mineral composition was reported in some plant species such as *Passiflora edulis* (Monteiro et al. 2000) and *Corylus colurna* (Nas and Read 2004). In the present study, we hypothesized that a medium formulated on the basis of mineral proportions in mature cotyledons would favor carob shoot growth and proliferation. In fact, cotyledons of epigeous species store mineral nutrients that are remobilized during seed germination and translocated to the developing seedlings (Kramer and Kozlowski 1979 Marschner 2011).

The mineral composition of carob cotyledons presented in Table 1 highlights an accumulation of particularly high levels of P, Fe, Mn, and Zn, as compared with other carob plant organs, in the absence of specific data related to cotyledons. These nutrients are present in carob pods, leaves, and flowers at the following ranges: 0.7–3 g P kg^{-1}; 18.8–74 mg Fe kg^{-1}; 1.5–42.7 mg Mn kg^{-1}; 5.5–24 mg Zn kg^{-1} (Gonçalves et al. 2005; Ayaz et al. 2007; Custódio et al. 2007; Oziyci et al. 2014). The N amount is greater (1.5-fold) than that found in carob seeds (Oziyci et al. 2014), leaves (over twofold) (Gonçalves et al. 2005), and pods (over five-fold). The Ca

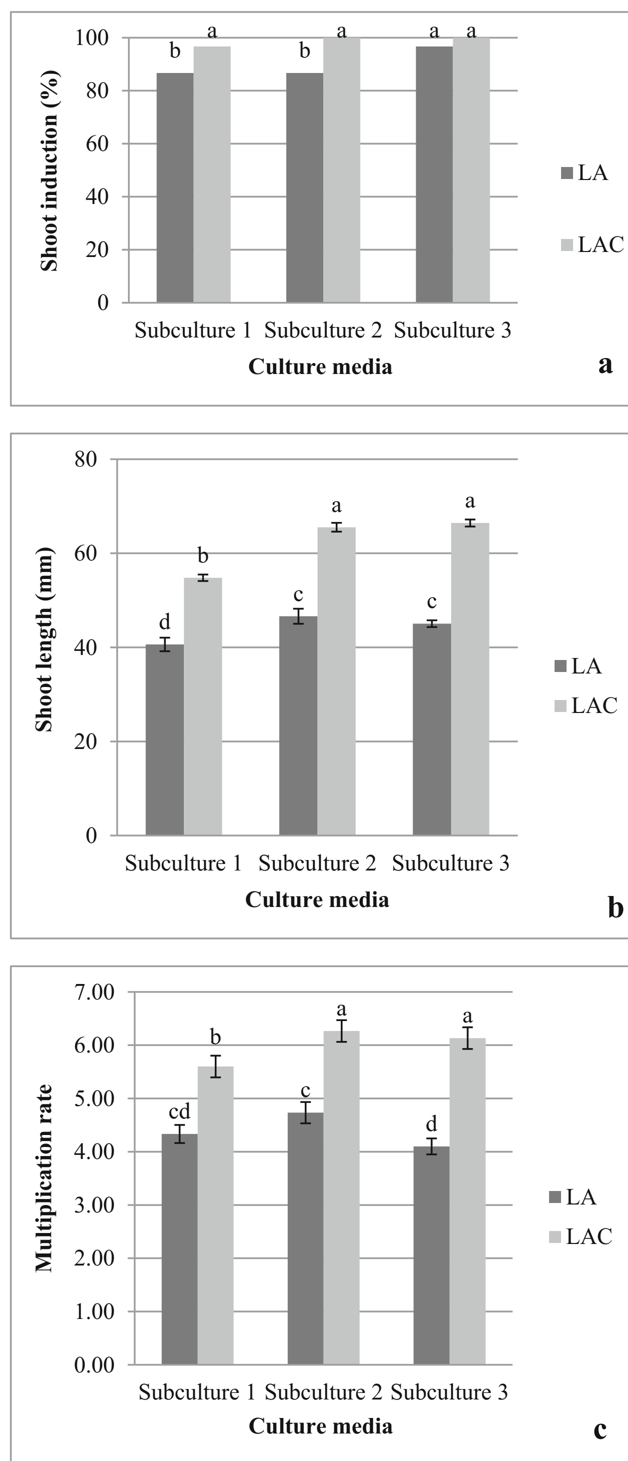

Figure 2. Effect of different liquid media compositions on *C. siliqua* (**a**) shoot induction, (**b**) shoot length, and (**c**) multiplication rate, in the three subcultures. Each subculture duration is 4 wk. Mean values within a *column* followed by the same *letter* are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

and K levels are, however, within the ranges reported in the literature (El-Shatnawi and Ereifej 2001; Gonçalves et al. 2005; Custódio et al. 2007; Oziyci et al. 2014).

The newly formulated LA macronutrient composition (Table 2) differs from that of MS; the major differences being lower N concentration (0.7-fold) as well as NO_3^-/NH_4^+ ratio (1.6), lower K level (0.6-fold), and higher P (2.9-fold) and Ca (2.0-fold). In the case of micronutrients, Fe concentration was increased up to twofold that of MS as previously mentioned.

The results of the first experiment showed that the mineral composition of the culture medium significantly affected all the measured growth and proliferation parameters; the best responses being obtained in the new LA composition. The interaction between the culture system and the mineral composition was significant only in the case of shoot length ($P \leq 0.01$) and multiplication rate ($P \leq 0.001$). Using LA and a liquid culture system for carob micropropagation showed a maximum multiplication rate (4.0) which was about 1.6-fold that observed in MS liquid medium. Similarly, the highest average shoot length (38.1 mm) was recorded in LA liquid culture system. In both media, the lowest responses were noticed in the solid culture system (Table 3).

An increasing number of studies indeed found that carob, as well as many other woody plant species, is sensitive to high N levels present in MS medium, and a reduced concentration is required for maximum shoots proliferation (McCown and Sellmer 1987; Beyl 2005; Gonçalves et al. 2005; George and De Klerk 2008). Regarding phosphorus, increasing its level in MS-induced adventitious shoots and increased shoot multiplication rate in other plant species (George and De Klerk 2008). Furthermore, Correia and Martins-Loução (1997) previously noted a significant retranslocation of P from leaves to the growing shoots in mature carob trees under field conditions. Calcium is another important macronutrient for plant growth and morphogenesis, and is required for explant response to plant growth regulators (Moshkov et al. 2008). It was observed, under hydroponic cultivation conditions, that calcium deficiency reduces carob shoot growth in addition to the production of deformed young leaves due to the role of this element in cell wall synthesis and membrane permeability (Correia et al. 2003). The low Ca level in MS medium was found to be related to the increase of shoot-tip-necrosis level in carob (Gonçalves et al. 2005; El Bouzdoudi et al. 2017) and in several other plant species such as *Pistacia vera* (Abousalim and Mantell 1994), *Portulaca grandiflora* (Srivastava and Joshi 2013), and *Harpagophytum procumbens* (Bairu et al. 2009). Iron deficiency was also proved to be related to the appearance of this physiological disorder (Christensen et al. 2008). These findings would explain the significant reduction ($P \leq 0.01$) of shoot-tip-necrosis level we observed under *in vitro* conditions when LA medium was used as compared with MS (Table 3). Importantly, these disorders were absent in LA liquid culture system in comparison with LA solid medium (15%). A similar tendency was also observed when a liquid medium was used in the case some woody tree species such as *Pyrus* (Thakur and Kanwar 2011) and *Dalbergia sissoo* (Fabaceae) (Vibha et al. 2014). This response might be due to better uptake of nutrients as compared with a solid medium (Ascough et al. 2004).

Carob shoot induction and length as well as the multiplication rate were significantly improved ($P \leq 0.001$) in the liquid medium with about fourfold increase in shoot length and a twofold increase in multiplication rate (Fig. 1). Such growth enhancement was also reported for several species (Jo et al. 2008; Pati et al. 2011; Rathore et al. 2014). These responses might be the result of a better nutrient and phytohormone uptake due to the close contact of the explants with the liquid medium (Sivanandhan et al. 2013). Furthermore, such culture system reduces the deleterious effect of toxins that became rapidly diluted in the culture medium (Ascough et al. 2004).

The remarkable absence of hyperhydric shoots throughout the experiment period is in favor of using the liquid system for carob micropropagation, which usually is associated with the development of this major physiological disorder. Using cotton plugs in our experiments to seal test tubes and flasks might have prevented the development of hyperhydricity symptoms. It was stated that this physiological malformation is mainly associated with a lack of gas exchange (Rossetto et al. 1992). Cotton plugs were reported to provide excellent aeration and reduce the accumulation of gaseous that can lead to hyperhydricity (Bhojwani and Dantu 2013). In addition to the benefits of using cotton plugs, we observed a substantial increase in carob microcutting leaf size as compared with the common transparent plastic caps (data not shown). Similarly, de Santana et al.

Table 4. Comparative response of rooting in ½LAC and ½MS using semisolid and liquid media. Observations were taken 4 wk after culture

Salt composition	Type of culture medium	Rooting (%)	Root number	Root length (mm)
½MS	Semisolid	41.7[a]	7.6 ± 1.4	36.0 ± 3.2[b]
½MS	Liquid	11.1[b]	6.7 ± 0.9	58.7 ± 11.9[ab]
½LAC	Semisolid	46.3[a]	7.0 ± 0.6	45.8 ± 6.1[b]
½LAC	Liquid	5.6[b]	6.8 ± 0.9	70.7 ± 10.4[a]

Mean values within a *column* followed by the same *letter* are not significantly different by Duncan's multiple range test (5%)

Table 5. Effect of auxins (IBA of NAA) in *ex vitro* rooting in regenerated shoots of *C. siliqua*

IBA (μM)	NAA (μM)	Rooting (%)	Root number	Root length
0	0	0.0	0.0	0.0
4.8	0	69.4^{bc}	5.0 ± 0.9^{bc}	9.7 ± 0.8$_{b}$
9.6	0	83.3^{ab}	5.5 ± 0.6^{b}	13.9 ± 1.6^{b}
14.4	0	91.7^{a}	8.3 ± 0.9^{a}	31.5 ± 1.2^{a}
0	4.8	50.0^{d}	3.1 ± 0.2^{c}	4.0 ± 0.8^{b}
0	9.6	61.1^{cd}	4.9 ± 0.4^{bc}	9.6 ± 1.6^{b}
0	14.4	89.2^{a}	5.3 ± 0.5^{b}	12.6 ± 1.2^{b}

Mean values within a *column* followed by the same *letter* are not significantly different by Duncan's multiple range test (5%)

(2011) observed a significant increase in leaf size and dry weight with the use of cotton plug seals in *Annona glabra* micropropagation.

Experiment 2: Optimization of LA and formulation of LAC basal medium The optimized LAC basal medium, deriving from the formulated LA mineral composition, significantly ($P \leq 0.001$) improved all the assessed growth parameters during the three successive subcultures (Fig. 2 a, b, c). Satisfactory shoot induction percentages (96.7 to 100%) were obtained in LAC. Recorded shoot length and multiplication rate during the second and third subcultures were, respectively, > 65 mm and > 6 as compared with LA (< 47 mm and < 4.8) as shown in Fig. 2.

LAC medium is distinguished by its higher NO_3^-/NH_4^+ ratio (2.6), an important parameter for nutrient uptake and pH regulation (Yan et al. 2009; Zhao et al. 2016). Interestingly, it was shown that carob seedlings growing under hydroponic cultivation produced more biomass and more sucrose when supplied with nitrate as compared with those supplied with ammonium (Cruz et al. 1997). In the case of several Fabaceae culture media, it was also reported that NO_3^-/NH_4^+ ratio is greater than that in MS (1.9) medium (George and De Klerk 2008). A higher level of NO_3^- makes the medium less acid and preserves the charge balance of plant tissues (Ismail and Othman 1995; George and de Klerk 2008). In the case of ammonium, its uptake results in an acid nutrient solution due to the release of H^+ proton into the medium (Ramage and Williams 2002; George and De Klerk 2008). On the other hand, the accumulation of excess NH_4^+ ions in plant tissues may induce toxicity symptoms such as reduced plant growth and leaf chlorosis (Esteban et al. 2016) and inhibits the uptake of cations such as magnesium and calcium and thereby induces deficiency of those elements in plants (Britto and Kronzucker 2002; Zhao et al. 2016). A suitable balance between the two nitrogen forms is, therefore, required.

In the present study, carob shoots were found to grow best when increasing the amount of NO_3^- to NH_4^+ from 1.6 to 2.6 (while keeping the same amount of N). Interestingly, during the three passages, the formulated media produced vigorous shoots, showing no signs of hyperhydricity or necrosis. Repeated subcultures usually increase the shoot multiplication rate of several woody species such as *Cyclea peltata* (Abraham et al. 2010), *Boscia senegalensis* (de Santana et al. 2011), and *Pterocarpus santalinus* (Prakash et al. 2006).

Experiment 3: *In vitro* and *ex vitro* rooting of shoots *In vitro* rooting of shoots The solid medium expressed significantly higher ($P \leq 0.001$) rooting percentages (46.3 and 41.7%) in both salt compositions ½LAC and ½MS as compared with the liquid culture system (< 12%) as shown in Table 4. However, the latter system significantly ($P \leq 0.05$) increased root length especially in ½LAC medium (70.7 mm). With regard to root number, a slight increase was noticed in the case of solid media (7 to 7.6) but with no significant difference. Gonçalves et al. (2005) obtained a higher percentage of root induction (80%) in carob shoots of a distinct genotype, but

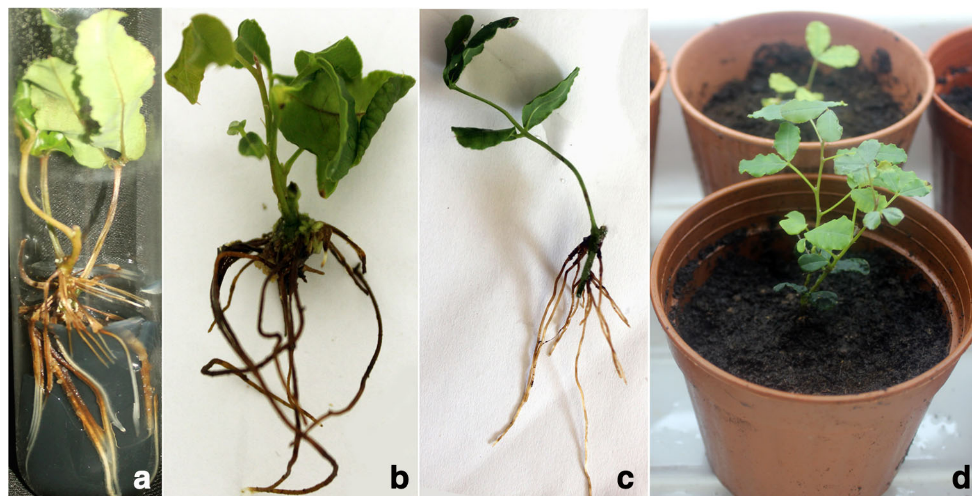

Figure 3. Roots initiation in regenerated shoots of *C. siliqua*, in (**a**) LAC semisolid medium and (**b**) in LAC liquid medium, (**c**) *ex vitro* rooted shoot, and (**d**) hardened plants in pots.

lower root number (4.3) and length (27.3) compared with our results using solid culture media. Using a liquid medium was, in contrast, found more effective for root initiation in the case of other species such as *Alocasia amazonica* (Jo et al. 2008) and *Catharanthus roseus* (Pati et al. 2011). This difference might probably be due to auxin dilution following their culture in liquid media.

***Ex vitro* rooting of shoots** Shoots treated with 14.4 μM IBA exhibited the highest rooting percent (91.7%) with an average of 8.3 roots per shoot, length of 31.5 mm (Table 5). NAA was less effective when compared with IBA, especially in the case of root number and root length ($P \leq 0.01$, Table 5). The effectiveness of IBA was also reported *in vitro* in the case of carob (Romano and Barros 2002; Radi et al. 2013). *Ex vitro* rooted shoots were successfully acclimatized showing more than 90% survivability. Most importantly, rooted plants under *ex vitro* conditions showed normal morphological appearance with lateral roots and no callus formation (Fig. 3). In contrast, *in vitro* induced roots were thick and fragile. A similar observation was made for *Siraitia grosvenorii* rooted shoots (HuaBing et al. 2010).

Conclusions

The present findings demonstrate that carob shoots growth and multiplication were significantly improved in the newly formulated LAC basal medium in comparison with MS. The new carob medium was first developed on the basis of mineral proportions in carob cotyledons before it was taken up for further optimization. The liquid culture system was also confirmed more efficient in promoting shoot growth and multiplication than the solid medium. Furthermore, the present study describes, for the first time in *C. siliqua*, an efficient protocol of micropropagation using *ex vitro* rooting with simultaneous hardening. Combining the *ex vitro* rooting method and shoot multiplication in a liquid culture system offers the opportunity to reduce the overall production cost of micropropagated carob plants. The established protocol could be considered cost-effective, time-saving, and appropriate for the multiplication of carob shoots, and its application to adult-derived explants is the following step of the present applied research.

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Compliance with ethical standards

Conflict of interest A provisional patent application based on the technology described in this work has been filed.

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