BIOLOGIA PLANTARUM 49 (2): 277-280, 2005

BRIEF COMMUNICATION

A new medium formulation for *in vitro* rooting of carob tree based on leaf macronutrients concentrations

S. GONÇALVES*, P.J. CORREIA*, M.A. MARTINS-LOUÇÃO** and A. ROMANO*[1]

*Faculdade de Engenharia de Recursos Naturais, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal**
*Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal***

Abstract

Experiments were performed to optimize the macronutrients concentrations for *in vitro* rooting of *Ceratonia siliqua* micropropagated shoots. Several dilutions of Murashige and Skoog (MS) medium were tested: full-strength MS, half-strength MS (½MS), and ½MS + full N. The frequency of *in vitro* rooting was enhanced when the ½MS was used (50 % rooted shoots). Mature leaves from 20 - 30 year-old carob trees and from 2 year-old micropropagated plants were collected and the concentrations of macronutrients (N, P, K, Ca, Mg) assessed. Based on the mineral composition of the leaves a new medium was formulated and compared with the previous ones showing an increment of the rooting frequency to 80 %. Moreover, shoots rooted in the new medium did not show symptoms of apical necrosis that occurred in the other tested media.

Additional key words: *Ceratonia siliqua*, culture medium, micropropagation, nutrient medium.

The success of *in vitro* tissue culture is strongly dependent on the chemical composition of the culture medium (Ružić *et al*. 2004). Macronutrient salts are indispensable for the growth of higher plants *in vitro*, namely N, P, K, Ca, Mg and S. From these, nitrogen is the most important component of the basal medium (Chawala 2002). The deficiency of minerals in plants can cause biochemical, physiological and morphological changes, according to the nutrient and to the level of deficiency (Preece 1995, Monteiro *et al*. 2000). The optimization of mineral composition of the culture medium has been frequently reported. Morard and Henry (1998) used minerals in proportions found in shoot and root tissues of seedlings. More recently, the use of nutrients present in leaves (Monteiro *et al*. 2000, Terrer and Tomás 2001) or seeds (Nas and Read 2004) was proposed to optimize culture medium composition.

Carob (*Ceratonia siliqua* L.) is an evergreen legume tree commonly cultivated in the Mediterranean area since historic times. This species is of substantial importance for the locust bean gum obtained from its pods and used in the food industry. The Portuguese cultivar Mulata is economical and ecologically interesting (Battle and Tous 1997). As the traditional carob propagation methods failed to meet the market request, the use of micropropagation seems to be appropriate in order to fulfil the increased demand for propagating this tree (Romano *et al*. 2002). With the objective of improving the rooting frequency and the shoots quality, a new specific medium, based on the nutrient analysis of leaves of mature carob trees and 2 year-old micropropagated plants, was formulated and assayed.

For leaf nutrient analysis six adult carob (*Ceratonia siliqua* L., cv. Mulata) 20 - 30-year-old trees were selected in an orchard located in southern Portugal on a sandy loam soil. The trees were fertilized with 0.9 kg N and 1.4 kg K_2O $tree^{-1}$ $year^{-1}$, and were irrigated in summer during last 4 years. Irrigation was based on water loss by evaporation from a class A pan (100 %). Leaf samples, (30 - 40 mature leaves per tree) homogenous in terms of age and canopy position, were randomly collected in the selected trees during four years (19 sampling dates, throughout all the phenological stages of the crop).

Received 29 March 2004, *accepted* 14 December 2004.
Abbreviations: BA - benzyladenine; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog (1962) medium.
[1] Author for correspondence; fax: (+351) 289 818419, e-mail: aromano@ualg.pt

Micropropagated plants of one adult clone of cv. Mulata were produced according to Romano *et al*. (2002). Plants were grown for 2 years in the glasshouse, and were fertilized once a month with a NPK fertilizer. Forty mature leaves were randomly collected from 30 micropropagated plants of the same clone.

Leaf nitrogen concentration was determined by the Kjeldahl method, P was analyzed spectrophotometrically and K, Ca, and Mg, by atomic absorption spectroscopy (*Pye Unicam*, Cambridge, UK), following the guidelines of the AOAC. Concentration values were expressed on a dry mass basis.

To compare leaf mineral composition of mature trees and micropropagated plants with mineral composition of the culture media, the balance among macronutrients was calculated, taking N concentration value as the reference element. The balance of macronutrients in leaves was expressed as the ratio between each element and N. The same procedure was applied to the culture media using the content in macronutrients. Therefore, the results are dimensionless and comparable among them.

Using N concentration of half-strength Murashige and Skoog (½MS) medium as a reference a new medium was formulated (the carob medium) (Table 1). To define the mineral composition of this culture medium, the concentration of each nutrient was calculated multiplying N by the mean of the balances obtained in the leaves of healthy mature trees and micropropagated plants (Table 2). These values were converted into amounts of salts [g dm^{-3}] to add in the culture medium.

Table 1. Concentrations of macronutrients and balance using nitrogen as reference for calculations of macronutrients in leaves of mature carob trees and 2 year-old micropropagated plants.

Nutrients	Mature trees [g kg^{-1}(d.m.)]	balance	Micropropagated plants [g kg^{-1}(d.m.)]	balance
N	18.0	1.00	19.2	1.00
P	0.9	0.05	1.2	0.06
K	7.6	0.43	13.1	0.68
Mg	3.0	0.17	3.3	0.17
Ca	12.9	0.72	9.2	0.48

The establishment and multiplication of shoot cultures, starting from buds of mature carob trees cv. Mulata, have been previously described (Romano *et al*. 2002). For root induction the basal ends of the shoots, 3 cm in length, were harvested at the end of the multiplication stage on full-strength MS medium supplemented with 2.22 µM benzyladenine (BA), were dipped in 4.9 mM indole-3-butyric acid (IBA) for 3 min, followed by culture on one of the following growth-regulator-free media: full-strength MS, ½MS, ½MS + full N (½MS+N) and carob medium (Table 2). All media contained micronutrients and vitamins of MS medium, 2 % (m/v) sucrose and 0.7 % (m/v) agar. The pH of the media was adjusted to 5.8 before autoclaving at 121 ºC for 20 min.

Shoots were grown in test tubes (32 × 200 mm) containing 20 cm^3 of medium, capped with aluminum foil. Shoots were grown in the dark for one week and then transferred to a 16-h photoperiod, with an irradiance of 60 µmol m^{-2} s^{-1}, produced from cool-white fluorescent lamps, and grown for three weeks. Rooting was evaluated 1 month after induction and was expressed in terms of rooting frequency, root number, and the longest root length per plantlet. The results presented are the mean ± standard error (SE) of 30 plantlets per experiment. All the experiments were carried out three times. The results were compared through analysis of variance (*ANOVA*) and Duncan's New Multiple Range Test.

Table 2. Content of macronutrients [mg dm^{-3}] in culture media tested during *in vitro* rooting of carob tree micropropagated shoots.

Nutrients	MS	½MS+N	½MS	Carob medium
N	840.9	840.9	420.4	420.4
P	37.8	19.4	19.4	23.7
K	782.4	758.0	391.2	233.9
Mg	36.1	18.1	18.1	71.2
Ca	120.6	60.3	60.3	219.8

The concentration of macronutrients of MS basal medium played important role on rooting response of carob shoots. When the concentration of the macronutrients was lowered (½MS+N and ½MS) the rooting frequency was enhanced to 40 and 50 %, respectively. The mean number of roots was higher in MS or ½MS+N, however the roots developed in ½MS medium were longer ($P < 0.05$) (Table 3).

The preference of carob cultures for a low total ionic strength agrees with the results obtained for a great number of other woody plants and with the general rules recommended for tree culture (Bonga and Von Aderkas 1992, Chawala 2002, Channuntapipat *et al*. 2003, Walia *et al*. 2003). Alorda and Medrano (1996) have previously reported a high rooting frequency of carob shoots using ½MS medium. More recently, Romano *et al*. (2002) observed high rooting frequency in carob cv. Galhosa using the reduced strength GD (Gresshoff and Doy 1972) medium.

The concentrations of macronutrients in leaves of mature trees indicated lower proportion of P, K, and Mg in relation to N (Table 1). In leaves of 2 year-old micropropagated plants the pattern is similar for P and Mg, however, the amount of K is higher and Ca lower than in mature trees. In the culture media (MS, ½MS and ½MS+N) the balances among the different macronutrients are different (Table 2). Generally, culture media

presented a higher amount of K and a lower amount of Mg and Ca (Table 2) in comparison with leaves.

Since it was previously shown that carob is sensitive to high content of N in the medium (Martins-Loução and Duarte 1987, Cruz *et al*. 1993, Martins-Loução and Cruz 1999), the concentration of N in ½MS was used as reference to formulate a new medium (carob medium) according to leaf analysis (Table 2). The most important differences between ½MS and carob medium in terms of elemental composition are a decrease in K concentration and an increase in the concentration of Ca and Mg.

It is known that high levels of K reduce the Mg inflow (Troyanos *et al*. 2000) and carob tree rootstocks showed multiple deficiency symptoms in the absence of Mg in spite of the development of special adaptations mechanisms like the increase in root length, swelling of subapical zones and increment in root ferric chelate-reductase activity (Correia *et al*. 2003).

Table 3. Effect of culture media on the rooting frequency, mean number of roots and length of the longest root. Values represent means ± SE of tree replicates with 30 shoots. Different letters indicate significant effects at $P < 0.05$.

Culture medium	Rooting frequency [%]	Mean number of roots	Longest root length [mm]
MS	30 ± 1 a	10.0 ± 1.1 a	13.7 ± 5.2b
MS+Ntotal	40 ± 3 b	7.1 ± 1.2 ab	11.0 ± 4.5b
½MS	50 ± 2 c	3.1 ± 0.5 c	38.3 ± 5.2a
Carob medium	80 ± 4 d	4.3 ± 0.9 bc	27.3 ± 3.3a

In a non-circulating nutritive solution, occurs a recombination between $H_2PO_4^-$ and Ca^{2+} responsible for an insoluble form of calcium (Morard *et al*. 1987). In addition, calcium availability is low in media, because of its low solubility in water. Therefore, Morard and Henry (1998) proposed a new mineral composition for *in vitro* culture of *Solanum paludosum* with a larger amount of Ca than K. Also, in *Eucalyptus tereticornis* high Ca concentration in the rooting medium has been successfully used (Sharma and Ramamurthy 2000).

When carob medium was used the rooting frequency was enhanced to 80 % and the formed roots were longer (Table 3). The increase of root number, observed in MS and ½MS+N (Table 3), may also be a result of nutrient imbalance since carob is very plastic in changing morphology more than in biomass partitioning, depending on growth conditions (Cruz *et al*. 1997, Correia *et al*. 2003).

Differences in culture media affected the quality of plants at the end of rooting period. Plantlets rooted in MS, ½MS+N or ½MS exhibited symptoms of apical necrosis, being these symptoms absent in plants rooted in carob medium. Since shoot-tip necrosis is a typical symptom of calcium deficiency in tissue cultures (McCown and Sellmer 1987), this could be due to the low Ca contents in those media, as compared to carob medium (Table 2). Calcium depletion effects were always visible in young leaves, although photosynthetic efficiency was not affected (Correia *et al*. 2003). A high dependency of carob on Ca has already been observed during studies with seedlings in hydroponic culture (Martins-Loução and Duarte 1987).

The high air humidity in the culture vessels might also interfere in Ca absorption, which occurs in a passive way (Monteiro *et al*. 2000). In addition, more than one third of the Ca^{2+} supplied by the culture medium is required by agar to create its net (Scholten and Pierik 1998), so the increase of Ca concentration in carob medium may compensate this reduction of calcium concentration in the culture medium. A new medium formulation based on the mineral composition of leaves was also developed by Monteiro *et al*. (2000) in order to eliminate calcium deficiency symptoms in *Passiflora edulis*.

From the results obtained we could conclude that the *in vitro* rooting of carob shoots is better in medium with lower N and K concentration and higher Mg and Ca concentration than in standard medium. The usefulness of medium based on leaves mineral analysis for *in vitro* rooting of carob was confirmed in terms of rooting results and shoots appearance. The procedure used for the definition of this culture medium could be further used in other carob cultivars or other woody species.

References

Alorda, M., Medrano, H.: Micropropagation of *Ceratonia siliqua* L.: a method to clone mature carob trees selected from the field. - In: Book of Abstracts of the 3rd International Carob Symposium. P. 13. Tavira 1996.

Batlle, I., Tous, J.: Carob tree. *Ceratonia siliqua* L. - Institute of Plant Genetics and Crop Plant Research, Gatersleben / International Plant Genetic Resources Institute, Rome 1997.

Bonga, J., Von Aderkas, P.: *In vitro* Culture of Trees. - Kluwer Academic Publishers, Dordrecht 1992.

Channuntapipat, C., Sedgley, M., Collins, G.: Micropropagation of almond cultivars Nonpareil and Ne Plus Ultra and the hybrid rootstock Titan×Nemaguard hockpisit, argaret and raham. - Sci. Hort. **98**: 473-484, 2003.

Chawala, H.S.: Introduction to Plant Biotechnology. - Science Publishers, Enfield 2002.

Correia, P.J., Pestana, M., Martins-Loução, MA.: Nutrient deficiencies in carob (*Ceratonia siliqua* L.) grown in solution culture. - J. hort. Sci. Biotechnol. **78**: 847-852, 2003.

Cruz, C., Martins-Loução, M.A., Lips, H.: Growth and nutrition of carob (*Ceratonia siliqua* L.) plants as affected by nitrogen sources. - J. Plant Nutr. **16**: 1-15, 1993.

Cruz, C., Lips, S. H., Martins-Loução, M.A.: Changes in the morphology of roots and leaves of carob seedlings induced

by nitrogen source and atmospheric carbon dioxide. - Ann. Bot. **80**: 817-823, 1997.

Gresshoff, P.M., Doy, C.H.: Development and differentiation of haploid *Lycopersicon esculentum* (tomato). - Planta **170**: 161-170, 1972.

Martins-Loução, M.A., Duarte, P.: Effect of ammonium and nitrate nutrition on the growth of carob (*Ceratonia siliqua* L.) plants. - In: Ullrich, W.R., Aparicio, P.J., Syrett, P.J., Castillo F. (ed.): Inorganic Nitrogen Metabolism. Pp. 251-253. Springer-Verlag Berlin 1987.

Martins-Loução, M.A., Cruz, C.: The role of N source on carbon balance. - In: Srivastava, H.S., Rana, P. (ed.): Modes of Nitrogen Nutrition in Higher Plants. Pp. 231-282. Oxford & IBH Publishing Co., New Delhi 1999.

McCown, B., Sellmer, J.: General media and vessels suitable for woody plant culture. - In: Bonga, J., Durzan, D. (ed.): Cell and Tissue Culture in Forestry. Pp. 4-16. Martinus Nijhoff, Dordrecht 1987.

Monteiro, A.C.B., Higashi, E.N., Gonçalves, A.N., Rodriguez, A.P.M.: A novel approach for the definition of the inorganic medium components for micropropagation of yellow passionfruit (*Passiflora edulis* sims. *flavicarpa* deg.). - *In Vitro* cell. dev. Biol. Plant **36**: 527-531, 2000.

Morard, P., Henry, M.: Optimization of the mineral composition of *in vitro* culture media. - J. Plant Nutr. **21**: 1565-1576, 1998.

Morard, P., Vallles, V., Anglard, M.P.: Assessment of the availability of ions to plant roots from the determination of their chemical activity in nutrient solutions. - J. Plant Nutr. **10**: 539-551, 1987.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue culture. - Physiol. Plant. **15**: 473-497, 1962.

Nas, M.N., Read, P.E.: A hypothesis for the development of a defined tissue culture medium of higher plants and micropropagation of hazelnuts. - Sci. Hort. **101**: 189-200, 2004.

Preece, J.E.: Can nutrient salts partially substitute for plant growth regulators? - Plant Tissue Culture Biotechnol. **1**: 26-37, 1995.

Romano, A., Barros, S., Martins-Loução, M.A.: Micropropagation of the mediterranean tree *Ceratonia siliqua* L. - Plant Cell Tissue and Organ Cult. **68**: 35-41, 2002.

Ružić, D., Sarić, M., Cerović, R., Ćulafić, Lj.: Contents of macronutrients and growth of sweet cherry rootstock *in vitro*. - Biol. Plant. **47**: 463-465, 2004.

Scholten, H.J., Pierik, R.L.M.: Agar as a gelling agent: chemical and physical analysis. - Plant Cell Rep. **17**: 230-235, 1998.

Sharma, S.K., Ramamurthy, V.: Micropropagation of 4 year-old elite *Eucalyptus tereticornis* tree. - Plant Cell Rep. **19**: 511-8, 2000.

Terrer, J.C., Tomás, D.F.: Determination of macronutrients to be included in *in vitro* culture media according to leaf concentrations. - J. hort. Sci. Biotechnol. **76**: 484-8, 2001.

Troyanos, Y.E., Hipps, N.A. Moorby, J., Kingswell, G.: The effects of external potassium and magnesium concentrations on the magnesium and potassium inflow rates and growth of micropropagated cherry rootstocks, 'F.12/1' (*Prunus avium* L.) and 'Colt' (*Prunus avium* L.) *Prunus pseudocerasus* L.). - Plant Soil **225**: 73–82, 2000.

Walia, N., Sinha, S., Babbar, S.B.: Micropropagation of *Crataeva nurvala*. - Biol. Plant. **46**: 181-5, 2003.