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In vitro propagation of the medicinal plant *Morinda royoc* L.

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ABSTRACT
Morinda royoc L., is a specie threatened by over collection due to its importance as medicinal plant. The *in vitro* propagation of *M. royoc* may help to solve the increasing demand of stock plants for field cultivation and also would contribute with *in vitro* conservation strategies and further breeding programs. The aim was to regenerate *in vitro* plants of *M. royoc* from shoot tips and nodal explants. Shoot cultures were established starting from tips and nodal segments from greenhouse grown plants. In the multiplication phase the highest multiplication rate was achieved when the culture medium was supplemented with 4.4 µM 6-benzylaminopurine (BA) and 2.9 µM indole-3-acetic acid (IAA). Addition of IAA to rooting medium influenced the quality of the plantlets and the survival rate after transplant to acclimatization stage. Survival 96.4% was achieved in plants cultured in the medium containing 2.9 µM IAA. The *in vitro* regeneration of *M. royoc* plants could be used either for the commercial clonal propagation of the species or for future studies on the production of secondary metabolites for pharmaceutical use given the medicinal properties of the species.

Keywords: acclimatization, micropropagation, rooting, shoots multiplication

RESUMEN
Morinda royoc L., es una especie amenazada por la sobrerrecolección debido a su importancia como planta medicinal. La propagación *in vitro* de *M. royoc* puede ayudar a resolver la creciente demanda de plantas madre para el cultivo del campo y también contribuir con las estrategias de conservación *in vitro* y otros programas de mejoramiento genético. El objetivo de este trabajo fue regenerar *in vitro* plantas de *M. royoc* a partir de yemas apicales y explantes nodales. Se establecieron *in vitro* brotes a partir de plantas crecidas en casa de cultivo. En la fase de multiplicación el mayor coeficiente de multiplicación se logró cuando en el medio de cultivo se incluyó 4.4 µM de 6-bencilaminopurina (BA) y 2.9 µM de ácido indolacético (AIA). La adición de AIA al medio de cultivo de enraizamiento tuvo influencia sobre la calidad de las plántulas y el porcentaje de supervivencia después del trasplante a la fase de aclimatación. Se logró un 96,4% de supervivencia en las plantas que se cultivaron en medio de cultivo con 2,9 µM de IAA. La regeneración *in vitro* de plantas de *M. royoc* podría ser utilizada para la propagación clonal comercial de la especie y para futuros estudios sobre la producción de metabolitos secundarios de uso farmacéutico debido a las propiedades medicinales de esta especie.

Palabras clave: acclimatización, enraizamiento, micropropagación, multiplicación de brotes

INTRODUCTION

Morinda royoc L. (family *Rubiaceae*) is a medicinal plant indigenous to the West Indies. This species is distributed all over seacoasts of Cuba. Also, it was reported in the Bahamas, Florida, Jamaica, Santo Domingo, Central America, Venezuela, Curazao and Aruba. In Cuba, the species *M. citrifolia* L. and *M. moamensis* Alain are also present (Roig and Mesa, 1974).

M. royoc is a small, shrubby plant with small, white, odorous flowers. The fruits, leaves, and roots are used in traditional medicine to treat a wide variety of ailments such as menstrual

disorders, impotence, respiratory problems, ease urinary problems, lumbago, stomach pains, dysentery and as laxative (Roig and Mesa, 1974).

Extracts from the plant have shown stimulating, revitalizing and anti-stress activity (Rodríguez *et al*., 1999; Scull *et al*., 2000). The plants roots are used to elaborate an alcoholic extract, which is recommended as a nutritional supplement (Scull *et al*., 2000). However, the chemical constituents of this plant have not been well studied and a comprehensive scientific characterization of its phytochemical properties is still needed.

The low viability of the seeds limits the natural reproduction and commercial propagation of *M. royoc*. Additionally, due to its coastal habitat, there are not extensive fields cultivated. This limits the availability of raw material and also threatens natural populations due to indiscriminate over collection.

The *in vitro* propagation of *M. royoc* might help to solve the increasing demand of stock plants for field cultivation and also would contribute with *in vitro* conservation strategies and further breeding programs.

On the other hand, the interest of the scientific community for the use of *in vitro* culture techniques for the production of high value plant-derived compounds has increased. Such systems may solve many of the problems faced, during industrial scale-up, on the extraction of these compounds when using plants cultivated in the field or collected in wild (Wilken *et al*., 2005).

Several reports describe the application of *in vitro* culture techniques in *Morinda* species, with emphasis on the production of anthraquinones using cell suspension cultures, i.e *M. citrifolia* (Hagendoom *et al*., 1994; Bassetti *et al*., 1995), *M. elliptica* (Abdullah *et al*., 1998; 2000; Chong *et al*., 2004) and *M. lucida* (Igbavboa *et al*., 1985). Recently, Borroto *et al*. (2008) reported the production of anthraquinones from root cultures of *M. royoc*. However, to our knowledge, no previous experiences on the *in vitro* propagation of *M. royoc* have been described.

Based on the above mentioned precedents, this research was performed with the aim to regenerate *in vitro* plants of *M. royoc* from shoot tips and nodal explants.

MATERIALS AND METHODS

Plant material and explant source

Morinda royoc L. plants were collected in natural populations in the north coast of Havana Province. Then they were maintained in a greenhouse stock plants collection at IBP (Figure 1 A).

Plants were grown in 15 l plastic pots filled with a substrate of compost and zeolite in the ratio 3:1 (v/v) and sprayed regularly (once a week) with a Benlate® solution (1 g l^{-1}). Tips and nodal segments (1cm length aprox.) were dissected, rinsed in tap water and washed with 1% (v/v) commercial detergent. Then surface was sterilized in a 2% (w/v) sodium hypochlorite solution for 15 minutes and rinsed twice in sterile distilled water.

Shoot initiation

Isolated apical and axillary buds (Figure 1 B) were placed on Murashige and Skoog (MS) liquid medium (Murashige and Skoog, 1962). The pH of the media was adjusted to 5.8 before autoclaving at 121°C and 1.1 kg $cm^{»2}$ pressure for 15 min. Ten mililiters of liquid culture medium were added per culture tube (25 x150 mm) with paper bridges to avoid total inmersion of the buds and plugged with nonabsorbent cotton wrapped in cheese cloth. All cultures were incubated in sun light growth rooms at 26 ± 2ºC. Shoots were subcultured after 4 weeks.

Influence of plant growth regulators on shoot multiplication

The effect of different concentrations of 6-benzylaminopurine (BA) (0, 2.2, 4.4, 6.7 and 8.9 µM) and indole-3-acetic acid (IAA) (0, 2.9 and 5.7 µM) on shoot multiplication were assessed. Five shoots were inoculated per culture flask containing 40 ml of basal MS medium gelled with 0.7% (w/v) agar (Plant agar, Duchefa). Ten culture flasks were inoculated per treatment and the experiment was repeated twice. The cultures were incubated as previously described. After six weeks of culture, the shoot length, the number of shoots and the number of nodal segments per shoot were recorded. Multiplication rate (number of explants produced per inoculated explant) was calculated based on the number of shoots and the number of nodal segmentes produced per shoot.

Table 1. Multiplication of *M. royoc* shoots on MS medium with different concentrations of BA and IAA.

Plant growth regulators (µM)	Shoot length (cm)	No of nodal segments/shoot	No of shoots/explant	Multiplication rate
0.0 BA + 0.0 IAA	3.39 bcd	1.5 fg	1.07 f	1.60 f
0.0 BA + 2.9 IAA	3.52 abc	1.25 g	1.15 f	1.44 f
0.0 BA + 5.7 IAA	3.44 abc	0.82 h	0.55 g	0.45 g
2.2 BA + 0.0 IAA	3.74 a	1.75 ef	1.65 d	2.89 de
2.2 BA + 2.9 IAA	2.85 e	0.55 h	0.55 g	0.30 g
2.2 BA + 5.7 IAA	3.18 cd	2.40 abc	2.35 a	5.64 a
4.4 BA + 0.0 IAA	3.21 cd	1.75 ef	1.80 bcd	3.15 cd
4.4 BA + 2.9 IAA	3.69 ab	2.80 a	2.45 a	6.86 a
4.4 BA + 5.7 IAA	3.17 d	1.67 ef	1.29 ef	2.15 e
6.7 BA + 0.0 IAA	3.20 cd	2.60 ab	1.60 d	4.16 bc
6.7 BA + 2.9 IAA	3.17 d	1.95 de	1.70 cd	3.31 cd
6.7 BA + 5.7 IAA	3.36 bcd	2.25 cd	2.41 a	5.42 a
8.9 BA + 0.0 IAA	3.31 cd	2.70 a	1.55 de	4.19 bc
8.9 BA + 2.9 IAA	3.20 cd	2.23 cd	1.95 bc	4.35 b
8.9 BA + 5.7 IAA	2.80 e	1.77 ef	2.00 b	3.54 bc

0.05)

This multiplication system proved to be very efficient allowing a fast increase in the number of propagules and shortening the *in vitro* multiplication period for the production of a considerable amount of plantlets.

Root induction and acclimatization

Addition of IAA to rooting medium influenced greatly the quality of the plantlets and the survival rate after transplant to acclimatization stage. All culture media supplemented with IAA showed increased values for the number of nodal segments, percent of rooted shoots, root length and survival rate in greenhouse compared to the control (Table 2).

Rooting was induced in 71% of the shoots with 4.2 nodal segments per plantlet and an average root length of 6.3 cm when 2.9 µM IAA was added to the culture medium. Plants cultured in a medium supplemented with 2.9 µM IAA showed 96.4% survival when transferred to acclimatization stage (Figure 1E).

Table 2. Rooting and acclimatization of *M. royoc* plantlets cultured in media with different IAA concentrations.

	No of nodal segments/shoot	Rooted shoots (%)	Root length (cm)	Survival rate in greenhouse (%)
IAA 0.0 µM	3.74 b	47.0 c	3.6 b	64.3 c
IAA 2.7 µM	4.26 a	71.0 a	6.3 a	96.4 a
IAA 5.7µM	3.90 ab	53.0 bc	6.2 a	78.5 b
IAA 11.4 µM	4.10 ab	61.0 ab	7.5 a	82.1 b
SE	0.074	3.81	0.067	4.03

**Values followed by the same letter in the same column are not significantly different by Duncan test ($p< 0.05$).*

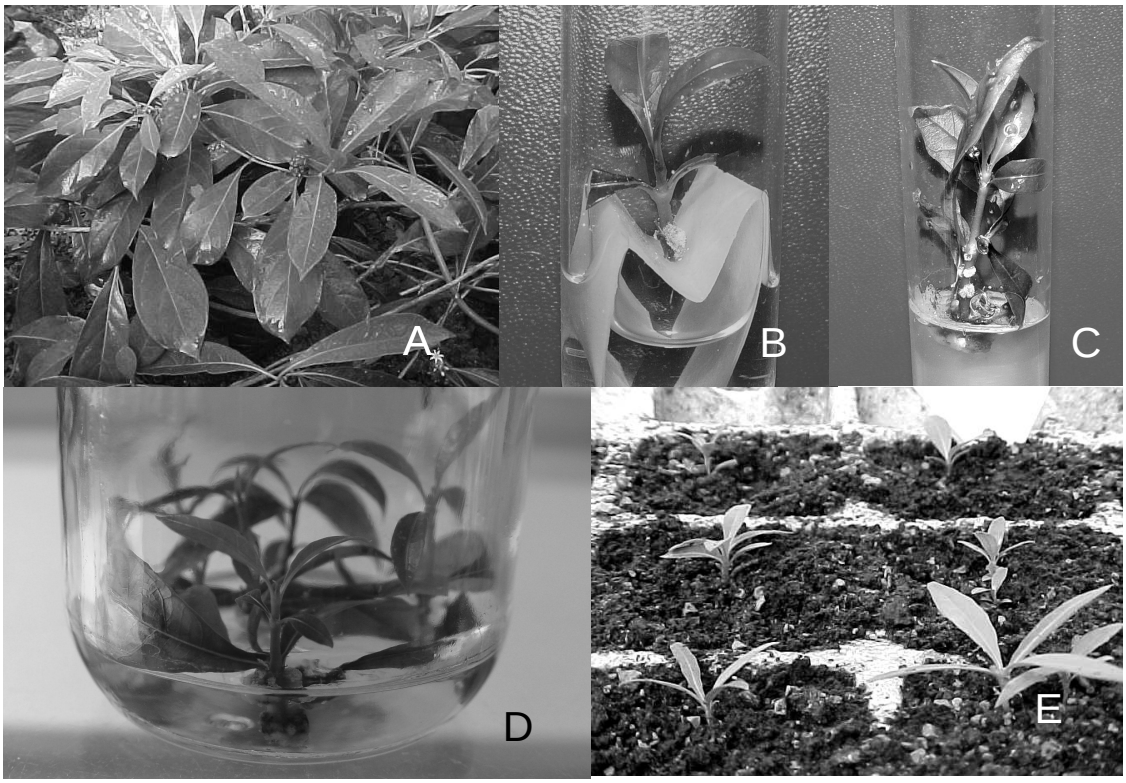

Figure 1. *In vitro* propagation of *Morinda royoc* L. (A) Donor plants cultivated in the greenhouse. (B) Excised shoot tip cultured in liquid medium with paper bridge. (C) Regenerated shoot after four weeks culture. (D) Shoots multiplied in agar-gelled culture medium for six week. (E) Acclimatized plants 15 days after transplant to soil mixture.

The *in vitro* regeneration of *M. royoc* plants reported here is characterized by a rapid growth and proliferation of shoots. A high multiplication rate was achieved, but also plantlets were easily acclimatized to the external environment from tissue culture undergoing normal morphological development. This is of great advantage for the commercial propagation and also for the conservation of this species. It could be the basis for future studies on the *in vitro* production of secondary metabolites for pharmaceutical use given the medicinal properties of the species. Little work on *in vitro* propagation, by using tissue culture techniques, were carried out in this genus up to day (Huang *et al*., 2006; Selvaraj et al., 2006). To our knowledge, this is the first report of *in vitro* regeneration of *Morinda royoc* L.

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