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Management of Light During *In Vitro* Culture of *Garcinia aristata* (Griseb.) Borhidi

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ABSTRACT

Garcinia aristata (Griseb.) Borihidi seedlings growth *in vitro* under dark and light conditions was compared. Shoot tips and single node explants from both etiolated and non-etiolated seedlings were also compared for shoot formation. Light significantly inhibited plant height, stem elongation, the number of nodes per seedling and the protein content. However, the content of phenolics (free and cell wall-linked) and the peroxidase activity of light-treated explants at day 35 of culture were significantly higher than those observed under darkness. Interaction between light and 6-benzyladenine (BA) concentration significantly affected shoot induction. The maximum percentage of shoot formation for shoot tips and single nodes were achieved always in the dark on Murashige and Skoog culture media supplemented with 4.44 and 6.67 μM of BA, respectively. Etiolation also promoted adventitious bud regeneration on the internode segments for all concentrations of the cytokinin evaluated. This is the first report ever of the propagation of *G. aristata in vitro*.

Keywords: peroxidases, phenolics, protein, rigidity
Abbreviations: BA, 6-benzyladenine; **EDTA**, ethylenedinitrilotetraacetic acid; **IAA**, indole-3-acetic acid; **MS**, Murashige and Skoog medium

INTRODUCTION

Garcinia aristata (Griseb.) Borhidi, belonging to the *Clusiaceae*, is a slow-growing woody species native to Cuba. This tree is valuable for traditionally medicine since its latex, cork and leaves are frequently used to fight several diseases such as asthma and tetanus (Roig 1974), although metabolites with biological activity have not yet been elucidated and only a few phytochemical studies focusing on this goal exist (Cuesta-Rubio *et al*. 2001). *G. aristata* has been overexploited due to its medicinal properties, which, together with an ongoing habitat loss and degradation caused by humans, have led to its inclusion in the list of endangered plant species (Rankin and Areces 2003).

G. aristata is sexually propagated in nature, but the number of seeds per fruit is low and also there is a lack of fruits related to a low fruit production and also to a decrease in the population of trees. On the other hand, there is no report of successful vegetative propagation due to the slow rooting process. Therefore, *in vitro* culture techniques will be a useful tool to develop a micropropagation protocol for conservation and sustainable utilization of this species. However, propagation protocols for this species *in vitro* have not yet been reported. Research has been reported for woody species belonging to *Clusiaceae* which, despite a proven recalcitrance, responded positively to tissue culture in many cases. Some of these studies have been conducted on the genus *Callophylum* (Nair and Seeni 2003; Thengane *et al*. 2006) but the most of them focused on species from the genus *Garcinia* (Te-chato and Lim 1999; Kulkarni and Deodhar 2002; Malik *et al*. 2005) using seeds and young leaves from *in vitro*-grown seedlings as explants for the establishment of cultures, mainly through direct regeneration processes. Nevertheless, similar studies using leaf explants taken from field-grown seedlings and mature trees has also been reported (Goh *et al*. 1990; Nair and Seeni 2003). With this scope, one goal of this study was to achieve the *in vitro* establishment of shoot culture from seed material.

On the other hand, it is well known that light is able to affect plant growth and development (Kim *et al*. 2002; Symons and Reid 2003). Hence, it is an important factor to be taken in account during the *in vitro* culture of plants. In this sense, several *in vitro* studies aiming to achieve higher shoot proliferation rates have investigated the interaction between plant growth regulators and light (Mohamed *et al*. 1992; Muleo *et al*. 2001; reviewed in Teixeira da Silva 2006). Thus, this work also aimed to evaluate the effect of light on *in vitro* response of plant material.

MATERIALS AND METHODS

Plant material

Mature fruits of *Garcinia aristata* (Griseb.) Borhidi were collected in February from field-grown plants originated from a forest near Modesto Reyes town, Ciego de Ávila, Cuba. Mature seeds were then selected from fruits.

Seed sterilization and *in vitro* germination

Mature seeds were carefully washed with commercial detergent, rinsed with plenty of water three times and dried at room temperature for a week. Later, seeds coats were removed and the resulting endosperms (including embryos) were surface-sterilized in 0.1% mercuric chloride ($HgCl_2$, BDH, UK) for seven minutes and then rinsed three times for 10 minutes with distilled water. Uncoated seeds (**Fig. 1A, 1B**) were germinated in the dark on germination medium consisting of MS basal medium (Murashige and Skoog 1962) (Duchefa, The Netherlands) supplemented with 30.0 $g.L^{-1}$ sucrose (Sigma, USA), 50.0 $mg.L^{-1}$ cysteine (BDH) and 2.5 $g.L^{-1}$ Phytagel (Sigma). In all cases, the pH was adjusted to 5.8 before autoclaving at 121°C and 118 kPa for 20 minutes. Cultures were incubated at 26°C. In the experiments that involved light, cultures were placed under a 16-h photoperiod and photon flux of 30-40 $\mu mol.m^{-2}.s^{-1}$ (cool white fluorescent light).

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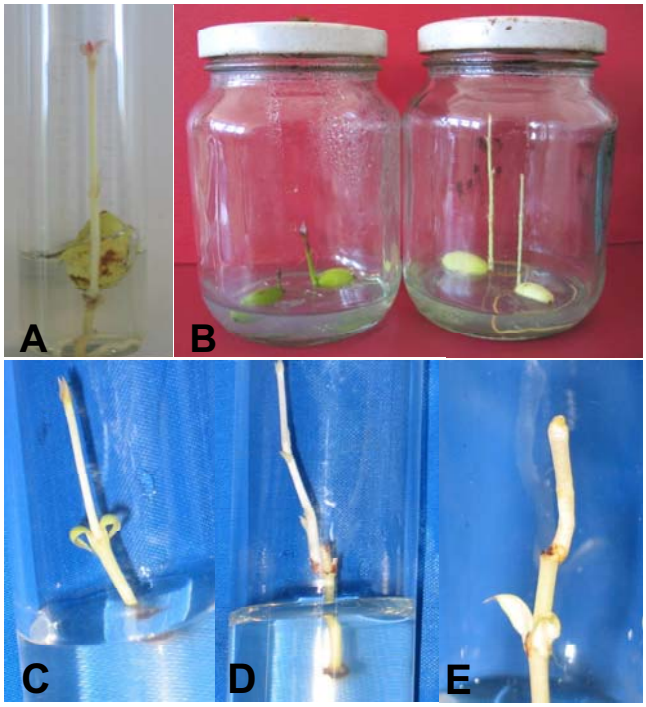

Fig. 1 Seed germination *in vitro*, shoot tip and adventitious shoot development. (**A**) Seed germinated *in vitro*. (**B**) Seedlings grown *in vitro*, under light (left) and darkness (right) conditions. Note the morphological differences (color, plant height, number of nodes). (**C**) Shoot tip established on MS germination medium supplemented with BA. (**D**) Single node established on MS germination medium supplemented with BA. (**E**) Adventitious shoot formed at the cut zone on the internode.

Management of light and physiological stage of explants

The first week of culture was in darkness and then two treatments were established: seedlings growing in germination medium under either darkness or light conditions for another four weeks. After 35 days of cultures, plant height (cm), internode length (cm) and the number of nodes per plant were determined as morphological parameters of *in vitro* growth.

For the biochemical determinations, leaves were removed from 35-days-old seedlings taken from both light and dark conditions. Stems bearing shoot tips and three nodes were used as samples and stored in liquid nitrogen until analysis. Each determination started from three independent pooled samples (250 mg). In all cases, they were finely ground with a mortar and pestle in liquid nitrogen. Peroxidase activity was measured according to Pascual *et al*. (1983). Extraction was performed in 2.5 ml of 10 mM tris-HCl (Sigma) buffer (pH 7.0) and the reaction mixture included 100 µL of enzyme extract, 1.0 mL of 10 mM tris-HCl buffer (pH 7.0), 150 µL 100 mM guaiacol (Sigma) and 20 µl 50 mM hydrogen peroxide (H_2O_2, Sigma). Activity was determined by measureing the increase in absorbance at 470 nm. Phenolics (free and cell-wall linked) were determined according to Gurr *et al*. (1992). Extraction was done with 0.5 ml methanol (Merck, Germany). Samples were briefly vortexed and centrifuged (12,100 × *g*, 15 min). The pellet was submitted to two additional cycles of methanol-mediated extractions as described above (supernatant was always collected and regarded as containing free phenolics). The pellet was then incubated with 0.25 ml sodium hydroxide (2.0 $mol.l^{-1}$, Sigma) for 16 h (70°C). Hydrochloric acid (0.25 ml, 2.0 $mol.l^{-1}$, Sigma) was added after incubation. Samples were centrifuged (12,100 × *g*, 15 min). The pellet was discarded and supernatant was considered as containing cell wall-linked phenolics. In order to quantify free and cell wall-linked phenolic levels, 20.0 µl of supernatants were mixed with 980.0 µl distilled water. Folin-Ciocalteau's phenol reagent (100.0 µl, BDH) was added and samples were incubated for 5 min. Sodium bicarbonate (600.0 µl, saturated with sodium hydroxide (0.1 $mol.l^{-1}$), Sigma) was supplemented and samples were incubated for 2.5 h. Absorbance (725 nm) was recorded using a spectrophotometer (Pharmacia LKB, USA). Protein extraction was performed with 1.6 ml of pre-cooled extraction buffer consisting of 50 $mmol.l^{-1}$ potassium phosphate buffer (pH 7.8) (Sigma), 1 $mmol.l^{-1}$ EDTA (Sigma), 1 $mmol.l^{-1}$ ascorbic acid (Sigma), 0.02 $mol.l^{-1}$ sodium bisulphite (Sigma), 20% sorbitol (Sigma) and 2% polyvinylpolypyrrolidone (Sigma). Protein concentration was determined according to Bradford's method (1976).

Establishment of shoot cultures

Shoot tips and single nodes (0.5-1.0 cm) obtained from 35-days old (both, dark and light-treated) seedlings were used to establish shoot cultures. To test the effect of BA on shoot initiation, various concentrations (0.00, 2.22, 4.44, 6.67 and 8.89 µM, Duchefa) of this growth regulator were added to germination medium. Explants were orientated in a vertical position on the culture media and experiments were incubated in the dark (**Fig. 1C-E**). Each treatment was replicated 30 times. Data of percentages of shoots formation and adventitious shoots formation in the internodes were recorded after 45 days.

Statistical analysis

All statistical analyses were carried out using SPSS version 11.5. Variations among treatments means in the experiments involving tissue culture, were analyzed using ANOVA followed by a Tukey's test at the 5% level. The results of enzymatic activities, phenolics determination and morphological parameters of seedlings were analyzed using the Student's *t*-test at the 5% level.

RESULTS AND DISCUSSION

Management of light and physiological stage of explants

Light treatments significantly affected the morphological parameters measured. Plant height, internode length of seedlings and the number of nodes per plant (**Fig. 1C-E**) were about two-fold higher in seedlings grown under dark conditions. Moreover, the content of proteins observed in dark-grown seedlings were significantly higher than that from plants under light treatment. In contrast, light enhanced the peroxidase activity and the content of free and cell wall-linked phenolics, being statistically different to dark-treated seedlings (**Table 1**). In addition, the plant material showed a fast and extremely marked increase in rigidity as it was exposed to light.

Results obtained in this work – when plant height and internode length parameters measured – indicate the negative influence of light on stem elongation, which is consistent with its widely reported inhibitory effect (Reid *et al*. 2002; Symons and Reid 2003). However, the stimulatory action of etiolation on the number of nodes per plant, suggesting its direct influence on the shooting capacity of seedlings, is a light-related morphological response not described before in the literature for any woody species (at least to our knowledge). The latter result, together with the strengthening process of the plant material suggests an im-

Table 1 Morphological parameters, phenolics (free and cell wall-linked), protein content and peroxidase activity during *in vitro* growth of seedlings under light and darkness conditions.

	Culture conditions	
	Light	**Darkness**
Plant height (cm)	3.18 b	6.63 a
Number of nodes per plants	2.70 b	4.20 a
Internode length (cm)	0.99 b	1.47 a
Content of free phenolics (µg g^{-1} fresh mass)	228.83 a	22.57 b
Content of cell wall-linked phenolics (µg. g^{-1} fresh mass)	490.71a	19.37 b
Peroxidase activity (Ug^{-1} fresh mass)	3.42 a	2.56 b
Protein content (mg g^{-1} leaf fresh mass)	0.17 b	0.18 a

Means with different letters are significantly different according to T-test at the 5% level.

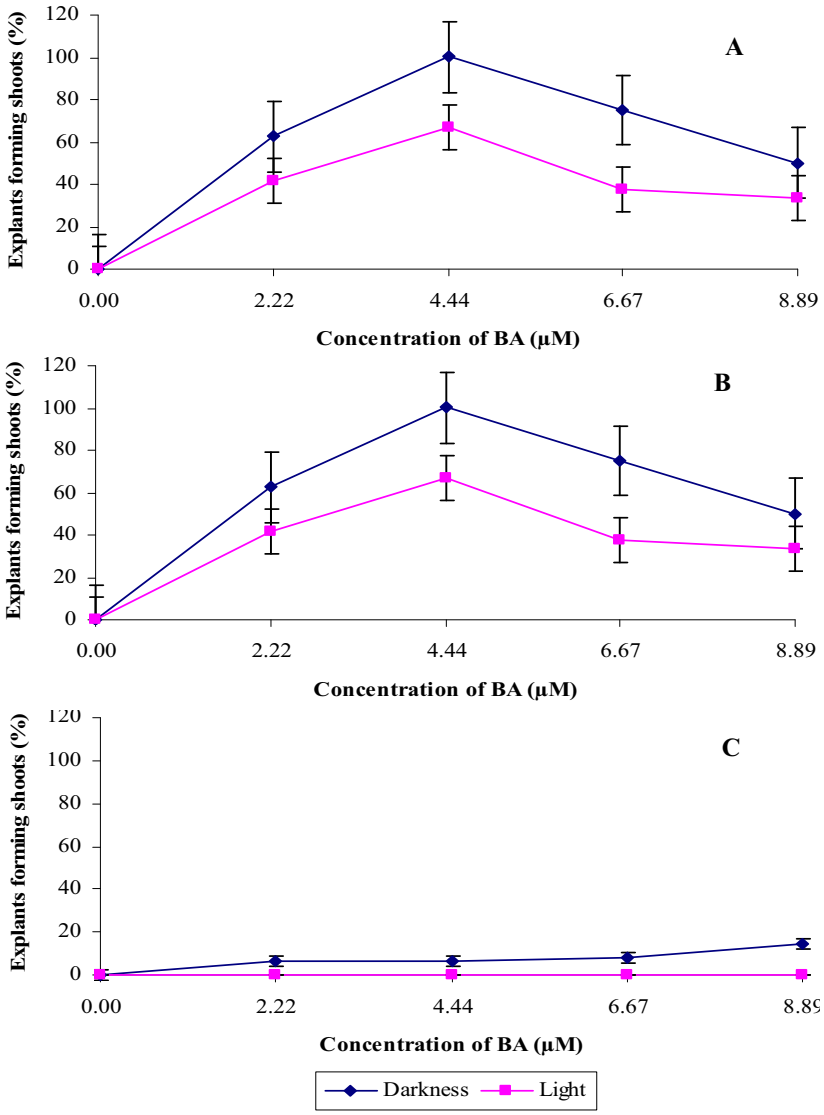

Fig. 2 Effect of light on the percentages of shoot formation on germination medium supplemented with various concentrations of BA, during the *in vitro* establishment of (**A**) shoot tip culture and (**B**) single node culture. (**C**) Percentages of adventitious shoot formation on the internodes segments during the experiment with single nodes. Statistical analysis according to ANOVA followed by a Tukey's test at the 5% level.

portant role of light on the slow-growing process of *G. aristata*, through a mechanism involving the induction of rigidity.

Previous reports had shown changes in the content of phenolics and peroxidase activity accompanied by morphogenetic programs under light control (Cvikrova and Hrubcova 1999). Furthermore, a light-induced augmentation in the latter biochemical parameters has been associated with higher levels of lignin in soybean hypocotyls which became more rigid when exposed to light (Zin-Huang *et al*. 1996). Hence, the process of lignification could explain in part the increment in rigidity of light-treated seedlings in our work, despite not having measured the contents of lignin and its specific phenolic precursors. However, this is a theory to prove in further studies.

Establishment of shoot cultures

Interaction among light and concentrations of BA negatively affected the percentages of explants forming shoots. Dark-treated shoot tips cultured in germination medium supplemented with 4.44 and 6.67 µM of BA showed significantly higher percentages of shoot formation than the treatments with light (see **Fig. 2A**). In addition, etiolation significantly induced shoots formation in nodes (**Fig. 1D**) but only when 2.22 µM of BA was added to the culture medium (**Fig. 2B**). Furthermore, the regeneration of adventitious buds in the internodes segments was only observed in dark-treated explants (**Fig. 2C**).

The positive effect of darkness on *in vitro* morphogenetic response of explants has been shown previously through the use of etiolation as a method for rejuvenation of plant material (Rodríguez *et al*. 2005). In addition, etiolation has been shown to enhance shoot regeneration during *in vitro* studies (Kiss *et al*. 1995; Liu *et al*. 1998). Despite the mechanisms involved in these processes not yet being well described, results obtained in this experiment indicate a relation between the loss of morphogenic capacity of explants and the light-dependent induction of rigidity in 35 days old seedlings of *G. aristata*. On the other hand, the higher levels of peroxidase activity observed in light-treated seedlings could be associated with a decrease in endogenous IAA content in explants, due to the involvement of peroxidases in auxin catabolism (Ros Barceló and Muñoz 1992; Duarte-Vázquez *et al*. 2007).

In conclusion, light diminished the shooting capacity of *in vitro*-grown seedlings. The *in vitro* culture of *Garcinia aristata* (Griseb.) Borihidi was achieved by using shoot tips and nodes from seedlings as explants to establish shoot culture on MS basal culture medium supplemented with BA. Dark-treated shoot tips and nodes showed higher percentages of shoot formation in media cultures supplemented with 4.44 and 6.67 µM of BA. Etiolation promoted adventitious bud regeneration on the internode segments at all concentrations of the cytokinin evaluated.

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