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IN VITRO FLOWERING OF *PHYLLANTHUS TENELLUS* ROXB. CULTURED UNDER DIFFERENT LIGHT QUALITIES AND GROWTH REGULATORS

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Summary. Tissue cultures of *Phyllanthus tenellus* Roxb. were maintained on modified MS to evaluate *in vitro* flowering under light of different qualities: white (control), blue, green, red, yellow, UV-A plus white and darkness; as well as different growth regulators at concentrations (μM): IBA 0.98, KIN 0.46, KIN 2.3, IBA 0.98 + KIN 0.46, IAA 1.14 + KIN 0.46. The events from vegetative to reproductive growth were observed under *in vitro* conditions. Flowering was verified from 15 days in all light treatments. Plantlets grown under darkness presented 6% of flowered plantlets within 60 days. White and blue lights showed the highest *in vitro* flowering percentage (70% and 56%, respectively). A reduction of flowering was observed under red, yellow and green light exposure. IBA added at 0.98 μM concentration induced a greater number of flowers per branch (4.4±0.5). *In vitro* fruiting was obtained within 60 days.

***Key words**:* light spectra, micropropagation, Phyllanthaceae, plant growth regulator, tissue culture.

Abbreviations: MS – Murashige and Skoog; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; KIN – kinetin.

INTRODUCTION

Flowering obtained with *in vitro* cultures is a pre-requisite for many genetic manipulations and can also improve knowledge about physiology of flowering. Studies on *in vitro* flowering using different light qualities, photoperiods and plant growth regulators have been reported for several species (Zhang and Leung, 2000; Sudhakaran and Sivasankari, 2002). The main advantages of *in vitro* flowering procedures are isolation of external signals from environmental, acquisition of aseptic cultures and standardized conditions that permit repeating several cycles in a short time (Zhang and Leung, 2000). Additionally, *in vitro* cultures turn easy and fast the studies about flowering.

The initiation of reproductive phase generally requires that plant perceives and responds to the appropriate environmental conditions (Samach and Wigge, 2005). Light is the most important environmental factor

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that induces changes in plant physiology and morphology, regulating flowering season cycles (Victório et al. 2007a; Victório et al. 2007b; Kerbauy, 2008). Plants sense their ambient light conditions through a group of photoreceptors as the phytochrome and cryptochrome families responsible for red and blue/UV light-absorbing, respectively (Smith 2000; Franklin, 2008). Light qualities as green and yellow have been insufficiently investigated. Classical literature has reported sporadic evidence about the effects of green light or characterizes it as innocuous to growth although previous studies have shown that cryptochromes and phytochromes readily absorb green light to initiate photomorphogenic responses (Folta and Maruhnich, 2007). UV radiation can alter many aspects of plant responses at the physiological levels. Considering UV radiation, there are few studies about UV-A effects on plant development because its radiation is attenuated by ozone layer (Lautenschlager et al., 2007). Plant growth regulators represent essential factors that regulate induction, evocation and development of flowers. Cytokinins are associated with floral induction. Auxins, according to several studies, may either inhibit or promote flowering (Ferreira et al., 2006).

Phyllanthus tenellus Roxb. (Phyllanthaceae, recently dismembered taxon of Euphorbiaceae), commonly known as "quebra-pedra" in Brazil, is often used for treatment of kidney and urinary bladder disturbances due to its therapeutic potential (Calixto et al., 1998; Samuel et al., 2005). *P. tenellus* is a herbal plant with a cylindrical stem from growing two kinds of lateral branches: perennial (without flowers) and deciduous (with flowers) (Webster, 1970). The reproductive phase has an impact on the secondary metabolites profiles of plants and indirectly may influence therapeutic effects of medicinal plants (Spitaler et al., 2006; Nejad Ebrahimi et al., 2008). The aim of the present work was to verify the effects of different light qualities and growth regulators on *in vitro* flowering of *P. tenellus*.

MATERIALS AND METHODS

Cultures were initiated from *P. tenellus* seeds (Victório et al., 2009) and samples of matrix plants were identified and deposited in the Herbarium of National Museum of Rio de Janeiro under number R 200872. From 12-week-old seedling, segments were isolated and cultured for 60 days on a modified MS medium (Murashige and Skoog, 1962) reduced to half of NH_4NO_3 and KNO_3 solution (MS½N). Media were supplemented with 3% (w/v) sucrose, vitamins, myo-inostol and 0.78% (w/v) agar and pH was adjusted to 5.8 with 0.1N KOH before autoclaving at 121°C for 15 min. Different treatments with light qualities and growth regulators were applied. One genotype was chosen and cultures were maintained at 25±2°C and photoperiod of 16 h. One lamp per shelf for each light quality was used: white (control, 20 μmol $m^{-2}s^{-1}$), blue (17 μmol $m^{-2}s^{-1}$), green (12 μmol $m^{-2}s^{-1}$), red (12 μmol $m^{-2}s^{-1}$), yellow (12 μmol $m^{-2}s^{-1}$), UV-A plus white light (20 μmol $m^{-2}s^{-1}$) and darkness. All light qualities were obtained from Sylvania® fluorescent tubes (F20 W T-12) and light intensities were measured by a quantameter (Biospherical Instruments Inc., QSL-100). Plantlets maintained in darkness were exposed to light for short periods within 20 and 40 days. To study the effects of growth regulator, explants were cultured on MS½N supplemented with IBA 0.98 μM,

KIN 0.46 and 2.3 μM, IBA 0.98 μM + KIN 0.46 μM, IAA 1.14 μM + KIN 0.46 μM, under white light. Within 60 days, plantlets were evaluated according to the following criteria: regenerated frequency of shoot (1, 2 and 3 shoots per explant), ratio number of lateral branches/number of shoots, flowering percentage and number of flowers per branch. For each treatment three experiments were conducted with about 15 replicates, using a completely randomized design. Some flowers were observed through the magnifying glass (Carl Zeiss Stemi SV 11).

Percentage values were analyzed by test of the difference between two percentages using Statistica 6.0 software, and LSD for all pairs comparison at P<0.05 level was calculated using Student's *t*-test. Data on number of lateral branches and number of flowers were analyzed using analysis of variance (ANOVA) and the averages were evaluated at the 5% significance level using Tukey`s test.

RESULTS AND DISCUSSION

Data on percentage of plantlets, lateral branches, flowering and number of flowers are presented in Table 1. Plantlets cultured under white (Fig. 1) and blue light showed the best results for flowering percentage and number of flower per branch: white light (70 %, 3.6) and blue light (56 %, 3.6). Both criteria evaluated showed that the presence of light was essential. Well-developed rooted plantlets were obtained at all tested light treatments. Any environmental variables are potential factors that control the transition to flowering. Changes from vegetative to reproductive phase depend on light, temperature and photoperiod (Bernier, 1993). The first flowers were developed *in vitro* within 20 days under light exposure. There was good correlation between the number of lateral branches and flowering. Within 60 days, only 6 % of plantlets presented flowers under darkness (Table 1), suggesting a previous

Table 1. *In vitro* flowering of *Phyllanthus tenellus* under different light qualities and darkness, within 60 days, photoperiod 16 h, 25°C. Average ± SE, $n = 45$. Means followed by the same letters were not significantly different at $P < 0.05$ (Tukey`s test). RF- regenerated frequency, 1S - 1 shoot per explant, 2S - 2 shoots per explant and 3S - 3 shoots per explant. *Data did not obtain.

Light qualities	RF [%]			No lateral branches/ No shoot	Flowered plantlets [%]			No flowers/ branch
	1S	2S	3S		20d	40d	60d	
White	60^{b}	31^{a}	6^{a}	4.8^{a}	27^{ab}	36^{ab}	70^{a}	3.6 ± 0.4^{a}
Blue	86^{ac}	19^{ab}	*	4.4^{ac}	32^{ab}	38^{b}	56^{ad}	3.6 ± 0.4^{a}
Red	71^{bc}	24^{ab}	*	3.1^{b}	15^{bc}	19^{c}	25^{bd}	1.9 ± 0.2^{b}
Yellow	74^{bc}	22^{ab}	5^{a}	2.9^{b}	24^{b}	10^{cd}	25^{bd}	2.3 ± 0.2^{ab}
Green	66^{b}	35^{a}	8^{a}	3.8^{bc}	47^{a}	35^{ab}	12^{bc}	2.2 ± 0.3^{ab}
UV-A+White	90^{a}	10^{bc}	*	3.6^{b}	3^{c}	7^{d}	35^{d}	2.4 ± 0.3^{ab}
Darkness	97^{a}	3^{c}	*	2.5^{d}	*	*	6^{c}	(*n* not enough)

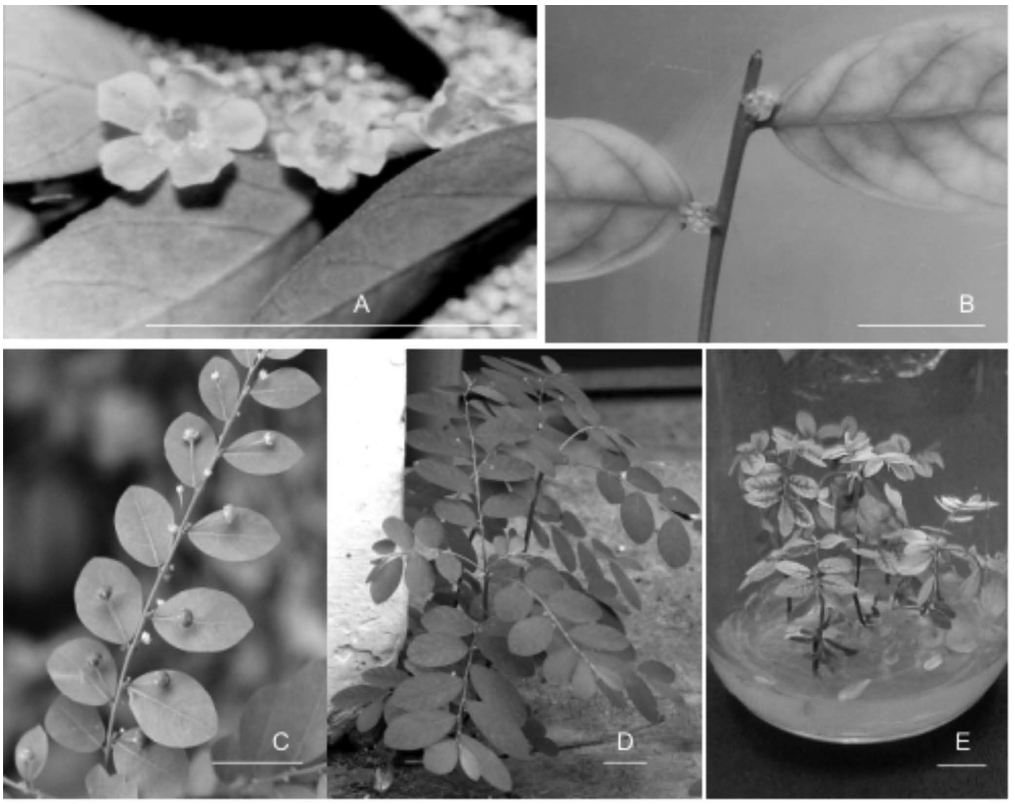

Fig. 1. (*A*) *Phyllanthus tenellus* Roxb. flowers of field-grown plants in detail. (*B*) Flowers of 60-day-old plantlets cultured *in vitro*. (*C*) Lateral branch showing flowers and fruits of field-grown plants. (*D*) Field-grown plants at the reproductive phase. (*E*) 60-day-old plantlets cultured on MS½N, under white light. Bar = 1 cm.

floral evocation. Green light induced lower response (12%) of flower production in *P. tenellus* plantlets, the results being similar to dark treatment. However, taking into account the number of flowers per branch, there were no statistical differences of green light compared with white, blue, yellow and UV-A radiation. It was verified that green light was able to induce *P. tenellus* development until flowering. White and blue light qualities produced major flowered plants compared with the other light qualities tested.

The number of flowers per branch was reduced under red light when compared to white and blue lights. Red, yellow and green lights showed decreased flowers production. Furthermore, higher green light exposure led to flower senescence and a reduction in the number of flowered plantlets within 60 days.

Ohtani et al. (2000) applied a method to control flowering in plants of genus *Pelargonium*, *Petunia* and *Euphorbi*a (*Euphorbiaceae*) using different light qualities without using any chemical resource. As a result yellow light influenced morphogenesis of flowers of *Euphorbia* inducing changes in the number and length of bracts. In this study, yellow light showed effects statistically equal to those of red and green lights.

Flowers produced from tissue cultures systems presented normal morphological aspects. They were monoecious and differentiated from lateral branches as field-grown plants (Fig. 1). Besides, anthesis was observed in floral buds development.

Table 2. *In vitro* flowering of *Phyllanthus tenellus* in the presence of different plant growth regulators, within 60 days, photoperiod 16 h, 25°C. Means ± SE, $n = 45$. Means followed by the same letter were not significantly different at $P < 0.05$ (Tukey`s test). RF- regenerated frequency, 1S- 1 shoot per explant, 2S- 2 shoots per explant and 3S- 3 shoots per explant. *Data not obtained.

Growth regulators [μM]	RF [%]			No lateral branches/ No shoot	Flowered plantlets [%]	No. flowers/ branch
	1S	2S	3S			
MS (control)	54^{bc}	33^{a}	4^{a}	2.7^{b}	21^{bc}	2.8±0.4^{a}
IBA 0.98	77^{ac}	23^{ab}	*	6.0^{a}	58^{a}	4.4±0.5^{a}
KIN 0.46	73^{ac}	18^{ab}	2^{a}	4.9^{ab}	31^{b}	4.0±0.6^{a}
KIN 2.3	48^{b}	33^{a}	12^{a}	3.6^{b}	15^{bc}	(*n* not enough)
IBA 0.98+KIN 0.46	66^{ab}	24^{ab}	3^{a}	4.5^{ab}	*	4.3±0.5^{a}
IAA 1.14+KIN 0.46	83^{a}	10^{b}	*	5.3^{ab}	9^{c}	(*n* not enough)

Flowers self-fertilized and *in vitro* fruiting was also found.

The increased flowered plantlets within 60 days might be associated with the high level of ethylene in the vessels of cultures (Table 1), verified through accelerated senescence of leaves. Ethylene may act on flower induction as reported by Dukovsky et al. (2006) and is also involved in plant senescence responses. Probably, this hormone contributed to the reduction of the number of flowered plantlets of *P. tenellus* under green (60 days) and yellow lights (40 days).

Flowering responses were verified for all growth regulators tested. However, the addition of 0.98 μM IBA induced a greater number of branches, flowering (%) and flowers per branch, showing a promoted effect of auxin (Table 2). Auxins may induce suppression of lateral bud development, differentiation, and definition of lateral meristems into more complex floral tissues. In addition, high auxin concentrations tend to promote ethylene biosynthesis. Opposite effects of auxins on flowering have been widely observed depending on species. This phytohormone has a remarkable action in flowering, and can also prevent flower abscission. In addition, its high concentration in species with monoecious flowers favors the development of female flowers (Takahashi and Jaffe, 2003).

In conclusion, in the current study, the role of different light qualities, UV-A and phytohormones in the regulation of flowering was verified. This protocol represents also an easy way to produce plantlets of *P. tenellus* from nodal segments in a short period of time (60 days) covering the whole life cycle.

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