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Full Length Research Paper

Evaluation of extract from leaves of *Calophyllum brasiliense* Cambess. (Clusiaceae) using micronucleus assay in mouse bone-marrow cells

Lilian Lucy dos Santos¹, Mislaine Adriana Brenzan², Paula Akemi Honda², Roberto Barbosa Bazotte², Carlos Eduardo Oliveira², Mirian Ueda Yamaguchi¹, Lucia Elaine Ranieri Cortez¹ and Diógenes Aparício Garcia Cortez¹*

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Received 12 March, 2015; Accepted 8 May, 2015

This study evaluated the chromosomal damage potential of the extract from leaves of *Calophyllum brasiliense* Cambess. (Clusiaceae) in mouse bone-marrow cells. The extract was analyzed by high-performance liquid chromatography (HPLC) to assess the concentration of coumarin (-) mammea A/BB, a compound with antileishmanial activity. The chromosomal damage potential of the extract was evaluated by the micronucleus test in erythrocytes of mouse bone marrow. The animals were treated with cyclophosphamide (50 mg) as positive control, DMSO (1%) as negative control, or the crude extract from *C. brasiliense* (100 or 200 mg/kg). The HPLC analyses showed that the extract contained 25.97 ± 0.91 µg of (-) mammea A/BB per mg of extract. The extract of *C. brasiliense* did not show chromosomal damage potential at the concentrations used for the preparation of creams with antileishmanial activity. This study may contribute to the registration of a topical phytomedicine containing the extract of *C. brasiliense* to treat cutaneous leishmaniasis.

Key words: *Calophyllum brasiliense*, genotoxicity assay, (-) mammea A/BB, high-performance liquid chromatography (HPLC), antileishmanial activity, medicinal plants.

INTRODUCTION

*Calophyllum brasiliense* Cambess. (Clusiaceae), popularly known as "guanandi", is a large tree that grows mainly in the Brazilian Atlantic rainforest, and is distributed in South America from Brazil to Mexico. This tree has been used in folk medicine for the treatment of several diseases such as rheumatism, varicose veins, hemorrhoids and chronic ulcers (Corrêa et al., 1978). Previous studies showed that *C. brasiliense* is a rich

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source of bioactive compounds, including coumarins (Ito et al., 2003; Reyes-Chilpa et al., 2004), xanthones (Sartori et al., 1999; Ito et al., 2002), triterpenoids (Reyes-Chilpa et al., 2004), and biflavonoids (Da Silva et al., 2001).

The compounds isolated from C. brasiliense showed cytotoxic activity against some tumor cell lines (Kimura et al., 2005; Ito et al., 2006), mainly the coumarin mammea A/BB (Reyes-Chilpa et al., 2004; Ruiz-Marcial et al., 2007). Previous studies have also demonstrated that extracts, fractions, and especially the coumarin (-) mammea A/BB isolated from C. brasiliense leaves show significant molluscidal activity against Biomphalaria glabrata (Gasparrotto et al., 2005) and potent in vitro and in vivo leishmanicidal activity against Leishmania amazonensis and Leishmania braziliensis (Brenzan et al., 2007; 2008a; Honda et al., 2010; Tiuman et al., 2012). Honda et al. (2010) demonstrated that footpad lesions of mice infected with L. amazonensis decreased in size when the mice were treated topically with 10% dichloromethane extract or intraperitoneally with 100 mg/kg and 200 mg/kg of the hexane fraction of C. brasiliense.

This study contributed to the development of a topical phytopharmaceutical formulation containing compounds isolated from C. brasiliense, to treat cutaneous leishmaniasis (patent BR200904042-A2). Intraperitoneal administration of the extract at doses of 100 and 200 mg/kg also revealed healing lesions. The parasite load in the popliteal lymph nodes was significantly reduced in mice administered 50 mg/kg, 100 mg/kg, and 200 mg/kg of the extract. In addition, the extract at doses of 100 mg/kg and 200 mg/kg of the crude extract from C. brasiliense showed significant antileishmanial activity against Leishmania amazonensis and Leishmania braziliensis (Brenzan et al., 2007; 2008a; Honda et al., 2010; Tiuman et al., 2012). Honda et al. (2010) demonstrated that footpad lesions of mice infected with L. amazonensis decreased in size when the mice were treated topically with 10% dichloromethane extract or intraperitoneally with 100 mg/kg and 200 mg/kg of the hexane fraction of C. brasiliense.

MATERIALS AND METHODS

Plant

Aerial parts of C. brasiliense were collected at the Botanical Institute of São Paulo, Brazil, on 13/09/2012. The plant material was identified by Prof. Dr. Maria Claudia M. Young, and a voucher specimen (SP 363818) was deposited and authenticated at the herbarium of this institute. The leaves were dried at 45°C in a circulating-air oven and triturated in a knife mill (Usi Ram®), and the resulting powder was stored in a dry location in the dark.

Plant extraction

The powdered leaves (825.0 g) were extracted by exhaustive maceration in ethanol/water (9:1) at room temperature, until depleted of all compounds. The extract was filtered and concentrated under vacuum at 40°C to obtain an aqueous extract and a dark-green residue. The residue from the extract, stored in glass bottles, was dissolved with dichlormethane, and the solvent was then completely evaporated at room temperature; this extract was termed the crude extract (31.5 g). The aqueous extract was lyophilized (148.0 g). Both were stored at -10°C, in the dark, until use (Brenzan et al., 2007).

HPLC analyses

The HPLC analyses of the crude extract from C. brasiliense leaves were carried out according to Brenzan et al. (2010). A Shimadzu LC-10 liquid chromatography equipped with a quaternary pump (LC-10 AD), automatic injection valve (Rheodyne) with a 20 µl loop, degasser (DEU-14), thermostatted column compartment from (CTO-10A), and a detector UV/vis (SPD-10A), controlled by the software CLASS LC-10 were used. The sample extracts were prepared in methanol at 3 mg/ml and filtered through a Millipore 0.45 µm membrane filter (Millipore, Brazil); then, 20 µl was injected into the HPLC system. A Metasil ODS column, 5 mm, 150 × 4.6 mm maintained at 30°C was used in the chromatographic analysis. The separation was performed in a gradient system, using as mobile phase a mixture of acetonitrile-water 5:95 v/v at 55:45 (0 to 10 min), 55:45 v/v at 80:20 (10 to 20 min), 80:20 v/v at 100% acetonitrile (20 to 30 min), and 100% acetonitrile (30 to 40 min) with a flow rate of 0.6 ml/min. The detection of the compounds was carried out at 254 nm and the run time was 40 min. Three determinations were carried out for each sample.

Animals and treatments

The experimental protocol was approved by the Animal Ethics Committee of Maringá State University (protocol number 037/2011).

The in vivo assay for chromosomal damage was carried out in female albino Swiss mice, approximately 7 to 8 weeks old and with a mean weight of 39.8 g. The mice were obtained from the Animal Facility of the State University of Maringá. The animals were kept in polyethylene cages with controlled temperature (25°C) in a 12:12 h light-dark cycle. Food and water were provided ad libitum. The feed was a standard commercial rodent chow (Nuvilab®).

The mice were housed in groups of six per cage and randomly divided into four groups, which received via orogastric gavage: (1) 1% DMSO (negative control), (2) 50 mg/kg of Cyclophosphamide (positive control), (3) 100 mg/kg of crude extract from C. brasiliense and (4) 200 mg/kg of crude extract from C. brasiliense. After 24 h, the animals were anesthetized through intraperitoneal injection of ketamine (60 mg/kg) and xylazine (12 mg/kg). The bone marrow was removed from the femur, and the animals were euthanized.

Micronucleus assay

The genotoxic effects of the C. brasiliense extract were evaluated in bone-marrow cells of mice by the micronucleus test, according to Schmid (1975). Immediately after the animals were anesthetized, the femurs were removed. The bone marrow was removed, emulsified, and transferred to centrifuge tubes containing 2 ml of fetal bovine serum (FBS). The bone-marrow suspension was centrifuged at 1000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in one drop of FBS, and one drop of this suspension was applied to a slide to prepare the
Figure 1. Chromatogram of crude extract from Calophyllum brasiliense leaves. (1) (-) mammea A/BB (Rt: 26.2 min). Chromatographic conditions: Metasil ODS column; mobile phase: acetonitrile:water 5:95 v/v at 55:45 (0–10 min), 55:45 v/v at 80:20 (10–20 min), 80:20 v/v at 100% acetonitrile (20–30 min) and 100% acetonitrile (30–40 min); flow rate of 0.6 ml/min; temperature: 30°C; detection: 254 nm.

smears. After 24 h of drying at room temperature, the smears were stained (May-Grunwald Giemsa) and the slides were again dried at room temperature.

The smears were examined under an optical microscope (Olympus, Japan) at 1000 × magnification. The criteria for counting micronuclei were based on the diameter, shape and coloration of micronuclei, according to Krishna and Hayashi (2000). To assess the induction of micronuclei formation (MN), the number of micronucleated polychromatic erythrocytes (MNPCE) in 1000 polychromatic erythrocytes (EPC) per animal and per slide was determined (Schmid, 1975).

Statistical analysis

The results were expressed as mean ± standard deviation (SD), to compare the frequencies of MNPCE between the treated and control groups. The micronucleus assay data were statistically analyzed by ANOVA followed by Tukey’s test. P ≤ 0.05 was considered statistically significant.

RESULTS

HPLC analysis of the C. brasiliense extract

In the chromatogram of the extract from C. brasiliense leaves, with a retention time of 26.2 min, the coumarin (-) mammea A/BB was identified as the majority compound (Figure 1). This extract showed a concentration of 25.97 ± 0.91 µg of (-) mammea A/BB per mg of crude extract. The content of this compound in the C. brasiliense extract was analyzed according to the methodology proposed by Brenzan et al. (2010), and the results were in agreement with this previous study.

Micronucleus test

The frequencies of micronucleated polychromatic erythrocytes (% MNPCE ± standard deviation (SD)) of the negative control and the groups that received 100 or 200 mg/kg of crude extract of C. brasiliense were 2.34 ± 1.6, 1.84 ± 1.2, and 3.0 ± 3.0%, respectively. The frequencies of MNPCE for mice treated with 100 or 200 mg/kg of the crude extract were similar to the negative control (Tukey's ANOVA Test, p ≤ 0.05). The frequency of MNPCE for the positive control group (16.83 ± 8.4%) (Figure 2) differed significantly (p ≤ 0.05) from the negative control group and from the groups treated with 100 or 200 mg/kg of the crude extract of C. brasiliense.
Figure 2. Micronuclei in bone-marrow cells of mice 24 h after the administration (orogastric gavage) of crude extract from *Calophyllum brasiliense* leaves. MN: Micronuclei.

**Table 1.** Frequencies of micronuclei in the mouse femur bone-marrow cells treated with the crude extract from *Calophyllum brasiliense* leaves.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>nMN/1000 erythrocytes</th>
<th>Mean % (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. brasiliense</em> extract (100 mg/kg)</td>
<td>6</td>
<td>2,0,2,3,3,1</td>
<td>1.84 (±1.2)</td>
</tr>
<tr>
<td><em>C. brasiliense</em> extract (200 mg/kg)</td>
<td>6</td>
<td>9,3,2,2,0,2</td>
<td>3.0 (±3.0)</td>
</tr>
<tr>
<td>Cyclophosphamide (50 mg/kg)</td>
<td>6</td>
<td>19,26,24,12,3,17</td>
<td>16.83* (±8.4)</td>
</tr>
<tr>
<td>DMSO 1%</td>
<td>6</td>
<td>4,2,0,1,3,4,</td>
<td>2.34 (±1.6)</td>
</tr>
</tbody>
</table>

*p*≤0.05. (ANOVA-Tukey’s) compared to the control (1% DMSO). n: Number of animals. nMN: number of micronuclei. SD: standard deviation.

**DISCUSSION**

Coumarins are heterocyclic molecules that have been associated with beneficial effects in reducing the risk of cancer, diabetes, and cardiovascular and brain diseases. These effects are related to the elimination of free radicals, due to their antioxidant activities. Gonçalves et al. (2013) demonstrated that dichloromethane extracts and extracts obtained by supercritical fluid from *C. brasiliense* leaves showed a significant content of the coumarin (-) - mammea A/BB, with high antioxidant activity.

Micronuclei in interphase cells result from chromosome breakage or delayed chromosomal and DNA damage; in eukaryotic organisms or individual cells, this damage is often evaluated with the comet assay (Garcia et al., 2004). The high content of (-) mammea A/BB in the dichloromethane extracts obtained from leaves of *C. brasiliense* may be effective in reducing oxidation of DNA by free radicals.

The crude extract of *C. brasiliense* leaves, with a concentration of (-) mammea A/BB at 25.97 ± 0.91 µg/mg of extract was used in two doses, that is, 100 and 200 mg/kg, to evaluate the induction of micronuclei in erythrocytes from bone marrow. This is the primary in vivo genotoxicity test recommended by regulatory agencies worldwide, as part of the safety assessment of chemicals...
chemicals and natural products. This test, when performed correctly, detects both clastogenic and aneugenic effects (Krishna and Hayashi, 2000).

The micronuclei in young erythrocytes arise mainly fromacentric fragments or chromosomes that are unable to migrate following the mitotic spindle during cell division of hematopoietic tissue (Salamone and Hedde, 1983; Ouanes et al., 2003). The increase in the frequency of MNPCE in animals treated with different compounds is an indication of induced chromosome damage (Krishna and Hayashi, 2000).

The frequency of MNPCE in groups treated with the crude extract from *C. brasiliense* was significantly lower than the frequencies observed in the positive control. Cyclophosphamide has been widely used as a positive control in the micronucleus test with rodents (Krishna and Hayashi, 2000), because of its ability to induce MNPCE. However, the decrease in frequency of MNPCE compared to the controls suggests that the crude extract from *C. brasiliense* leaves does not act as a genotoxin, and it may also contain compounds that have an antigenotoxic effect.

This study was the first to evaluate the potential for chromosomal damage of the *C. brasiliense* extract in mouse bone-marrow cells. The results indicate that the compounds present in this extract did not cause a significant increase in the number of cells with micronuclei at doses of 100 and 200 mg/kg. These doses are higher than that received by human patients using creams with antileishmanial activity (Honda et al., 2010, 2011).

**Conclusion**

The results of this study demonstrated that the extracts from *C. brasiliense* are safe, at least in terms of the potential for chromosomal damage.

**ACKNOWLEDGEMENTS**

This study was supported by the Coordenação de Capacitação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

**Conflicts of interest**

The authors declare that they have no conflicts of interest.

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The effect of light quality, temperature and substrate on seed germination and epicotyl development of *Carapa guianensis*, a multi-use neotropical tree

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Received 15 January, 2015; Accepted 8 May, 2015

The neotropical tree *Carapa guianensis* has several economic and medicinal applications, and is valued for both the high-quality oil extracted from its seeds and its mahogany-like wood. Recruitment and expansion of *C. guianensis* depend mainly on sexual reproduction. However, germination conditions for *C. guianensis* are poorly studied. This makes the understanding of its germination requirements vital for conservation and management. Laboratory studies were conducted to investigate the effects of three environmental variables (temperature, substratum and light quality) on the seed germination of *C. guianensis*. Our results showed that for seed germination, a temperature range of 30 to 40°C was the most favorable, and fertilized soil and water were more favorable than sand. Seeds in the dark germinated in higher percentages and in a shorter time. Removal of the seed coat accelerated the germination process for all conditions tested, except for green and red light, and darkness. Green and blue light retarded and red light stimulated seed germination. Plants under green light always grew taller than in the other treatments. Plants grown under blue light and in the dark showed the same profile after four weeks. Compared with sand and water, fertilized soil was the best substrate for epicotyl development. It was suggested that measurement of light quality can be used to estimate forest stand density, impact of silvicultural treatment and structural impact of anthropogenic effects on tropical forest diversity.

**Key words:** Neotropical species, seedling development, spectral light, abiotic effects, seed floatation.

**INTRODUCTION**

*Carapa guianensis* Aublet (Meliaceae) is native to Northern Brazil (Ferraz et al., 2002), with an Amazonian center of origin and subsequent diversification and migration to the Pacific coast of South and Central
America (Scotti-Saintagne et al., 2013). In Brazil, this tree is known as andiroba or mahogany (Fournier, 2003). *C. guianensis* is a sub-canopy and canopy, shade-tolerant tree inhabiting seasonally flooded forests (Ferraz, 2002; Lopez and Kursar, 2003).

Andiroba has several economic and medicinal applications, and is valued for both the high-quality oil extracted from its seeds and its mahogany-like wood (Amaral and Fierro, 2013). Andiroba trees were intensively harvested during the industrial-scale logging boom in the Amazon, from 1950s until 1990s (Fortini and Zarin, 2011).

The oil from seeds of *C. guianensis* has many uses, from illumination and soap production to a makeup base, insect repellent (Prophiro et al., 2012) and arthritis treatment (Ferraz et al., 2002; Das Graças Henriques and Penido, 2014). Andiroba-seed oil is one of the most important natural products exported from Brazil (Tonini et al., 2008). This economic potential has prompted the Brazilian government to target *C. guianensis* as one of its priority species for sustainable development (Ministry of Environment, 2009), and to include it in the National List of Medicinal Plants of Interest to the Ministry of Health (Amaral and Fierro, 2013).

Due to the wide range of commercial applications, the exploitation of *C. guianensis* is inevitable and will intensify, especially in the old-growth forests of central Amazonia, with the likelihood of reducing the species’ population and, in many cases, of degradation of the logged area. *C. guianensis* occurs naturally in high densities with a clumped distribution, which favors sustainable management (Schwartz et al., 2014), except for the wide variations in its population throughout the Amazon (Klimas et al., 2012). Klimas et al. (2012) showed that combined management of andiroba wood and seed is viable only in occasionally flooded areas, where a collection rate of 10% of the annual seed production is compatible with exploitation of 100% of individuals with diameter at breast height DBH ≥ 50 (≈ 2 trees.ha⁻¹) and a cutting cycle twice in a century. Despite this potential for multiple use, sustainable management of andiroba is not yet a reality. Seed collection and andiroba oil extraction in natural forests have been conducted by local communities with no analysis of population structure (Guarino et al., 2014). Population recruitment and expansion of *C. guianensis* depends solely on sexual reproduction. However, very little is known about the specific effect of light quality, temperature and soil type on plant physiology, on seed germination and seedling growth regulation under culture conditions. This makes the understanding of *C. guianensis* germination requirements vital for conservation and management.

In the Amazon Forest, *C. guianensis* germination can occur in water, during the flood season and in nearby rivers, or in flood-free sites (terra firme) (McHargue and Hartshorn, 1983; Scarano et al., 2003). Germination and survival of seedlings are important parameters in forest regeneration and in forestation programs. However, seed germination is a complex process controlled both by the internal properties of seeds and by an array of environmental factors including light quality, soil moisture, temperature, and soil chemistry (Tiansawat and Dalling, 2013). Temperature is an important trigger for seed germination, and most fresh-water species require light to achieve high germination rates (Xiao et al., 2010). Substrate type is also an important factor influencing seed germination and growth of aquatic plants (Jarvis and Moore, 2008).

Assuming that flotation, different bands of the radiation spectrum, different temperatures and substrates can influence physiological aspects of plants in otherwise standard culture conditions, this study evaluated the effect of alterations in the light quality, temperature and substrates on the germination and epicotyl growth of *C. guianensis*. These analyses may extend the knowledge of ecological and physiological aspects of *C. guianensis*, collaborating with conservation and management of andiroba.

**MATERIALS AND METHODS**

**Plant**

*C. guianensis* Aublet (andiroba) seeds were harvested in the city of Rio de Janeiro (Jardim Botânico do Rio de Janeiro), from under identified parent trees, with prior authorization from the Botanical Garden.

**Light experiment**

Two randomized groups of 60 seeds (three replicates each of 20 seeds/ light treatment), each seed weighing around 20 to 30 g, one group with integuments (seed coats) and the other without, were washed, soaked in water for 24 h, and then placed in 200-ml plastic bottles with equal volumes of sterile fertilized soil mixed with plant humus (Sabino), watered twice a week, and maintained in individual growth chambers (Controlled Environments). The growth chambers were equipped with Sylvania Cool 60 F20-T12-Cor fluorescent tubes, providing photosynthetically active radiation (PAR) at approximately 20 μmol.m⁻².s⁻¹. The tubes were used for the continuous light-quality experiments, to provide red light (RL), green light (GL), white light (WL) or blue light (BL) (Macedo et al., 2011). WL and continuous-darkness (D) conditions were used as control treatments to assess the effect of light on seeds and epicotyls under the same culture conditions. A 16-h Light: 8-h Dark photoperiod was used for all treatments, and the cultures were maintained at 27 ± 1°C for 70 days.

**Temperature and substratum experiment**

Three germination substrata (well-washed sand, fertilized soil, and water) were tested for their effects on the germination of *C. guianensis* seeds (Scarano et al., 2003). Other studies indicate that temperature, substrate and light can be important determinants of seed germination of aquatic plant. Their potential interactive effects necessitate a joint assessment (Yin et al., 2013). The grain size of the sand was approximately 0.1 mm and was measured with a
caliper rule. Fifty milliliters of tap water was added to the plastic bottles with sand or fertilized soil twice a week. The water in the water experiments was changed twice a week to prevent fungus contamination (Scarano et al., 2003). The germination experiments were carried out in the dark, in growth chambers with constant temperatures (20, 30 or 40°C). Each replicate contained 20 seeds/seed treatment. The seeds were placed on the three types of substratum in the same bottles described earlier. All treatments lasted 70 days and were replicated three times (Scarano et al., 2003). For all experiments were used in a completely randomized design.

Assessment of seed germination and epicotyl growth

In all assays, germination was considered as the emergence of the radicle. The effects of the different light qualities and darkness, different substrates and temperatures on the germination of andiroba were analyzed based on: (a) the amount of time between inoculation and the observation of the protrusion of the small radicle; and (b) number of germinated seeds, in each treatment, within the germination-time intervals (1 to 21, 22 to 35, 36 to 49 and 50 to 63 days). Germination percentage was calculated after 21 or 63 days of germination, by dividing the total number of germinated seeds by the total number of seeds spread and multiplying by 100. Measurements are means of three replicates, with error given as SE of the mean. Epicotyl growth (n=60/treatment) was assessed weekly, for one month, by measuring the epicotyl elongation with a caliper ruler (Scarano et al., 2003).

Statistics

The statistical tests were conducted with the Statistica for Windows software (StatSoft, Inc. 2005, STATISTICA - data analysis software system, version 7.1). The results for each treatment are shown as the mean and standard error (SE) of three independent experiments. The data were analyzed by one-way analysis of variance (ANOVA). The assumptions of ANOVA were fulfilled in all cases. The statistical significance of differences among groups was evaluated by analyses of variance, followed by a multiple-comparison Tukey's test. P values lower than 0.05 were considered significant.

RESULTS

Light-quality and dark effects

For C. guianensis, the absence of light promoted germination, compared to the other light treatments, in terms of both the number of germinated seeds and the speed of germination (Table 1). Of all the seeds sown in the dark, over half, 52.5% germinated, and of those that germinated, 62.5% had germinated by day 21 (Table 1). All other treatments resulted in significantly lower and slower germination rates (Table 1).

Of the spectrum bands tested, green and blue light inhibited and red light stimulated germination of the andiroba seeds, compared to white light (Table 1). Of the 60 seeds sown under red light, 46.7% germinated by the end of the experiment. However, under blue and green light, only about 38% of the seeds germinated by day 60 (Table 1). The results with blue and green lights were statistically similar to the control (WL). However, seeds under green light showed a significantly faster germination rate (47.8% by day 21) than seeds under blue and red lights (Table 1).

More seeds with a tegument, as opposed to those without it, germinated in the dark, under red light or under green light. The opposite was observed for white light, where 66.5% of the seeds without a tegument germinated, and only 37.5% of those with a tegument did so (Table 1). Under blue light, the presence or lack of a tegument did not affect the germination rate significantly, which was 50% in both cases (Table 1).

For majority of the treatments, the seeds without a tegument germinated faster (Figure 1). Under blue or red light or in the dark, most seeds without a tegument germinated by the 35th day of cultivation. Seeds with a tegument were still germinating up to the 63rd day, especially for blue light and in the dark (Figure 1). However, the results for white and green light showed that these spectral bands had different effects than the other treatments. The seeds with a tegument germinated faster under GL, and seeds under WL germinated at the same rate with or without a tegument (Figure 1).

After germination, epicotyl growth was measured weekly for four weeks. All spectral bands stimulated growth after 30 days, because all the results exceeded the response under the positive control, white light (Figure 2). During the four weeks, the epicotyls of plants grown under green light were always taller than in the other treatments (Figure 2). However, at 30 days of epicotyl development, green and red light induced statistically similar results, with a mean height of around 33 cm (Figure 2). Plants grown under red light showed accelerated growth, exceeding those grown under white and blue light after two weeks and in the dark after three weeks (Figure 2). Plants grown under blue light and in the dark showed the same profile during four weeks, and the growth rate stabilized in the fourth week (Figure 2). Under white light, epicotyls grew faster in the first two weeks, and slowed during the second and third weeks. After the third week, plants under white light resumed their previous growth rate (Figure 2).

Temperature and substratum effects

Temperature had a significant effect on seed germination of C. guianensis (Table 2). Temperatures of 30 and 40°C produced the highest germination percentages, mainly in the fertilized-soil and water treatments (Table 2). In contrast, seed germination percentage was significantly lower at 20°C in all substrates assayed (Table 2). The substratum also had a significant effect on seed germination (Table 2). The germination percentages in water and fertilized soil were significantly higher than in sand, under all temperatures, except at 20°C (Table 2). At the same time, there were no significant differences between sand and water at 30 and 40°C.
Germination frequency in the different periods was also significantly affected by substrate and temperature conditions (Figure 3). In all treatments, the same profile of seed germination was observed (Figure 3). Majority of the seeds germinated in the first 21 days of culture and the germination rate then progressively declined (Figure 3). The exception was the 20°C-water assay, where majority of the seeds germinated at the end of the experiment (Figure 3). At 20°C, in water, the seeds tended to germinate later (Figure 3). In the other temperature treatments and compared to the other substrates assayed, for seeds in water, the germination rate did not decline as rapidly on succeeding days of cultivation, particularly at 40°C.

After germination, the epicotyl growth was measured weekly for four weeks. At 20 and 30°C, in the three substrates assayed, the epicotyl reached similar heights, between 54 and 68 cm, and showed no significant difference in final height in each temperature (Figures 4 and 5). However, epicotyl growth at 20 and 30°C in water was slightly slower than in the other substrates (Figures 4 and 5). At 40°C, a different epicotyl growth profile was observed (Figure 6). The epicotyls grew best on fertilized soil, reaching 58 cm, followed by sand (30 cm) and water (29 cm) (Figure 6). As in the other temperature experiments and also at 40°C, the epicotyls showed the slowest growth rate in water (Figure 6).

### DISCUSSION

**Light-quality and dark effects**

The absence of light is the ideal condition for germination of *C. guianensis* seeds, but not for epicotyl growth (Table 1 and Figure 2). Light is an important environmental signal triggering germination or promoting dormancy, depending on the species. The results observed on Table 1 and Figure 2 diverge from other studies on others species such as andiroba, with large reserves to sustain prolonged periods of sapling growth in the dark (e.g., underground), do not require light to germinate (Milberg et al., 2000).

Our results showed that *C. guianensis* seeds germinated in all light conditions tested (Table 1). Our finding concords with Tiansawat and Dalling (2013) review. In their recent review, it was noted that in tropical environments, large seeds are independent of light for germination but are affected by the diurnal temperature on *C. guianensis*. *C. guianensis* is a partially shade-tolerant (PST) species, and its germination and seedling survival can be affected by the amount of light.

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**Table 1. Percentages of seed germination under different light treatments.**

<table>
<thead>
<tr>
<th>Light treatment</th>
<th>Total seed germination to day 63 (%)</th>
<th>Total seed germination to day 21, including only germinated seeds (%)</th>
<th>Total seed with tegument, germination to day 63, including only germinated seeds (%)</th>
<th>Total seed without tegument, germination to day 63, including only germinated seeds (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White light</td>
<td>38.3 ± 1.7c</td>
<td>39.3 ± 2.7c</td>
<td>37.5 ± 2.3d</td>
<td>66.50 ± 1.0a</td>
</tr>
<tr>
<td>Dark</td>
<td>52.5 ± 1.4a</td>
<td>62.5 ± 1.7a</td>
<td>57.5 ± 0.9b</td>
<td>42.5 ± 0.8c</td>
</tr>
<tr>
<td>Blue light</td>
<td>38.4 ± 1.6c</td>
<td>25.1 ± 1.1d</td>
<td>50 ± 2.5f</td>
<td>50 ± 7.5b</td>
</tr>
<tr>
<td>Red light</td>
<td>46.7 ± 1.3b</td>
<td>31.2 ± 2.2e</td>
<td>55 ± 1.0b</td>
<td>45 ± 0.5f</td>
</tr>
<tr>
<td>Green light</td>
<td>35.0 ± 2.5c</td>
<td>47.8 ± 2.5b</td>
<td>52.9 ± 0.8h</td>
<td>47.1 ± 1.2c</td>
</tr>
</tbody>
</table>

Mean ± standard error for three groups of 20 explants each replicate. Mean values with the same letter are not significantly different, based on ANOVA followed by Tukey’s test at P<0.05.

**Table 2. Germination percentages of *Carapa guianensis* seeds in three different substrates and three different temperatures.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Temperature</th>
<th>20°C</th>
<th>30°C</th>
<th>40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>18.33±3.28866d</td>
<td>80.4533 ±18.1368b</td>
<td>68.4200a</td>
<td></td>
</tr>
<tr>
<td>Fertilized soil</td>
<td>10.00d</td>
<td>90.00±10.00a</td>
<td>100a</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>41.6666±2.8867c</td>
<td>95.00a</td>
<td>90.00a</td>
<td></td>
</tr>
</tbody>
</table>

Mean standard error for three groups of 20 explants each replicate. Mean values with the same letter are not significantly different, based on ANOVA followed by Tukey's test at P<0.05.
Figure 1. Frequency of germination at different periods of time. Values are the germination results after four different periods of time (days): 1-21 days (open bars); 22-35 days (bars with diagonal lines); 36-49 days (closed bars) and 50-63 days (bars with parallel lines). Germinability (mean ± standard error) of Carapa guianensis Aublet seeds under different continuous light treatments, at 27 ± 1°C with a 16-h Light: 8-h Dark photoperiod. WL: White light, seeds without tegument; WLt: white light, seeds with tegument; D: dark, seeds without tegument; Dt: dark, seeds with tegument; BL: blue light, seeds without tegument; BLt: blue light, seeds with tegument; RL: red light, seeds without tegument; RLt: red light, seeds with tegument; GL: green light, seeds without tegument; GLt: green light, seeds with tegument. Measurements are means of three replicates, with error bars representing standard error of the mean. Mean values with the same letter are not significantly different, based on ANOVA followed by Tukey’s test at P ≤ 0.05.

penetration (Schwartz et al., 2012), and probably also the light quality. The same authors suggested that light is probably not the best indicator of habitat quality for large seeds, since the seedlings can emerge successfully from below the depth to which light penetrates into the soil, or from beneath layers of surface leaf litter.

The quality and quantity of solar radiation are crucial for growth and competition in forest ecosystems. The spectral waveband between 350 and 800 nm, termed morphogenetically active radiation (MAR) (Combes et al., 2000), triggers or inhibits processes such as seed germination, stem growth, leaf expansion and orientation, flowering, and dormancy (Capers and Chazdon, 2004). Spectral ratios such as red/far red (R/FR) and blue/red (B/R) give important information about the light quality within stands (Hertel et al., 2011).

In our study, green and blue lights had no effect, and red light increased the germination rate of andiroba seeds (Table 1). These responses probably occurred because andiroba has evolved specific strategies adapted to germinate in the dense Amazon Forest, beneath the canopy or buried by rodents, while on terra firme. These shaded conditions are more influenced by red than by green or blue light. Gkika et al. (2014) also observed that the final percentage of seed germination was higher under red-light conditions for Erysimum naxense and Erysimum krendlii, compared with four other light regimes tested. Other studies have recorded similar results. Rattan and Tomar (2013) observed that green light, and to a lesser degree blue light, negatively affected negatively seed germination of Hippophae salicifolia, a deciduous tree; red light produced the highest germination
Seeds subjected to blue light had higher dormancy than those under other light spectra (Figure 1 and Table 1). In the Amazon rainforest, seeds can also fall into the river, and not in the soil, on terra firme. In this environment, blue light predominates due to the more-open environment. This may be the reason that the seeds take longer to germinate under blue light, to await anchorage in the soil or the arrival of the dry season. Although Scarano et al. (2003) argued that seed dormancy can increase under floating; the quality of light can be an important factor triggering germination.

Similarly to the conclusions of Barrero et al. (2012), working with Brachypodium genotypes, on the soil surface a predominantly blue light would inhibit germination. After the first few millimeters, where blue light disappears, seeds would find an appropriate germination environment, as the ratio of red to far-red would be high. Finally, deeper in the soil, the ratio of red to far-red would be pushed toward far-red, again inhibiting germination. Previous studies showed that C. guianensis decreased in density after logging, probably affected by light incidence, and that additional silvicultural treatments could be useful to increase the density (Schwartz et al., 2012).

Our results concerning the effect of darkness on germination also agree with those of Arias-Le Claire (2001), who reported data showing that burial of C. guianensis seeds by rodents, under natural conditions, increases germination. Thus, these data suggest that the successful regeneration of C. guianensis during the dry season, in both flooded and non-flooded forests, depends on the preservation of these rodent populations (Ferraz et al., 2002; Nyiramana et al., 2011). In another study, seed-coat fragments of C. grandiflora as well as cotyledons with rodent tooth marks were observed in the majority of experimental sets (Nyiramana et al., 2011). This could explain why seeds without a tegument germinated faster and in larger numbers in the dark (Figure 1 and Table 1).

According to the seed classification presented by Takaki (2001), C. guianensis seems to be insensitive to light. Therefore, C. guianensis germination must be controlled by phytochrome A (phyA) through a very-low-fluence response (VLFR). This type of response does not display the classical red/long red reversibility that is a typical survival mechanism of small-seed plants. Through this mechanism, seeds will only germinate in optimal light conditions, with subsequent successful development and growth of the sapling (Milberg et al., 2000).

Very-low-fluence responses, can also be induced by weak green light (Takaki, 2001), as seems to be the case with andiroba. However, green and blue light retarded and red light stimulated germination of the andiroba seeds.

Figure 2. Epicotyl growth (epicotyl heights) with time (four weeks) of C. guianensis under different light qualities and in the dark. Values are means (n=60) ± standard error. Significant difference between treatments at P ≤ 0.05 is denoted by different letters. WL: White light; D: dark; BL: blue light; RL: red light; GL: green light.
(Table 1). Previous studies have indicated that the active form of the phytochrome may increase the embryo's growth potential and promote the activity of degrading enzymes, thus reducing the mechanical resistance of tissues near the micropyle region (De Miguel et al., 2000). The inhibitory effect of blue light on germination could be due to the low levels of red radiation, resulting in failure to activate the phytochrome (Dissanayake et al., 2010).

More seeds with a tegument than without it, germinated in the dark, under red light or green light; the opposite was observed for white light (Table 1). These data agree with those of Ferraz et al. (2002) for andiroba seeds under white light.

Under our conditions, the presence of a tegument, and dark, red-and blue-light treatments were dormancy-promoting factors in the \textit{C. guianensis} seeds, compared to the other experiments (Figure 1). Under these conditions, germination occurred up to day 63. In contrast, under green light, seed dormancy was stronger in the absence of a tegument (Figure 1). Seed dormancy was always weaker if seeds were under white light, with or without a tegument, compared to all other experimental conditions (Figure 1). Our observations of weaker dormancy expression under white light contrast with previous reports for grasses such as the \textit{Brachypodium} genotypes wild oats, barley and wheat (Barrero et al., 2012), thus indicating that the seed size and light conditions of the environment where the plant has developed are important features affecting seed responses to light quality.

Some authors have reported that when tegument integrity is lost, the control of seed germination changes, with the activation of phy A or phy B. These two phytochromes are evolutionarily different and have distinct effects (Takaki and Gama, 1998). Our data for a shorter germination time in seeds without a tegument (Figure 1) agree with those of Suda and Pereira (1997) and Oliveira et al. (2003). Suda and Pereira (1997) suggested that the germination-inducing mechanism is activated faster in the absence of a tegument, independently of the light wavelength. This would pro-
probably occur due to an increased supply of respiratory gases and water to the embryo, to a change in light and temperature sensitivities, or to the elimination of inhibitory substances (Bewley and Black, 1994). A study of guayule seed germination provided evidence of an inhibitory effect of the seed coat, because removing the seed coat significantly increased germination (Dissanayake et al., 2010). However, this is relevant to our work only for germination velocity or dormancy break (Figure 1), but not for germination rate (Table 1), where the presence of a tegument increases the number of germinating seeds.

When the epicotyls emerge, green and red light stimulate growth in the same manner (Figure 2). In shade, under the canopy, green and yellow-green bands prevail due to the reflection of light and transmission through leaves (Théry, 2001). This “forest” spectrum is most similar to the green and red lamps used in our experiments. Epicotyls grown under blue light were repressed (Figure 2). Under the vegetation cover, “the blue part of the spectrum, present in the canopy, disappears as the distance from the canopy increases (Théry, 2001). It is logical to assume that blue light repressed the growth of the andiroba epicotyls.

In agreement with Théry (2001), it was suggested that light-quality measurement can be used to estimate the structural impact of forest exploitation, and this will provide the information necessary for a functional explanation of anthropogenic effects on tropical-forest diversity. Studies of the effects of human disturbance on forest light and species diversity show that it is crucial to investigate the use, distribution and importance of light environments in conservation projects.

**Temperature and substratum effects**

In this present study, the germination rate of *C. guianensis* seeds was higher at 30 and 40°C (Table 2), in contrast to the ideal germination temperatures of 20 to 30°C found for other Brazilian species (Kissman and Scalon, 2011). However, some tropical-forest species such as *Acacia catechu*, *Acacia nilotica*, *Albizia lebbek*, *Dalbergia sisso* and *Tectona grandis* show a similar trend of a lower germination rate at 20°C and higher germination
Figure 5. Epicotyl growth (epicotyl heights) with time (four weeks) of *C. guianensis* at 30°C on three different substrates. Values are means (*n* = 60) ± SE. Significant difference between treatments at *P* ≤ 0.05 is denoted by different letters.

The assay of epicotyl growth resulted in the same...
Figure 6. Epicotyl growth (epicotyl heights) with time (four weeks) of *C. guianensis* at 40°C on three different substrates. Values are means \((n = 60) \pm SE.\) Significant difference between treatments at \(P \leq 0.05\) is denoted by different letters.

Findings as for germination speed. Temperature increase affected the growth response. Epicotyl growth was higher in fertilized soil than in water, although not significantly so (Figures 4 and 5). Epicotyl growth was significantly higher in fertilized soil compared to water and sand, only at 40°C (Figure 6). Therefore, for epicotyl growth, the effects of water and fertilized soil were similar, but temperature had a promoting effect. These findings contrast with those of Scarano (2003), where *C. guianensis* seedling growth was delayed in water compared to vermiculite, at 20 to 30°C. Scarano (2003) reported that epicotyl growth was more accentuated in the hard substrate than in water, possibly because of nutrients in the substrate. Seeds germinate and plantlets start to develop in water because of the nutrients provided by the endosperm (Manasse, 1990). Our results suggested that water availability and drainage are more important for epicotyl growth, but only in higher temperatures.

In sand, the germination rate was lower than in the other substrates, mainly at 20°C (Table 2), probably due to the poor water environment. This species requires appropriate moisture conditions because of its thick seed coat and large size (Khera and Singh, 2005). In sand, water drains rapidly, which prevents ideal moisture conditions for germination.

This study provided important information about the best conditions for appropriate development of *C. guianensis* seeds and establishment of plantlets. Conditions similar to the Amazon habitat, including high temperatures and moist soil, are ideal. However, low humidity and temperatures will result in low germination rates. In addition, we suggest that light-quality measurement be used to estimate the structural impact of forest exploitation, providing information for a functional explanation of anthropogenic effects on tropical-forest diversity, since germination and epicotyl growth are influenced by different light qualities.

**ACKNOWLEDGEMENTS**

The authors are indebted to the Universidade Federal do
Estado do Rio de Janeiro (UNIRIO) and to the Instituto de Pesquisas, Jardim Botânico do Rio de Janeiro. This article is part of the dissertation of the Post Graduate Program in Biological Sciences (Neotropical Biodiversity) from UNIRIO.

Conflict of interests

The authors declare that they have no conflict of interests.

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