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Full Length Research Paper

Evaluating Growth Regulator Effects on Two Differently Cultured Explants of Carapa guianensis in vitro

Larisse Lobo de Oliveira[1], Anaíze Borges Henriques[2], Andrea Furtado Macedo[1,3]*

[1]Laboratório Integrado de Biologia Vegetal, Departamento de Botânica, Centro de Ciências Biológicas e da Saúde, Universidade Federal do Estado do Rio de Janeiro, Brazil.
[2]Laboratório de Fisiologia do Desenvolvimento Vegetal, Departamento de Botânica, Centro de Ciências e da Saúde, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Brazil.
[3]Centro de Inovação em Espectrometria de Massas do Laboratório de Bioquímica de Proteínas, Centro de Ciências Biológicas e da Saúde, Universidade Federal do Estado do Rio de Janeiro, Brazil. *Corresponding author. E-mail: andreafm22@yahoo.com.br Tel: +55 21 2530-2551.

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***Carapa guianensis* Aubl. (Meliaceae), known locally as andiroba, is a multi-use species from Amazonia. Andiroba oil is considered an important natural product in the Brazilian market, and international demand is increasing due to its cosmetic and pharmaceutical potential. *C. guianensis* trees produce seed irregularly over different harvest periods, leading to inconsistent oil production and difficulties with supply. No management plans or protocols have been developed for *in vitro* or clonal production of *Carapa* seedlings and the maintenance of genetic resources. The objective of this study was to assess the effect of growth regulators on explants (young leaves, old leaves and apical buds). Explants consisting of leaf segments 1 cm on a side were cultivated in MS medium with and without growth regulators. Evaluation was based on fresh and dry weight of the explants after 20 days. In the media with 2,4-dichlorophenoxyacetic acid (5, 15, 35 or 45 µM), changes were observed in weight and explant appearance (callus). Bud breakage and development of shoots were achieved using 5 µM of 6-benzylaminopurine. Overall, the results showed that 2,4-dichlorophenoxyacetic acid stimulates callus formation on andiroba foliar explants, while 6-benzylaminopurine was superior to thidiazuron for the initial development of shoots.**

Key words: Growth regulators, *Carapa guianensis*, *in vitro*, tissue culture, organogenesis.

INTRODUCTION

Carapa guianensis Aubl. (Melicaceae), commonly known as andiroba, is a neotropical tree distributed throughout South and Central America, as well as the Caribbean Islands (Cloutier et al., 2007). It is a multi-use species, the main product being seed oil used for medicinal purposes due to its significant limonoid content (Mendonça and Ferraz, 2007; Henriques and Penido, 2014). The bio-oils obtained from *C. guiannesis*

seed have physical and chemical properties that make them acceptable renewable diesel fuels (Iha et al., 2014). Andiroba oil is used as an insect repellent (Freire et al., 2006) and in the manufacture of cosmetics, due to its high level of unsaturated triacylglycerols (TAG) (Cabral et al., 2013). Additionally, its wood is valued for the construction of buildings and furniture (Guariguata et al., 2002), and cultivated andiroba trees have the potential to recover degraded land.

The exploitation of *C. guianensis* is inevitable and is intensifying, mainly in central Amazonia where seed extraction leads to population reduction, as seed dispersal is the main reproductive mode. Evidence indicates that the size of felled logs has been decreasing for decades (Fortini and Zarin, 2011).

Andiroba trees produce seed irregularly over different harvest periods (Tonini et al., 2008). This variability, which does not allow continuous oil production, generates management difficulties, resulting in periods with low seed and oil production (Frankie et al., 1974; McHargue and Hartshorn, 1983). Industries that use *C. guianensis* need a constant source of homogeneous raw plant material.

Plant regeneration by tissue culture, through either organogenesis or somatic embryogenesis, is a prerequisite for potential clone propagation, genetic transformation and *in vitro* preservation for germplasm from timber trees, including andiroba (Handley, 1995; Park et al., 1998; Minocha and Jain, 2000). Clone propagation in aseptic conditions is an alternative method of propagation for some medicinal plant species with large-scale production issues, accelerating the conventional propagation process and producing genetically identical plants (Zhou and Wu, 2006).

This study evaluated the effects of growth regulators on *C. guianensis* foliar explants and apical buds, with a view toward shoot induction and providing guidelines for optimizing andiroba cultivation.

MATERIALS AND METHODS

Plant

Seeds of *C. guianensis* Aublet (andiroba) were collected in the city of Rio de Janeiro (Jardim Botânico do Rio de Janeiro) beneath identified parent trees, with previous authorization from the institution. Seeds, weighing around 20 to 30 g were washed, soaked in water for 24 h, placed in 200 ml plastic bottles with equal volumes of sterile soil fertilized with plant humus, and watered twice a week. Three kinds of explants were used: (a) young leaf explants (less than 1 week old), when they were pink-colored; (b) old leaf explants, when each leaf was at most 2 weeks old and green; and (c) shoot apical buds. The younger leaves for explants were simply cut into three parts (apex, middle and base). Leaf fragments (1 cm^2) were obtained from the older green leaves. Apical buds were collected from the same seedlings when the apical segment was still green and soft.

Surface sterilization of explants

The surfaces of the explants from young and old leaves were sterilized with a 50% (v/v) commercial bleach solution for 1.5 min and then washed three times for 1 min each in sterile distilled water. Surface-sterilized explants were placed with their adaxial or abaxial surfaces firmly in contact with the medium in culture flasks.

The apical bud explants were surface-sterilized with a 50% (v/v) commercial bleach solution, for 2 min, followed by a quick dip in 70% (v/v) ethanol solution, and then washed 3 times with sterile distilled water. The exposed ends of the explant were trimmed aseptically and then inoculated on the medium.

Tissue culture

To assay the effectiveness of growth regulators (GR), surface-sterilized leaf explants were inoculated on sterile MS medium (Murashige and Skoog, 1962). The medium was supplemented with 30 g/L sucrose, 7 g/L agar and vitamins, with or without growth regulators (MS 0) (Macedo et al., 1999).

To determine if foliar explants would respond to medium supplemented with one GR at a time, the following GRs were used: indoleacetic acid (IAA) (1, 5, 15, 35 or 45 µM), 6-benzylaminopurine (BA) (1, 5, 15, 35 or 45 µM), 2,4-dichlorophenoxyacetic acid (2,4-D) (1, 5, 15, 35 or 45 µM), and thidiazuron (TDZ) (0.5, 1 or 5 µM) (Figure 1).

Combinations of BA and IAA (1 + 1; 1 + 5 or 5 + 5 µM) were also tested. The plant material was observed for 2 months. For each treatment, 12 explants were used, and the experiments were repeated three times. The explants were placed on the medium with either the abaxial or the adaxial surface turned up. In order to determine if callus obtained from foliar explants would undergo indirect organogenesis, callus explants were subcultivated on control, IAA, BA and TDZ media. Then, calli developed on the lowest and the highest (5 and 45 µM) 2,4-D medium concentrations were subcultivated (Figure 1). Explants cultured for 4 weeks in 2,4-D supplemented medium with callus formation were transferred to fresh MS 0 medium or to medium supplemented with IAA (1 or 5 µM), BA (1 or 5 µM), 2,4-D (1 or 5 µM) or TDZ (0.5, 1 or 5 µM). Explants alive after 4 weeks were subcultivated on MS 0 supplemented with 3 g/L charcoal (Figure 1).

For bud growth experiments, the explants were inoculated in MS medium with IAA, BA, TDZ (1 or 5 µM) or no GR, and with or without 3 g/L charcoal (Figure 1). After 4 weeks, the bud explants were transferred to MS medium supplemented with cytokinins and auxins, in an attempt to achieve organogenesis: IAA (0.5, 1 or 5 µM), BA (0.5, 1 or 5 µM), TDZ (0.5, 1 or 5 µM) and combinations of IAA and BA (1 + 5 µM; 5 + 1 µM; 1 + 1 µM) (Fig. 1). Assays performed on a small number of samples with higher concentrations of IAA and BA (15, 35 or 45 µM) and a combination of IAA and BA (5 + 5 µM) produced brown and dry explants. Therefore, these GR combinations were not tested for bud explants.

All experiments were performed in a climate-controlled room equipped with white fluorescent lamps (Osram F20T12/CW) (approximately 20 µmol m^{-2} s^{-1} photosynthetically active radiation, PAR). For all treatments, a 16-h photoperiod was used. Cultures were maintained at 25±1°C.

Assessment of leaf explant development

After the culture periods detailed above, the effect of each treatment was evaluated by dry and fresh explant mass. For dry weight, the leaf explants were individually oven-dried in aluminum

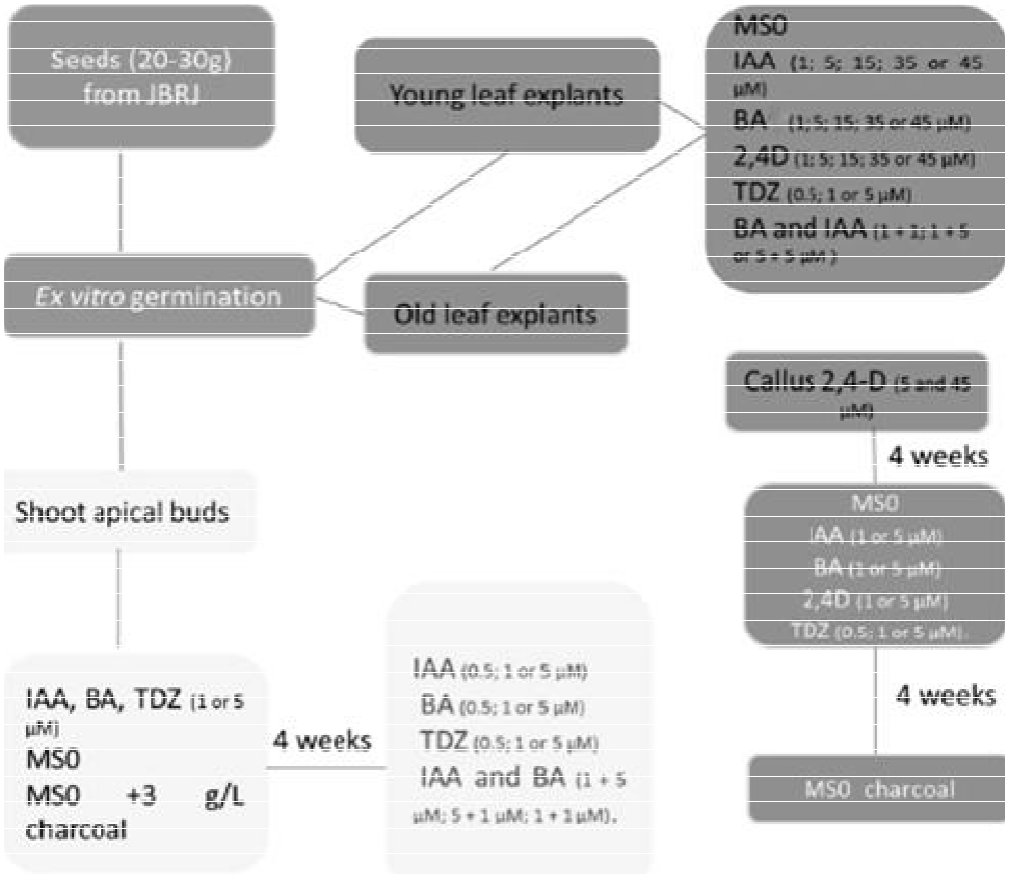

Figure 1. sequence of assays

vessels at 40°C to constant mass and then weighed. To measure fresh weight, the material was removed from the culture flasks and immediately weighed to prevent dehydration.

Assessment of apical bud explants development

After the culture period, the height of explants and leaf length were measured weekly with a ruler. Fresh weight was determined immediately after each explant was removed from the culture flask after 8 weeks of culture; the dry weight was determined after the explant was oven-dried at 40°C to constant mass.

Statistics

The results were analyzed by Analysis of Variance (ANOVA), followed by Tukey's test with a significance level set at $\alpha = 0.05$, using Statistica 7 software for Windows. Means ± standard error (SE) are presented.

RESULTS

A method for organogenesis, either direct or indirect, was developed. Indirect organogenesis involves the production of organs by callus stage, whilst direct organogenesis is related to the formation of organs directly on the surface of cultured intact explants (Us-Camas et al., 2014). The purpose of this study was to produce *in vitro* shoots as an alternative to sexual propagation for *C. guianensis*. Sexual propagation is limited by the tendency of andiroba seeds to lose their power of germination soon after harvest, as a result of dehydration. Micropropagation of selected phenotypes of *C. guianensis* is also desirable since propagation by seed yields high levels of genetic variability, a limiting factor for its commercial use.

Old leaf explants

No callus formation was observed from old leaf-tissue explants cultured in media supplemented with IAA, BA, TDZ or IAA-BA combinations (Figure 2). However, friable whitish callus was formed in all explants cultured in 2,4-D media, except the medium supplemented with 1 µM 2,4-

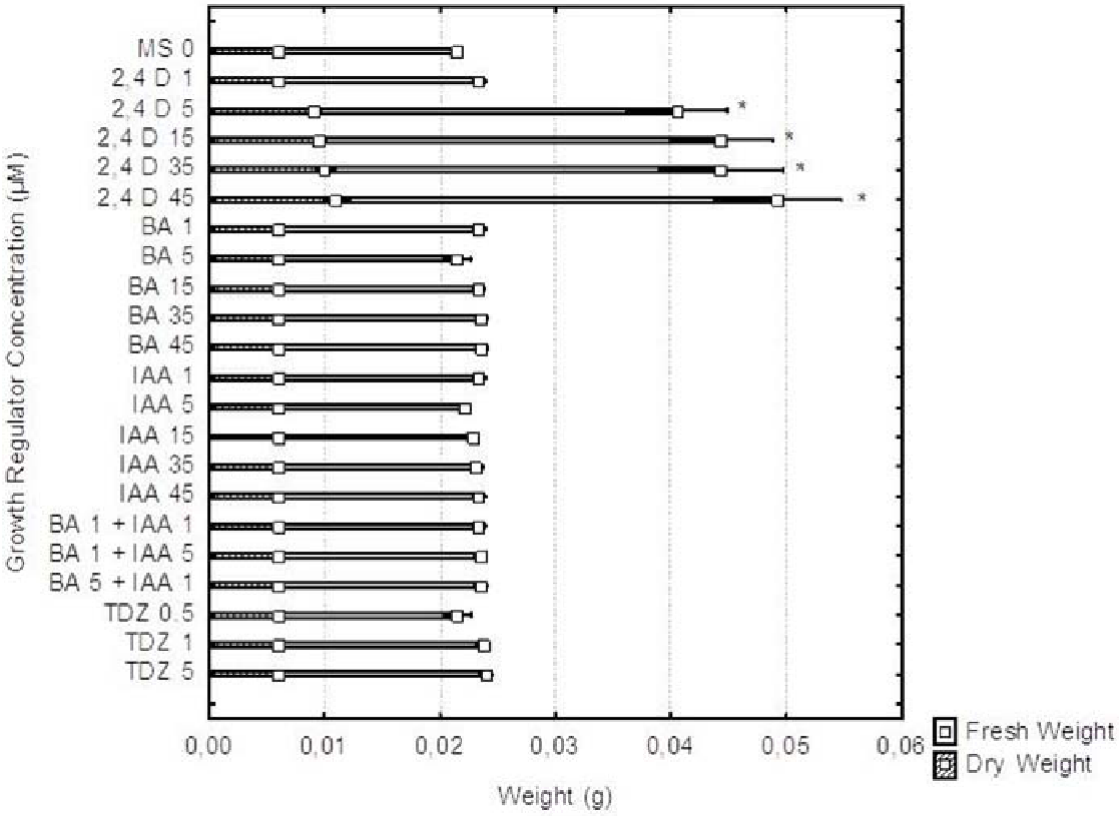

Figure 2. Fresh and dry weight of explants from old leaves of *Carapa guianensis* cultured.

D (Figures 2 and 3). After 4 weeks of observation, a significant difference, according to Tukey's test, in explant fresh and dry weight was observed when comparing explants cultured in 2,4-D supplemented medium with others cultured in MS 0 or with IAA, BA, TDZ or IAA-BA supplemented medium (Figure 2). No difference was observed between the orientations of explants (abaxial or adaxial surface turned up) in differently supplemented MS medium or MS 0 for 4 weeks.

After calluses were obtained on 2,4-D supplemented medium, the effects of different subculture medium conditions on callus development were investigated (Figure 1). Therefore, calluses obtained from old leaf explants, after 4 weeks of culture in 2,4- D medium (5 or 45 µM of 2,4-D), were transferred to fresh medium. Calluses obtained with 5 µM of 2,4-D and then transferred to medium with 1 or 5 µM of 2,4-D or 0.5, 1 or 5 µM of TDZ survived and showed increase in callus mass (Figures 4 and 5.). These calli acquired a brighter green color (Figure 5). The calli that were subcultivated on MS 0 or MS supplemented with IAA or BA did not survive after 4 weeks. These explants turned brown and became dry. Calli subcultivated on 2,4-D and TDZ that showed no or very few signs of brown parts after 4 weeks were transferred to MS 0 supplemented with 3 g/L of charcoal (Figure 1). Only a few calli (30%) that came from 1 µM 2,4-D produced very small thin roots after 4 weeks (Figure 6). The calli that came from TDZ in all concentrations merely maintained their green color.

Callus obtained with 45 µM of 2,4-D and then transferred to medium with 1 or 5 µM of 2,4-D or 0.5, 1 or 5 µM of TDZ also showed increases in callus mass (Figure 7). The calli that were transferred to MS 0 or MS supplemented with IAA or BA did not survive after 4 weeks. These explants turned brown and became dry, as shown subsequently. Rhizogenesis was not observed in any calli that were first subjected to 2,4-D 45 µM supplemented medium and then transferred to MS 0 supplemented with charcoal, as observed with the callus from 2,4-D. They merely maintained the green callus mass.

Young leaf explants

Young, pink-colored leaf explants were tested to compare the results obtained using old leaf explants, and to

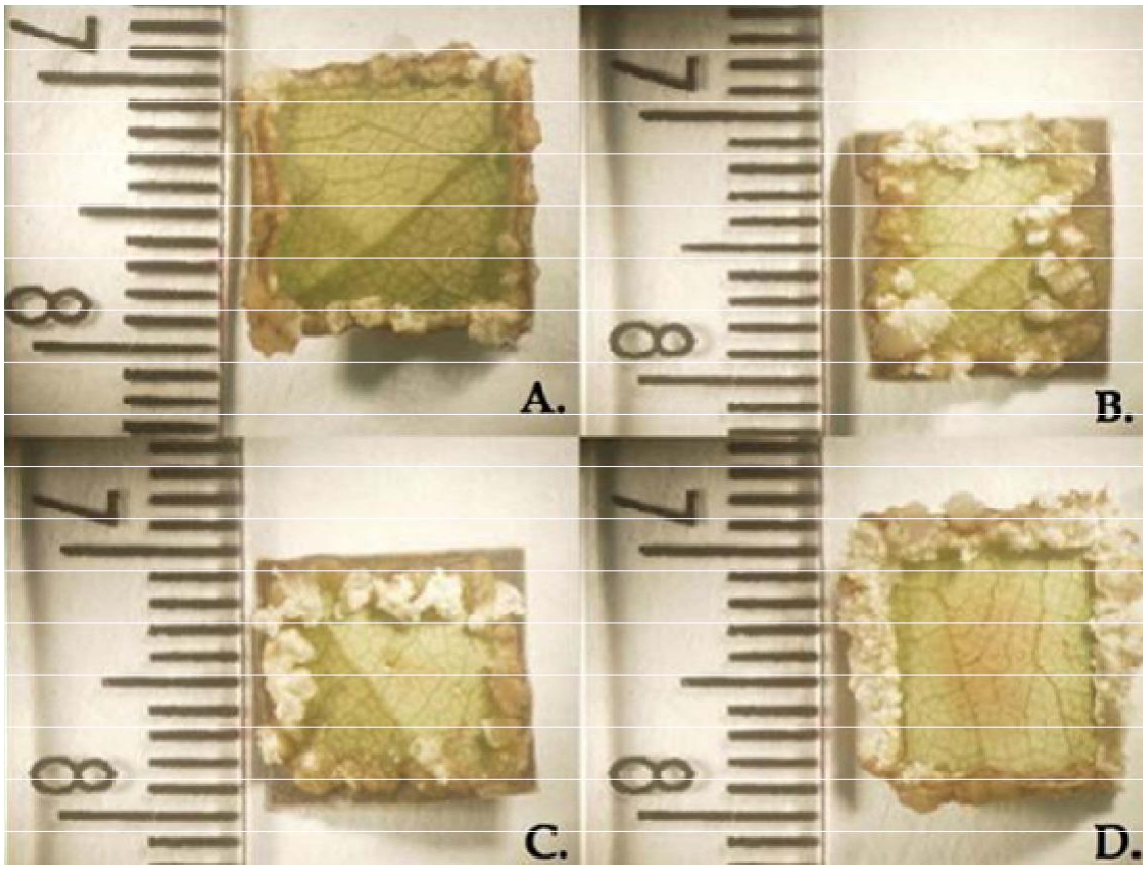

Figure. 3 Four- week-old old leaf explants of *Carapa guianensis* on medium with differe nt concentrations of 2,4-D. (A) 5 µM; (B) 15 µM; (C) 35 µM; (D) 45 µM

determine if they would respond to GR better than older leaves from the same plants (Figure 1). However, these explants did not survive more than 4 weeks on any culture medium. After the first week of incubation in all media, explants turned from pink, to a pale yellow to green, and then to brown. There was no difference in response among the three parts of the leaf (apex, middle and base).

Apical shoot bud explants

Apical bud explants were cultured in MS 0 and with cytokinins and auxins to check their development (Figure 1). Bud breakage was 100% successful only in MS 0 supplemented with charcoal, but shoots did not grow longer than 1 cm. A mean of 3 to 4 small shoots were obtained per bud explant (Figure 8). On the other media, no bud explant development was observed.

Shoots developed only on BA supplemented medium (Figure 8), after the initial growth on MS 0. On the other GR supplemented media, the explants did not develop and the shoots maintained the size that they had reached on the first medium (MS 0). No signs of rooting were observed. When the medium was not supplemented with 3 g/L of charcoal, all the explants turned brown and died. The plantlets reached 2.55 ± 0.36 cm in height (mean ± standard error) at the end of 2 months. The leaves reached 3.66 ± 0.38 cm in length (mean ± standard error).

DISCUSSION

The present results for callus culture contrast with those obtained by Da Costa Nunes et al. (2002) and Rocha and Quoirin (2004). Using a cotyledonary node culture, Da Costa Nunes et al. (2002) found that callus formation in *Cedrela fissilis* Vell., a woody species of Meliaceae, occurred with the growth regulators naphthalene acetic acid (NAA) and BA. These authors obtained the largest increase of fresh weight in treatments with combinations

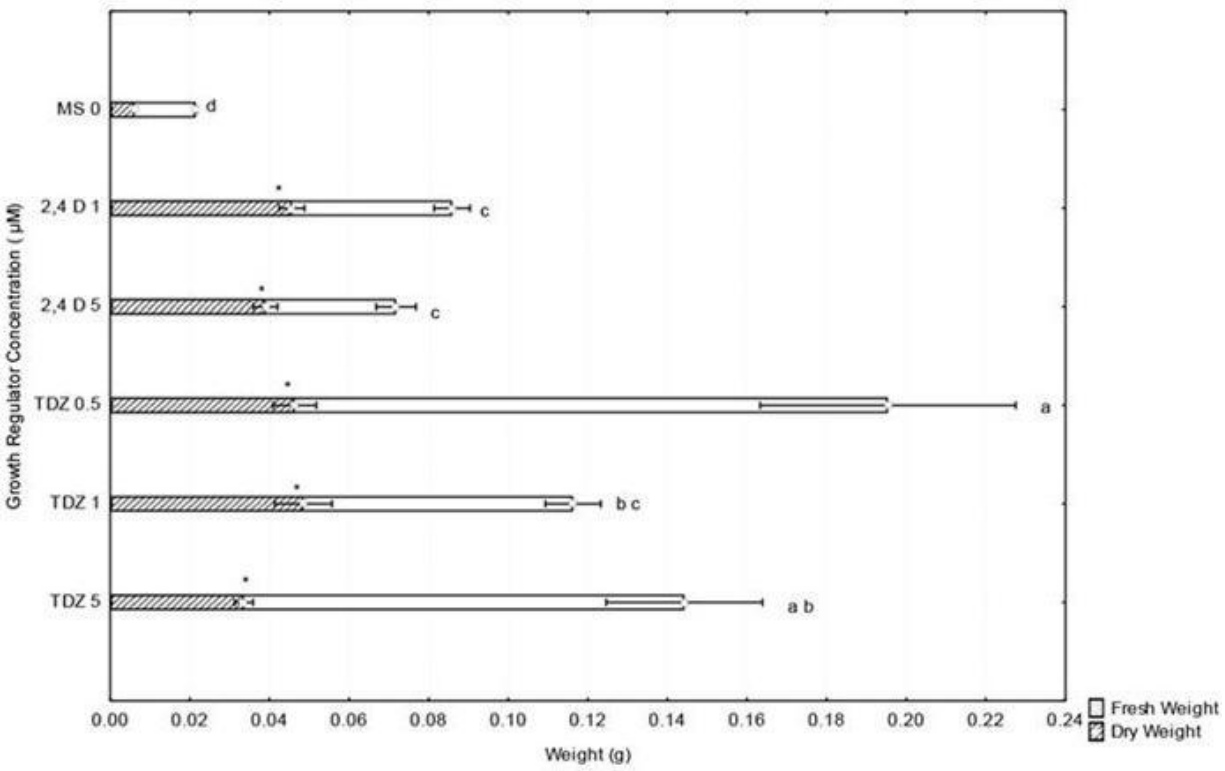

Figure. 4 Fresh and dry weight of *Carapa guianensis* callus obtained in 5 µM of 2,4-D supplemented medium and subcultivated on MS0; 1 or 5 µM of 2,4-D or 0.5, 1 or 5 µM of TDZ supplemented medium. Data represent mean values per treatment, and bars indicate SE of 36 explants/medium. Mean values with the same letter or * are not significantly different based on ANOVA followed by Tukey's test at $P \leq 0.05$.

of 6-BA at 1.25, 2.5 and 5.0 µM with 2.5, 1.25 to 5.0 or 5.0 µM of NAA, respectively. Rocha and Quoirin (2004) observed callus formation in mahogany (*Swietenia macrophylla* King), using BA. However, Vila et al. (2009) reported that 2,4-D induces callus formation in *C. fissilis* and somatic embryos were formed after 6 months, reducing the concentration of GR in the medium. In this present study, although the entire plant did not regenerate, there was a morphogenic response with the appearance of roots. In general, the absence or reduction of plant growth regulators led to the development and differentiation of somatic embryos or their conversion into plantlets (Merkle, 1995; Hu et al., 2008; Kumar et al., 2008; Yang et al., 2008).

In the present study, *C. guianensis* leaf explants developed *in vitro* in different 2,4-D concentrations and showed callus formation. In agreement with our results, Vila et al. (2007) noted that callus mass in zygotic embryo cultures of *Melia azedarach* L. (Meliaceae) was induced by high concentrations of 2,4-D and Picloran. To differentiate embryos from calli originating from hypocotyls or immature cotyledons in *Azadirachta indica* A. juss. (Su et al., 1997), it was necessary to use medium supplemented with IAA. Thus, it is evident that callus production in different woody species of Meliaceae is induced by different growth regulators, in varied concentrations. Furthermore, the type of auxins and cytokinins used in the culture media was shown to strongly influence callus formation.

Cytokinins, principally BA, have been reported to be a positive influence to break dormancy from buds and increase its development, as seen on Husain and Anis (2009), where MS medium with 5 µM of BA was the best condition for multiple shoots growth and the increase of length. BA is naturally present in plant tissues, plus its stability in comparison to other cytokinins (Letham and Palni, 1983), may be an explanation for the better response from explants using BA.

In the present work, rhizogenesis was only obtained in callus and not in plantlets. Rhizogenesis was observed when GR was reduced in MS 0 medium. These results agree with that of Basto et al. (2012). It was not possible in the same medium to develop roots from plantlets. A different response was also observed by Da Costa et al.

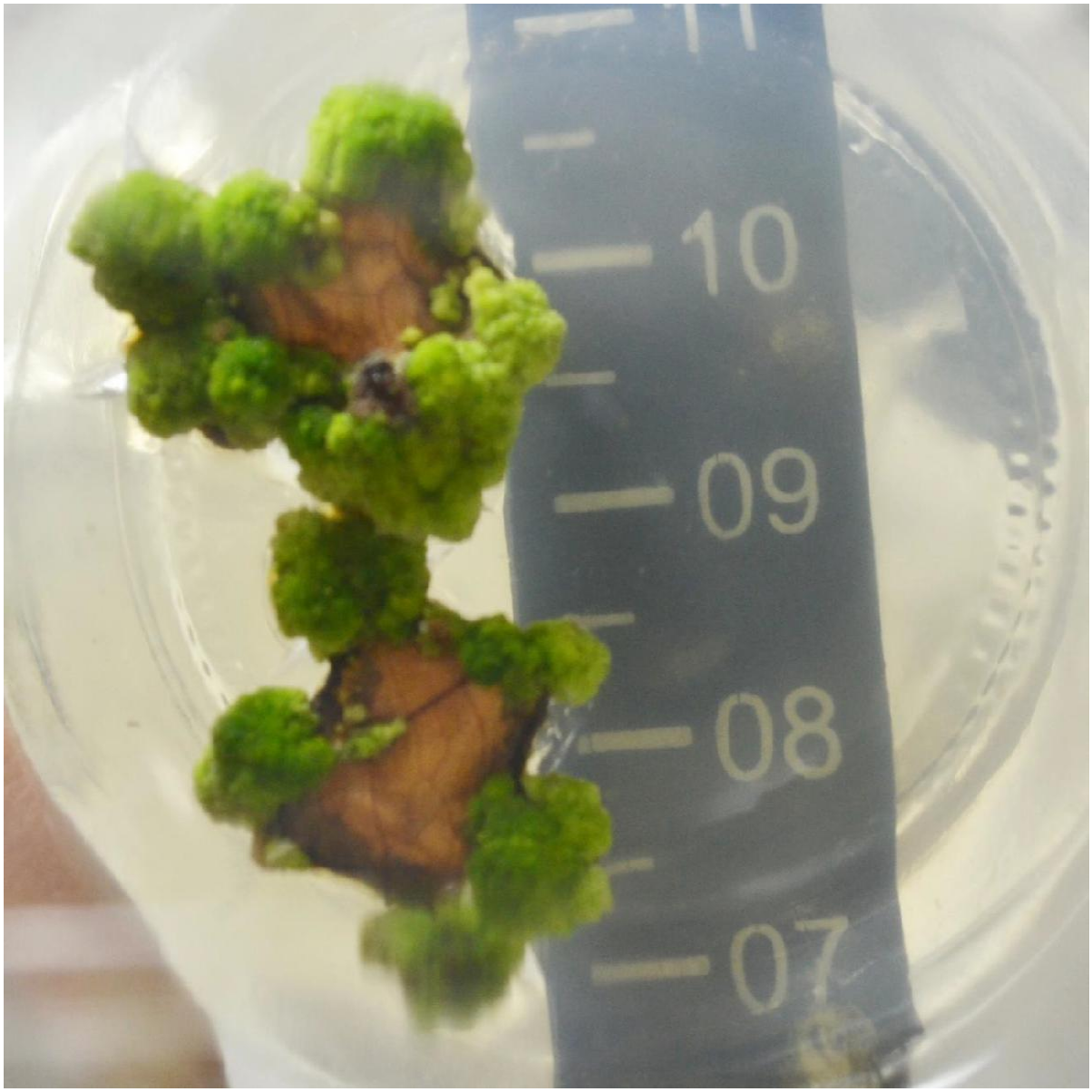

Figure. 5 Two-month- old callus of *Carapa guianensis* first obtained in 5 µM of 2,4-D- supplemented medium and then subcultivated on 0.5 µM of TDZ-supplemented medium. Ruler in cm.

(2002) , who reported rooting rates of over 87% of *C. fissilis* node cuttings without growth regulators, and with Millán-Orozco et al. (2011) regarding *C. odorata* shoots from seeds germinated *in vitro.*

The success of *in vitro* regeneration relies on the rooting percentage and survival of the plantlets in field conditions. Future studies can focus on achieving rhizogenesis by using media with indole-3- butyric acid (IBA). The IBA improved rooting efficiency and the superiority of IBA in rhizogenesis was also envisaged by other workers (Chiruvella et al., 2011). Rooted plantlets with 4 to 6 fully expanded leaflets will be transferred into plastic cups containing sterilized soil, sand and water to acclimatization tests.

In conclusion, although the induction of callus in *C. guianensis* has been achieved and bud breakage was inducted, further research is required to confirm the efficiency of embryogenic tissue or bud induction. However, the protocol described here may be suitable for clonal propagation and genetic transformation of *C.*

Figure. 6 Roots on callus of Carapa guianensis subcultivated a second time on MS0 supplemented with 3 g/L of charcoal (red arrow indicates root).

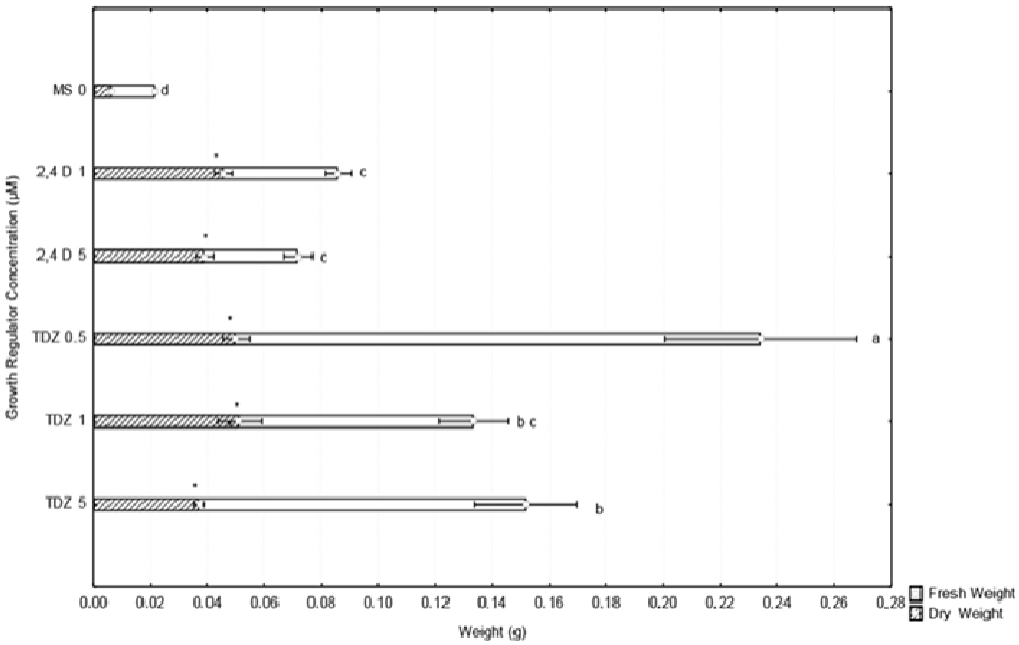

Figure. 7 Fresh and dry weight of *Carapa guianensis* callus obtained in 45 µM of 2,4-D supplemented medium and subcultivated on MS0; 1 or 5 µM of 2,4-D or 0.5, 1 or 5 µM of TDZ supplemented medium. Data represent mean values per treatment, and bars indicate SE from 36 explants/medium. Mean values with the same letter or * are not significantly different based on ANOVA followed by Tukey's test at P≤ 0.05.

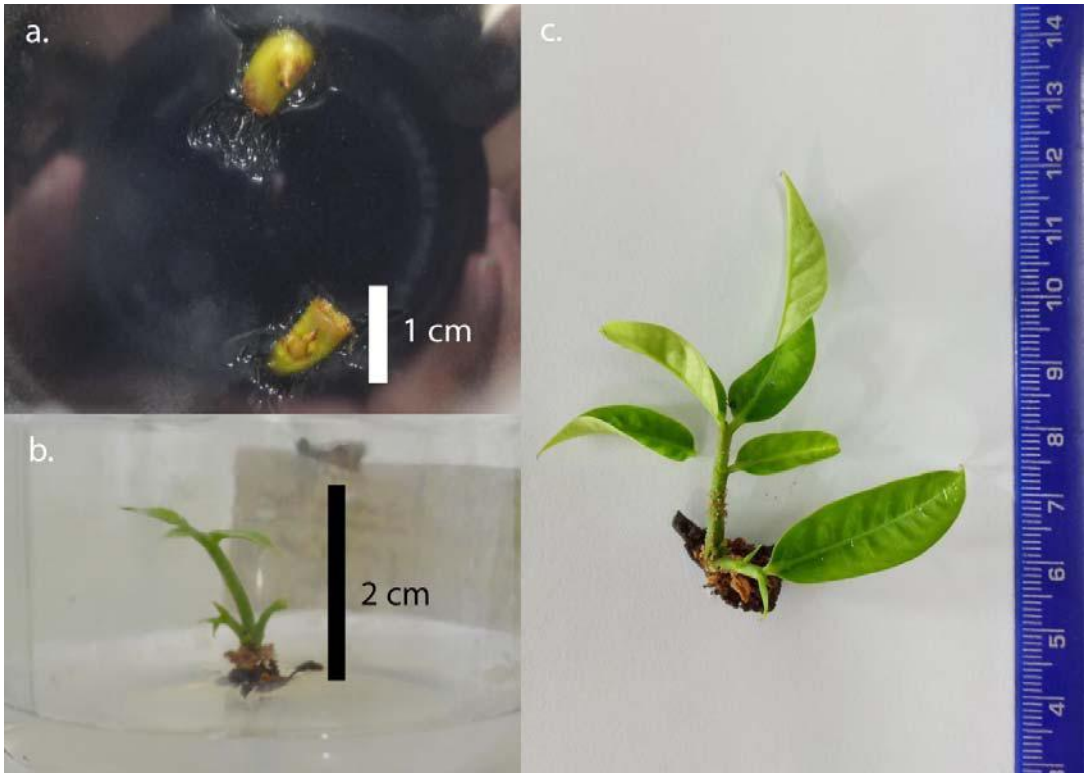

Fig. 8 In vitro establishment of plantlets of *Carapa guianensis* (a) Apical buds on MS0 medium supplemented with 3 g/L charcoal, (b) Plantlet after 4 weeks of subculture on MS supplemented with 5 µM of BA, (c) Plantlet after 8 weeks on MS supplemented with 5 µM of BA.

guianensis.

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Competing interests

We have no conflicting or competing financial interests.

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