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CELLULAR AND MOLECULAR BIOLOGY

Germination and in vitro development of mature zygotic embryos and protein profile of seedlings of wild and cultivated *Hevea brasiliensis*

JOSIANE C. DE CARVALHO, GLEISSON DE O. NASCIMENTO, ANA CLAUDIA L. DA SILVA, MARIA DAS GRACAS R. FERREIRA, WAGNER L. ARAÚJO & JOSÉ FRANCISCO DE C. GONÇALVES

Abstract: The main factors governing *Hevea brasiliensis* germination and seedling establishment remains unclear. We examined the effect of growth regulators Indole 3-Acetic Acid (IAA) and 6-Benzylaminopurine (BAP), and their interactions on germination and the development of mature zygotic embryos (MZE) and protein profile of *Hevea brasiliensis* seedlings from wild and cultivated (clone PB 250) genotypes. Embryonic axes excised from seeds (wild and clone PB 250) were inoculated in Murashige and Skoog medium (control) and supplemented with IAA (3 µM) and BAP (6 µM) individually and their combination (3 µM IAA + 6 µM BAP). For both genotypes, the mature embryos displayed a high percentage of germination and establishment, and the seedlings were characterized by protein bands ranging from 7 to 30 kDa. Notably, the wild genotype showed proteins in the 14 kDa range, which may be associated with one of the major rubber elongation factors (REF). The wild and clone genotypes presented different behavior and strategies in relation to the protein profile in the presence of different growth regulators. Although the latex biosynthetic pathway and its mechanisms of regulation still remain largely unknown, our results aid in our understanding of the dynamics of proteins in different rubber tree clones in vitro.

Key words: Clone, growth regulators, micropropagation, rubber tree, seeds.

INTRODUCTION

The species *Hevea brasiliensis* belongs to the family Euphorbiaceae whose center of dispersion is the Amazon region, and its main product (latex) is the raw material for rubber production (Priyadarshan 2017). Moreover, it is one of the most economically important species of the genus *Hevea*, since its high productivity and the quality of its latex make this specie the primary source of natural rubber in the world (Ighere et al. 2011, Gireesh et al. 2017, Fan et al. 2020).

Although rubber trees are perennial plants with a long cycle of conventional reproduction (Tan et al. 2019), they are traditionally propagated by grafting clones from aboveground organs and rootstock derived from illegitimate seeds. These seeds have recalcitrant characteristics, and easily lose viability and germinative power (Bonome et al. 2011, Theodoro & Batista 2014, Souza et al. 2018). In addition to the difficulties related to the recalcitrancy of seeds, this is a long-term process since clones require one to two years before plants can be transplanted into the field (Mendanha et al. 1998, Alvarenga & Carmo 2014, Martin et al. 2018). Three selection stages covering approximately 30 years are generally required until the final choice of clones is ready

for large-scale planting (Gouvêa et al. 2013), and this long period is one of the main limitations in *Hevea* breeding programs (Rahman et al. 2017). However, recent studies on the relationship between yield variables and anatomical and morphological characteristics in rubber tree progenies revealed that characteristics such as yield, circumference, shell thickness, and the number of laticiferous vessels can be used for early selection (Sant'Anna et al. 2020).

Studies have been carried out to understand the stress tolerance (physiological characterization) and functional genome of *H. brasiliensis* (Cheng et al. 2015, Lau et al. 2016, Sathik et al. 2018, Nascimento et al. 2019). Taken together, these results have clearly increased our current understanding of the mechanisms associated with the stress response of *H. brasiliensis* and have further generated compelling genomic information for potential biotechnological applications of the rubber tree. It seems reasonable to assume that the propagation of *H. brasiliensis,* which can take place in vitro, is a promising technique for large-scale plant production. This fact aside, the efficiency of embryo culture in vitro depends on different genotypes and may present different responses in the embryo stage, and in the formulation of culture media (Razdan 2004, Kothari et al. 2010, Manzur et al. 2013).

Plant tissue culture has emerged as a technique with the potential not only for cloning and mass propagation of plant species, which are required in large numbers in reforestation programs but can also be extended in order to study the conservation of important and rare species, as well as in the identification of biochemical markers such as expression of proteins or enzymes with emphasis on functional genomics of biotic and abiotic stresses (Pawłowski & Staszak 2016, Lau et al. 2016, Khalid & Aftab 2020). In recent years, there has been an increasing interest in micropropagation, with the aim of rapidly obtaining genetically homogenous and uniform plant material suitable for more specialized plantings (Gianguzzi et al. 2019).

The use of in vitro cultivation of zygotic embryos from recalcitrant seeds, such as rubber seeds, can not only increase the viability of germination and embryonic development, but it can also enable the use of cryopreservation studies and germplasm banks (Fonseca & Freire 2003, Nakkanong & Nualsri 2018). For instance, the line 2HA of *Medicago truncatula* exhibited a 500-fold greater capacity to regenerate plants by somatic embryogenesis than wild-type Jemalong, which was associated with the somatic embryogenesis of *M. truncatula* at the differential protein expression (Imin et al. 2005). Plant growth and development are controlled by growth regulators (e.g. auxins, cytokinins, gibberellins, abscisic acid, ethylene, among others), while the actions of growth regulators can be mediated by peptides (Jiménez 2005, Wang et al. 2016, Bao et al. 2017). Thus, growth regulators can influence protein metabolism and increase protein synthesis in the germination and initial seedling growth process. Moreover, growth regulators promote cell division, influence cell differentiation, and stimulate the biosynthesis of secondary metabolites in plants (Costa et al. 2019).

By exploiting the physiological traits and functional genome information of *H. brasiliensis*, coupled with techniques and strategies associated with the cultivation of cells and tissues of this species, it is expected that cell tissue culture there will be major advances in rubber tree propagation and breeding programs. The main limitations of the *H. brasiliensis* species are the narrow genetic base, non-synchronized flowering, low fruit yield, long period of fruit formation, and the absence of fully-reliable early selection parameters

(Venkatachalam et al. 2004, Rahman et al. 2017). Furthermore, the species present seeds with recalcitrant characteristics, which lose viability in a short period of time. Rubber tree breeding programs can be improved by the use of in vitro techniques for large-scale propagation of elite genotypes, transformation or conservation of genetic resources, and metabolite production. In the present study, we hypothesize that different culture media formulations can modify the protein expression from wild and cultivated (hereafter clone) genotypes. Thus, we examined the effect of Indole 3-Acetic Acid (IAA) and 6-Benzylaminopurine (BAP) growth regulators individually and combined on the germination and development of mature zygotic embryos and protein profile of *H. brasiliensis* seedlings of different genotypes. These results obtained are discussed in the current context of our understanding of the significance and implications of specific proteins related to latex synthesis.

MATERIALS AND METHODS

Plant material

Seeds were obtained from five matrices of *Hevea brasiliensis* from different origins. First, wild seeds were collected in an area located 17 km up the Tatajuba byway, in the municipality of Altamira in the Pará state, Brazil. Second, seeds from the clone PB 250 were collected on a rubber-tree plantation located near the BR 163 highway, in the municipality of Belterra-PA, meso-region of the Lower Amazon, Para state, Brazil. Right after the collection of seeds, how they were taken to the laboratory to determine the degree of absorption since these seeds are recalcitrant and lost due to rapid viability. The moisture content of seeds was determined by the oven method at 105 ° C ± 3 °, for 24 hours (Brasil 2009).

The seeds underwent two processes of asepsis, inside and outside the laminar flow chamber. The seeds with integument presented moisture content of 39 and 36% for wild seeds and clones, respectively. These seeds were washed with water and detergent, immersed in 5% sodium hypochlorite for 20 min, then the seed coatings were removed and the second asepsis was performed with seeds without tegument, with a moisture content of 36 and 32% for wild seeds and clone, respectively. Right after the asepsis was carried out in a laminar flow chamber, where they were immersed in 70% alcohol for 2 min under constant stirring and washed with autoclaved distilled water. Subsequently, disinfestation in 2% hypochlorite was carried out for 5 min under constant stirring and, finally, a triple wash with autoclaved distilled water.

The culture medium used was MS (Murashige & Skoog 1962), supplemented with 3% sucrose and vitamins plus growth regulators as the follows: MS (control, without growth regulator), IAA (3 µM), BAP (6 µM) and their respective combination (3 µM IAA + 6 µM BAP). Subsequently, the pH of the solutions was adjusted to 5.8, and then 1.8 g of Phytagel® was added to the growth media in each of the solutions. Then, autoclaving was carried out for 15 min at 121° C.

Inoculation of the embryonic axes

The mature embryonic axes were excised from the seeds, transversal sections were performed in the center and inoculated individually into each test tube containing 20 ml of the culture media. After inoculation, the tubes were then sealed with parafilm the cultures were maintained with a photoperiod of 16 hours/light, a light intensity of 50 µmol m^2 s^{-1}, and temperature alternating (day and night) temperatures of 20-30 °C.

Conducting the experiment and collecting data

The experiment was conducted for over 40 days and the following variables were evaluated: the percentage of germination of embryos axes (all the swollen embryos); hypocotyl emission; secondary root; primary root; epicotyl; eophyll, as well as counting the number of leaves, roots and root length (the distance between the end of the root and the end of the hypocotyl) (cm), hypocotyls (the distance between the beginning of the root and the end of the epicotyl) (cm), the diameter of the lap and epicotyl (the distance between the neck of the plant and the end of the terminal bud) (cm). The evaluation, depending on the variable, was performed daily. After the inoculating of the mature embryonic axe, the percentages of germination (G %), the germination speed index (Maguire 1962), and the mean germination time (MGT) were obtained (Labouriau 1983).

Morphology of germination

The morphological characterization of *H. brasiliensis* embryos was carried out in samples at each stage of germination, development, and seedling formation. The observations were performed using a 10 x 75 mm hand-magnifier. Morphological characteristics of the embryonic axes (shape, position, and hypocotyl-radicle axis) and development of mature zygotic embryos (MZE) (secondary, primary, hypocotyl, epicotyl, and eophyll emission) were described.

Extraction and quantification of soluble proteins

The finely pulverized material of the seedling tissues, aerial part, and root of the different treatments (MS, IAA, BAP, and IAA x BAP) were extracted in 0.15 M NaCl (10% w/v). Extraction took place under homogenization for two hours at room temperature, followed by centrifugation at 11.000 x *g* for 20 min at 10 °C. The supernatant was dialyzed against distilled water for 72 hours to remove the salt and then lyophilized for 96 hours, which resulted in the protein extract.

The protein extract (2 mg) was resuspended in 1 mL of distilled water and the protein concentration was estimated from the incubation of 100 µL of the extract with 3 ml of the Bradford reagent (BioAgency) for 10 min, using bovine serum albumin (BSA, BioAgency), according to the method described by Bradford, 1976. The protein content was determined by spectrophotometric readings at 595 nm (UV/Visible Ultrospec 2100 pro, Amersham Biosciences).

SDS-PAGE electrophoresis

The concentration and separation gels were obtained from a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) N-N'-methylenebisacrylamide. The 5% concentration gel was prepared in 0.5 M Tris-HCl buffer, pH 6.8, and the 12.5% separation gel in 1.5 M Tris-HCl buffer pH 8.8, both in 20% (w/v) SDS. Polymerization was achieved by the addition of TEMED and 10% (w/v) PSA (Laemmli 1970).

Electrophoresis was performed in 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% SDS, 200 volts, 15 mA/gel running buffer for 2 hours. Molecular mass markers (Promega) were used with a range of 10 kDa - 100 kDa. After the runs, the gels were stained with Coomassie Brilliant Blue dissolved in 0.1% (v/v) acetic acid, ethanol, and 1: 4: 5 v/v/v distilled water for 2 hours and then bleached in acid solution acetic acid, ethanol, and distilled water 1: 4: 5 (v/v/v) for 1 hour.

Experimental design and statistical analysis

The experimental design was completely randomized (CRD), using two genotypes (wild and clone) and MS media (control) with three different treatments of growth regulators (IAA, BAP, and IAA x BAP combination) which constituted a factorial scheme of 2x4, with 4

replicates for each treatment, each replicate being constituted by three test tubes containing an explant. The statistical analyzes of the data were submitted to the normality test (Shapiro Wilks) and homogeneity of variance (F test). Based on these assumptions, evaluations were further performed for analysis of variance (ANOVA), and the means were compared by the Tukey test at the level of 5 % probability. All statistical analyses were performed using the algorithm embedded into the program Sigma Plot 12.0 (Systat Software Inc., Germany).

RESULTS AND DISCUSSION

Morphological characterization in response to growth regulators

By analyzing the germination and in vitro development of mature zygotic embryos of *Hevea brasiliensis* from the wild and clone we observed the presence of an embryo with a short hypocotyl radical, and a straight, cylindrical axis. Accordingly, the germination process started with the swelling of the embryonic axis at 2 and 3 days, and the onset of a greenish pigmentation. Next, the formation of the hypocotyl started at 4 and 5 days with 0.7 cm for both wild and clone zygotic embryos, and with greenish coloration in the basal region and, subsequently, it presented structures at its extremity that gave rise to root tissues (Figure 1a).

At 5 and 8 days, the elongation of the secondary roots started in the wild and clone zygotic embryos, presenting a size of 2.0 and 2.5 cm, respectively, with yellowish coloration. At 7 and 8 days, the elongation of the primary roots started when they had 4 and 6 cm, respectively, with a light green coloration.

At 8 and 9 days the epicotyl emission started, at 4 and 5 cm, and possessed a dark-green color, at 10 and 12 days, the greenish-colored apical bud appeared, which gave rise to the eophyll.

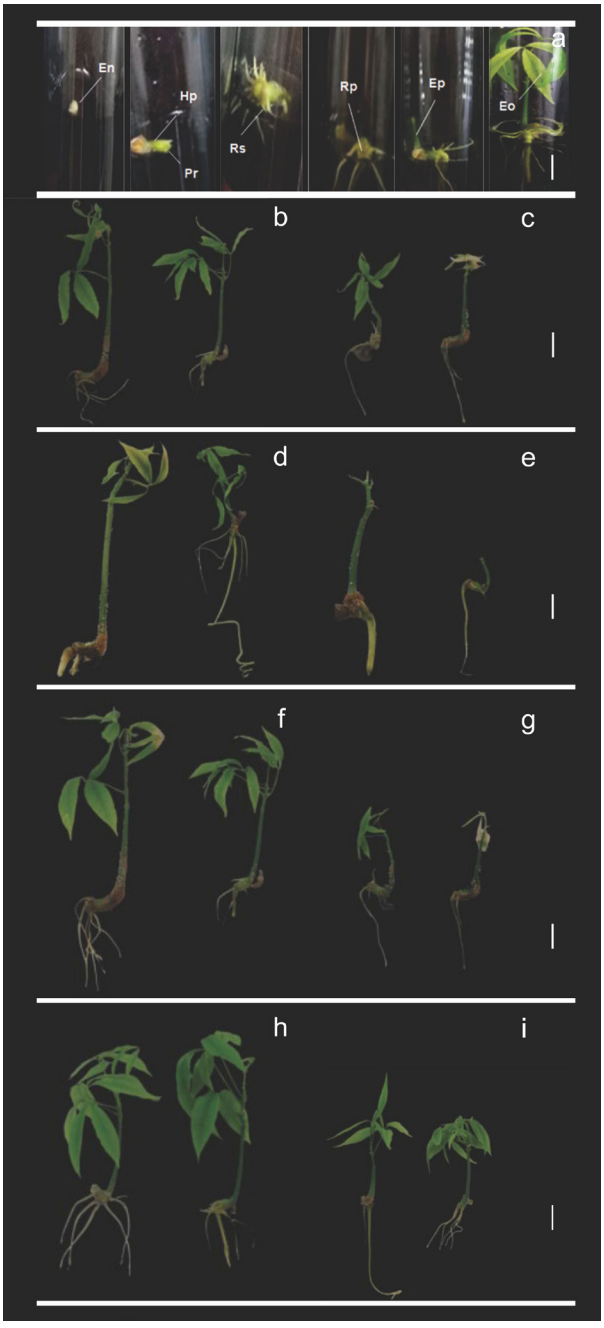

Figure 1. (a)- Representative images of stages of germination and development of *Hevea brasiliensis* mature zygotic embryos (MZE) wild and clone were taken in different culture media after 40 days of cultivation. (b-c) - Murashige & Skoog (MS). (d-e) - Indole 3-Acetic Acid (IAA). (f-g) - 6-Benzylaminopurine (BAP). (h-i) - Interaction IAA + BAP. Abbreviations: En, embryonic axis; Hp, hypocotyl; Pr, root primordia; Rp, primary root; Rs, secondary root; Ep, epicotyl; Eo, eophyll. Scale bars: 1 cm.

From 13 to 15 days, the eophylls, which were light green and 1.2 and 1.8 cm size, followed its leaf expansion with dark-green eophylls (Figure 1a).

Our results revealed that during the germination and in vitro development of mature zygotic embryos of *Hevea brasiliensis* from the wild and clone we observed the presence of an embryo with a short hypocotyl radical, and a straight, cylindrical axis. Accordingly, the germination process started with the swelling of the embryonic axis at 2 and 3 days, and the onset of a greenish pigmentation. In vitro seedlings development, both wild and clone zygotic embryos showed no morphological differences, despite the minor changes in plant biometrics (height and diameter), most likely due to limited space for seedling growth. The morphological characteristics of zygotic embryos in vitro, as well as the germination process, are important features to interpret which one may be associated with the viability of the species. Notably, in vitro conditions not only compromise leaf morphology, leaf expansion, and dry mass but these characteristics also negatively affect the ability of ex vitro acclimatization; yet, such characteristics may vary according to the in vitro environment and the plant species under study (Kozai et al. 1992, Llorente & Apóstolo 1998). All together, these factors are able to induce disturbances in overall plant development and photosynthetic performance, which can cause the formation of seedlings with abnormal morphology, anatomy, and physiology (Darwesh 2015, Hoang et al. 2020).

Germination is affected by growth regulators

In general, after two days of inoculation, we observed the swelling of the mature embryos in both wild and clone. The percentage data of germination and mean germination time in the different substrates/growth media are additionally presented in figure 2 and table I, respectively. The highest percentage value of germination in wild zygotic embryos occurred in the MS media (67%) and, for the clone, higher mean values were observed in BAP and IAA + BAP (83 and 75%, respectively), which were significantly different between wild and clone zygotic embryos ($p<0.042$) (Figure 2a).

The percentage of root emission was higher in both IAA and IAA + BAP for both the wild and clone embryos, presenting a statistical difference ($p <0.001$) in MS media with BAP growth regulator (Figure 2b). The secondary and primary root elongation in wild-type zygotic embryos had a mean of 50% in IAA, whereas in the clone it had 67% in BAP and IAA + BAP media, and the mean values were also different for these treatments ($p<0.005$) (Figure 2c and d). The emission of the epicotyl and eophyll presented the highest averages in MS, and were 33% for wild zygotic embryos and in clones in IAA + BAP media, demonstrating significant differences between wild and clone embryos in media with either BAP or IAA + BAP ($p<0.041$) (Figure 2e and f).

In their natural environments, *H. brasiliensis* seeds are usually characterized by relatively low germination rates (58-80%), due to the recalcitrant characteristics of the seeds coupled with a high rate of infestation by microorganisms, and therefore lose their viability rapidly (Garcia & Vieira 1994, Paula et al. 1997). Our results revealed that the cultivation of zygotic embryos in vitro reached germination rates close to ones observed in their natural environment, which is an advantage for the large-scale propagation of this species since the inoculated embryos are all viable, mature, and undergo an aseptic process. It has been further demonstrated that the younger the embryos, the more difficult their in vitro culture will be because of their small size, the damage that may occur during their removal, and their nutritional requirements which are

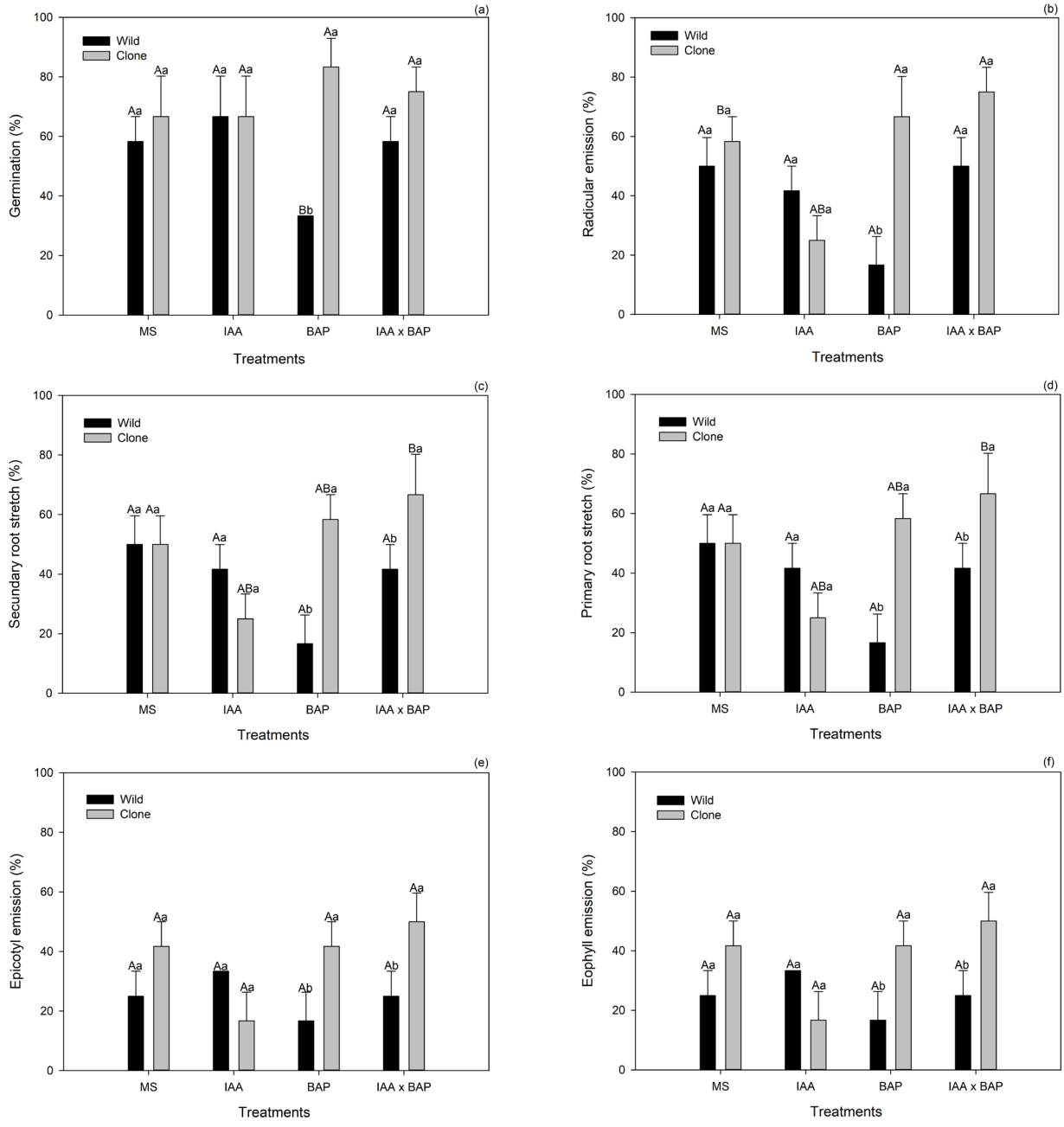

Figure 2. Germination and morphological changes of in vitro MZE of *Hevea brasiliensis* different growth regulators. (a) germination, (b) radicular emission, (c) secondary (d) and primary (c) root elongation, (e) epicotyl (f) and eophyl emission (n = 12). Lower case letters refer to the difference between species in the same treatment and upper-case letters refer to the difference between treatments in the same species. The Tukey test was applied at a 5% probability level.

more complex (Hu & Ferreira 1998, Mohan et al. 2011). Indeed, *Jatropha curcas* embryos, which belong to the same family as the investigated species, had a low (2-5%) embryo germination rate (Paula et al. 1997, Mohan et al. 2011).

Higher germination velocity was observed for wild and clone zygotic embryos in the MS media with the addition of either IAA or IAA + BAP and showed significant differences ($p < 0.030$). The highest root emission velocities were in MS

Table I. Comparison of germination time of *Hevea brasiliensis* MZE wild and clone in different culture media. Mean germination time, root primordia, secondary and primary root length, epicotyl and eophyll emission.

Variables analyzed	Treatments							
	MS		IAA		BAP		IAA + BAP	
	Wild	Clone	Wild	Clone	Wild	Clone	Wild	Clone
(%)								
Germination	66.7 Aa	66.7 Aa	58.3 Aa	66.7 Aa	33.3 Bb	83.3 Aa	58.3 Aa	75.0 Aa
Root primordia	41.7 Aa	25.0 Ba	50.0 Aa	58.3 ABa	16.7 Ab	66.7 Aa	50.0 Aa	75.0 Aa
Secondary root	41.7 Aa	25.0 Aa	50.0 Aa	50.0 ABa	16.7 Ab	58.3 ABa	41.7 Ab	66.7 Ba
Primary root	41.7 Aa	25.0 Aa	50.0 Aa	50.0 ABa	16.7 Ab	58.3 ABa	41.7 Ab	66.7 Ba
Epicotyl	33.3 Aa	16.7 Aa	25.0 Aa	41.7 Aa	16.7 Ab	41.7 Aa	25.0 Ab	50.0 Aa
Eophyll	33.3 Aa	16.7 Aa	25.0 Aa	41.7 Aa	16.7 Ab	41.7 Aa	25.0 Ab	50.0 Aa
(TM)								
Germination	6.12 Aa	7.37 Aa	6.25 Aa	4.12 ABa	7.00 Aa	6.37 ABa	6.50 Aa	3.12 Bb
Root primordia	7.75 Aa	9.87 Aa	4.50 Aa	4.33 Ba	7.50 Aa	7.41 ABa	6.75 Aa	5.12 Ba
Secondary root	8.75 Aa	8.00 Aa	5.00 Ba	6.00 Aa	6.50 ABa	6.37 Aa	6.37 ABa	2.50 Ba
Primary root	8.75 Aa	8.00 Aa	5.00 Ba	6.00 Aa	6.50 ABa	7.62 Aa	6.37 ABa	2.50 Bb
Epicotyl	8.33 ABa	8.75 Aa	8.00 Aa	8.00 Aa	10.50 Ba	11.25 Ba	6.00 Ca	5.37 Ca
Eophyll	8.33 ABa	8.75 Aa	8.00 Aa	9.00 Aa	10.50 Ba	11.25 Ba	6.00 Ca	10.37 ABb

***Averages followed by the same letter do not differ statistically from each other. Lower case letters refers to the difference between species in the same treatment and upper case letters refers to the difference between treatments in the same species. The Tukey test was applied at the 5% probability level.**

media with IAA for wild ($p < 0.225$) and clone ($p < 0.298$) embryos, with no significant differences for treatments. The highest secondary and primary root elongation velocity values for wild ($p<0.200$) and clone ($p<0.417$) zygotic embryos occurred in MS media with IAA and IAA + BAP, with significant difference ($p < 0.001$) in IAA + BAP. For epicotyl emission, the highest mean velocities were found in the MS media with IAA + BAP for wild ($p<0.175$) and clone ($p<0.188$) embryos and showed no significant difference between treatments ($p > 0.05$).

The eophyll emission rate for wild embryos was higher in media supplemented with IAA + BAP ($p<0.175$) whereas for clone embryos it was in MS media without regulators ($p<0.115$), and showed a significant difference between genotypes studied only in media with IAA + BAP interaction ($p<0.001$). In the germination time, no difference was observed between MS media and in the presence of either IAA or BAP for both wild zygotic embryos and the clone ($p>0.05$) (Table I).

Embryo contamination occurred between 3 and 6 days after inoculation and between 5 and 8 days and it was observed for the wild genotype (8%) and for the clone (12%). In addition to the contamination, oxidation of some embryos was observed for the wild embryos (6%) and for the clone (10%). Similar results were also found by Ighere et al. 2011 working with the same species, *Hevea brasiliensis,* despite little contamination observed in the explants. Mohan et al. 2011, working with *J. curcas*, which is from the same family of the species under study, also observed a high percentage of contamination and abnormal seedling formations. It is important to mention, however, that this is seemingly associated with the type of genotype studied. In fact, a successful tissue culture initiation often begins with an effective explant sterilization technique and, in the micropropagation of *H. brasiliensis,* the establishment of an axenic culture is clearly

one of the most challenging steps. It should be kept in mind that during the micropropagation of *H. brasiliensis* the occurrence of oxidation of the embryos is usually observed (Moradpour et al. 2016). Further studies are required to fully elucidate whether the oxidation is involved in cell division induction or paralyzation since it has been previously demonstrated that a higher accumulation of reactive oxygen species promotes an arrest of the cell cycle (Foyer et al. 2019).

Impacts of growth regulators on seedling growth

We further evaluated seedling growth by measuring the following variables: height, length of secondary and primary roots, hypocotyl, epicotyl, the diameter of the lap, number of leaves, fresh shoot, and root mass (Table II). In Figure 1b-i the images of *H. brasiliensis* seedlings wild and clone in culture media with different growth regulators are presented. The highest shoot length was observed in BAP (3.35-3.50 cm), followed by MS culture media and IAA + BAP interaction, although there were no significant differences for wild and clone zygotic embryos ($p>0.05$). Higher height averages (2.55 cm) with the use of the BAP were previously found with *H. brasiliensis* (Asseara et al. 1998, Ighere et al. 2011). Nevertheless, by using the species *J. curcas* cultivated in MS media with and without BAP, it was observed the formation of seedlings (Hu & Ferreira 1998); however, the most promising results were found with the induction of cytokinin (BAP), which is similar to the results found here with *H. brasiliensis*.

The highest values for the secondary and primary root lengths were observed in the presence of IAA individually and significant differences were observed between wild and clone zygotic embryos for secondary roots ($p<0.032$) and primary roots ($p<0.004$). Similar results were found in MS culture media supplied with IAA in *Euphorbia heterophylla*, which belongs to the same species family under study, with the same efficiency in root formation (Colussi et al. 2008). It should be further noted that natural or synthetic auxins do not always induce root primordia in explants in vitro (Jardim et al. 2010).

Table II. Mean aerial part length (APL) and secondary root (SR), primary root (PR), hypocotyl (H), diameter of the lap (DL), number of leaves (NL), Fresh mass of the aerial part (FMAP) and root portion (RP) of *Hevea brasiliensis* seedlings in different culture media after 40 days of cultivation.

Variables	Treatments							
	MS		IAA		BAP		IAA x BAP	
	Wild	Clone	Wild	Clone	Wild	Clone	Wild	Clone
Height (cm)	2.23 Bb	4.00 Aa	1.60 Ca	1.80 Da	3.46 Aa	3.21 Ba	2.70 Ba	2.63 Ca
Secondary root (cm)	2.16 Bb	3.80 Aa	5.20 Aa	2.30 Bb	2.49 Ba	2.04 Ba	2.43 Ba	2.50 Ba
Primary root (cm)	3.33 Bb	3.93 ABa	9.07 Aa	4.43 Ab	2.03 Ca	2.00 Ca	2.30 Ca	2.63 Ba
Hypocotyl (cm)	0.65 Bb	0.78 ABa	0.65 Ba	0.65 Ba	0.70 ABa	0.71 Ba	0.75 Ab	0.83 Aa
Diameter of the lap (mm)	2.53 Ba	2.33 Bb	3.00 Ab	3.53 Aa	2.49 Ba	2.28 Bb	2.61 Ba	2.33 Bb
Leaves (number)	2.33 Bb	3.33 ABa	4.00 Aa	3.83 Aa	2.00 Cb	3.00 Ba	2.33 Bb	2.66 Ba
Fresh Mass PA (g)	0.14 Bb	0.17 Ba	0.14 Ba	0.12 Cb	0.21 Aa	0.12 Cb	0.21 Aa	0.20 Aa
Fresh Mass PR (g)	0.11 Ba	0.10 Ba	0.21 Aa	0.13 Ab	0.11 Ba	0.12 ABa	0.12 Ba	0.10 Ba

***Averages followed by the same letter do not differ statistically from each other. Lower case letters refer to the difference between species in the same treatment and upper case letters refer to the difference between treatments in the same species. The Tukey test was applied at the 5% probability level.**

No differences were observed for hypocotyl length, epicotyl diameter, and the number of leaves. Similarly, Costa et al. (2015) also observed that the supply of either BAP or IAA did not promote significant differences in the number of leaves in *Occidental basilicum*. This is most likely associated with the low plasticity of this physiological variable throughout the development of seedlings in vitro, in comparison with other biometric variables including height and diameter.

We observed higher fresh mass in seedlings in the MS culture medium with IAA + BAP. Seedlings in this medium showed significant differences in relation to the other treatments ($p<0.042$) (Table II), which can also be observed in figure 1h-i, where the greatest plant development in the interaction of cytokinin and auxin is observed. In fact, the growth regulator BAP is often added to the culture medium in order to improve micropropagation, which results in an increase in the number of buds and leaves, leads to an increase in fresh mass production, and improves the quality of cultivated plants (Rubin et al. 2007). In good agreement with the results from Rubin et al. (2007), higher fresh mass was observed in the root portion of seedlings treated with IAA growth regulators.

Changes in soluble proteins and seedlings' protein profile in response to growth regulators

The concentrations of soluble proteins in the aerial part of *H. brasiliensis* seedlings from explants obtained from the wild embryos and the clone are significantly different ($p<0.001$) for the medium which contained the culture and growth regulator IAA, and the highest levels were found in the medium containing IAA. Briefly, the proteins present in the aerial part of clone embryos were larger when compared to the wild. Regarding the soluble proteins in the root portion of *H. brasiliensis* seedlings, there were also differences between wild and clone embryos in the MS medium with the addition of IAA ($p<0.021$). Notably, the highest levels of proteins in the root part were observed in the MS culture media (Figure 3a and b). Thus, changes observed suggested that an extensive reprogramming in protein metabolism may be occurring in our growth conditions, and as a consequence, we extended our analysis to proteomic analysis. Proteins play important roles in the composition of living organisms and in various life activities (Hao et al. 2019). Synthesis and degradation of proteins occur constantly and this allows plants to effectively use amino acids by altering their protein content during development and thus adapt to new environmental conditions (Piza et al. 2003, Hildebrandt et al. 2015).

Since we have demonstrated that our growth conditions have substantially altered levels of total protein, we carry out SDS-PAGE analysis and observed that proteins present in the tissues of the shoots and roots of *H. brasiliensis* are mostly in the range of 7 to 30 kDa. In the shoot and root tissues for wild and clone explants, clear protein bands with an apparent molecular mass of ~ 12, 20, and 30 kDa were observed in the different growth regulators. Proteins with an apparent molecular mass of ~30 kDa were very evident in shoot tissue, with either IAA or IAA + BAP interaction, but when in either MS or BAP culture media fewer proteins were evident. However, proteins with a molecular mass of ~30 kDa were less evident in root tissues of seedlings cultivated in the different culture media for both embryos (Figure 4a-b).

Our results revealed that proteins in the aerial tissues are primarily in the range between 20 and 30 kDa in the presence of the IAA growth regulator. In comparison to the protein expression profile in the root tissues, regardless of the growth regulators used, a pattern in the range of 20 and 30 kDa were additionally observed. Collectively,

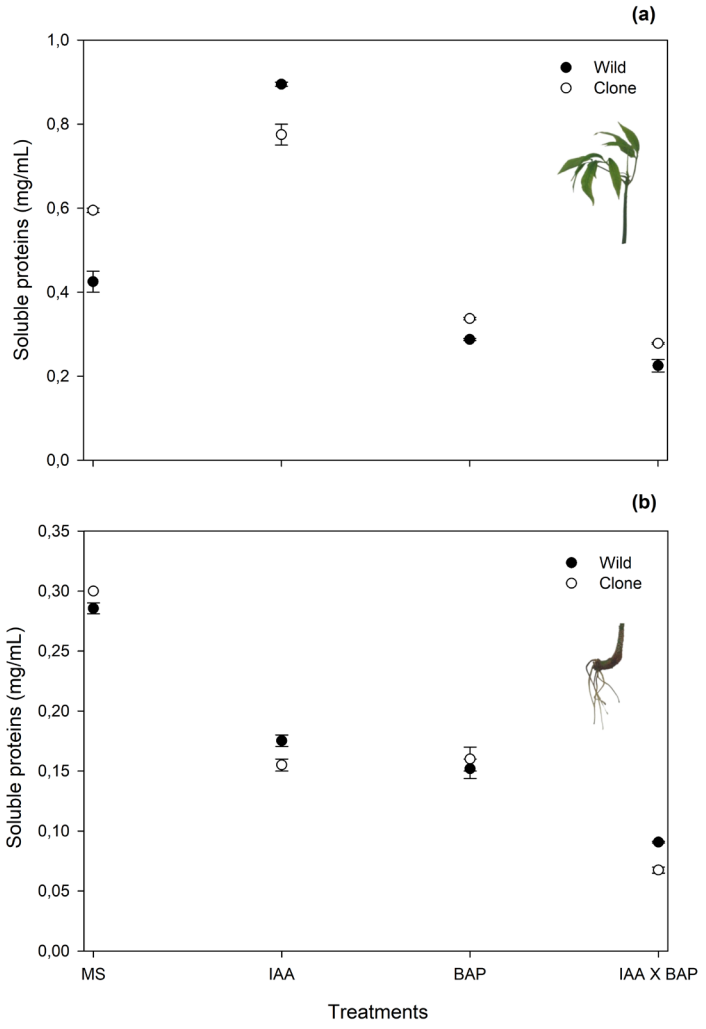

Figure 3. Soluble proteins are differentially altered in *Hevea brasiliensis* seedlings tissues in vitro different culture media. (a) Aerial part. (b) Root portion. Wild (●) and Clone (○). Values are means ± standard deviation (*n*=12).

it allows us to infer that this range of proteins is more easily expressed in the aerial part in the presence of auxin whereas in the root part it is expressed independently of auxin and cytokinin.

It has been previously postulated that the mevalonate pathway is most likely the main route for latex synthesis (Berthelot et al. 2014). Additionally, among the proteins with a fundamental role in this route are the rubber elongation factor (REF) proteins that have a molecular mass of 14.7 kDa and the small rubber particle protein (SRPP) of 22.3 kDa. Accordingly, the presence of proteins in the range of 20 kDa, both in the aerial part and in the radicular for both genotypes, was observed. This indicated that proteins of this range could be associated with SRPP, a protein associated with the synthesis of the latex, while the presence of proteins in the 14 kDa range, which may be associated with one of the major REF proteins was also observed.

Differences in the protein profile of an organism can be attributed to tissue types, developmental stages, and internal and external conditions (Shepard et al. 2000). This fact aside, plant tissue culture may be the most suitable system for producing small and medium amounts of high purity proteins of high value and with various applications. Thus, the interactions that occur between the seed's information and the parent plant are rather complex, making it difficult

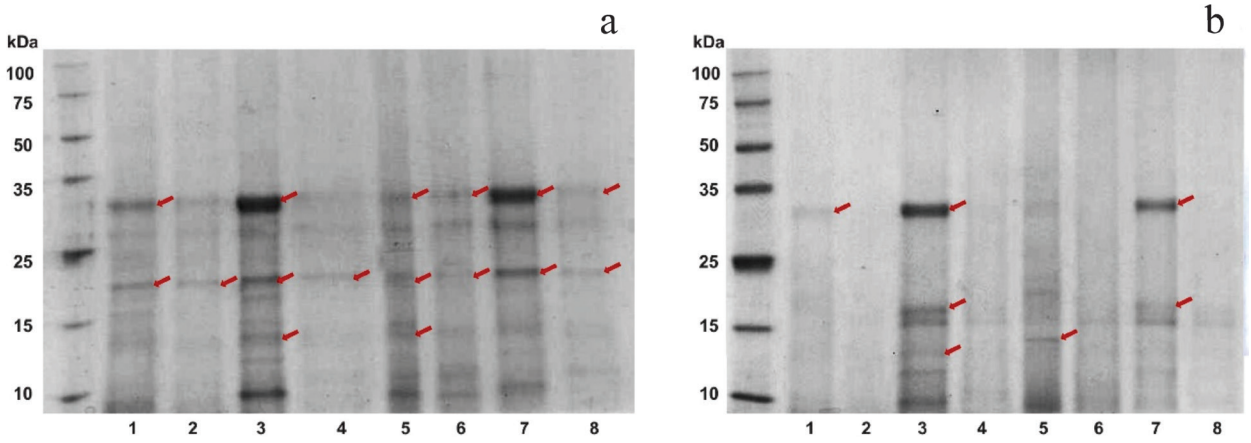

Figure 4. Total protein extracts the seedlings were electrophoretically separated by Blue native/SDS–PAGE and after gels were Coomassie stained. (a) wild and (b) clone. (1) MS-aerial part. (2) MS-root portion. (3) IAA-aerial part. (4) IAA-aerial part. (5) BAP-aerial part. (6) BAP-root portion. (7) IAA x BAP- aerial part. (8) IAA x BAP- root portion. Molecular masses of standard protein (M) are given in the left of the gel (in kDa). The experiments were performed in triplicate and repeated three times with similar results.

to identify which plant hormones are involved in seed development and in the accumulation of proteins (Nascimento & Mosquim 2004). Nevertheless, it seems reasonable to suggest that plant hormones act not only influence seed growth and development but may also induce protein synthesis as observed here. In close agreement, protein synthesis in pea seeds was observed within six hours of application of auxins and gibberellins (Van Huizen et al. 1996). Furthermore, Saravitz & Raper Jr (1995) verified that the increase of glutamine concentration of the medium from 0.6 to 6 mM increased the protein content in soybean seeds. This evidence reinforces the idea that the culture medium formulations can alter the protein synthesis during in vitro propagation.

CONCLUSIONS

By using rubber tree wild and clone genotypes we observe the differential behavior and genotypic strategies used by seedlings in relation to protein expression in response to specific growth regulators. Although the precise nature of such adjustments could not be solved in the present study, it it remains an exciting topic for further enhance our understanding of the dynamics of proteins in different Amazonian rubber tree genotypes in vitro, with emphasis on latex biosynthesis. While the mechanisms involved in the synthesis and expression patterns of proteins remains unclear in embryonic tissue from rubber tree seeds, our findings indicate that an adequate manipulation of culture media composition on regeneration of rubber tree may influence protein profiles of the target tissues from seedlings.

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JOSIANE C. DE CARVALHO[1]
https://orcid.org/0000-0001-7986-4693

GLEISSON DE O. NASCIMENTO[2]
https://orcid.org/0000-0003-4303-0042

ANA CLAUDIA L. DA SILVA[1]
https://orcid.org/0000-0002-7954-8989

MARIA DAS GRACAS R. FERREIRA[3]
https://orcid.org/0000-0002-3412-6387

WAGNER L. ARAÚJO[4]
https://orcid.org/0000-0002- 4796-2616

JOSÉ FRANCISCO DE C. GONÇALVES[1]
https://orcid.org/0000-0001-9197-4617

[1]Instituto Nacional de Pesquisas da Amazônia-MCTI-INPA, Laboratório de Fisiologia e Bioquímica Vegetal, Campus III (prédio 152), Av. HI, s/n, Conj. Morada do Sol, Aleixo, 69060-062 Manaus, AM, Brazil

[2]Universidade Federal do Acre/UFAC, Centro Multidisciplinar, Campus Floresta, Estrada do Canela Fina, Km 12, Gleba Formoso, Lote 245, Colônia São Francisco, 69980-000 Cruzeiro do Sul, AC, Brazil

[3]Embrapa Cocais, Av. Praça da República, 147, Diamante, 65065-470 São Luís, MA, Brazil

[4]Universidade Federal de Viçosa/UFV, Departamento de Biologia Vegetal, Edif. CCB II, Centro de Ciências Biológicas II, Campus Universitário, Av. Purdue, s/n, 36570-900 Viçosa, MG, Brazil

Correspondence to: **José Francisco de Carvalho Gonçalves**
E-mail: jfc@inpa.gov.br

Author contributions

J.F.C and J.C de C planned and designed the research. J.C de C; A.C.L da. S performed the experiments. J.C de C and G de. O.N analyzed the data. J.F.C and J.C de C wrote the manuscript with the support of M das. G.R.F and W.L.A.