

In Vitro Propagation of the Amazonian Medicinal Plant Guayusa (*Ilex Guayusa*) and Effects of Light in the Growth and Development of this Shade Tolerant Plant

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1 ***In vitro* propagation of the Amazonian medicinal plant guayusa (*Ilex guayusa*) and**
2 **effects of light in the growth and development of this shade tolerant plant**

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1 **Keywords**

2 *Ilex guayusa*, axillary bud culture, light regulatory effects, Amazon region, indigenous groups.

3

4 **Abstract**

5 Guayusa (*Ilex guayusa*) is an endemic plant from the Amazon with potential medicinal applications. Indigenous
6 people are familiar with such applications and use guayusa based on ancestral knowledge. There is a growing
7 interest in guayusa-based products in urban areas of Ecuador and internationally. The supply cannot meet the
8 demand. Currently, traditional practices are used for guayusa growth and the potential use of the protected forest
9 is foreseen. This work describes a protocol for the *in vitro* propagation of guayusa, a sustainable solution to
10 generate high quality plants in reduced space. Stakes obtained from stems were used as explants. Chemical
11 sterilization with ethanol and sodium hypochlorite resulted in 100% surface-sterilized stakes. The growth
12 medium mWPM resulted in favorable outcomes regarding shoot development and elongation, as well as rooting.
13 Supplementation with activated charcoal resulted in reduced browning during the elongation phase. The majority
14 of shoots were able to develop roots spontaneously. Medium supplementation with the auxin indole-3-butyric
15 acid, IBA, may be considered when rooting does not occur spontaneously. Acclimatization was performed in soil.
16 The protocol was tested under different light spectra, revealing that guayusa growth is affected by light quality.
17 The photobiology of this shade tolerant plant requires further characterization, but the data uncovered a potential
18 role for green and far-red light in root development.

19

20 **Key message**

21 Guayusa was propagated on mWPM medium supplemented with activated charcoal. Spontaneous root
22 development occurred in most shoots. Light quality affected plant development, green and far-red light could
23 influence root growth.

24

25 **Abbreviations**

26 **AC:** activated charcoal

27 **B:** blue light

28 **FR:** far-red light

29 **G:** green light

- 1 **HY5:** ELONGATED HYPOCOTYL 5
- 2 **IBA:** indole-3-butyric acid
- 3 **LED:** light-emitting diode
- 4 **MS:** Murashige & Skoog
- 5 **mWPM:** modified Woody Plant Medium
- 6 **NAA:** 1-naphthaleneacetic acid
- 7 **R:** red light
- 8 **STN:** shoot tip necrosis
- 9 **W:** white light
- 10 **WPM:** Woody Plant Medium

11

12 **Introduction**

13 The Amazon rainforest is home to thousands of medicinal plants. Indigenous groups rely on ancestral
14 knowledge to use and propagate these plants for diverse therapeutic uses (Thomas et al. 2011; Innerhofer and
15 Bernhardt 2011; Giovannini 2015; Robles Arias et al. 2020). A considerable part of the general population of
16 countries within the Amazonian region also depends on herbal medicine for basic healthcare needs (Leone et al.
17 2007), and there is a growing interest from the pharmaceutical and food industries and from international
18 consumers to benefit from Amazonian plants (Gu et al. 2014). Cultivation practices remain however often
19 primitive and result in low yields. Increasing areas of forestland are used, which disturbs wildlife habitats and
20 poses a threat to the delicate and essential Amazonian ecosystem (Thomas et al. 2011). The development of
21 sustainable and efficient cultivation practices is critical to respond to the growing demand of medicinal plants and
22 simultaneously protect the Amazonia.

23 Guayusa (*Ilex guayusa*) is an evergreen dioecious tree from the upper Amazon in Ecuador, Peru and Colombia. It
24 is largely cultivated by indigenous groups and the most significant medicinal plant among the Kichwa
25 (Innerhofer and Bernhardt 2011). Guayusa leaves have antioxidant, antibacterial, anti-parasitic, and anti-
26 inflammatory properties (Kapp et al. 2016; Radice, M. et al. 2017; García-Ruiz et al. 2017; Pardau et al. 2017;
27 Gamboa et al. 2018; Gan et al. 2018; Chianese et al. 2019). They are used for many purposes, such as, boosting
28 energy and alertness, protection against snakebites, treatment for gastritis, or inducer of female fertility. These

1 traditional therapeutic applications correlate with biochemical analyses of guayusa leaves that have identified the
2 presence of several secondary metabolites, such as theobromine and other alkaloids, flavonoids and other
3 phenolic compounds, as well as caffeine (Kapp et al. 2016; García-Ruiz et al. 2017; Pardau et al. 2017; Gan et al.
4 2018; Chianese et al. 2019).

5 Indigenous groups typically propagate guayusa in household gardens called “chakras”, together with other
6 medicinal and edible plants and subsistence crops (Perreault 2005; Krause and Ness 2017). “Chakra” production
7 is a marker of cultural identity for indigenous groups and traditionally ensured household food security.
8 Nowadays some indigenous groups in Ecuador have established small organizations that use “chakra” production
9 for the commercialization of guayusa leaves together with other products (Wiñak Association 2020). A few
10 companies, such as Runa and Wa, acquire these guayusa leaves to produce and sell tea-based products and other
11 beverages. Indigenous groups in Ecuador are amongst the most vulnerable people in poverty and such activity
12 has helped them reaching better economic status (El Comercio 2018; Fundación Futuro Latinoamericano and
13 Grupo FARO 2020). However, the expansion of cultivated areas in the forest may not be sustainable. It has been
14 estimated that over 2,000 hectares of the Amazonia are being cultivated with guayusa, which poses a burden on
15 the forest (El Comercio 2018). The continuous preference of the same cultivars over other varieties may also
16 negatively impact ecosystems and their sustainability (FAO 2008; Isbell et al. 2017).

17 The practice of *in vitro* propagation is an alternative and sustainable solution to replace greenhouse or outdoor
18 nursery operations; optimal protocols can yield high numbers of vegetative propagules using less space and fewer
19 resources. It allows the conservation of plant genetic diversity by not converting natural space to propagation
20 nursery space (Yokoya and Yoneshigue-Valentin 2011; Opabode 2017; El-Sherif 2019). It can be a solution to
21 propagate plants with reduced seed fertility, such as is the case for guayusa (Dolce et al. 2011; Dueñas et al.
22 2016). Plant *in vitro* propagation is performed in indoor conditions, where environmental factors that affect plant
23 growth, yield and quality can be controlled with precision. Light is an environmental factor of particular interest
24 in such approaches. Light regulates gene expression, plant growth, hormone signaling, physiology and
25 metabolism at different stages of development (Wu 2014; Gelderen et al. 2018b; Wang et al. 2019). From UV to
26 far-red light, discrete wavelengths are sensed by plant photoreceptors that activate and regulate specific internal
27 pathways (Fankhauser and Christie 2015; Galvão and Fankhauser 2015; Legris et al. 2019; Tripathi et al. 2019;
28 Yadav et al. 2020). The usage of LEDs in indoor conditions allows for the design of specific light conditions to

1 modulate plant growth and quality (Darko et al. 2014; Landi et al. 2020). Fundamental knowledge on plant
2 photobiology was first explored and described in *Arabidopsis thaliana* (Paik and Huq 2019). While some of these
3 mechanisms translate to crops, it has been evident that some light regulatory effects are plant species-specific
4 (reviewed in Carvalho and Folta 2014). It is important therefore that plant response to light is individually
5 assessed on case-by-case studies. The role of light in the growth and development of guayusa has not been
6 described. This plant typically grows in the dense Amazonian forest, where the solar spectrum is enriched in
7 green and far-red light, compared to full sunlight conditions (Ballaré and Pierik 2017). The molecular
8 mechanisms behind the shade tolerance of guayusa are unknown. Unraveling such mechanisms can allow the
9 development of strategies to facilitate the indoor propagation of this plant and to improve its quality, namely in
10 the accumulation of leaf secondary metabolites.

11 The current work describes an *in vitro* protocol for the propagation of guayusa. We couple plant tissue culture
12 with specific light regimen to test the hypothesis that guayusa growth and quality can be manipulated with light.
13 The usage of specific light treatments revealed novel details of the photobiology of guayusa and specific roles of
14 light during the development of this plant. Our results highlight the importance of conclusive photobiological
15 assays in order to understand the effect of light on plant physiology and optimize the growth and cultivation of
16 individual species.

17

18 **Materials and Methods**

19

20 **Overview of the protocol**

21 The *in vitro* propagation of guayusa includes three main stages: shoot bud induction, shoot elongation and rooting
22 (Fig. 1). In the first stage, sterilized stakes with axillary buds were cultured on shoot bud induction medium (Fig.
23 1a). Thirty day-old shoots were then separated from the stake and transferred to fresh medium to allow elongation
24 (Fig. 1b), shoot development (Fig. 1c) and rooting (Fig. 1d).

25

26 **Plant material**

27 Forty-five, two-year-old, guayusa plants from Runa Foundation nurseries in Tena, Ecuador, were transferred to
28 the Plant Biotechnology Laboratory at Universidad San Francisco de Quito. These plants were used as source

1 material for establishing the axillary bud culture protocol. Shoots obtained *in vitro* were used for elongation,
2 rooting, acclimatization, and light quality assays.

3

4 **Sterilization protocol**

5 Apical stem segment explants were harvested from two-year-old guayusa plants. The segments were washed in
6 running tap water for 2-4 min. The leaves were dried and removed off, and the stems were cut into 1 cm stakes
7 with one axillary bud each. The explants were sterilized by submersion in 70% ethanol for 2 min, followed by
8 2,5% sodium hypochlorite + five drops of Tween®-20 for 25 min. Finally, the stakes were washed five times
9 with sterile distilled water.

10

11 **Axillary bud culture**

12 Shoot regeneration from axillary buds was initially tested in two culture media: Murashige & Skoog with 1/4 of
13 the original salt concentration (1/4 MS) supplemented with 3% sucrose (pH 5.8); and modified Woody Plant
14 Medium (mWPM) (Lloyd and McCown's, 1981) with 3% sucrose (pH 5.2) – Table 1. Explants were incubated at
15 $23 \pm 2^\circ\text{C}$ under a 16 h photoperiod for 47 d. Growth rates were calculated based on shoot size measurements
16 every three days for two months. The final medium used for shoot regeneration was mWPM supplemented with
17 activated charcoal (AC) at 2 g l^{-1} .

18

19 **Elongation and rooting**

20 Shoots obtained from the axillary bud culture ($> 0.5 \text{ cm}$) were separated from the stakes and cultivated in mWPM
21 + AC (2 g l^{-1}). Shoots were grown at $23 \pm 2^\circ\text{C}$ in a 16 h photoperiod. Data were collected every four weeks for
22 six months regarding shoot development, plantlet height, and leaf number and length. Length measurements were
23 performed using a size standard and analyzed in ImageJ (Image processing software).

24 Root development was scored with the analysis of primary and secondary root number and length. Plantlets that
25 rooted spontaneously were transferred to soil for acclimatization. Plantlets that did not develop roots
26 spontaneously were transferred to mWPM + $4.5 \mu\text{M}$, $9.1 \mu\text{M}$ IBA (indole-3-butyric acid) to gather preliminary
27 data on the effect of IBA on rooting induction. Plantlets that developed browning were discarded.

28

1 **Acclimatization**

2 Plantlets that did not show browning and that developed spontaneous roots were transferred from *in vitro*
3 conditions to autoclaved soil, and covered with plastic wrap in order to gradually reduce the relative humidity.
4 Plants were then kept in the tissue culture room under a 16 h photoperiod of white light at 23 ± 2 °C for 30 d.
5 Growth and development was assessed over these 30 d by recording leaf area, shoot length, and root
6 development.

7
8 **Light treatments**

9 Light was provided by LED sources (Light Emitting Computers, Victoria, BC, Canada) with four independent
10 channels: 450 nm (Blue – B), 520 nm (Green – G), 660 nm (Red – R), and 735 nm (Far-Red – FR). The assays
11 were conducted in enclosed wooden boxes covered with aluminum foil. Light was applied at various fluence
12 rates with a 16 h photoperiod. Fluence rates within the visible range were measured with a full-spectrum quantum
13 meter (Apogee, model MQ-500) and far-red fluence rates with an International Light meter (model IL1400A).
14 Seven light treatments were tested (Control - T7): Control: cool fluorescent white light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$); T1: R
15 ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) + B ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$); T2: R ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) + B ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) + G ($5 \mu\text{mol m}^{-2} \text{s}^{-1}$); T3: R
16 ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) + B ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) + FR1 ($4 \mu\text{mol m}^{-2} \text{s}^{-1}$); T4: R ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) + B ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) +
17 FR2 ($16 \mu\text{mol m}^{-2} \text{s}^{-1}$); T5: R ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) + B ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) + G ($5 \mu\text{mol m}^{-2} \text{s}^{-1}$) + FR1 ($4 \mu\text{mol m}^{-2} \text{s}^{-1}$);
18 and T6: R ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) + B ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) + G ($5 \mu\text{mol m}^{-2} \text{s}^{-1}$) + FR2 ($16 \mu\text{mol m}^{-2} \text{s}^{-1}$) – Table 2. Eight
19 shoots were grown and evaluated per light treatment, as described above. Data were recorded for each plantlet
20 and average numbers were calculated under each light treatment.

21
22 **Statistical analyses**

23 Differences between culture media used for axillary bud propagation were evaluated based on a t-test for each
24 variable with an α -value of 0.05 ($n = 48$). In shoot elongation, root development, and plant acclimatization assays
25 data were analyzed using Ordinary one-way ANOVA Multiple comparisons. The six LED treatments were
26 compared to white light. Under the seven light regimens and *in vitro* conditions results are representative of seven
27 to eight plants per treatment. After transfer to soil results are representative of four to six plants per light

1 treatment. Box plots were created in GraphPad Prism. The composite images were configured and labeled in
2 Microsoft PowerPoint.

3

4 **Results**

5 **Effects of culture media on shoot regeneration from axillary buds**

6 An initial assay was performed to establish an optimal medium for shoot bud induction. Sterilized stakes were
7 cultivated on 1/4 MS and mWPM media during a period of 47 d (Fig. 1a). Shoot bud induction was evaluated
8 every two or three days by recording shoot length. Although no significant differences were seen between the two
9 tested media (Fig. S1), our visual observations suggested that mWPM resulted in slightly longer shoots than 1/4
10 MS. Based on these observations, and on reports in the literature (Mccown and Sellmer 1987), mWPM was
11 selected as the growth medium for axillary bud regeneration and shoot elongation, and it was supplemented with
12 activated charcoal (AC), Fig. 1.

13

14 **Shoot elongation and leaf development over four subcultures**

15 Shoot elongation was assessed starting with 30-d-old shoots on mWPM + AC under white light. Four subcultures
16 were performed during 150 d (Fig. 2). In each subculture, shoots were transferred to fresh growth medium. The
17 material used in the first subculture showed on average a shoot length of 0.6 cm (Fig. 2a). The second subculture
18 was performed at 90 d, and shoots had almost doubled their length since the first subculture (Fig. 2b). The third
19 subculture was performed 60 d later, and shoot length showed an increase of 50%. The fourth and last subculture
20 was performed at 180 d. At this stage, shoot length had reached 2 cm.

21 Leaf emergence initiated after the first subculture (from 60 to 90 d). Leaf length was therefore assessed over the
22 three remaining subcultures (Fig. 3). At 90 d, shoots showed a leaf length with 1 cm on average (Fig. 3a). Leaf
23 length did not change considerably over the remaining two subcultures (Fig. 3b and 3c). At 180 d, leaf length was
24 1.2 cm (Fig. 3c).

25

26 **Root formation**

27 During the fourth and fifth subcultures, 69% of the plantlets had developed roots spontaneously (Fig. 4). A
28 preliminary assay tested the effect of IBA supplementation on root induction with plantlets that did not root

1 spontaneously. IBA was tested at two concentrations: 4.5 μM and 9.1 μM . Analysis of root development pointed
2 to 4.5 μM IBA as the most effective concentration for rooting induction (Fig. S2).

4 **Acclimatization**

5 Plantlets with roots that developed spontaneously were transferred to soil for acclimatization (Fig. 1d, Fig. 5).
6 Most plants under treatments T3 and T4 did not develop spontaneous roots and were not acclimatized. During the
7 first 30 d after the beginning of acclimatization, plants that were grown under white light conditions in *in vitro*
8 conditions roughly maintained a shoot length of 2 cm (Fig. 6). Leaf area in contrast increased 89%, from 0.39 to
9 3.56 cm^2 (Fig. 7). Root development was assessed at the beginning of acclimatization in plants that had
10 spontaneously developed roots. Plants grown under white light showed about 14 main roots (Fig. 8a) and five
11 secondary roots (Fig. 8b). Main roots had 0.37 cm and secondary roots had 0.21 cm in length (Fig. 9).

13 **Effects of light quality on the *in vitro* propagation of guayusa**

14 The described protocol for the *in vitro* propagation of guayusa was tested under different light conditions (Table
15 2). Treatments T1 and T2 resulted in a shoot length similar to white light conditions during the four subcultures
16 (Fig. 2), except during the last subculture. At this point shoots grown under T1 showed an average length of 1.44
17 cm, 28% less than under white light (Fig. 2d). More notable differences were observed when comparing
18 treatments T3 to T6 and white light. During the second subculture shoots grown under T3 to T6 measured from
19 0.54 to 0.71 cm (Fig. 2b). These numbers are reduced 45% to 30% compared to white light. At 180 d of growth,
20 during the last subculture, treatments T3 to T6 maintained a similar trend and resulted in shorter shoots, with 1.1
21 to 1.3 cm in length (a 47% to 37% reduction compared to white light-grown shoots). When assessing leaf
22 development during three subcultures, no differences were detected between the white light control and
23 treatments T1 to T6 (Fig. 3 and Fig. S3).

24 The effects of specific light treatments were further assessed within the first 30 d of acclimatization (Fig. 6-10) in
25 plants that spontaneously developed roots. Treatments T3 and T4 were excluded from this analysis as the
26 majority of plants under these light regimes did not develop roots spontaneously. Both at the beginning of
27 acclimatization and 30 d later, shoots grown *in vitro* under T1, T2, T5, and T6 were similar in length to shoots
28 grown under white light (Fig. 6). No differences were seen in leaf area, and LED-grown plants showed similar

1 trends in leaf growth compared to the white light control (Fig. 7). In terms of root development at the beginning
2 of acclimatization all light conditions except T5 resulted in similar numbers of main and secondary roots per
3 plant (Fig. 8). Under T5 the number of main roots was reduced 65% when compared to white light – five main
4 roots per plant in T5 in contrast to 14 main roots in C (Fig. 8a). Also at the stage of plant transfer to soil,
5 treatments T1, T5, and T6 showed longer main roots than the white light control (Fig. 9a). T1 resulted in main
6 roots with 0.68 cm, T5 in 0.78 cm, and T6 in 0.68 cm, which is 46%, 53% and 46% longer than white light
7 conditions, respectively. Secondary root length was similar in all light conditions (Fig. 9b).

8

9 **Discussion**

10 The Amazon rain forest is home to thousands of potentially therapeutic plants and is an important resource to
11 screen for novel drugs (Schultes 1994; Skirycz et al. 2016). A number of these plants have been described but a
12 large fraction remains to be explored and characterized. Traditional medicine in the Amazonian countries largely
13 depends on local plants but native people use outdated cultivation practices that pose a threat to the forest (World
14 Health Organization 2002; Bussmann 2013). At large scale, the systematic usage of plant natural compounds has
15 been limited by factors such as supply problems and low yields (Skirycz et al. 2016). This situation presents a
16 perilous situation, as plants may be subject to un-sustainable harvesting from natural areas, or their natural areas
17 may be compromised to facilitate their growth and propagation. The sustainable usage of Amazonian plants
18 requires the development and implementation of efficient propagation and growth strategies.

19 The *in vitro* propagation of plants is a sustainable solution to propagate plants that can achieve high yields and
20 year-round predictable products (Opabode 2017; El-Sherif 2019). The current work reports a protocol for the *in*
21 *vitro* propagation of the Amazonian medicinal plant guayusa. Guayusa is largely used in Ecuador but its
22 international market is rapidly growing (Wise and Negrin 2020). To facilitate entry of guayusa-based products in
23 restricted markets, safety and risks to human health have been assessed, and adverse effects have not been
24 reported (Wise and Negrin 2020). These observations can reassure regulatory entities and consumers but do not
25 solve key issues in production systems. Current guayusa cultivation practices are still based on low yield
26 techniques. Our protocol may help solve this problem and reducing ecological impacts of growth areas used in
27 the forest. The next major challenge will be to implement the protocol within local producers. This may be

1 achieved by establishing a communication platform that involves producers, companies that sell guayusa-based
2 products, and academia.

3 *In vitro* guayusa plants were obtained from axillary buds using stakes as initial material. The sterilization protocol
4 showed to be effective, and after 14 days of culture, 100% of the stakes were sterile. 90% of the stakes developed
5 shoots while 10% developed browning. Explant browning has been described in other woody plants and shrubs,
6 including some *Ilex* species (Mroginski et al. 1999). The production of brown substances is predominantly caused
7 by enzymatic oxidation of phenolic compounds, and often causes explant death. The toxic substances and the
8 obstruction of oxidized tissues restrain nutrient absorption from the growth medium (Tarragó et al. 2012).

9 Previous studies were able to reduce oxidation with AC supplementation, a carbonaceous adsorbent of aromatic
10 compounds (de Cássia Tomasi et al. 2019). Similar results were observed in this study, and 2 g l⁻¹ of AC in the
11 growing media significantly reduced oxidation up to 90%.

12 Two culture media compositions were tested for shoot regeneration and elongation. WPM was first developed for
13 shoot culture of woody plants and bushes and for tree propagation, and is currently the second most used medium
14 for *in vitro* culture (Jain and Häggman 2007). WPM is characterized by having low salt concentrations, since
15 many woody species are known to be sensitive to NaCl. WPM minimizes chloride levels by using sulfate salts
16 (McCown and Sellmer 1987). Moreover, WPM has low levels of ammonium nitrate, which prevents shoot
17 vitrification (Huang and Dai 2011) and decreases the percentage of nodal browning in woody species (Mroginski
18 et al. 1999). The MS medium provides similar nutritional values as WPM but presents high NH₄NO₃ and KNO₃
19 levels. A reduced basal concentration of this medium (1/4 MS) benefits shoot regeneration and decreases
20 browning (Mroginski et al. 1999). In shoot regeneration of *Quercus ilex* L. (holm oak), a recalcitrant woody
21 species of difficult propagation, MS medium yielded small leaves, while WPM resulted in larger shoots
22 (Martínez et al. 2017). These observations are consistent with our observations where, despite the absence of
23 significant differences, plant vigor was more evident on mWPM.

24 Plantlets that presented three to five extended leaves and spontaneously developed roots were successfully
25 acclimatized in soil. Plants that did not root spontaneously often showed one of two main characteristics: shoot
26 tip necrosis (STN) or less developed shoots. STN is a physiological condition that can emerge in *in vitro* plantlets
27 or shoots and affect propagation by causing tissue death (Bairu et al. 2009). STN has been reported in several
28 arboreal and shrubby plants (Teixeira da Silva et al. 2020). Furthermore, the usage of short shoots (less than 0.5

1 cm in length) affects the elongation phase and delays roots appearance, which can lead to nutrient deficiency in the plant (Martínez et al. 2017).

3 Plantlets that do not root spontaneously and do not show STN or less developed shoots may be transferred to 4 rooting induction conditions. Phytohormones are routinely needed in such approaches. Specific compounds and 5 their concentrations largely vary within woody species and depend on the type of cutting (Haissig 1986). Still, it 6 has been suggested that rooting in woody plants is responsive to IBA, and to a lesser extent to NAA (1- 7 naphthaleneacetic acid) (Azad et al. 2005). IBA is the most commonly auxin used for root formation in woody 8 species due to its higher stability (Rathore et al. 2005). For instance, vegetative propagation of *Ilex khasiana*, as 9 in other woody plants, is slow and root formation is the most challenging step (Dang et al. 2011). An effective 10 treatment for root formation is 9.84 μM IBA, resulting in 93.33 % of developed roots in 4-week-old shoots (Dang 11 et al. 2011). Similar findings have been observed in other shrubs and woody species, such as *Ilex aquifolium* 12 (Dennis Thomas and Yoichiro 2010). In our preliminary assay that analyzed the impact of IBA on rooting 13 induction we tested a similar amount of IBA (9.1 μM) and roughly half of it (4.5 μM). We obtained better results 14 with 4.5 μM IBA, getting overall higher number of roots per shoots (Fig. S2). Further work with additional 15 samples is needed.

16 The protocol for the *in vitro* propagation of guayusa can be modulated with selective light conditions, instead of 17 using a traditional white light source. Light modulates plant growth and development and knowledge often 18 obtained from the model *Arabidopsis* has facilitated crop manipulation and improvement with light environments 19 (Paik and Huq 2019; Landi et al. 2020). Translation of such approaches to underexplored crops such as guayusa 20 is limited by the poor knowledge of how this species responds to light. Guayusa grows in dense vegetation and is 21 tolerant to shade, in contrast to *Arabidopsis*. It is expectable that guayusa and *Arabidopsis* may differ in their 22 responses to similar light conditions.

23 Leaf number per shoot was recorded from subcultures two to four in plants grown under the seven light 24 conditions (Fig. S3). Handling of the plant material during each subculture leads however to a few leaves falling 25 off from the shoots, making it impossible to accurately quantify leaf emergence and to assess effects of light 26 quality on plant development based on leaf number.

27 The major impact of light depicted in this experimental approach was in root development. Most shoots grown 28 under T3 and T4 treatments did not develop roots spontaneously and were therefore transferred to growth

1 medium supplemented with IBA to induce rooting. These plants were excluded from the analysis of acclimatized
2 plants (Fig. 6 to 9). Both T3 and T4 have FR light added to a background of R and B at two different red:far-red
3 (R:FR) ratios (T3: 6.3 and T4: 1.6). Supplemental far-red light at the referred R:FR ratios may therefore inhibit
4 root development of guayusa. At the initiation of acclimatization, treatments T1, T5, and T6 resulted in longer
5 main roots than the control and other LED conditions. In addition, T5 reduced the number of main roots and
6 caused longer secondary roots compared to the other regimens. T5 and T6 have G light added to R/B (T1) and FR
7 light at the R:FR ratios 6.3 and 1.6, respectively. Since no major differences were observed in T2 (G light added
8 to R/B), and given the delayed root development under T3 and T4, it may be possible that the results observed in
9 T5 result from a synergistic effect of G and FR light on root development. Both G and FR light have been
10 implicated in the modulation of root development and morphology, although the role of the latter has been more
11 explored and established at molecular levels (Webb 1981; Gelderen et al. 2018b; Klem et al. 2019; Xu et al.
12 2020; Mølmann et al. 2020). In Arabidopsis, a shoot-to-root communication system senses supplemental FR
13 enrichment (ratio R:FR 0.1) and reduces lateral root emergence and density through the modulation of the
14 transcription factor ELONGATED HYPOCOTYL 5 (HY5) activity, which regulates auxin signaling (Gelderen et
15 al. 2018a). This regulation of root growth by reduced ratios of R:FR occurs in dense vegetation, where shade-
16 avoidance responses are activated in shade intolerant plants such as Arabidopsis (Morelli and Ruberti 2000;
17 Pierik and de Wit 2014). Green light is also an important environmental cue in shade avoidance (Zhang et al.
18 2011). Green and FR light control shade avoidance in Arabidopsis through the activity of several photoreceptors,
19 including phytochrome, cryptochrome and an unknown green sensor (Sellaro et al. 2010; Zhang et al. 2011;
20 Gelderen et al. 2018b). The activity of such light sensors may be explored in guayusa in future studies in order to
21 gain insight on the mechanisms that have evolved in this Amazonian plant.

22 The current study used $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ as the background fluence rate because it is the fluence rate provided by
23 the white light sources currently installed in our plant tissue room. Our current LED system limited the creation
24 of additional light environments with different wavelength ratios. In the future we aim at testing higher fluence
25 rates with various wavelengths on guayusa propagation. It will be particularly relevant to analyze the impact of
26 reduced R:FR ratios (<0.7) together with G light enrichment and B light depletion to simulate dense vegetation
27 conditions (Morelli and Ruberti 2000; Gelderen et al. 2018b).

1 Guayusa leaves accumulate several compounds of interest, including phenolic compounds, terpenoids, and the
2 methylxanthines caffeine and theobromine (Wise and Negrin 2020). Light quality and quantity will be tested on
3 leaf characteristics in the future with the aim of enhancing guayusa properties. Leaf metabolites have been
4 manipulated with light in aromatic and medicinal species such as basil, parsley, tea, and water mint (Fu et al.
5 2015; Carvalho et al. 2016; Ascrizzi et al. 2018; Pennisi et al. 2019; Zheng et al. 2019; Wang et al. 2020; Nazari
6 and Zarinkamar 2020). Identifying proper light conditions to stimulate leaf quality will facilitate obtaining
7 reliable products unaffected by environmental fluctuations. Fluctuations of light cues occur in forests and affect
8 leaf metabolite synthesis (Zhang et al. 2018a, b). It is interesting to note that treatments T2, T4 and T6 resulted in
9 lower variances in each trait studied, suggesting these light conditions may be used to obtain consistent guayusa
10 plants.

11

12 **Conclusions**

13 Guayusa was propagated from axillary buds on mWPM supplemented with AC. After 180 days plants that
14 developed roots spontaneously were ready for acclimatization in soil. In shoots with delayed or absent rooting,
15 root initiation may be stimulated with supplementation of 4.5 μm IBA. Light quality was tested on plant
16 propagation, and an effect of green and far-red light was observed on root growth. Future studies will further
17 explore the impact of light of guayusa development, with an emphasis on leaf quality and the onset of flowering.
18 This study can sustain similar projects in other potential medicinal plants and facilitate drug discovery with
19 sustainable approaches.

20

21 **Declarations**

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25

26 **Conflicts of interest**

27 The authors have no conflicts of interest to declare.

28

1 **Availability of data and material**

2 The datasets generated during and/or analyzed during the current study are available from the corresponding
3 author on reasonable request.

4

5 **Code availability**

6 Not applicable.

7

8 **Authors' contributions**

9 MLT supervised the project. MLT, SC conceptualized the project. SC, MaO, and MLT designed the experiments,
10 examined results. MaO, MiO, and MR performed the experiments, compiled and analyzed the data. KF made
11 available the light sources and provided insight in result interpretations. SC and MaO prepared and wrote the
12 manuscript. MLT provided feedback and editions during manuscript preparation. SC and MLT obtained funding
13 for the study, oversaw experiments. All authors approved the manuscript.

14

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23

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37

1 **Tables**

2

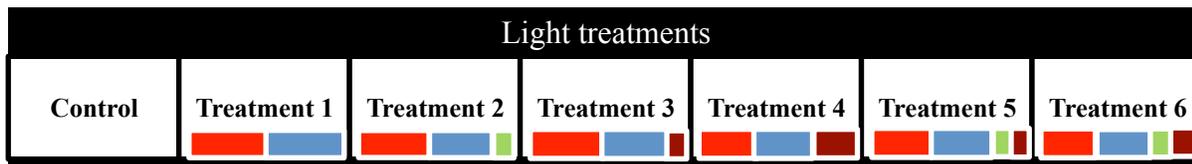
3 **Table 1.** Components of the modified Woody Plant Medium used in this study and Lloyd and McCown's original Woody
 4 Plant medium (Trigiano and Gray 1999; Schuchovski and Biasi 2019).

Components	Modified WPM (g l⁻¹)	Original WPM (g l⁻¹)
Macronutrients		
NH ₄ NO ₃	5.4	0.4
KNO ₃	3.9	-
CaCl ₂ ·2H ₂ O	2.8	0.096
MgSO ₄ ·7H ₂ O	3.7	0.37
KH ₂ PO ₄	1.7	0.17
K ₂ SO ₄	-	0.99
Ca(NO ₃) ₂ ·4H ₂ O	-	0.556
Micronutrients		
H ₃ BO ₃	0.62	0.0062
MnSO ₄ ·H ₂ O	1.69	0.0223
ZnSO ₄ ·7H ₂ O	1.05	0.0086
KI	0.083	-
Na ₂ MoO ₄ ·2H ₂ O	0.025	0.025
CoCl ₂ ·6H ₂ O	0.0025	-
CuSO ₄ ·5H ₂ O	0.0025	0.025
FeSO ₄ ·7H ₂ O	2.78	0.0278
Na ₂ - EDTA	3.73	0.0373

5

1 **Table 2.** Light spectrum and photon flux density in seven treatments.

2



3

White (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Red (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Red (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Red (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Red (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Red (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Red (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)
	Blue (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Blue (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Blue (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Blue (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Blue (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Blue (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$)
		Green (5 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Far red 1 (4 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Far red 2 (16 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Green (5 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Green (5 $\mu\text{mol m}^{-2} \text{s}^{-1}$)
					Far red 1 (4 $\mu\text{mol m}^{-2} \text{s}^{-1}$)	Far red 2 (16 $\mu\text{mol m}^{-2} \text{s}^{-1}$)

4

1 **Legends to figures**

2

3 **Fig. 1** *In vitro* propagation of guayusa from axillary buds using stakes as initial material. Cuttings grown in
4 mWPM + AC medium and exposed to light treatment T2 are shown as representative examples. (a) Developing
5 bud on a stake. (b) 30 d-old shoots ready to be separated from the stake. (c) Shoots after 150 d. (d) Developed
6 roots in 180 d-old shoots

7

8 **Fig. 2** Shoot length in four subcultures. Box plots represent the control and six light conditions, T1 to T6. Results
9 are representative of seven to eight plants per condition. (a) First subculture, 30 d. (b) Second subculture, 90 d.
10 (c) Third subculture, 150 d. (d) Fourth subculture, 180 d. Letters denote significantly different values (one-way
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12

13 **Fig. 3** Leaf length in three subcultures. Box plots represent the control and six light conditions, T1 to T6. Results
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16

17 **Fig. 4** Spontaneous root development after 180 d. Five plantlets grown under light treatment T2 are shown as
18 representative examples

19

20 **Fig. 5** Acclimatization of guayusa plants. (a) Representation of the process used for data collection from
21 acclimatized plants. (b) Guayusa plant 30 d after acclimatization

22

23 **Fig. 6** Shoot length of acclimated guayusa plants. Box plots represent the control and four light conditions, T1,
24 T2, T5, T6. Results are representative of four to six plants per treatment. (a) First day of acclimatization after
25 180 d of growth. (b) 30 days after the beginning of acclimatization. Letters denote significantly different values
26 (one-way ANOVA, $p < 0.05$)

27

1 **Fig. 7** Leaf area of acclimated guayusa plants. Box plots represent the control and four light conditions, T1, T2,
2 T5, T6. Results are representative of four to six plants per treatment. (a) First day of acclimatization after 180 d
3 of growth. (b) 30 days after the beginning of acclimatization. Letters denote significantly different values (one-
4 way ANOVA, $p < 0.05$)

5
6 **Fig. 8** Root development of acclimated guayusa plants that rooted spontaneously after 180 d. Box plots represent
7 the control and four light conditions, T1, T2, T5, T6. Results are representative of four to six plants per treatment.
8 (a) Number of main roots. (b) Number of secondary roots. Letters denote significantly different values (one-way
9 ANOVA, $p < 0.05$)

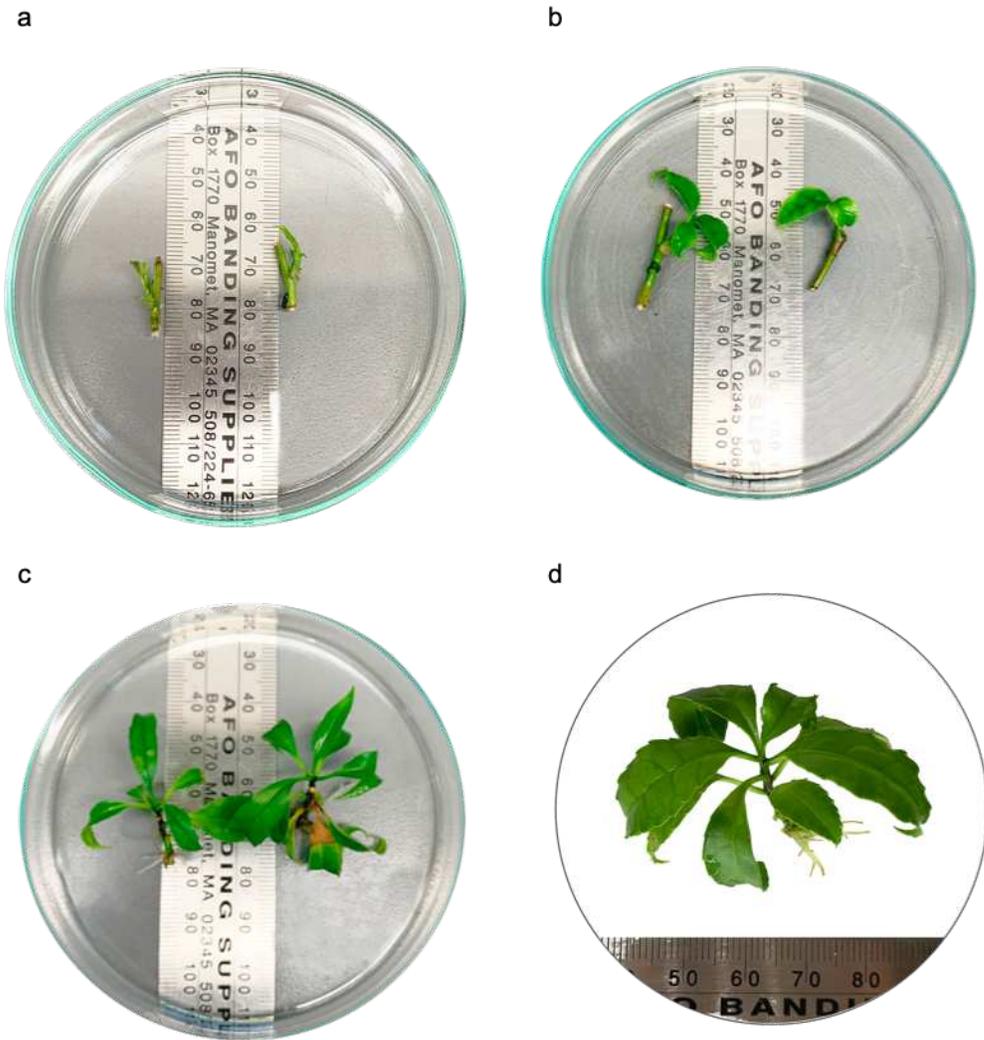
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11 **Fig. 9** Root length of acclimated guayusa plants that rooted spontaneously after 180 d. Box plots represent the
12 control and four light conditions, T1, T2, T5, T6. Results are representative of four to six plants per treatment.
13 (a) Main roots. (b) Secondary roots. Letters denote significantly different values (one-way ANOVA, $p < 0.05$)

14

1 **Figures**

2

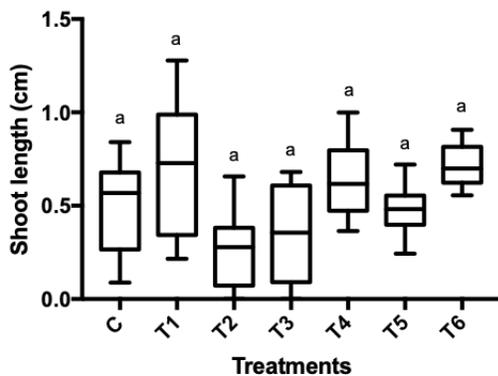
3 **Fig. 1**



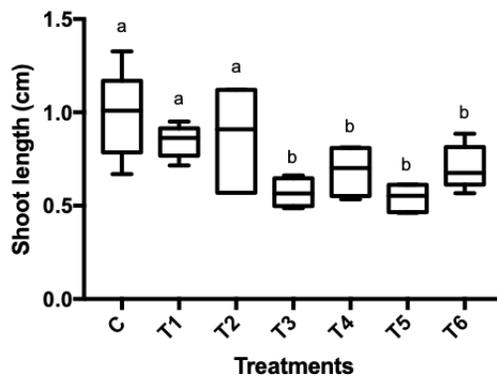
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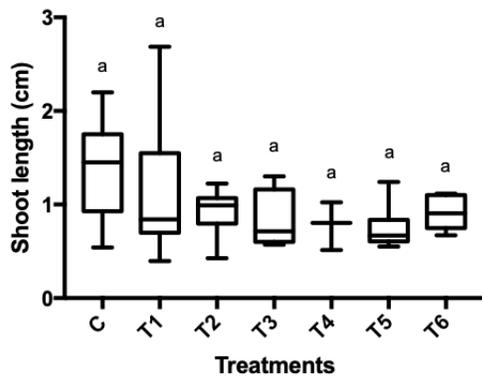
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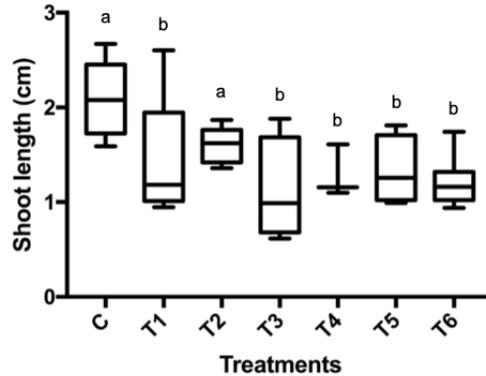
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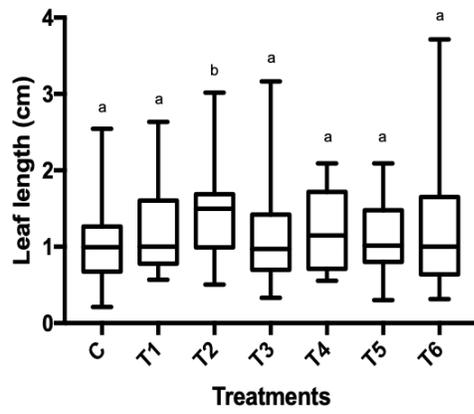
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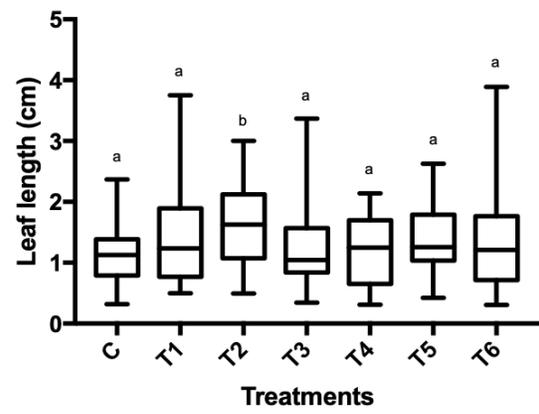
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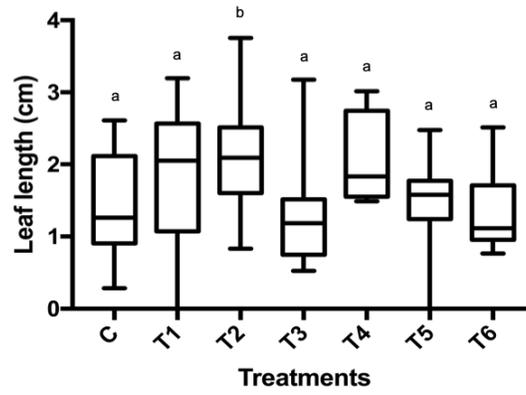
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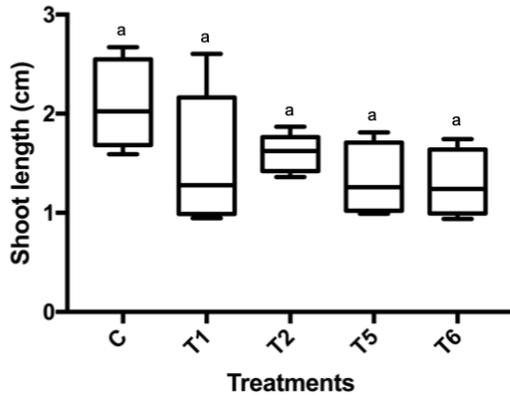
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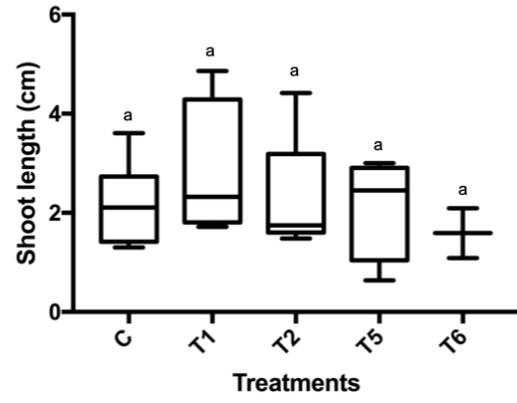
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1 Fig. 6

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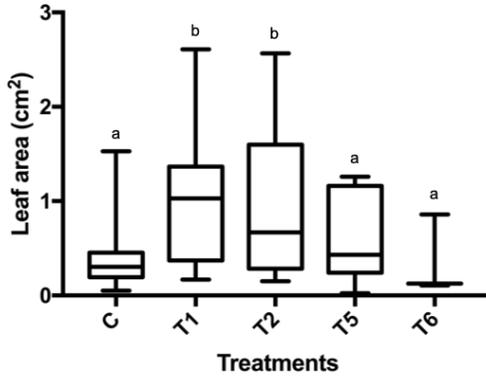


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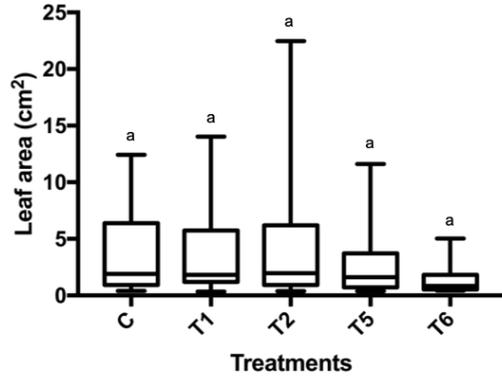
1 Fig. 7

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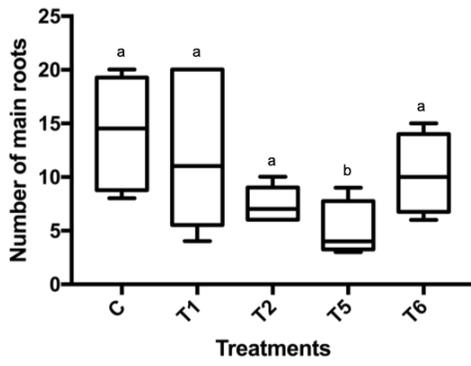
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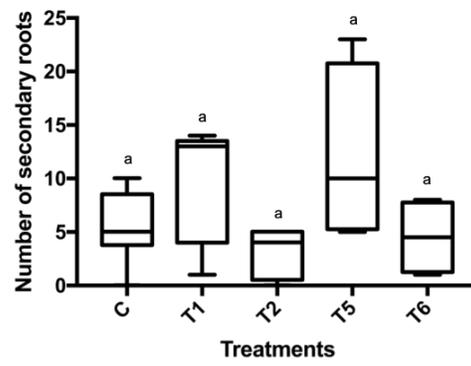
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1 Fig. 8

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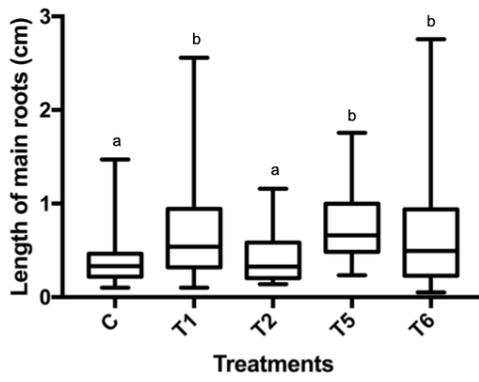
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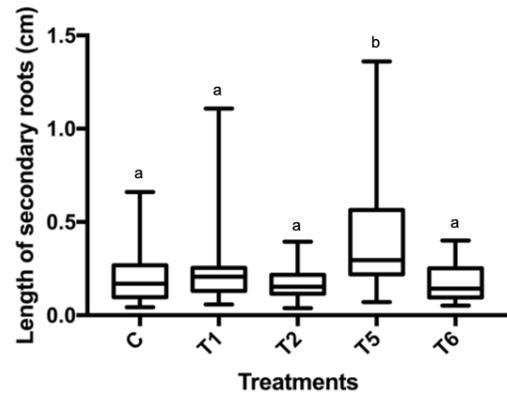
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1 Fig. 9

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b



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Figures

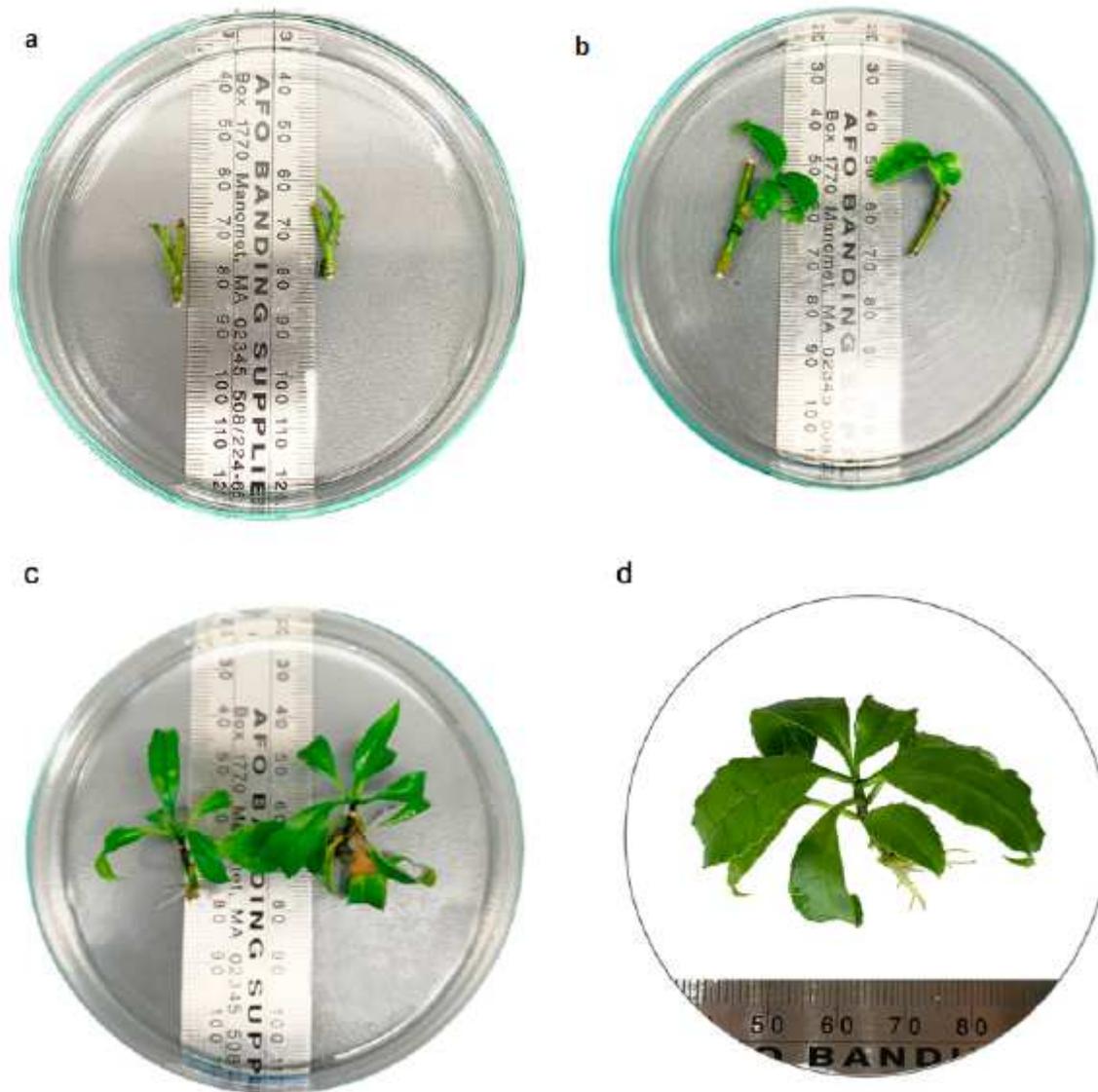


Figure 1

In vitro propagation of guayusa from axillary buds using stakes as initial material. Cuttings grown in mWPM + AC medium and exposed to light treatment T2 are shown as representative examples. (a) Developing bud on a stake. (b) 30 d-old shoots ready to be separated from the stake. (c) Shoots after 150 d. (d) Developed roots in 180 d-old shoots

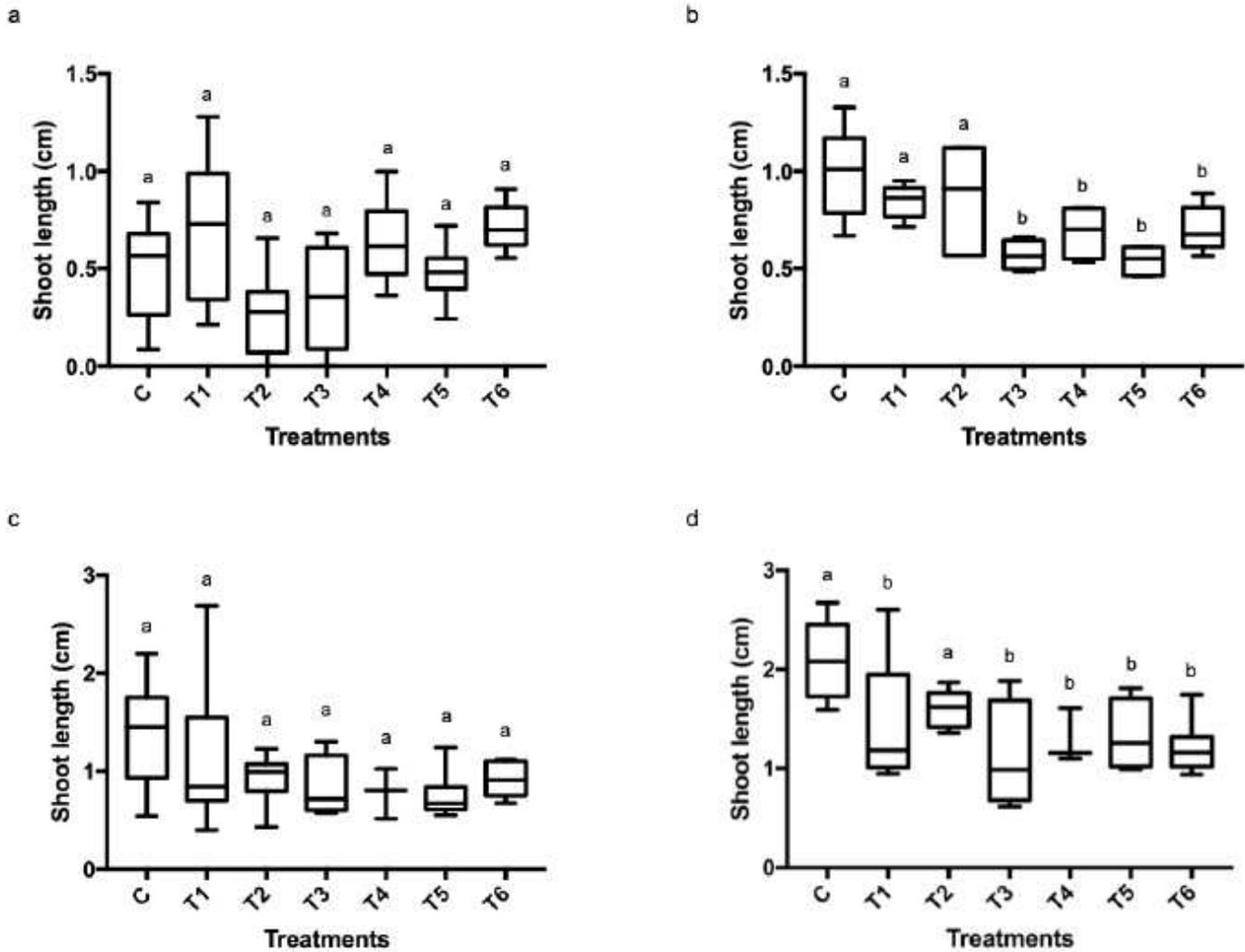


Figure 2

Shoot length in four subcultures. Box plots represent the control and six light conditions, T1 to T6. Results are representative of seven to eight plants per condition. (a) First subculture, 30 d. (b) Second subculture, 90 d. (c) Third subculture, 150 d. (d) Fourth subculture, 180 d. Letters denote significantly different values (one-way ANOVA, $p < 0.05$)

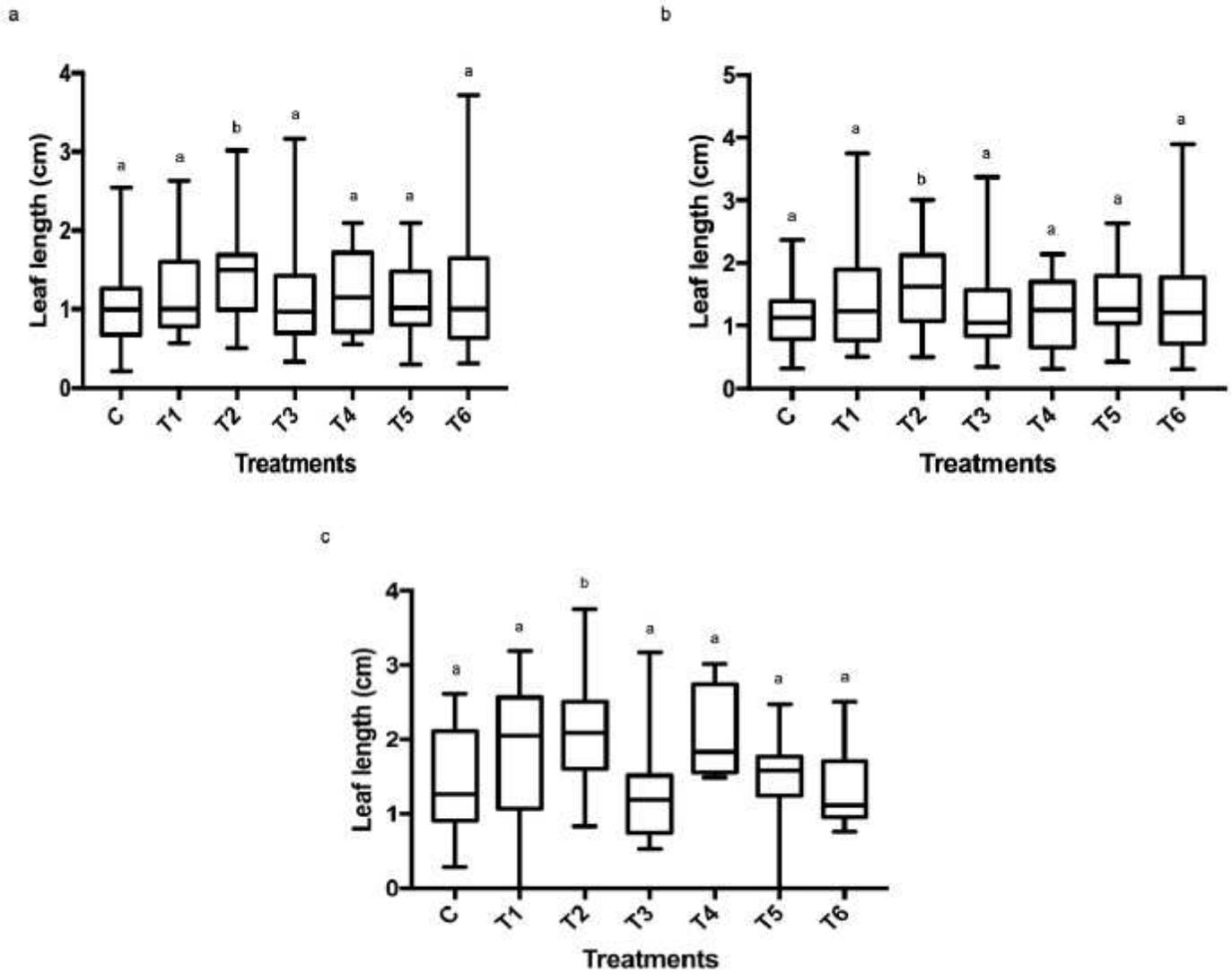


Figure 3

Leaf length in three subcultures. Box plots represent the control and six light conditions, T1 to T6. Results are representative of seven to eight plants per treatment. (a) Second subculture, 90 d. (b) Third subculture, 150 d. (c) Fourth subculture, 180 d. Letters denote significantly different values (one-way ANOVA, $p < 0.05$)



Figure 4

Spontaneous root development after 180 d. Five plantlets grown under light treatment T2 are shown as representative examples

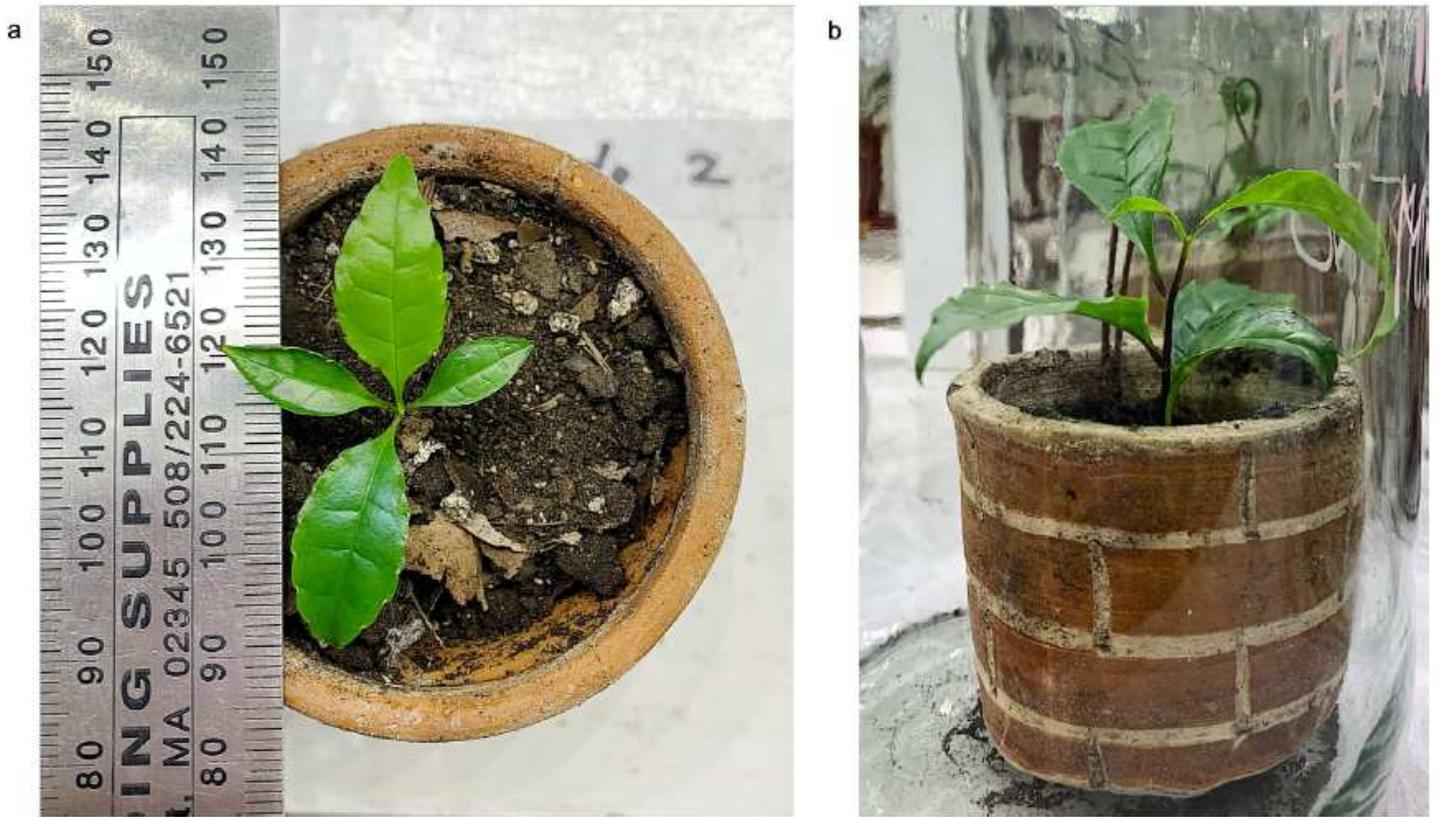


Figure 5

Acclimatization of guayusa plants. (a) Representation of the process used for data collection from acclimatized plants. (b) Guayusa plant 30 d after acclimatization

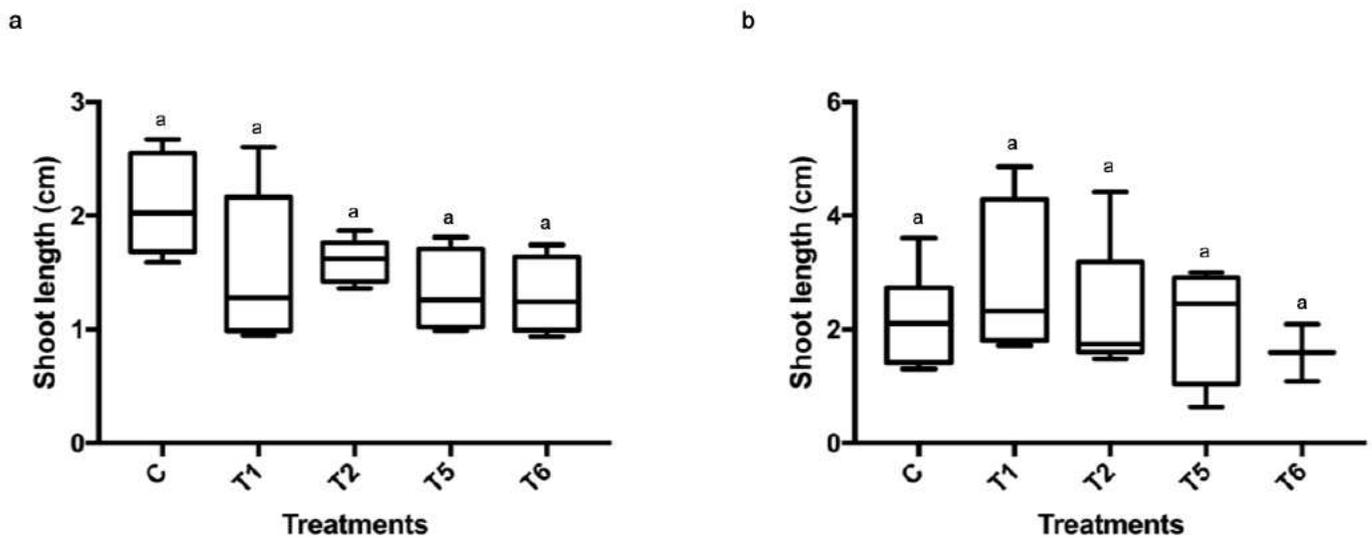


Figure 6

Shoot length of acclimated guayusa plants. Box plots represent the control and four light conditions, T1, T2, T5, T6. Results are representative of four to six plants per treatment. (a) First day of acclimatization

after 180 d of growth. (b) 30 days after the beginning of acclimatization. Letters denote significantly different values (one-way ANOVA, $p < 0.05$)

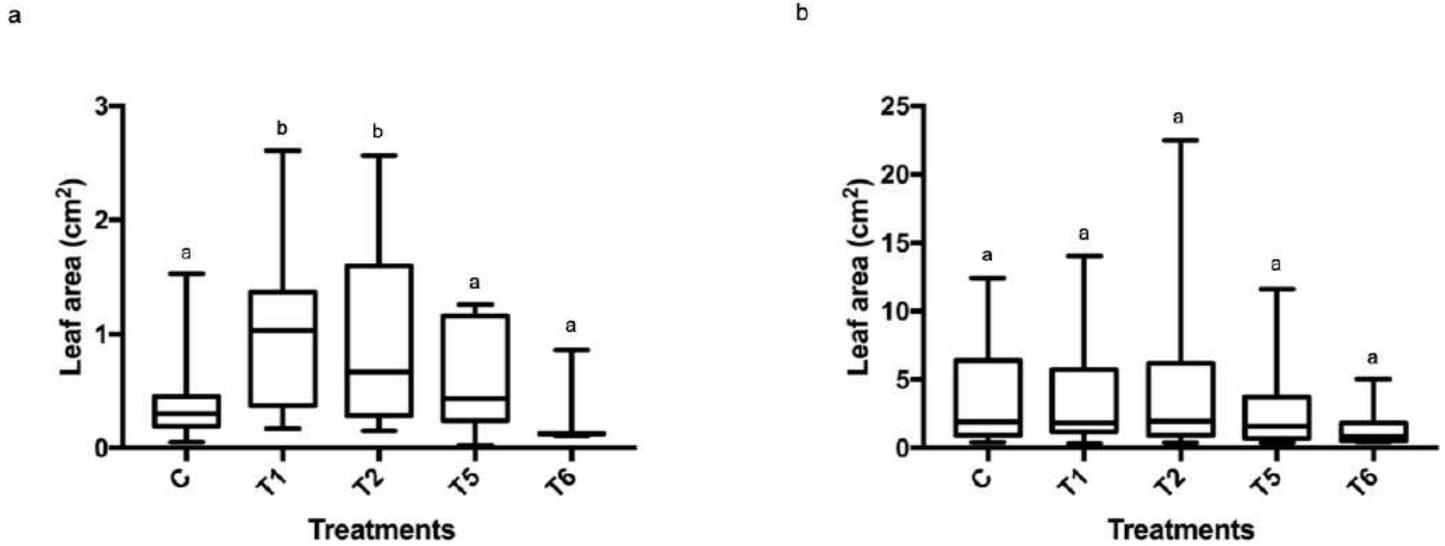


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Leaf area of acclimated guayusa plants. Box plots represent the control and four light conditions, T1, T2, T5, T6. Results are representative of four to six plants per treatment. (a) First day of acclimatization after 180 d of growth. (b) 30 days after the beginning of acclimatization. Letters denote significantly different values (one way ANOVA, $p < 0.05$)

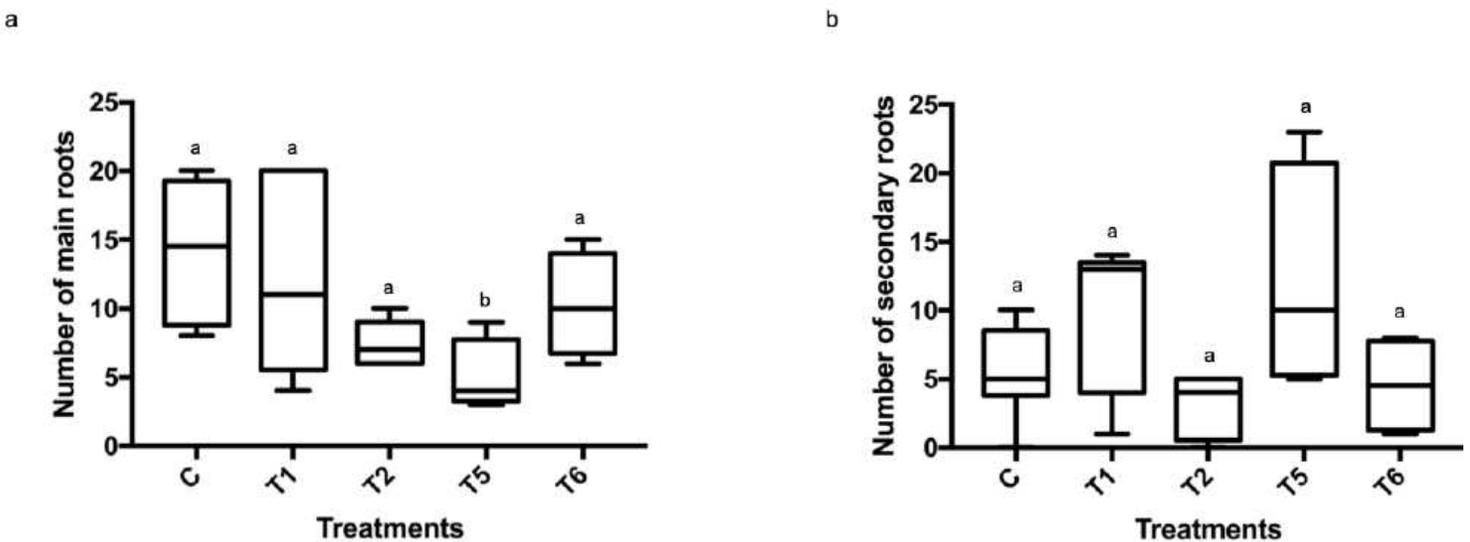


Figure 8

Root development of acclimated guayusa plants that rooted spontaneously after 180 d. Box plots represent the control and four light conditions, T1, T2, T5, T6. Results are representative of four to six plants per treatment. (a) Number of main roots. (b) Number of secondary roots. Letters denote significantly different values (one-way ANOVA, $p < 0.05$)

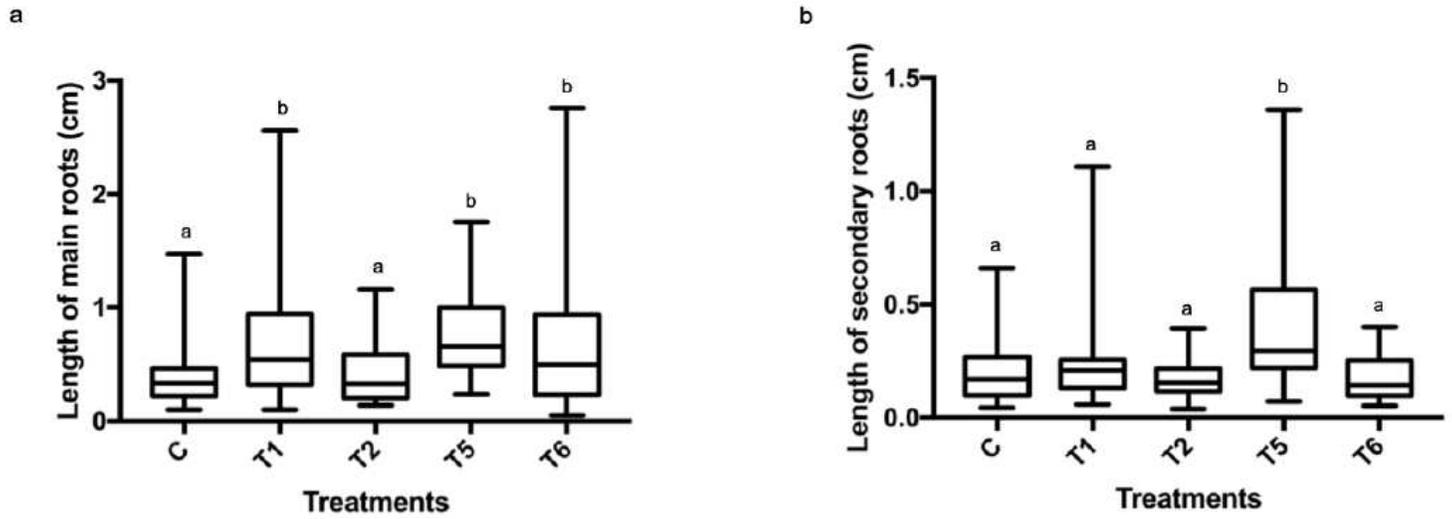


Figure 9

Root length of acclimated guayusa plants that rooted spontaneously after 180 d. Box plots represent the control and four light conditions, T1, T2, T5, T6. Results are representative of four to six plants per treatment. (a) Main roots. (b) Secondary roots. Letters denote significantly different values (one-way ANOVA, $p < 0.05$)

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