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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Keywords

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

Abstract

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

Key message

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

Abbreviations

AC: activated charcoal

B: blue light

FR: far-red light

G: green light

HY5: ELONGATED HYPOCOTYL 5

IBA: indole-3-butyric acid

LED: light-emitting diode

MS: Murashige & Skoog

mWPM: modified Woody Plant Medium

NAA: 1-naphthaleneacetic acid

R: red light

STN: shoot tip necrosis

W: white light

WPM: Woody Plant Medium

Introduction

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

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

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

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

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

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

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

Materials and Methods

Overview of the protocol

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

Plant material

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

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

Sterilization protocol

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

Axillary bud culture

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

Elongation and rooting

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

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

Acclimatization

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

Light treatments

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

Statistical analyses

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

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

Results

Effects of culture media on shoot regeneration from axillary buds

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

Shoot elongation and leaf development over four subcultures

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

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

Root formation

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

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

Acclimatization

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

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

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

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

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

Discussion

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

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

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

In vitro guayusa plants were obtained from axillary buds using stakes as initial material. The sterilization protocol showed to be effective, and after 14 days of culture, 100% of the stakes were sterile. 90% of the stakes developed shoots while 10% developed browning. Explant browning has been described in other woody plants and shrubs, including some *Ilex* species (Mroginski et al. 1999). The production of brown substances is predominantly caused by enzymatic oxidation of phenolic compounds, and often causes explant death. The toxic substances and the obstruction of oxidized tissues restrain nutrient absorption from the growth medium (Tarragó et al. 2012). Previous studies were able to reduce oxidation with AC supplementation, a carbonaceous adsorbent of aromatic compounds (de Cássia Tomasi et al. 2019). Similar results were observed in this study, and 2 g l^{-1} of AC in the growing media significantly reduced oxidation up to 90%.

Two culture media compositions were tested for shoot regeneration and elongation. WPM was first developed for shoot culture of woody plants and bushes and for tree propagation, and is currently the second most used medium for *in vitro* culture (Jain and Häggman 2007). WPM is characterized by having low salt concentrations, since many woody species are known to be sensitive to NaCl. WPM minimizes chloride levels by using sulfate salts (Mccown and Sellmer 1987). Moreover, WPM has low levels of ammonium nitrate, which prevents shoot vitrification (Huang and Dai 2011) and decreases the percentage of nodal browning in woody species (Mroginski et al. 1999). The MS medium provides similar nutritional values as WPM but presents high NH_4NO_3 and KNO_3 levels. A reduced basal concentration of this medium (1/4 MS) benefits shoot regeneration and decreases browning (Mroginski et al. 1999). In shoot regeneration of *Quercus ilex* L. (holm oak), a recalcitrant woody species of difficult propagation, MS medium yielded small leaves, while WPM resulted in larger shoots (Martínez et al. 2017). These observations are consistent with our observations where, despite the absence of significant differences, plant vigor was more evident on mWPM.

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

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

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

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

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

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

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

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

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

Conclusions

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

Declarations

Funding

This work was supported by Fundación Runa and by Universidad San Francisco de Quito USFQ, Colegio de Ciencias Biológicas y Ambientales COCIBA Grants – Hubi 12431.

Conflicts of interest

The authors have no conflicts of interest to declare.

Availability of data and material

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

Code availability

Not applicable.

Authors' contributions

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

Acknowledgments

The LED sources were installed in the plant tissue culture room with the dedicated contribution from the Physical Plant Team of USFQ. We thank Runa Foundation for sharing the guayusa plants used as source material, the Laboratory of Plant Biotechnology at USFQ for daily and continuous support during the development of this project, and Wa and David Garcia for sharing information on methods used for guayusa growth and details on production systems. We thank Venancio Arahana, Andrea Montero and María Mercedes Cobo for assisting in experimental design of preliminary propagation trials. We thank Raquel Carvalho for helpful discussion and reviewing of the manuscript.

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Tables

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

Components	Modified WPM (g l^{-1})	Original WPM (g l^{-1})
Macronutrients		
NH_4NO_3	5.4	0.4
KNO_3	3.9	-
$CaCl_2.2H_20$	2.8	0.096
$MgSO_4.7H_20$	3.7	0.37
KH_2PO_4	1.7	0.17
K_2SO_4	-	0.99
$Ca(NO_3)_2 \cdot 4H_2O$	-	0.556
Micronutrients		
H_3BO_3	0.62	0.0062
$MnSO_4.H_20$	1.69	0.0223
$ZnSO_4.7H_20$	1.05	0.0086
KI	0.083	-
$Na_2MoO_4.2H_20$	0.025	0.025
$CoCl_2.6H_20$	0.0025	-
$CuSO_4.5H_20$	0.0025	0.025
$FeSO_4.7H_20$	2.78	0.0278
Na_2 - EDTA	3.73	0.0373

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

Light treatments						
Control	**Treatment 1**	**Treatment 2**	**Treatment 3**	**Treatment 4**	**Treatment 5**	**Treatment 6**
White ($50\ \mu mol\ m^{-2}\ s^{-1}$)	**Red ($25\ \mu mol\ m^{-2}\ s^{-1}$)**	**Red ($25\ \mu mol\ m^{-2}\ s^{-1}$)**	**Red ($25\ \mu mol\ m^{-2}\ s^{-1}$)**	**Red ($25\ \mu mol\ m^{-2}\ s^{-1}$)**	**Red ($25\ \mu mol\ m^{-2}\ s^{-1}$)**	**Red ($25\ \mu mol\ m^{-2}\ s^{-1}$)**
	Blue ($25\ \mu mol\ m^{-2}\ s^{-1}$)	**Blue ($25\ \mu mol\ m^{-2}\ s^{-1}$)**	**Blue ($25\ \mu mol\ m^{-2}\ s^{-1}$)**	**Blue ($25\ \mu mol\ m^{-2}\ s^{-1}$)**	**Blue ($25\ \mu mol\ m^{-2}\ s^{-1}$)**	**Blue ($25\ \mu mol\ m^{-2}\ s^{-1}$)**
		Green ($5\ \mu mol\ m^{-2}\ s^{-1}$)	**Far red 1 ($4\ \mu mol\ m^{-2}\ s^{-1}$)**	**Far red 2 ($16\ \mu mol\ m^{-2}\ s^{-1}$)**	**Green ($5\ \mu mol\ m^{-2}\ s^{-1}$)**	**Green ($5\ \mu mol\ m^{-2}\ s^{-1}$)**
					Far red 1 ($4\ \mu mol\ m^{-2}\ s^{-1}$)	**Far red 2 ($16\ \mu mol\ m^{-2}\ s^{-1}$)**

Legends to figures

Fig. 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

Fig. 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, $p<0.05$)

Fig. 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, $p<0.05$)

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

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

Fig. 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, $p<0.05$)

Fig. 7 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, $p<0.05$)

Fig. 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, $p<0.05$)

Fig. 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, $p<0.05$)

Figures

Fig. 1

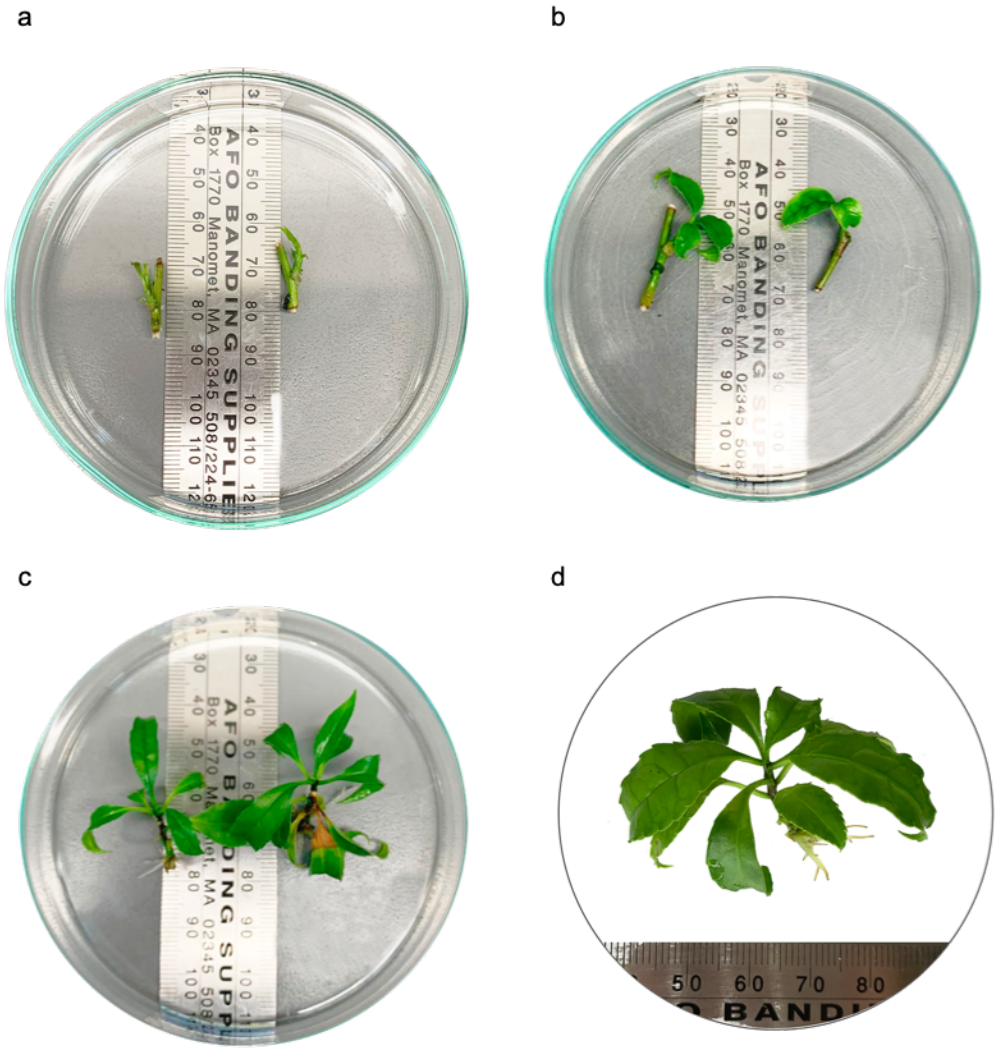


Fig. 2

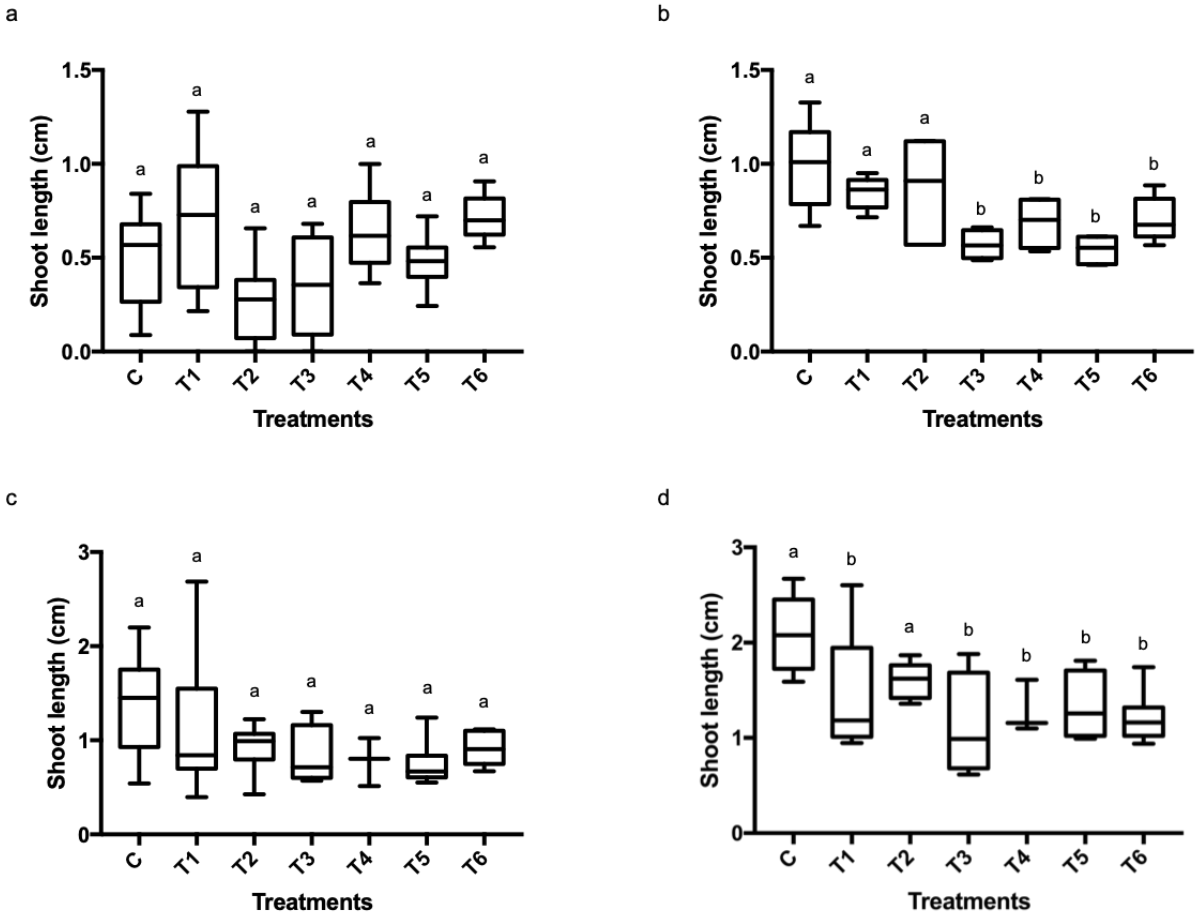

a
Shoot length (cm)
1.5
1.0
0.5
0.0
a a a a a a a
C T1 T2 T3 T4 T5 T6
Treatments
b
Shoot length (cm)
1.5
1.0
0.5
0.0
a a a b b b b
C T1 T2 T3 T4 T5 T6
Treatments
c
Shoot length (cm)
3
2
1
0
a a a a a a a
C T1 T2 T3 T4 T5 T6
Treatments
d
Shoot length (cm)
3
2
1
0
a b a b b b b
C T1 T2 T3 T4 T5 T6
Treatments

Fig. 3

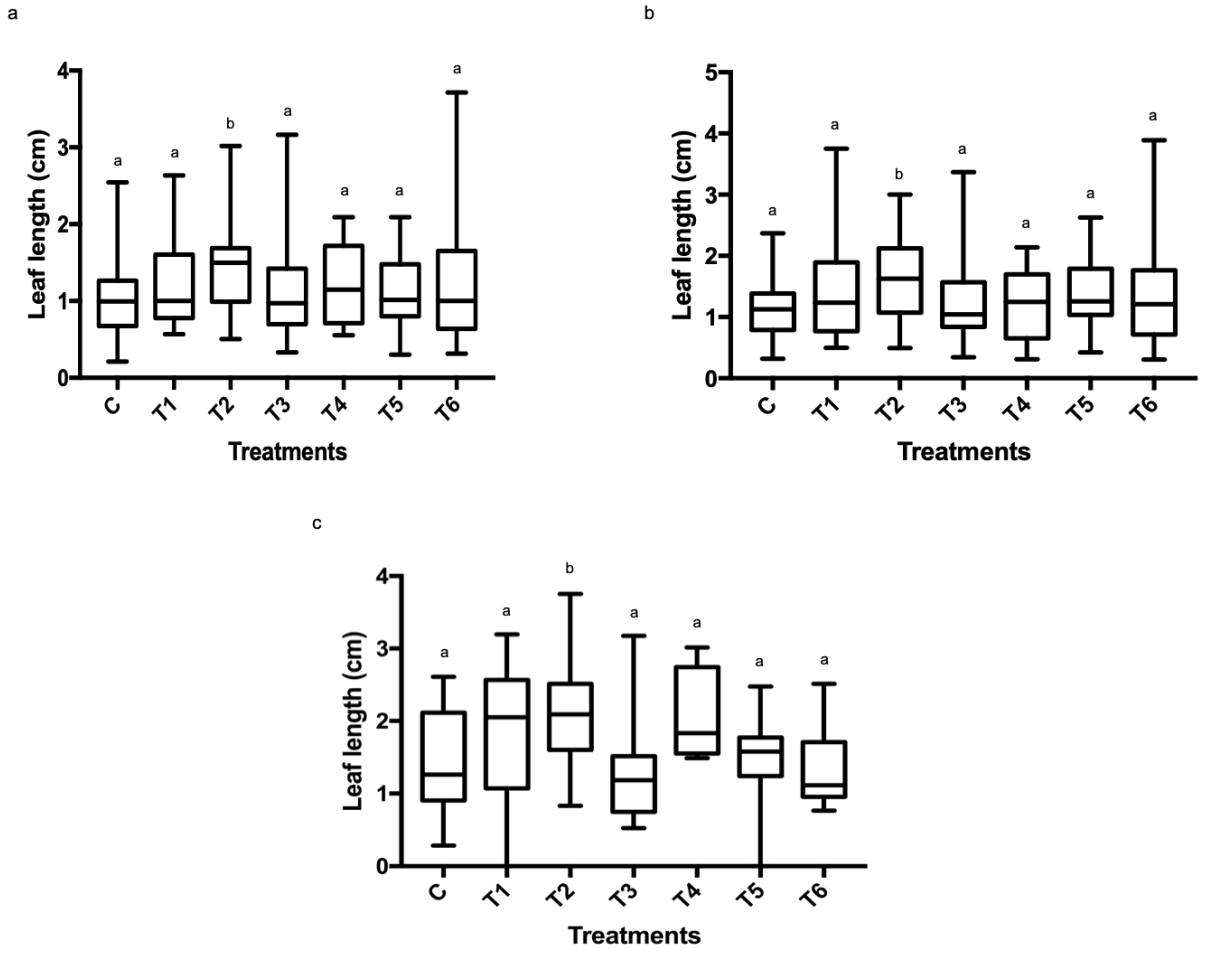

a
Leaf length (cm)
0
1
2
3
4
a a b a a a a
C T1 T2 T3 T4 T5 T6
Treatments
b
Leaf length (cm)
0
1
2
3
4
5
a a b a a a a
C T1 T2 T3 T4 T5 T6
Treatments
c
Leaf length (cm)
0
1
2
3
4
a a b a a a a
C T1 T2 T3 T4 T5 T6
Treatments

Fig. 4

Fig. 5

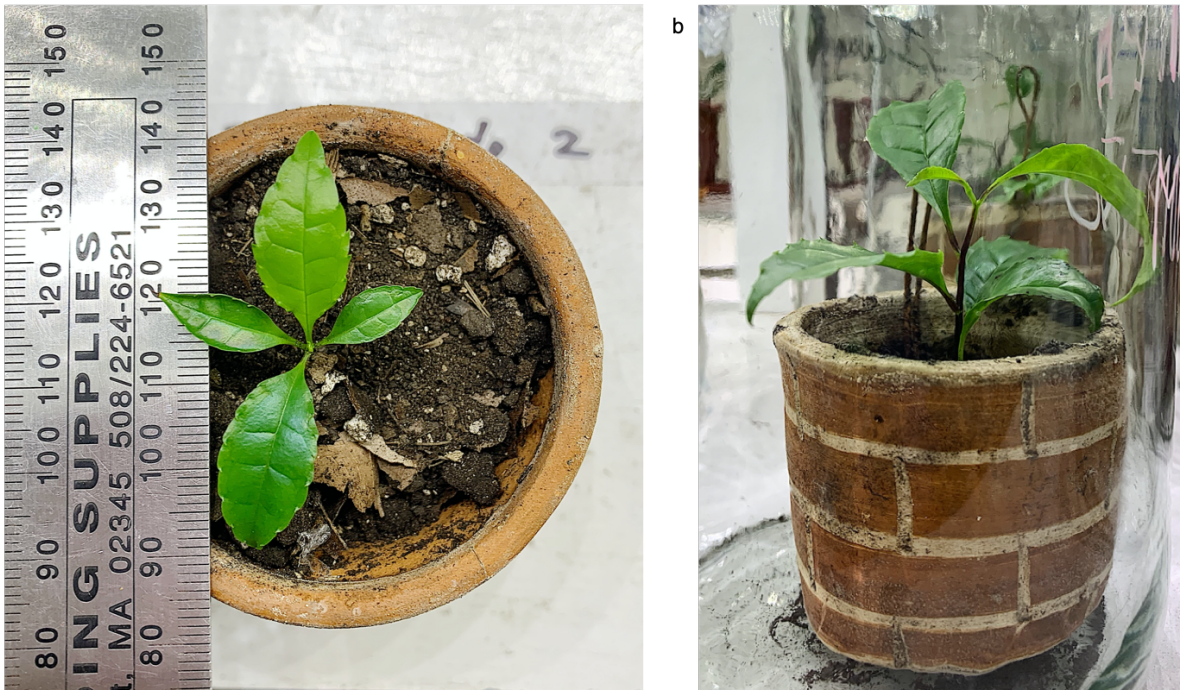


Fig. 6

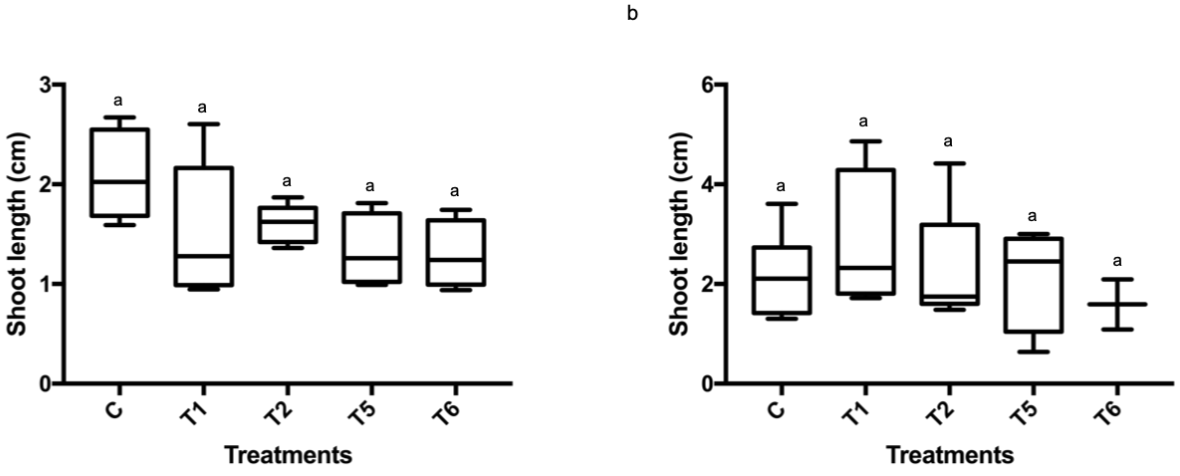


Fig. 7

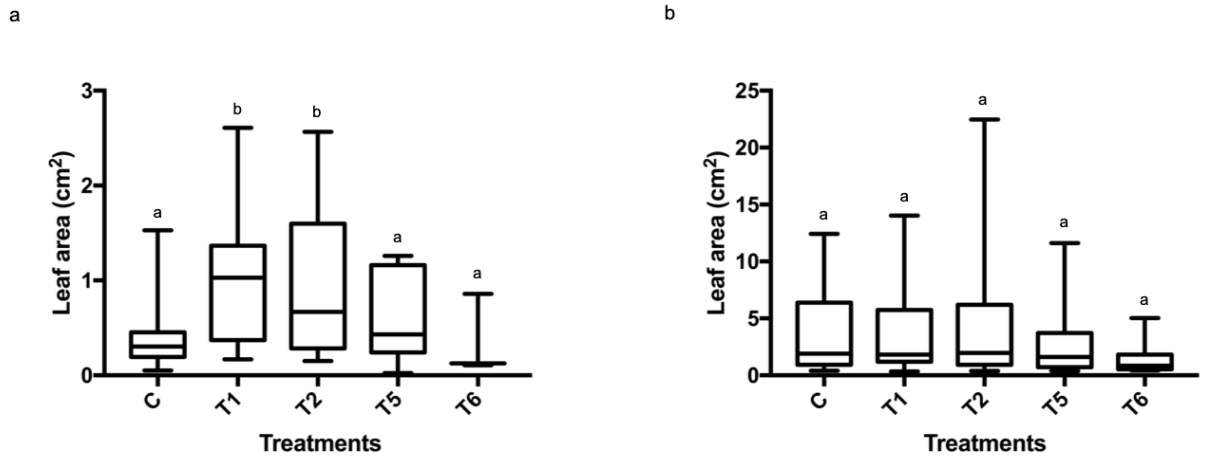


Fig. 8

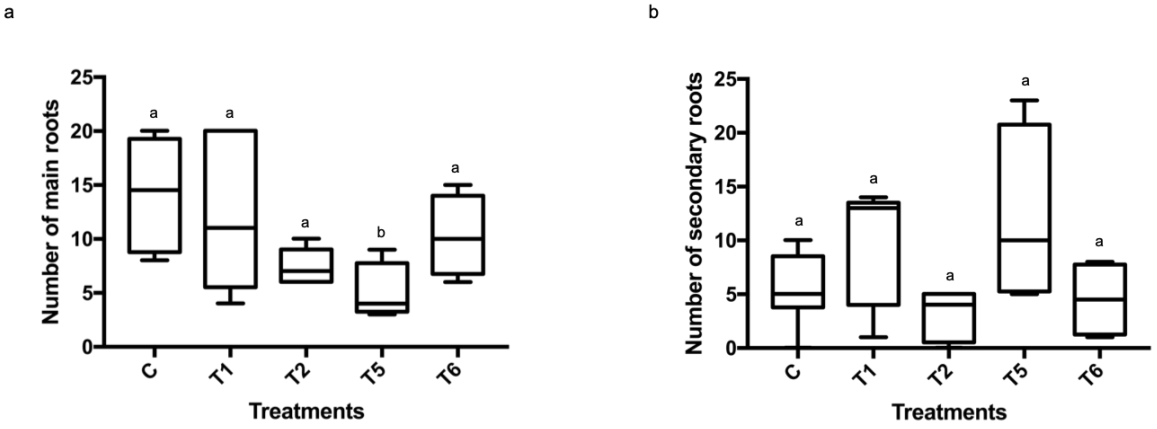
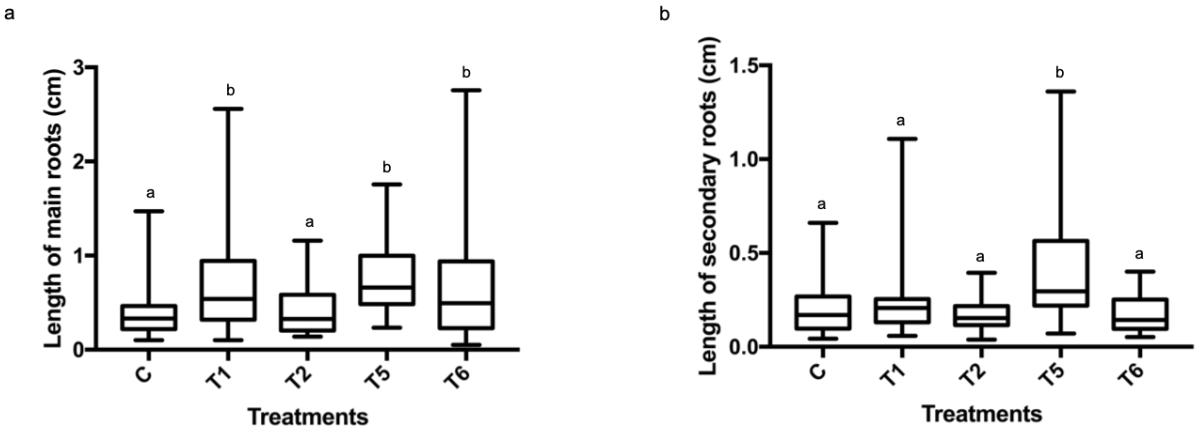

a
Number of main roots
25
20
15
10
5
0
a
a
a
b
a
C
T1
T2
T5
T6
Treatments
b
Number of secondary roots
25
20
15
10
5
0
a
a
a
a
a
C
T1
T2
T5
T6
Treatments

Fig. 9


a
Length of main roots (cm)
3
2
1
0
a
b
a
b
b
C
T1
T2
T5
T6
Treatments
b
Length of secondary roots (cm)
1.5
1.0
0.5
0.0
a
a
a
b
a
C
T1
T2
T5
T6
Treatments