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SHORT COMMUNICATION

Effects of leaflets and indole-3-butyric acid in the vegetative propagation by mini-tunnels of rubber tree (*Hevea brasiliensis*)

Geomar Vallejos-Torres[1] · Orlando Ríos-Ramírez[1] · Mike Anderson Corazon-Guivin[1] · Edgar Reátegui[2] · Francisco Mesén Sequeira[3] · César Marín[4,5]

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Abstract
Rooting induction of leafy stem cuttings of *Hevea brasiliensis* can be used as a relevant tool for the cultivation, conservation of genetic diversity, and rescue of this species in Peru, decimated by decades of extractive practices. In this study, we evaluated the effects of different numbers of leaflets and concentrations of indole-3-butyric acid (IBA) in the rooting capacity of leafy stem cuttings of *H. brasiliensis*. Cuttings 6–7 cm long were collected from stockplants in a germplasm bank; leaves were pruned to 2 or 5 leaflets, and the cutting base was treated with one of the following IBA concentrations: 0, 1000, 2000, 3000, 4000, or 5000 ppm. Cuttings were placed in trays with a sandy substrate and transferred to plastic mini-tunnels with fogging irrigation. After 29 days, cuttings with 5 leaflets and 2000 ppm of IBA showed the best results in survival (100%), rooting percentage (79%), and roots number (4.1) and length (2.9 cm). The high conversion rate of cuttings to plants indicates the feasibility of this technique as a simple and effective tool, both for the conservation and the commercial propagation of selected rubber tree genotypes.

Keywords Amazonas · Indole-3-butyric acid · Mini-tunnels · Peru · Rubber tree · Vegetative propagation

Introduction

The Euphorbiaceous genus *Hevea* is native to the Amazon plains, with native populations still found in Bolivia, Brazil, Colombia, and Peru [7]. *H. brasiliensis* ('jebe' or rubber tree) is the most commercially important species of the genus, due to the latex extracted from the tree. Latex is the raw material of a series of products that have an enormous impact on industrial, economic, and social development worldwide, as a component of a large number of essential products for society, including industrial and surgical gloves, hoses, footwear, adhesives, and car accessories and tyres for all types of transport [13]. Currently, commercial plantations of the rubber tree can be found in South America, Southeast Asia, and West Africa [34].

Latex was discovered by the ancient Olmecas, Mayas, and Aztecas, who used it to make balls, waterproof suits, and even handmade shoes [34]. The industrial production of natural rubber began in the mid-nineteenth century driven by the discovery of vulcanisation (1839), the invention of the rubber chamber tyres (1888), the manufacture of latex gloves (1890), and the emergence of the car industry in the late nineteenth century. During these earlier times, the main source of latex was the South American Amazon jungle. However, the increasing demand and price led to predatory practices of latex extraction by the end of the nineteenth century [17]. In the Peruvian Amazon jungle, the Tahuamanu Province, in the Madre de Dios region, is considered the productive area with the best quality of wild, native rubber [17].

✉ César Marín
cesar.marin@uoh.cl

1 Facultad de Ciencias Agrarias (UNSM) – Tarapoto, Universidad Nacional de San Martín, Jr. Maynas N° 177, Tarapoto 22160, San Martin, Peru

2 Cooperativa Agroindustrial Cauchera Caynarachi L.T.D.A., Km. 2, Carretera Pongo de Caynarachi – Barranquita, Lamas, Peru

3 Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), Turrialba, Cartago 30501, Costa Rica

4 Institute of Agri-Food, Animal and Environmental Sciences (ICA3), Universidad de O'Higgins, 3070000 San Fernando, Chile

5 Center of Applied Ecology and Sustainability, Pontificia Universidad Católica de Chile, 8320000 Santiago, Chile

Exploitation of the rubber tree in the Tahuamanu Province showed a steady decline in the last decades, but it was totally stopped in 1990 [17]. This was caused by several factors, viz. irrational extraction practices, the disappearance of the Peruvian Agrarian Bank that subsidised the rubber price, and an import of 99.99% of the rubber utilised from Asian plantations at lower prices, among other factors [30]. However, the latex extracted from Peruvian rubber trees is still used in modern rubber processing, and sometimes, it represents a substantial source of income for local farmers [34]. In Peru, small farmers commonly grow rubber trees in the regions of San Martín, Huánuco, and Madre de Dios [17].

There is a strong need to plant, conserve, and rescue rubber trees in the Peruvian Amazon. However, the use of heterogeneous and low-quality germplasm would only hinder these processes. Vegetative propagation by the induction of rooting of cuttings represents an effective, economic, and short-term option for massively replicating selected genotypes [38, 44], and constitutes an alternative for the rescue of this economically important species. In Peru, cuttings' rooting in plastic mini-tunnels has proven to be an effective technique in a large number of tropical species, such as *Calycophyllum spruceanum* [44], *Coffea arabica* [43], *Plukenetia volubilis* [35, 38], and *Simarouba amara* [39], among others. This technique allows to transform thousands of cuttings into plants in an efficient way, using little space and time [43, 44]. Similarly, it has been shown that the use of auxins combined with an optimal leaf area has been very successful in promoting the rooting of several tropical species [6, 9], as each species has an optimal leaf area for promoting rooting. Rooting reaches its maximum when cuttings are most photosynthetically active, as young leaves produce auxins and co-factors that are transported to the plant base, inducing rooting [27].

Clonal micro-propagation is a promising tool for the rescue of native rubber tree populations, as this species shows high level of hermaphroditism, aspect that deeply affects the reproductive strategies of this species. Rubber tree seeds are still widely used to reproduce this species in the Amazon, but usually yields are lower than expected due to the high variation in vigour and latex production [7]. Thus, unlike seed propagation, clonal propagation of rubber tree allows for the selection of bigger, more vigorous genotypes, with higher latex production, and which are less prone to an early production of lateral and axillary shoots [33]. This study aimed to evaluate the effects of different number of leaflets and different concentrations of indole-3-butyric acid (IBA) on the survival, roots number and length, and rooting percentage of leafy stem cuttings of *Hevea brasiliensis* grown in plastic mini-tunnels with fogging irrigation.

Materials and methods

Site and source of material

The study was conducted in the experimental nursery of the National University of San Martín—Tarapoto (UNSM-T), San Martin region, Peru, between July and August, 2019. The UNSM-T is located at 278 m.a.s.l. and has an average temperature of 24.6 °C, a monthly rainfall of 63.1 mm, and an average relative humidity of 45% [36].

Cuttings were collected from 24 rubber tree stockplants established in the UNSM-T germplasm garden, originated from seeds of different trees from a rubber tree plantation located in Tocache Province, San Martin region, Peru. Mother trees were selected based on their latex production capacity (Fig. 1a). Stockplants were planted in pots filled with a 3:1:1 forest soil:vermiculite:sand mixture (3 kg overall), in a greenhouse with 50% shade clothing and water irrigation twice a day. At the time of shoot harvesting, stockplants were 3 months old.

Preparation of cuttings

The collection of shoots was carried out in an orderly manner, carefully labelling each explant, and leaving at least one leaf in each stockplant. The two compound leaves on each shoot were pruned to half and shoots were transported to the propagation area in an icebox with water and fungicide (thiophonate methyl 2 g/L) to minimise the physiological stress they suffer after severance (Fig. 1b). Once in the propagation area, 6–7 cm-long cuttings were prepared making a straight cut just above the node and leaving 2 or 5 leaflets (75–100 cm^2/leaflet) in each cutting, accordingly. Prior to the application of auxins, cuttings were soaked for 5 min in a solution of sodium hypochlorite diluted (2%) in sterilised water, and then disinfected in a fungicide solution for 3 min.

Treatments and experimental design

The cuttings bases were treated with one of five concentrations of chemically pure indole-3-butyric acid (IBA) (Merck Co., USA) dissolved in 96% alcohol: 0, 1000, 2000, 3000, 4000, or 5000 ppm, directly applied in the cutting base using a 10-μl micropipette to ensure that all cuttings received the same amount of solution (Fig. 1c). Then, the alcohol was evaporated by exposing the cutting base to a stream of cold air for 20 s.

Cuttings were placed in trays of 40 cm × 50 cm × 5 cm filled with a sterilised substrate, inserting the cuttings at a 2-cm depth, and slightly pressing them it with the fingers to avoid leaving air between the hole and the cutting base.

Fig. 1 Growth process of *Hevea Brasiliensis.* **a** One selected tree. **b** Shoots harvested in the germplasm bank. **c** Application of 10 µl of indole-3-butyric acid (IBA) to the cuttings' base. **d** Cuttings establishment in sterilised substrate. **e** Rooted cuttings. **f** Acclimatisation of rooted cuttings

According to a granulometric analysis at the UNSM-T Laboratory of Soils, Geology, and Concrete, the substrate was composed of 0.12% gravel, 94.59% sand, and 5.29% silt and clay. The substrate was previously washed with running water, rinsed four times with sodium hypochlorite, sun-dried, and autoclaved for sterilisation (131 °C × 15 lb pressure for 2 h) (Fig. 1d).

Trays with cuttings were transferred to plastic mini-tunnels for the rooting phase. Mini-tunnels are currently being used in the UNSM-T for the rooting of coffee cuttings [43]. They are made of a galvanised metal frame with dimensions 1 m × 3 m × 0.60 m, with 1-m-long support legs, and covered with white transparent plastic which allows the passage of diffuse sunlight. The mini-tunnels had three internal nebulisers connected to a timer-controlled system, which produced 1-min fogging every 2 h from 7 a.m. to 5 p.m., equivalent to 21 L of water daily per mini-tunnel. This watering regime maintained a relative humidity above 75% and temperatures between 28 and 35 °C throughout the rooting phase.

The cuttings sprout after 29 days, after which they were extracted to evaluate the number of cuttings rooted, and the roots number and length (Fig. 1e). Rooted cuttings were planted in 3-L pots filled with a 3:1:1 forest soil:vermiculite:sand mixture, and transferred to a nursery for a 30-days period of acclimatisation. The nursery had a shade clothing that allowed the entry of 20% sunlight during the first 10 days, and 50% during the following 20 days, with frequent irrigation 3–5 times per day (Fig. 1f).

A completely randomised design with a 2×6 factorial arrangement was used, considering two values for the number of leaflets (2 and 5), and six IBA concentrations (0, 1000, 2000, 3000, 4000, and 5000 ppm), and 16 cuttings per treatment. Thus, 192 cuttings in total were analysed.

Table 1 Effects of (a) the number of leaflets and of (b) indole-3-butyric acid (IBA) dose on the vegetative propagation of *Hevea brasiliensis* by leafy stem cuttings, 29 days after rooting induction

	Survival (%)	Rooting (%)	Number of roots	Root length (cm)
a. Number of leaflets				
2	50.00^{a}	36.11^{b}	1.22^{b}	1.17^{a}
5	67.70^{a}	48.60^{a}	2.09^{a}	1.49^{a}
Mean	58.85	42.36	1.66	1.33
b. IBA dose (ppm)				
0	56.25^{ab}	37.50^{ab}	0.91^{b}	1.23^{ab}
1000	71.87^{ab}	48.96^{ab}	1.78^{b}	1.91^{a}
2000	81.25^{a}	63.54^{a}	2.91^{a}	2.03^{a}
3000	56.25^{ab}	43.75^{ab}	1.66^{b}	1.22^{ab}
4000	43.75^{b}	30.21^{b}	1.34^{b}	0.77^{b}
5000	43.75^{b}	30.21^{b}	1.34^{b}	0.87^{b}
Mean	58.85	42.36	1.66	1.33

Mean values in each column followed by different letters are statistically different, according to a Tukey test ($p < 0.05$)

Parameters evaluated and statistical analyses

Tukey tests ($p < 0.05$) were carried out for cuttings survival (number of successful cuttings), rooting percentage (proportion of cuttings that generated roots), and roots number and length. Analyses of variance were carried out in order to assess the effects of treatments interactions in the above-mentioned parameters. Prior to the analyses, data on rooting percentage and survival were transformed by the formula arcsin $\sqrt{\%}$, and the number of roots by the formula $\sqrt{x+1}$ [37]. Statistical analyses were performed using the statistical software InfoStat [10].

Results

After 29 days, there were no significant differences in the survival of rubber tree cuttings with 2 or 5 leaflets (Table 1a). Regarding the use of different indole-3-butyric acid (IBA) doses, the highest survival was obtained with 2000 ppm (81.25%), percentage significantly different from those of other doses (0, 1000, and 3000 ppm formed a group apart, also different from 4000 and 5000 ppm) (Table 1b). Overall, the highest survival (100%) was obtained in cuttings with five leaflets and a 2000 ppm IBA dose—being significantly different with the treatment of two leaflets at this dose (Fig. 2).

Rooting percentage varied according to the number of leaflets and IBA concentration, with the highest percentage (48.61%) in cuttings with 5 leaflets (Table 1a), or treated with 2000 ppm of IBA (63.64%) (Table 1b). Rooting had the

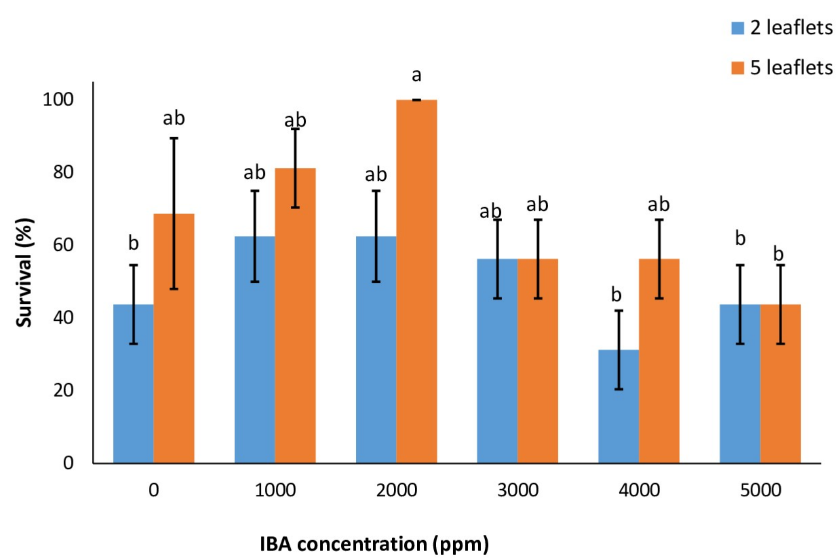

Fig. 2 Survival of *Hevea brasiliensis* cuttings according to the number of leaflets and indole-3-butyric acid dose, 29 days after rooting induction. Bars represent SD, letters represent significant differences by the Tukey test ($p < 0.05$)

exact same pattern as survival: 2000 ppm of IBA formed a group apart from 0, 1000, and 3000 ppm, also different from 4000 and 5000 ppm (Table 1b). Rooting showed a parabolic curve with respect to the IBA dose, reaching the highest percentage at 2000 ppm of AIB, and decreasing with either lower or higher doses (Table 1b; Fig. 3a). The highest rooting (79.1%) was obtained in cuttings with five leaflets at 2000 ppm of IBA, which was significantly different from the treatment with two leaflets at the same dose, and with all other treatment combinations (Fig. 3a).

Roots number and length had significant differences according to the number of leaflets and IBA dose, with a pattern very similar to the one for rooting percentage. On average, a larger number of roots per cutting and longer roots were obtained in cuttings with five leaflets (Table 1a) and 2000 ppm of IBA, which formed a group apart from all other doses (Table 1b). Overall, the highest number of roots (4.1, Fig. 3b) and root length (2.9 cm, Fig. 3c) were obtained with the treatment combination of five leaflets and 2000 ppm of IBA, combination that was significantly different from the rest.

The analyses of variance show that the number of leaflets significantly affected rooting and especially the number of roots, while the IBA dose just affected root length, and both factors—but not their interaction—affected plant survival (Table 2). The interaction between both factors (number of leaflets and IBA dose) also did not affect any other parameter measured (Table 2).

Discussion

We found a similar pattern of plant responses to auxin applications than in a significant number of other agroforestry species [3, 8, 16, 18, 21, 23, 32, 35]: an increased rooting capacity by increasing the auxin dose used until reaching a plateau, after which any increase in concentration results in a rooting decrease, due to the toxic effects of overdose. Our results are also similar to the first studies investigating the propagation of rubber tree in Malaya [41]. The positive effects of auxins on rooting have been known since the 1930s with the discovery of the natural auxin indolacetic acid (IAA), and the establishment of its role in plant rooting and growth [3]. The benefits of auxins as rooting promoters have been associated with an increase in carbohydrate transport and to other leaf co-factors to the site of application, as well as an augmentation in DNA synthesis in treated cells, which result in higher cell division [11, 16]. A greater cell division implies greater photosynthetic performance, spectral reflectance, water potential, cell tissue electrical conductivity, and chlorophyll content, all factors that strongly affect plant survival [46]. The concentration of 2000 ppm of indole-3-butyric acid (IBA) has also been found optimal for rooting cuttings of various eucalypt species [5, 15, 42], coffee plants [43], *Plukenetia volubilis* [35], and of a number of tropical broadleaves such as *Alnus acuminata, Bombacopsis quinata, Cedrela odorata, Gmelina arborea*, and *Swietenia macrophylla* [23].

The synthetic auxin IBA is by far the most used in rooting programmes [3, 16, 43]. It has the advantage that, contrary to the natural auxin IAA, it does not dissolve in water nor it is oxidised by light, therefore, it remains longer in the site of application and it maintains its effect over longer periods of time [16]. As with the rooting percentage, the response pattern of the number and length of roots of rubber tree cuttings followed a parabolic trend, which shows an optimal IBA dose of 2000 ppm, with a decrease with either sub-optimal or excessive doses of the auxin.

The number and length of roots in cuttings are highly influenced by their ability to supply carbohydrates from reserves or produced by photosynthesis, to the root formation area [14, 26, 31, 45]. Therefore, the presence of leaves in this type of juvenile cuttings is essential for successful rooting [19, 23]. Photosynthesis measurements during the propagation process have shown that rooting was maximised when cuttings were most photosynthetically active [24, 29]. In very small leaves, carbohydrates production is lower, which could be insufficient to support the growth of new roots. On the other hand, a large and photosynthetically active leaf also loses more water by transpiration and can suffer water stress, closing its stomata, and thus limiting photosynthesis [1, 19].

Therefore, a successful rooting requires a leaf area that achieves an optimal balance between the positive effects of photosynthesis and the negative effects of transpiration [19, 23]. The leaf area associated with this balance depends on the biology and morphology of each species, the environment prior and during the propagation process, the cutting age and its position in the shoot (lignification), among other factors [19]. In the case of *Hevea brasiliensis*, our results suggest that this balance was achieved in cuttings with five leaflets, which possibly promoted adequate photosynthesis, without excessive loss of water by transpiration. Our results are consistent with other studies for forest species as *Calycophyllum spruceanum* [44], *Prunus africana* [40], *Gmelina arborea* [9], *Plukenetia volubilis* [35], *Cabralea canjerana* [12], *Cheilocostus speciosus* [2], and *Prosopis alba* [8], among others. Overall, those studies show that a proper leaf area stimulated the rooting process [27], due to an increased photosynthetic activity during propagation. In the case of the rubber tree, we need further studies with more leaflets treatments to corroborate (or not) these results, and to calculate more precisely the optimal range of leaf area and IBA dose to achieve optimal rooting.

The use of plastic mini-tunnels, which in our study maintained a high relative humidity throughout the entire process,

Fig. 3 Root traits of *Hevea brasiliensis* cuttings, according to number of leaflets and indole-3-butyric acid concentration, 29 days after rooting induction. Bars represent SD, letters represent significant differences by the Tukey test ($p < 0.05$). **a** Rooting percentage. **b** Number of roots. **c** Root length

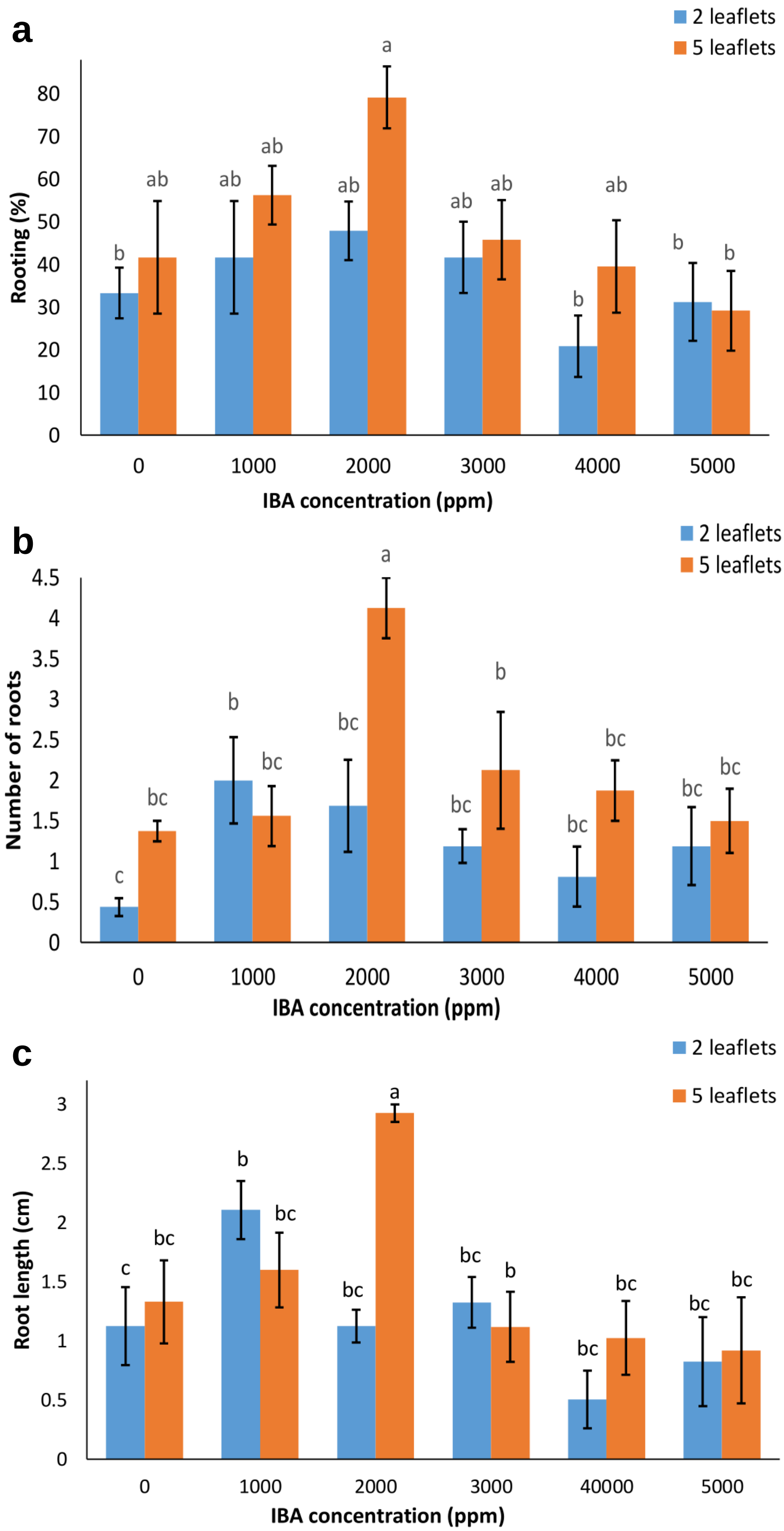

Table 2 Analyses of variance showing the effects of the number of leaflets, indole-3-butyric acid (IBA) dose, and their interaction on the vegetative propagation-related parameters of *Hevea brasiliensis*

Factor	Survival (%)	Rooting (%)	Number of roots	Root length (cm)
No. of leaflets	6.520*	4.383*	17.338***	3.368^{ns}
IBA dose	5.850*	3.381^{ns}	0.030^{ns}	10.286**
No. of leaflets: IBA dose	1.023^{ns}	0.297^{ns}	0.001^{ns}	0.002^{ns}
R^2	0.067	0.041	0.085	0.068

$F^{p-value}$ shown; $p<0.001$: ***, $p<0.01$: **, $p<0.05$: *, *ns* non-significant

was undoubtedly an essential tool in minimising the effects of transpiration on the cuttings and has also been successfully used in a wide variety of other species [20, 23, 25, 39, 43].

Since tissues with low lignification levels and an adequate physiological condition have a higher chance of emitting adventitious roots, another key factor in rooting success is the type of plant material used [22]. In studies with mini-cuttings of hybrid *Eucalyptus globulus*, Borges et al. [4] observed that a reduction in tissue lignification promoted higher rooting rates in such mini-cuttings. There is numerous literature about vegetative propagation using leafy stem cuttings that report a gradual change in plants from an easy-to-root juvenile state, to a mature state associated with low rooting capacity [19]. This reduction in rooting capacity is generally related to the inability of the tissues to return to the meristematic state as they mature, which is a necessary requirement for the emission of adventitious roots [16]. The frequent pruning carried out in stockplants in our germplasm garden aims at maintaining juvenility in the shoots, a benefit that was evident in the high rooting percentages obtained.

Murillo et al. [28] considers that 70% is the minimum rooting percentage required to justify a commercial clonal propagation programme. In our study, the high rooting percentage (79.16%) of rubber tree cuttings obtained with 5 leaflets at 2000 ppm of IBA indicates that the technique of rooted cuttings in mini-tunnels offers a viable alternative for the development of clonal programs of *H. brasiliensis*. With this treatment combination, probably greater photosynthetic and hormonal activities were promoted, enabling the rooting process and its nutritional needs.

Conclusion

The best rooting results in *Hevea Brasiliensis* 6–7-cm-long cuttings set to root in plastic mini-tunnels with fogging, were obtained with five leaflets and an application of 2000 ppm of indole-3-butyric acid (IBA). With this treatment combination, after 29 days, a rooting percentage of 79.16% was obtained, with an average of 4.1 roots per cutting, and an average root length of 2.9 cm. These results indicate the feasibility of propagating *H. brasiliensis* by leafy stem cuttings using mini-tunnels, which opens up great possibilities to start initiatives for the rescue of rubber trees with better characteristics, particularly with higher latex production, as well as germplasm conservation, and genetic improvement programmes. Particularly, we think that our results are applicable to other rubber tree germplasms, if they are under similar environmental conditions. Higher success rates could be expected with further research on other substrates, different leaf areas and leaflets numbers, cutting lengths, irradiance levels, among other factors.

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Availability of data and material The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest We declare that we do not have any conflict of interest.

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