Micropropagation of iraca palm (Carludovica palmata Ruiz y Pav)using a temporary immersion system

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Research Article

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Abstract

Carludovica palmata is a neotropical plant with a promising potential for the agroindustry. It is an important source of fibers used to manufacture hand-made goods. Colombia is one of the leading countries in the production this species, however, it lacks efficient techniques for its propagation. We developed a protocol for massive micropropagation of *C. palmata* using Temporary Immersion Bioreactor (TIB) system. Immersion frequency, immersion times, culture medium volume, and explant density were evaluated using a split-split-plot design. The variables evaluated were number and length of shoots and roots, number of leaves, and explants dry weight. The performance of three micropropagation systems, BIT, semi-solid medium, and liquid medium, were evaluated using generalized randomized block design. Murashige and Skoog (MS) culture medium with 1,0 mg L^{-1} of 6-Benzylaminopurine, 0,5 mg L^{-1} of 1-Naphthaleneacetic acid, and 20 g L^{-1} of sucrose added to it was used. Results obtained was frequency 12 h with immersion time 1 min improved the length of both shoots and roots, as well as the number of leaves and dry weight. Volume of 20 ml/explant was found to be adequate condition to increase the number and length of shoots, the number of leaves, and the dry weight. According to the optimization module, the factors levels that will maximize the evaluated variables are immersion frequency 12 h and immersion time 1 min, with 175 mL of culture medium per bioreactor and density of 10 explants. In conclusion, TIB was shown to be efficient for massive micropropagation of *C. palmata* compared with conventional methods.

Key Message

This investigation showed that In vitro iraca palm´s multiplication was highly efficient in a BIT system, with 12 h immersion frequency, 1 min immersion, and 20 mL/explant of culture media.

Introduction

Carludovica palmata Ruiz and Pav (iraca) is a plant belonging to the Cyclanthaceae family. It can be found in a large geographic area ranging from the center to the south of the American continent. It is an emblematic species of neotropical ethnobotanics, (Sehremmer 1982; Fadiman 2001). Fibers are its most widely used sub product. They are used as raw material for the manufacture of a wide variety of artisanal products among them the Panama hat (Iglesias 1999; Gálviz et al. 2019).

Nowadays, this species has acquired considerable economic importance due to its great versatility, since it can be used in the agroindustry as a dye, additive, and source of “palmito” (Murillo et al. 2021); by the chemical industry, to produce artisanal paper and as an important alternative for reinforcement of polymeric matrices (Moo et al. 2019); by the pharmaceutical industry, as a potential source of secondary metabolites such as capsorubin and phenolic compounds like coumarin, quercetin, chlorogenic acid, caffeic acid, and gallic acid, known among other things as anti-inflammatories, antioxidants, antimicrobial agents (Gálviz et al. 2021; Murillo et al. 2021), as well as aiding in the phytoremediation of contaminated soil (Garcés et al. 2017).

Sexual reproduction in *C. palmata* is slow and presents low rate of seed germination (Zambrano et al. 2022). For this reason, asexual reproduction using rhizomes is widely preferred (Gonzáles et al. 2004), however, such technique has its own limitations – for instance, it does not allow for high rates of multiplication (Graca et al. 2014) and it affects the integrity of the source plant due to the wounds caused by the extraction of the propagules, exposing it to severe infections by phytopathogens (Agrios 1998).

This calls for the need to provide seedlings as planting material of high phytosanitary quality for new agricultural projects (Muñoz y Tuberquía 1999; Gálviz et al. 2021). Even though, there is currently no efficient technique for the multiplication of *C. palmata*, which hinders the productivity of this species. The advancements that have been made in this area using techniques such as micropropagation have not yet reached the point where they would be enough to guarantee mass propagation (Hoyos et al. 2019). For that reason, it is imperative that more efficient methods for propagation and production of plants are developed to implement iraca crops on a larger scale.

Automatic Temporary Immersion Systems (ATIS) are technologic tools created to maximize the advantages of conventional micropropagation (Ramírez et al. 2019; Martínez et al. 2019). They function by temporarily generating contact between plant tissue and the culture medium (Välimäki et al. 2020), and regulating the supply of nutrients, bioregulators, oxygen (O_2), carbon dioxide (CO_2), and detrimental gasses such as ethylene (Etienne y Berthouly 2002; Vives et al. 2017), which generates optimal conditions for morphogenesis in the micropropagated plant material (Uma et al. 2021).

One of such systems is the Twin-Flask temporary immersion bioreactor (BIT®), which consists of two containers – one of them, which acts as a culture chamber, while the other stores the culture medium (De Carlo et al. 2021). Such a design favors the proper ventilation of the plant material and culture medium (Villamarín et al. 2019), improving the production of secondary metabolites, mixotrophic capacity and reduce cellular respiration which allows for the generation of larger, and great biomass plantlets with a more robust root system (Bello et al. 2021; Mancilla et al. 2021). The BIT® system has been successfully used in the micropropagation of several plant species, such as *Stevia rebaudiana* (Alvarenga y Salazar 2015), sugarcane (*Saccharum officinarum*) (Martínez et al. 2020), and taro (*Colocasia esculenta* L) (Arano et al. 2020), confirming its benefits.

The goal of this research was to establish a more efficient protocol for the massive propagation of *C. palmata in vitro* using a BIT® system, thus generating high-quality plant material to repopulate areas where this species is commonly grown.

Materials And Methods

The current research was developed in the Biotechnology Laboratories of the Faculty of Agricultural Sciences of the National University of Colombia in Medellín. The environmental conditions of the laboratory where the experiments were carried out were a constant temperature of 23°C +/− 2°C, a

luminosity of approximately 1500 lux, photosynthetically active radiation of 90 µmol of photons m^2 s^{-1} and a photoperiod of 12/12 h of light and dark.

Plant material

As starting material used was some seedling clones of *C. palmata* regenerated *in vitro* at the Plant Biotechnology laboratory of the National University of Colombia in Medellin. They were in a range of 5 to 7 months old with an average height of 8 cm and a stem diameter of at least 0.5 cm. From these seedlings, we isolated explants of stem segment with apical meristem of approximately 2 cm length.

Experimental step

For the experimental step in the BIT® system different volumes of liquid culture medium (50, 100 and 200 mL) were transferred to one of the bottles that comprise the bioreactor, after which they were sterilized in an autoclave (Trident, Taiwan) at 15 psi, 121°C for 15 min. Five or ten explants, depending on the treatment, were put in sterile conditions inside one of the bottles of the bioreactor.

For the other two techniques, namely liquid and semi-solid, 20 mL of culture medium were dispensed in sterile conditions in each of the glass containers (FLINT container, 245 CC B. 53, screw top). Five explants were put in each bottle, which were then closed hermetically and placed beside the bioreactors of the BIT® system.

The culture medium used in the three micropropagation systems was composed of full MS salts, supplemented with 1 mg L^{-1} of 6-Benzylaminopurine (BAP), 0.5 mg L^{-1} of 1-naphthaleneacetic acid (NAA) and 20 g L^{-1} of sucrose. For the semi-solid medium, 4 g L^{-1} of phytagel was added.

Experimental design

Four factors were considered to find the best experimental conditions for mass multiplication of *C. palmata* using the TIB system: density of explants per bioreactor (5 and 10 explants), volume of culture medium per bioreactor (50, 100, and 200 ml), time of immersion (1, 3, and 5 min), and frequency of immersion (6 and 12 h). The factors were assigned to the experimental units based on a split-split-plot design: the frequency of immersion was assigned to the whole plots (full action of the BIT®) based on a completely randomized design with two replications. Time of immersion were assigned to the split-plots (shelves), each conformed by six bioreactors. The number of explants and culture medium volume were assigned to the split-split-plots, that is, the bioreactors comprised of bottles of 1L capacity with explants of *C. palmata*. The response variables evaluated were number of shoots per explant, length of shoots, number of roots, length of roots, number of leaves and shoots dry weight. We chose the best two treatments of the BIT® system to compare them with the semisolid medium and liquid medium. Since neither the semisolid medium nor the liquid one allows controlling the factors associated with immersion (frequency and time), the comparison was performed using a generalized randomized block design, blocking in accordance with the running time.

Statistical analysis

All data were analyzed with the statistical software R, V. 4.1.3 (R Core Team 2022). For the TIB experimental data a split-split-plot model was fitted with the lmer{lmerTest} function. Normality of residuals was assessed using the Shapiro-Wilk test (shapiro.test{stats}). Differences between treatments were evaluated by the LSD test with Holm correction for multiplicity within each family (difflsmeans{lmerTest}). All tests were evaluated at a 0.05 significance level.

A Generalized Randomized Block Model was fitted (aov{stats}) to compare the three *in vitro* micropropagation methods. Differences between treatments were evaluated with a significance level of 0.05. Normal distribution and homogeneity were tested with Shapiro-Wilk and Levene test, respectively. Pairwise comparisons were performed by the HSD Tukey test.

Given that all the evaluated factors were numerical, a full second order response surface model was fitted to each of the dependent variables using Statgraphics Centurion, version XVI. We also used this software to generate the factor combinations levels which were expected to optimize the set of response variables evaluated in the micropropagation of *C. palmata.*

Results

The analysis of variance (ANOVA) carried out for the variables number and length of shoots and number and length of roots showed significant statistical differences for second order interactions. The ANOVA for the variables number of leaves and dry weight showed significant differences for first and third order interactions, respectively. Therefore, we analyzed the corresponding simple effects. Treatments that showed the highest mean of number of shoots per explant were 6 h as frequency, 5 min as the immersion time with 100 mL (10.60 shoots) and 200 mL (11.32 shoots) of the culture medium In contrast, the lowest mean of shoots were frequency of 12 h, immersion time of 1 min (2.90 shoots), 3 min (2.62 shoots) and 5 min (4.17 shoots) in 50 mL of culture medium. The treatments that showed the highest shoot length were 12 h as frequency, 200 ml of culture medium (20 and 40 ml/explant) and the immersion time of 1, 3 and 5 min with a mean of 2.21, 2.44 and 2.37 cm, respectively. On the other hand, the shortest shoot length was obtained at 6 h as frequency with 50, 100 and 200 ml of culture medium and the immersion time of 1, 3 and 5 min (Table 1).

The highest mean length for roots was obtained in the follow parameters: 12 h of frequency, 100 mL of culture medium and the immersion time of 1 min (1.57 cm) and 3 min (1.44 cm). On the hand, the lowest mean was 0.61 cm for a frequency of 6 h, 200 mL of culture medium and 1 min of the immersion time. Likewise, it was showed that treatments with a frequency of 6 h, volume of 50, 100 and 200 mL of culture medium and 5 min immersion time were the most inefficient parameters for increasing shoot and root length (Table 1).

Table 1
Simple effect of frequency, immersion time, volume of culture medium, mean over two explant densities on micropropagation of *C. palmate*

Treatments Frequency/volume/time	Number of shoots	Length of shoots (cm)	Length of roots (cm)
Fr6 / 50mL / 1min	4.02 Ab^{a}	0.89 Ab^{b}	0.92 Aba^{b}
Fr6 / 50mL / 3min	3.97 Ac^{a}	0.69 Ab^{b}	1.12 Aa^{a}
Fr6 / 50mL / 5min	4.43 Ab^{a}	0.62 Aa^{b}	0.70 Ba^{b}
Fr6 / 100mL / 1min	7.60 Ba^{a}	1.02 ABb^{b}	1.05 Aa^{b}
Fr6 /100mL / 3min	5.65 Cb^{a}	1.37 Aa^{b}	0.98 Aba^{b}
Fr6 / 100mL / 5min	10.60 Aa^{a}	0.64 Ba^{b}	0.68 Ba^{b}
Fr6 / 200mL / 1min	8.00 Aa^{a}	1.56 Aa^{b}	0.89 ABa^{a}
Fr6 / 200mL / 3min	8.05 Aa^{a}	1.48 Aa^{b}	1.09 Aa^{b}
Fr6 / 200mL / 5min	11.32 Ba^{a}	0.67 Ba^{b}	0.61 Ba^{b}
Fr12 / 50mL / 1min	3.90 Ac^{a}	1.42 Ab^{a}	1.19 Ab^{a}
Fr12 / 50mL / 3min	2.62 Ab^{a}	1.12 Ab^{a}	1.02 Ab^{a}
Fr12 / 50mL / 5min	4.17 Aa^{a}	1.16 Ac^{a}	1.10 Aa^{a}
Fr12 / 100mL / 1min	5.35 Ab^{b}	2.06 Aa^{a}	1.57 Aa^{a}
Fr12 / 100mL / 3min	4.91 Aa^{a}	2.11 Aa^{a}	1.44 Aa^{a}
Fr12 / 100mL / 5min	5.50 Aa^{b}	1.81 Ab^{a}	1.25 Aa^{a}
Fr12 / 200mL / 1min	6.87 Aa^{a}	2.21 Aa^{a}	0.89 Bc^{a}
Fr12 / 200mL / 3min	4.72 Aa^{b}	2.43 Aa^{a}	1.41 Aa^{a}
Fr12 / 200mL / 5min	4.55 Aa^{b}	2.37 Aa^{a}	1.20 Aa^{a}

Means labeled with the same letter are not significantly different ($\alpha = 0.05$) (Labels A, B, C for time of immersion with fixed levels frequency of immersion and volume; a, b, c for volume with fixed levels of frequency of immersion and immersion time; ^{a}, ^{b} for frequency of immersion with fixed levels of immersion time and volume of medium)

Explants density didn´t show significant differences for the variable number of shoots, except for the treatment, Fr12 / 200mL/10ex, where the density of 10 explants improved shoot production (5.96), in comparison with an inoculum of 5 explants (4.80). For the variable, number of roots, there were two treatments that showed a biggest mean of 13.26 roots, one then has the following parameters: 6 h of frequency, 100 mL of the culture media and 10 explants. The other, has 6 h of frequency, 200 mL of the culture media and 10 explants. In comparison with the same parameters of frequency, and volume, but 5 explants a mean of 10.75 root in the volume of 100 mL and 10.10 roots in a volume of 200 mL were obtained. On the other hand, the treatments with 12 h frequency, explant density did not show significant differences (Table 2).

Table 2
Simple effect of frequency of immersion, volume of culture medium, explant density mean over immersion time on micropropagation of *C. palmata*

Treatments **Frequency/volume/explant density**	**Shoot Number**	**Root Numbers**
Fr6 / 50ml /5ex	4.46 Ac^{a}	12.03 Aa^{a}
Fr6 / 50ml /10ex	3.82 Ab^{a}	9.85 Bb^{a}
Fr6 / 100ml /5ex	7.60 Ab^{a}	10.76 Ba^{a}
Fr6 / 100ml /10ex	8.30 Aa^{a}	13.26 Aa^{a}
Fr6 / 200ml /5ex	9.40 Aa^{a}	10.10 Ba^{a}
Fr6 / 200ml /10ex	8.85 Aa^{a}	13.26 Aa^{a}
Fr12 / 50ml /5ex	3.63 Ab^{a}	8.46 Ab^{b}
Fr12 / 50ml /10ex	3.50 Ab^{a}	8.46 Ab^{a}
Fr12 / 100ml /5ex	5.46 Aa^{b1}	11.83 Aa^{a}
Fr12 / 100ml /10ex	5.04 Aa^{b1}	11.38 Aa^{b}
Fr12 / 200ml /5ex	4.80 Bb^{a1}	9.26 Ab^{a}
Fr12 / 200ml /10ex	5.96 Aa^{b1}	9.45 Aab^{b}

Means labeled with the same letter are not significantly different ($\alpha = 0.05$) (Labels A, B, for explant density with fixed levels frequency of immersion and volume of medium; a, b, c for volume of medium with fixed levels of frequency of immersion and explant density; ^{a}, ^{b} for frequency of immersion with fixed levels of explant density and volume of medium)

Regarding the number of leaves per shoot, the most favorable conditions were a frequency of 12 h with 3 min of the explant immersion in a volume 200 mL of culture medium by bioreactor. In such a conditions, a mean of up to 3.03 leaves was achieved. In contrast, the lowest number of leaves was obtained with a frequency of 6 h and a time of immersion of 5 min, leading to a mean of 1.56 leaves per shoot. The number of explants by bioreactor showed no significant difference in number of leaves.

The last variable evaluated in this study was shoots dry weight. A 12 h frequency of immersion with a 1 min of the immersion time, 200 mL of culture medium and a density of 10 explants by bioreactor proved to be the most desirable treatment to increase the mean of plantlet biomass up to 1.36 g. Contrary, the lowest dry weight (0.26 g) was showed with a frequency of 6 h, an immersion time of 5 min in 50 mL of culture media, and a density of five explants by bioreactor (Fig. 1).

Three micropropagation systems of C. palmate in Vitro **systems**

To evaluate the multiplication efficiency of the three micropropagation systems, two best treatments of BIT® system were chosen and compared with the semisolid and liquid medium. Treatments in the BIT® system were 1 and 3 min of the immersion times, a frequency of 12 h, in 200 mL of culture medium and 10 explants. The semisolid culture medium showed the highest number of shoots per explant (10.29 shoots), followed by the BIT® system with 1 and 3 min immersion (7.29 and 6.05 shoots consecutively), and liquid culture medium which presented the lowest average (3.50 shoots) (Fig. 2-a). The longest shoot length was obtained with 1 and 3 min immersion (2.61 and 2.03 cm respectively), followed by the semi-solid and liquid culture system which evidenced the shortest shoot lengths (0.72 and 0.61 cm) (Fig. 2-b).

The BIT® system with 1 min of immersion time showed the highest number of roots (12.5), while the liquid medium showed the lowest number (4.18 roots) (Fig. 2-c). Evaluating root length, the BIT® system with 3 and 1 min immersion showed the greatest length with 1.44 and 1.11 cm, respectively, followed by the semi-solid medium (0.62 cm) and liquid medium, which showed the least root length (0.52 cm) (Fig. 2-d). The BIT® system with 3 and 1 min of immersion time reached the highest number of leaves (3.63 and 2.91 leaves), followed by the semi-solid and liquid medium, which did not differ statistically (Fig. 2-e). For the dry weight variable, BIT® system with 1 min immersion presented the highest mean (1.36 g) and the lowest values were for semi-solid medium (0.27 g) and liquid medium (0.20 g) (Fig. 2-f).

Discussion

Scientific research on micropropagation of *C. palmata* is extremely limited, therefore, the discussion, in part, is based on results obtained in other species. The results of this research indicate that a short frequency (6 h) increases the number of shoots and roots compared to a long one (12 h), this agrees with results obtained in carnation (*Dianthus caryophyllus* L) (Ahmadian et al. 2017) and banana (Rasthali AAB-Silk) (Uma et al. 2021) where short frequencies increase the number of shoots and roots per explant compared to a prolonged one, however, this condition is not always the most appropriate since they favor tissue hyperhydricity, a physiological disorder that is not observed in this research.

In contrast, shoot length, roots, leaf number and dry weight of *C. palmata* are favored by long frequency (12 h). This agrees with that reported by several researchers who obtain greater growth and development of shoots and roots with prolonged frequencies (12 h) (Mosqueda et al. 2017; Uma et al. 2021). Similar trend is reported by Aka et al. (2020) who achieve increased shoot length, roots, number of leaves and dry weight with prolonged frequency (8 h), when compared to a short frequency (4 h) in myrtle (*M. communis*). However, these frequencies are not always optimal to achieve a higher number of shoots and roots per explant (Arano et al. 2020; Pramita et al. 2018), concomitant with what was reported in our study. A possible explanation for the increase in shoot and root length with prolonged frequencies is that plants respond to water stress with the synthesis of abscisic acid, which is synthesized mainly in roots and leaves (Parkash y Singh, 2020). This phytohormone is responsible for giving plant material tolerance to drought stress (Taiz y Zeiger, 2006), therefore, it can be assumed that with a prolonged frequency, the plant material is exposed to dry periods, so it is assumed that the roots and leaves begin to synthesize abscisic acid, as a mechanism of tolerance to adverse conditions, which could stimulate an increase in the length of shoots and roots by the action of this phytohormone.

The findings of this research show that immersion time plays an important role in the micropropagation phase of *C. palmata*. It is evident that the number of shoots per explant is a function of longer immersion time (5 min). Similar trends are shown by Rachmi et al. (2019) and Alvarenga and Salazar (2015) who indicate that a prolonged immersion time is critical to increase the rate of shoot proliferation in *Aquilaria malaccensis* and *S. rebaudiana*, however, this immersion time is not always optimal to generate plant material of excellent morphological quality, since a prolonged immersion time can cause undesirable physiological disorders such as asphyxia, hyperhydricity and formation of reactive oxygen species, negatively affecting the quality of propagated plant material (Uma et al. 2021).

The results of this research show that a short immersion time (1 min) is the most suitable condition to increase shoot length, root system development, leaf number and crop dry weight. Similar results are reported by The et al. (2019) in carnation (*D. caryophyllus*) crop who point out that immersion of 60 and 90 s increases both shoot length, number and length of roots, as well as dry weight, compared to immersion times of 30, 120 and 150 s. Similarly, Regueira et al. (2018) achieve longer shoots with 1 min immersion in willow (*Salix viminalis*). Gatica and Weber (2013) obtain higher dry weight with 1 min immersion compared to 4 min in *H. lupulus*. The above indicates that the optimal immersion time will vary according to the species, therefore, it is essential to find the ideal immersion time for each one of them.

In this research, 10 and 20 mL/explant are shown to be the most suitable condition to increase the number and length of shoots and roots in *C. palmata* compared to volumes of 5 or 40 mL/explant which recorded the lowest averages. This same trend is reported by Uma et al. (2021) in banana (Rasthali AAB-Silk) where a volume of 40 mL/explant of culture medium improves the number and length of shoots compared to 16 and 83 mL that evidence lower multiplication rates and shoot development, using a BIT system; likewise, Arano et al. (2020) manage to increase the number and length of shoots with 25 or 50 mL/explant, whereas, a volume of 12.5 and 100 mL causes a decrease in the proliferation rate in *C.*

esculenta. The findings of this study show that a volume of 20 mL/explant of culture medium (200 mL) is the most suitable condition to increase the number of leaves and dry weight of *C. palmata* culture. Similar results are reported by Leyva et al. (2020) in the in vitro multiplication of orchid (*G. skinneri*) who obtained a higher number of leaves per explant, with a volume of 20 mL/explant.

A possible explanation for the results obtained in this research could be that the micropropagated plant material excretes some extracellular chemical compounds that stimulate the formation of new shoots, which are diluted when large volumes of culture medium are used, causing a decrease in the multiplication rate (Etienne y Berthouly 2002; Arano et al. 2020). Contrary to the above, when using a low volume of medium, the plant material could be subjected to increasing levels of stress, since this condition could hinder the uptake of nutrients, a rapid depletion of sucrose levels, mineral compounds, progressive decrease in pH negatively affecting the growth and development of micropropagated plant material (Oliveira et al. 2018; Gago et al. 2022).

In our work it was found that a density of 10 explants increases the number of shoots, number and length of roots and dry weight. Concomitant with our study, Bello et al. (2021) when evaluating densities of 5, 10, 15 and 20 explants/bioreactor, in the multiplication of pitahaya (*H. undatus*), suggest using a density between 10 and 15 explants/bioreactor with 50 and 33.3 mL of culture medium/explant, to reduce production costs, without affecting the multiplication rate and shoot development. On the other hand, explant density does not show significant statistical differences for the variables shoot length and number of leaves. Similar results are reported by Monja et al. (2021) in micropropagation of *Agave angustifolia*, who indicate that the inoculum of 20 and 40 explants per TIB bioreactor does not influence the growth rate of shoots and number of leaves. In some cases, the inoculum density is not decisive in physiological and morphological terms, but it is decisive in economic terms, since a low inoculum density causes a lower efficiency in the use of nutrients in the culture medium and waste of resources, with a consequent increase in the cost of seedlings (Uman et al. 2021).

In this study, the TIB system does not increase the number of shoots per explant of *C. palmata*, but it is determinant to obtain greater shoot length, number and length of roots, number of leaves and dry weight. These findings agree with those reported by Ghosh et al. (2021), who show that temporary immersion system does not generate a greater number of shoots per explant, but it does favor greater shoot growth and vigorous shoots in the micropropagation of blueberry (*Vaccinium spp* L) in comparison with semisolid medium. Similarly, Abahmane (2020) working with date palm (*Phoenix dactylifera* L) when comparing an immersion system with semisolid medium, indicates that the use of temporary immersion system is effective in increasing shoot length, roots and shoots dry weight, but does not induce a greater number of shoots per explant. Likewise, Vidal et al. (2015) and Corona et al. (2019) report that the TIB system was inefficient to increase the number of shoots in *Castanea sativa* and *Carica papaya* compared to semisolid medium, this agrees with the results obtained by Hoyos et al. (2019) who when micropropagating *C. palmata* using semisolid culture medium obtained a greater number and length of shoots.

The plant material micropropagation using a TIB system showed better morphological characteristics such as: larger shoots, adequate root development, greater number of leaves and higher dry weight. The results obtained in this work agree with those reported in most of the research, except for the number of shoots per explant. This is evidenced by what Mancilla et al. (2021) reported when comparing the TIB system with the semisolid medium, showing that the former presents a higher average for the variables number and length of shoots, roots, and leaves in *C. esculenta*. In this same sense, Kim et al. (2020) in the micropropagation of apple tree seedlings, evidenced greater shoot length, roots and better average biomass in the TIB system compared to the semisolid culture medium and liquid medium. These results may be since the TIB system influences physiological parameters such as nutrient uptake, bioregulators, chlorophyll synthesis, photosynthesis, stomatal regulation, and cellular respiration, which allows for adequate growth and development of plant material (Silva el al. 2018; Silva et al. 2020; San José et al. 2020). In addition, traditional culture systems (semi-solid and liquid culture medium) are believed to have poor growth because there is no gas exchange within the culture vessel (Hwang et al. 2022).

Conclusion

According to the results of this research, the frequency and the immersion time were the two critical factors for successful micropropagation of *C. palmata* using a BIT system. When evaluating the simple effect of the factors under study, it was concluded that the optimal culture condition in the BIT system was 12 h frequency, 1 min immersion, 200 mL of culture medium (20 mL/explant) with 10 explants. A frequency of 6 h with a prolonged immersion time of 5 min, showed a higher number of shoots.

The volume of the culture medium per explant was a factor that affected the proliferation and development of shoots, roots, leaves and dry weight of *C. palmata* when using a TIB system. A volume of 50 ml (5 ml/explant) was the least efficient condition and a volume of 200 ml (20 ml/explant) as the best condition for the micropropagation of *C. palmata*.

The results of this research indicate that the BIT system is efficient for mass propagation of *C. palmata* compared to conventional micropropagation systems (semi-solid or liquid medium). Liquid culture medium was the least suitable treatment for in vitro multiplication of *C. palmata*.

This is the first report on the micropropagation of *C. palmata* using a temporary immersion system.

Declarations

Author contributions

NMB Planned and development of the research, wrote the manuscript; RAHS Conception of the research and writing the manuscript. GACL: designed research, analyzed data, review the manuscript.

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Figures

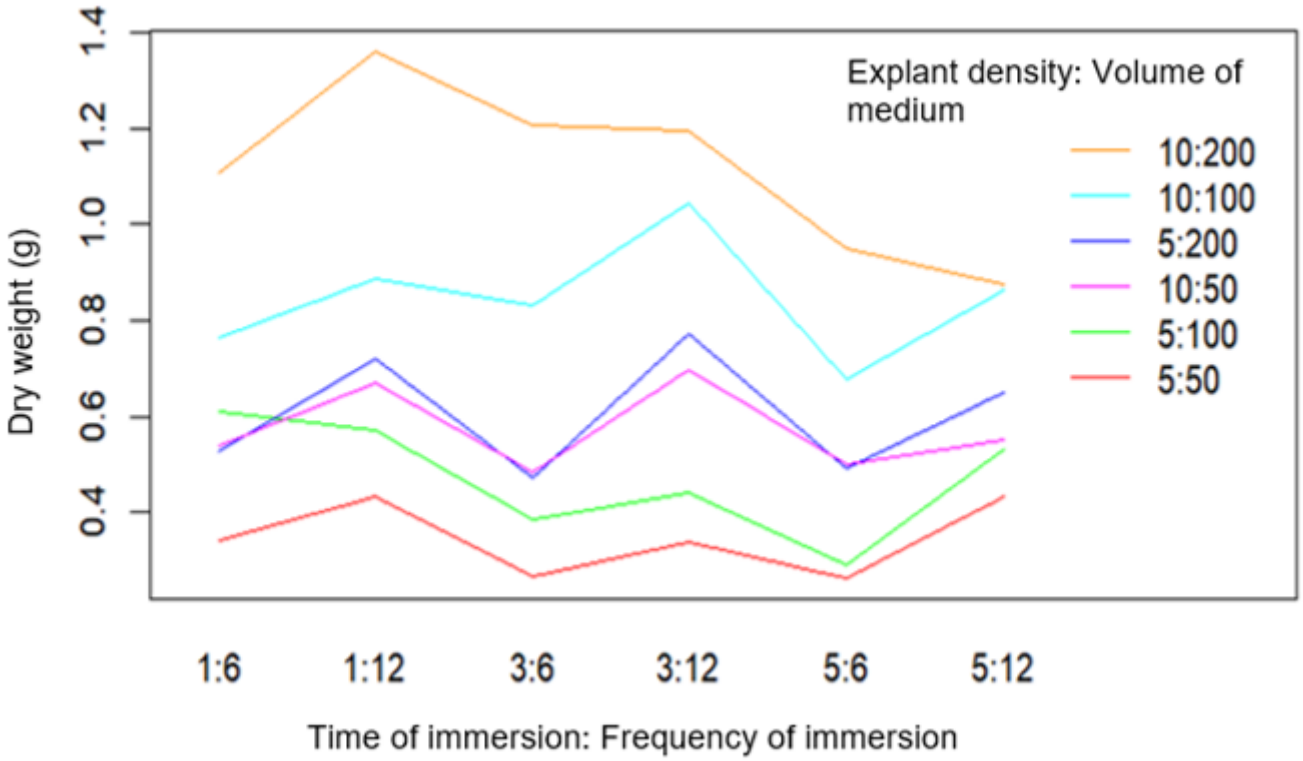

Figure 1

Third order interaction: frequency of immersion (Fr), time of immersion (Ti), number of plants per bioreactor (Np), and volume of culture medium per bioreactor (V) for dry weight

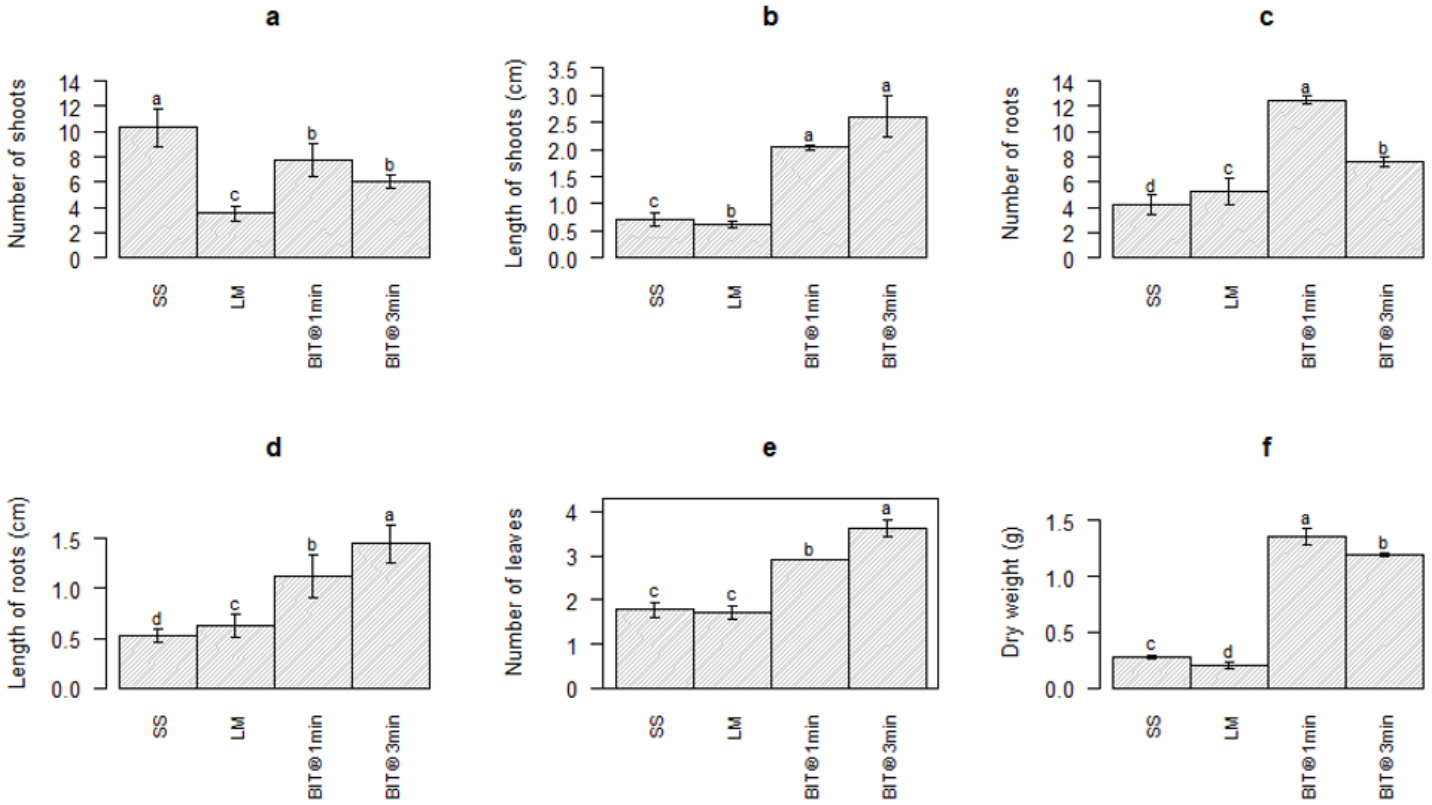

Figure 2

Response of the different variables to three culture systems in *C. palmata* micropropagation. **SS: semi-solid culture medium, LM: liquid culture medium, BIT® 1 min: immersion time of 1,** 200 mL of culture medium, 10 explants **y frequency of 12 h, BIT® 3** min: i**mmersion time of 3 min,** 200 mL of culture medium, 10 explants **y frequency of 12 h**. Means labeled with the same letter are not significantly different (Tukey, $\alpha = 0.05$)**. Note:** The plotted values of the variables shoot length and dry weight were non-transformed data. Significance letters were obtained from transformed data

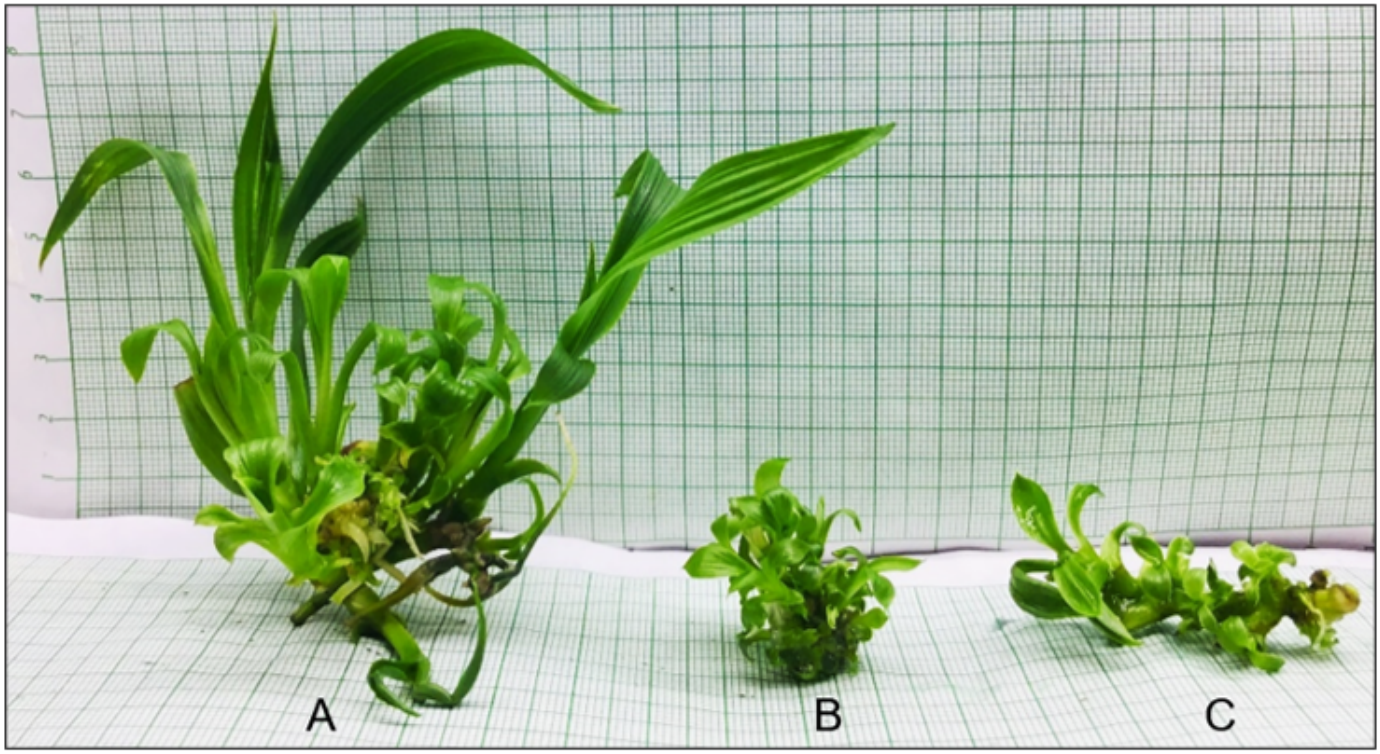

Figure 3

Size differences between shoots of *C. palmata* obtained from three different *in vitro* culture systems. **A:** shoots generated by the BIT® with frequency of 12 h, immersion time of 3 min in 200 mL of culture medium and 10 explants. **B:** Shoots generated within semi-solid medium. **C:** shoots generated within liquid medium. Lengths measured 60 days after the beginning of the experiment.