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

Nube Minchala-Buestán[1] · Rodrigo A. Hoyos-Sánchez[1] · Guillermo A. Correa-Londoño[1]

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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 handmade goods. Colombia is one of the leading countries in the production of this species; however, it lacks efficient techniques for its propagation. We developed a protocol for massive micropropagation of *C. palmata* using a temporary immersion bioreactor (TIB) system. Immersion frequency, immersion time, culture medium volume, and explant density were evaluated using a split-split-plot design. The variables evaluated were the number and length of shoots and roots, the number of leaves, and the dry weight of shoots. The performance of three micropropagation systems, TIB, semi-solid medium, and liquid medium, was evaluated using a 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. The results obtained show a frequency of 12 h with an immersion time of 1 min improved the length of shoots, roots, and number of leaves, and dry weight. A volume of 20 mL/explant was found to be an adequate condition to increase the number and length of shoots, the number of leaves, and the dry weight. According to the optimization module, the factor levels that will maximize the evaluated variables are a 1-minute immersion of the tissues in the culture medium with a frequency of 12 hours each time, 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.

Keywords *Carludovica palmata* · *In vitro* regeneration · Bioreactor · Toquilla straw

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 materials for the manufacture of a wide variety of artisanal products, among them the Panama hat (Iglesias 1999; Gálviz *et al*. 2019).

✉ Rodrigo A. Hoyos-Sánchez
rhoyos@unal.edu.co

Nube Minchala-Buestán
nminchala@unal.edu.co

Guillermo A. Correa-Londoño
gcorrea@unal.edu.co

[1] Facultad de Ciencias Agrarias, Universidad Nacional de Colombia - Sede Medellín, 050034 Medellín, Colombia

Nowadays, lately, this species has acquired considerable economic importance due to its great versatility, since it can be used in the agroindustry (Murillo *et al*. 2021) and as a dye, additive, and source of "palmito" (Ossa *et al*. 2023); in the chemical industry, to produce artisanal paper and as an important alternative for reinforcement of polymeric matrices (Moo *et al*. 2019); and in 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, and 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 a technique has some limitations: it does not allow for high rates of multiplication (Graca *et al*. 2014) and it affects the integrity of

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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 and 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 the propagation and production of plants are developed to implement iraca crops on a larger scale.

Automatic temporary immersion systems (ATIS) are technological 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 (Vives *et al*. 2017; Oliveira *et al*. 2018), which generates optimal conditions for morphogenesis in the micropropagated plant material (Etienne and Berthouly 2002; Uma *et al*. 2021).

One such system is the Twin-Flask temporary immersion bioreactor (BIT®), which consists of two containers, one of 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 and mixotrophic capacity and reducing cellular respiration which allows for the generation of larger and great biomass plantlets with a 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 *Phoenix dactylifera* L. (Abahmane 2020), 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 ± 2°C, a luminosity of approximately 1500 lx, photosynthetically active radiation of 90 µmol of photons m2 s^{-1}, and a photoperiod of 12/12 h of light and dark.

Plant material The starting material used consisted of 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 mo 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 segments with apical meristem of approximately 2 cm in length.

Experimental step In the BIT® system, bottles with volumes of liquid culture medium of 50, 100, and 200 mL were used after sterilized in an autoclave (Trident®, Taiwan-China) 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. The BIT® immersion system used in this research was built by engineers from the Universidad Nacional de Colombia.

For the other two techniques, namely liquid and semisolid, 20 mL of culture medium was 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 (Np5) and 10 explants (Np10); culture medium volume per bioreactor 50 (V50), 100 (V100), and 200 mL (V200); time of immersion 1 (Ti1), 3 (Ti3), and 5 min (Ti5); and frequency of immersion 6 (Fr6) and 12 h (Fr12). 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® system) based on a completely randomized design with two replications. Time of immersion was assigned to the split-plots (shelves), each conformed by six bioreactors. The number of explants and culture medium volume was assigned to the split-split-plots; that is, the bioreactors comprised of bottles of 1 L capacity with explants of *C. palmata*. The response variables evaluated were the number of shoots per explant, length of shoots, number of roots, length of roots,

number of leaves, and shoot dry weight. We chose the best two treatments of the TIB system to compare them with the semi-solid medium and liquid medium. Since neither the semi-solid 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. The 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 tests, respectively. Pairwise comparisons were performed by the HSD Tukey test (HSD.test{agricolae}).

Given that all the evaluated factors were numerical, a principal component analysis was performed and 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 combination levels which were expected to optimize the whole set of response variables evaluated in the micropropagation of *C. palmata.*

Results

Correlation structure Analyses of the most relevant correlations found among the six response variables were both positive and negative. There was a direct correlation ($r > 0.5$) among the variables root length (RL), shoot length (SL), and leaf number (LN); and a moderate correlation with crop dry weight (DW) ($0.3 < r < 0.5$). These variables are weakly inversely related to shoot number (SN) ($-0.4 < r < -0.2$). In contrast, the root number (RN) variable did not have a significant correlation ($p > 0.05$) with any other variable.

Likewise, there were some common conditions that stimulated the variables RL, SL, LN, and to some extent DW, although such conditions may not be the most suitable for SN (because of the negative correlation). This makes sense because SN is a variable clearly associated with quantity, while RL, LN, and DW account for the quality and vigor of the explants. The principal component analysis (PCA) of the 6 response variables collects 69.5% of the total variability in the first two dimensions. The more relevant combinations in the present study are highlighted in the biplot representation (Fig. 1).

Shoot length The analysis of variance (ANOVA) showed a statistically significant effect ($p = 0.026$) in the second-order interaction between Fr:Ti:V; therefore, the corresponding simple effects were analyzed. The highest shoot length averages were achieved with 12 h immersion frequency, which were significantly different ($p < 0.05$) in any combination of immersion time and culture medium volume. Volumes of 100 and 200 mL did not show statistically significant differences between them when evaluated with immersion times of 1 and 3 min. In each case, a volume of 200 mL per bioreactor was the most suitable condition to increase the shoot length; conversely, a volume of 50 mL was the least suitable condition for this variable. In general, the conditions that yielded the greatest shoot length were 12 h immersion frequency and 200 mL volume (20 and 40 mL/explant), at immersion times of 1, 3, and 5 min with averages of 2.21, 2.44, and 2.37 cm, respectively. Finally, promising conditions were found for Fr12:V200 and Fr12:V100 (Table 1), with no relevant levels of immersion time and explant density.

Root length The ANOVA showed a statistically significant effect ($p = 0.016$) in the third-order interaction (Fr:Ti:Np:V). The corresponding simple-effects analysis showed that the immersion frequency had no statistically significant differences when evaluated with immersion times of 1 and 3 min; 50, 100, and 200 mL of culture medium; and 10 explants. A volume of 100 mL of culture medium was the most suitable condition to increase the root length — up to an average of 1.82 cm — when evaluated with Fr12:Ti1:Np10 compared to a volume of 50 and 200 mL that yielded averages of 1.11 cm in both cases (Table 2). Immersion time and explant density showed no statistically significant differences in this variable except for Fr12:Ti1:V100; thus, a density of 10 explants shows statistically significant differences compared to 5 explants. The most promising combination to achieve longer root length was Fr12:Ti1:Np10:V100.

Leaf number The ANOVA showed statistically significant effects ($p < 0.001$) in the first-order interactions Fr:Ti and Ti:V; therefore, the corresponding simple effects were analyzed. In the Fr:Ti interaction, we observed that an immersion frequency of 12 h increased the number of leaves per shoot at each immersion time; in contrast, immersion time did not show a statistically significant effect on immersion frequency. In the Ti:V interaction, a volume of 200 mL was the most suitable condition to increase the number of leaves per shoot when evaluated with an immersion

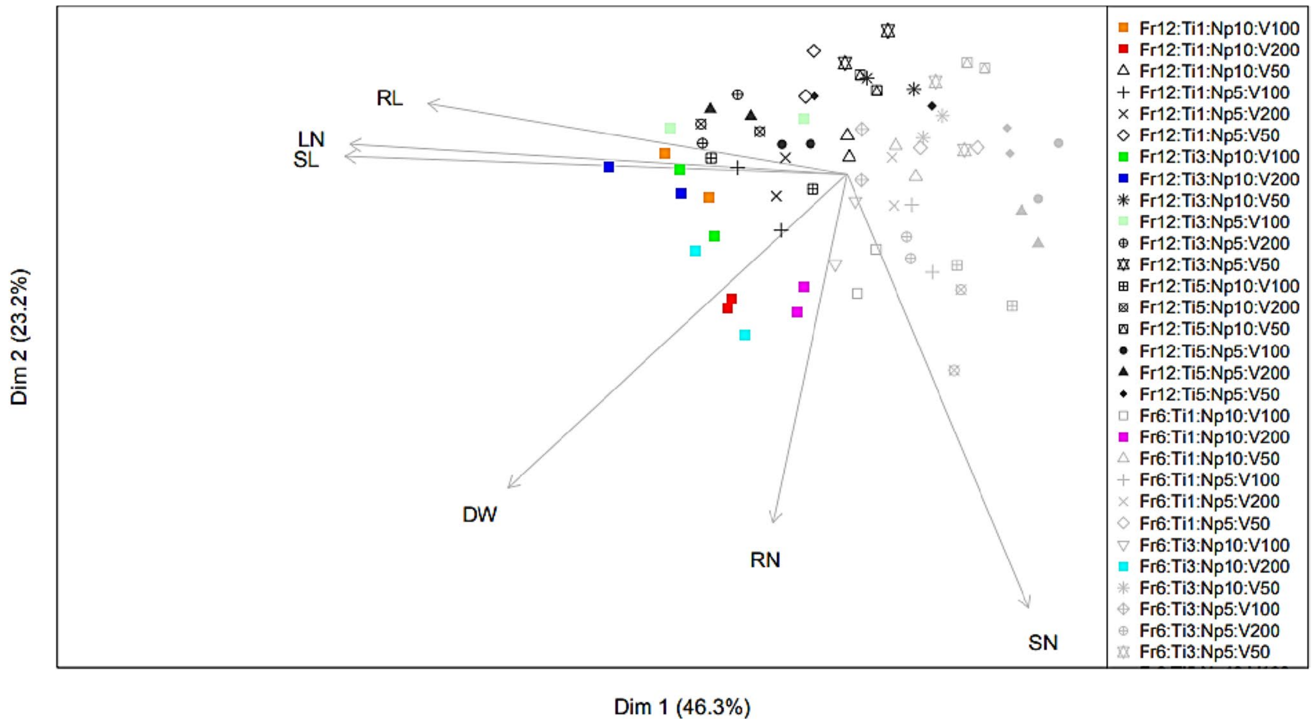

Figure 1. PCA-biplot representation highlighting the most relevant combinations between immersion frequency (Fr), immersion time (Ti), explant density (Np), and culture medium volume (V) for the response variables root length (RL), number of leaves (LN), shoot length (SL), shoots dry weight (DW), number of roots (RN), and number of shoots (SN).

time of 1 min. The most favorable condition for this variable was Fr12:Ti3:V200, which reached an average of 3.03 leaves. In contrast, the lowest number of leaves was obtained with Fr6:Ti5:V50 with an average of 1.56 leaves per shoot (Fig. 2). Since no significant effect was observed on the explant density factor, the 3 promising conditions for this variable were Fr12:Ti1:V200, Fr12:T3:V200, and Fr12:Ti3:V100.

Dry weight The ANOVA showed a statistically significant effect ($p = 6.963\text{e-}05$) in the third-order interaction (Fr:Ti:Np:V). When the corresponding simple effects were analyzed, we found that the immersion frequency of 12 h increased the dry weight of the culture. Immersion time of 1 min was the most favorable condition to increase dry weight when evaluated with 200 mL of culture medium and 10 explants. When studying the volume factor of the culture medium, 200 mL is the most suitable condition to increase dry weight. Regarding the explant density factor, a statistically significant difference was obtained using 10 units. The most favorable conditions to increase the dry weight of the culture were Fr12:Ti1:Np10:V200 and Fr12:Ti3:Np10:V200 with averages of 1.36 and 1.20 g, respectively. In contrast, the lowest dry weight (0.26 g) was obtained with Fr6:Ti5:V50:Np5 (Table 2).

Shoot number The shoot number variable presented statistically significant effects in the second-order interactions Fr:Np:V ($p = 0.03$) and Fr:Ti:V ($p = 0.000$). When analyzing the simple effects of the Fr:Np:V interaction, we found that 6 h immersion frequency is the most favorable condition to increase the number of shoots per explant when working with 100 and 200 mL of culture medium, with either 5 or 10 explants. Explant density was not statistically significant when evaluated at each immersion frequency and culture medium volume (Table 3). In contrast, when studying the Fr:Ti:V interaction, a similar trend was observed; a 6-h frequency increased the number of shoots per explant when working with 100 and 200 mL of culture medium in the three levels of immersion time evaluated, except for Ti1:V200, where no statistically significant difference was found between immersion frequencies. As for the immersion time, we observed that 5 min was the most suitable condition to increase the number of shoots when evaluated with an immersion frequency of 6 h. However, with a frequency of 12 h, an immersion time of 1 min yielded a greater number of shoots. The treatments that yielded the highest mean number of shoots per explant were Fr6:Ti5:V100 (10.60 shoots) and Fr6:Ti5:V200 (11.32 shoots) (Table 1).

Table 1. Simple effect of immersion frequency, immersion time, and culture medium volume in micropropagation of *C. palmata*

Treatments Frequency/volume/time	Shoots number	Shoots length (cm)
Fr6 / 50 mL / 1 min	4.02 Ab^{a}	0.89 Ab^{b}
Fr6 / 50 mL / 3 min	3.97 Ac^{a}	0.69 Ab^{b}
Fr6 / 50 mL / 5 min	4.43 Ab^{a}	0.62 Aa^{b}
Fr6 / 100 mL / 1 min	7.60 Ba^{a}	1.02 ABb^{b}
Fr6 /100 mL / 3 min	5.65 Cb^{a}	1.37 Aa^{b}
Fr6 / 100 mL / 5 min	10.60 Aa^{a}	0.64 Ba^{b}
Fr6 / 200 mL / 1 min	8.00 Aa^{a}	1.56 Aa^{b}
Fr6 / 200 mL / 3 min	8.05 Aa^{a}	1.48 Aa^{b}
Fr6 / 200 mL / 5 min	11.32 Ba^{a}	0.67 Ba^{b}
Fr12 / 50 mL / 1 min	3.90 Ac^{a}	1.42 Ab^{a}
Fr12 / 50 mL / 3 min	2.62 Ab^{a}	1.12 Ab^{a}
Fr12 / 50 mL / 5 min	4.17 Aa^{a}	1.16 Ac^{a}
Fr12 / 100 mL / 1 min	5.35 Ab^{b}	2.06 Aa^{a}
Fr12 / 100 mL / 3 min	4.91 Aa^{a}	2.11 Aa^{a}
Fr12 / 100 mL / 5 min	5.50 Ab^{b}	1.81 Ab^{a}
Fr12 / 200 mL / 1 min	6.87 Aa^{a}	2.21 Aa^{a}
Fr12 / 200 mL / 3 min	4.72 Aa^{b}	2.43 Aa^{a}
Fr12 / 200 mL / 5 min	4.55 Aa^{b}	2.37 Aa^{a}

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

Root number The ANOVA showed a statistically significant effect ($p = 0.009$) in the third-order interaction (Fr:Ti:Np:V). The simple-effects analysis showed that the immersion frequency did not have statistically significant difference when working with 100 mL of culture medium at any immersion time and density of explants evaluated. This same trend was observed when working with 200 mL of culture medium, immersion time 1 min, with 5 or 10 explants. When analyzing the simple effect of the immersion time, we found that 1 min generated a greater number of roots when evaluated under the most promising condition: frequency of 12 h, 200 mL of culture medium, with 5 or 10 explants. The explant density factor showed no statistically significant difference when evaluated at a frequency of 12 h.

Response surfaces Taking advantage of the numerical nature of the four factors evaluated, a complete quadratic model was adjusted for each response. Next, the multiple-response optimization module was used in Statgraphics to find the point that predicts the highest joint estimated response. Since the search for the point in question is carried out on a continuous surface, the values are not restricted to the levels evaluated in the present test. The point obtained was Fr12:Ti1.2:Np10:V165.8; this result is totally consistent with the previous analyses and confirms the convenience of working with a 12-h immersion frequency with 10 explants. The intermediate points obtained for Ti and V are also consistent with previous analyses, which showed as more promising points 1 and 3 for immersion time, and 100 and 200 for culture medium volume.

Three micropropagation systems of *C. palmata in vitro* To evaluate the multiplication efficiency of the three micropropagation systems, the two best treatments of the BIT system were chosen and compared with the semi-solid 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 semi-solid 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. 3*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) (Figs. 3*b* and 4).

The BIT system with 1 min of immersion time showed the highest number of roots (12.5), while the semi-solid culture medium showed the lowest number (4.18 roots) (Fig. 3*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 liquid medium (0.62 cm) and semi-solid medium, which showed the least root length (0.52 cm) (Fig. 3*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. 3*e*). For the dry weight variable, the 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. 3*f*).

Discussion

The scientific research on *C. palmata* in biotechnology, specifically on micropropagation, is limited. Therefore, the discussion is based on what is reported in the scientific literature for other species. The results of this research indicate that a long immersion frequency of 12 h is the most suitable condition to increase shoot and root length, leaf number, and dry weight of the crop, compared to a short frequency of 6 h. This is consistent with that reported by Mosqueda *et al*. (2017) and Uma *et al*. (2021), who

Table 2. Simple effect of immersion frequency, immersion time, culture medium volume, and explant density in the micropropagation of *C. palmata*

	Immersion frequency 6 h						Immersion frequency 12 h					
	Immersion time						Immersion time					
Explant density	1 min		3 min		5 min		1 min		3 min		5 min	
	5 Np	10 Np	5 Np	10 Np	5 Np	10 Np	5 Np	10 Np	5 Np	10 Np	5 Np	10 Np
Root number												
50 mL	12.20 Aa^{Aa}	13.85 Aa^{Aa}	11.90 Aa^{Aa}	10.70 Ab^{Aa}	8.00 Ab^{Aa}	7.00 Ac^{Ba}	9.00 Ba^{Ba}	10.25 Ba^{Aa}	8.50 Ba^{Aa}	6.90 Bb^{Ba}	7.90 Aa^{Aa}	8.25 Aab^{Aa}
100 mL	13.70 Aa^{Aa}	14.65 Aa^{Aa}	12.30 Aa^{Aa}	13.45 Aa^{Aa}	8.30 Ab^{Ab}	11.70 Ab^{Aa}	14.40 Aa^{Aa}	11.60 Aa^{Aa}	11.4 Aab^{Aa}	11.70 Aa^{Aa}	9.70 Ab^{Aa}	10.85 Aa^{Aa}
200 mL	9.10 Ab^{Bb}	13.80 Aa^{Aa}	13.40 Aa^{Aa}	14.35 Aa^{Aa}	7.80 Abc^{Ab}	11.85 Ab^{Aa}	10.1 Aa^{ABa}	12.50 Aa^{Aa}	9.70 Ba^{Aa}	7.60 Bb^{Ba}	8.00 Aa^{Aa}	8.25 Bb^{Aa}
Root length (cm)												
50 mL	0.84 Bab^{Aa}	0.99 Aab^{Aa}	1.11 Aa^{Aa}	1.13 Aa^{Aa}	0.68 Bb^{Aa}	0.72 Ab^{Aa}	1.28 Aa^{Aa}	1.11 Aa^{Ba}	1.10 Aa^{Aa}	0.96 Aa^{Ba}	1.24 Aa^{Aa}	0,97 Aa^{Aa}
100 mL	0.69 Ba^{Ab}	1.42 Aa^{Aa}	0.86 Ba^{ABa}	1.10 Aab^{Aa}	0.66 Ba^{Aa}	0.70 Bc^{Aa}	1.33 Aa^{Ab}	1.82 Aa^{Aa}	1.41 Aa^{Aa}	1.46 Aa^{Aa}	1.07 Aa^{Aa}	1.43 Aa^{Aa}
200 mL	0.83 Ba^{Aa}	0.96 Abc^{Aa}	0,68 Bab^{Ab}	1.44 Aa^{Aa}	0.49 Bb^{Aa}	0,74 Bc^{Aa}	1,06 Aa^{Aa}	1,11 Aa^{Ba}	1.38 Aa^{Aa}	1.44 Aa^{Aa}	1.21 Aa^{Aa}	1.19 Aa^{Aa}
Dry weight (g)												
50 mL	0.34 Ba^{Bb}	0.53 Ba^{Ca}	0,26 Bb^{Cb}	0.48 Ba^{Ca}	0,26 Bb^{Bb}	0.49 Ba^{Ca}	0.43 Aa^{Cb}	0,66 Aa^{Ca}	0,33 Ab^{Cb}	0,69 Aa^{Ca}	0,43 Aa^{Cb}	0.55 Ab^{Ba}
100 mL	0.57 Aa^{Ab}	0.76 Bab^{Ba}	0.38 Bb^{Bb}	0.83 Ba^{Ba}	0.28 Bc^{Bb}	0.67 Bb^{Ba}	0.61 Aa^{Bb}	0.88 Ab^{Ba}	0.53 Ab^{Bb}	1.04 Aa^{Ba}	0.53 Ab^{Bb}	0.86 Ab^{Aa}
200 mL	0.52 Ba^{Ab}	1.10 Ba^{Aa}	0.47 Ba^{Ab}	1.20 Aa^{Aa}	0.49 Ba^{Ab}	0.95 Ab^{Aa}	0.72 Aab^{Ab}	1.36 Aa^{Ca}	0.77 Aa^{Ab}	1.20 Ab^{Aa}	0.65 Ab^{Ab}	0.87 Ac^{Aa}

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

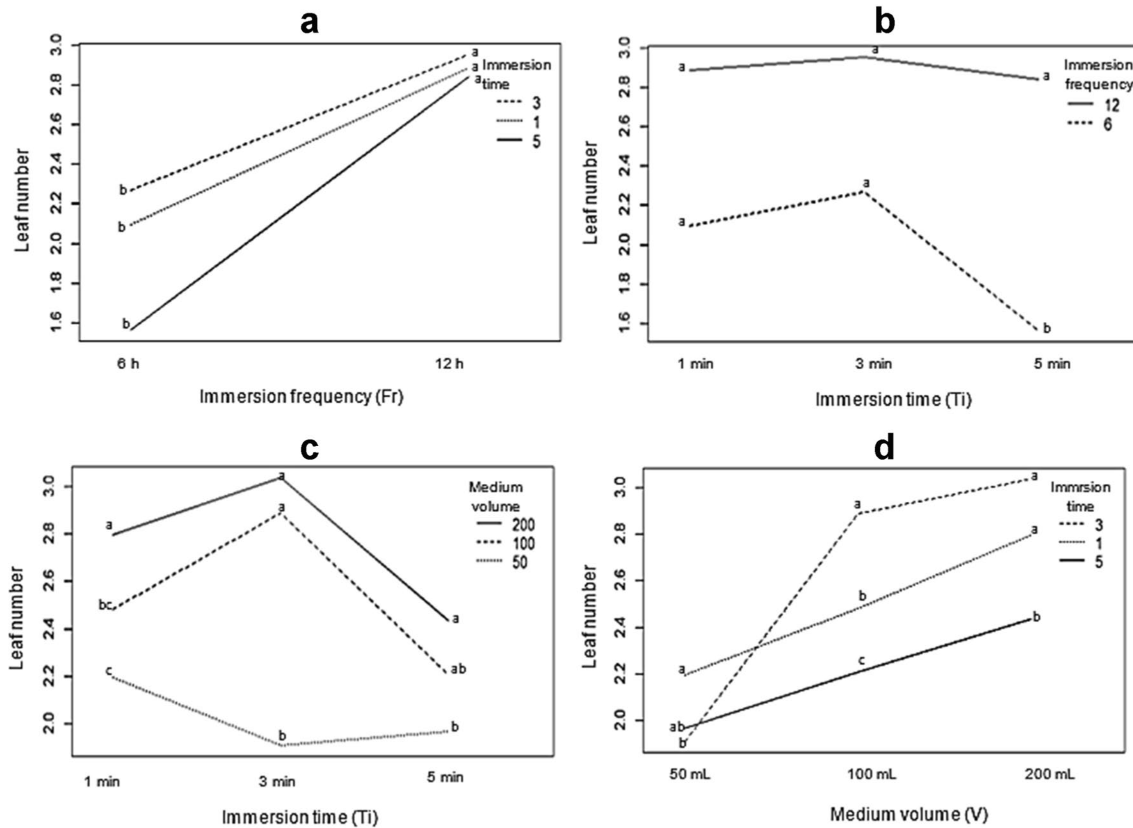

Figure 2. First-order interactions, between ***a***, ***b*** immersion frequency with immersion time (Fr:Ti) and ***c***, ***d*** immersion time with culture medium volume (Ti:V) for the variable leaf number in *C. palmata* micropropagation using temporary immersion system. Means vertically labeled with the same *letter* are not significantly different ($\alpha = 0.05$), according with the LSD test with Holm correction.

Table 3. Simple effect of immersion frequency, culture medium volume, and explant density in *C. palmata* micropropagation

Treatments Frequency/volume/explant density	Shoot number
Fr6 / 50 mL /5Np	4.46 Ac[a]
Fr6 / 50 mL /10Np	3.82 Ab[a]
Fr6 / 100 mL /5Np	7.60 Ab[a]
Fr6 / 100 mL /10Np	8.30 Aa[a]
Fr6 / 200 mL /5Np	9.40 Aa[a]
Fr6 / 200 mL /10Np	8.85 Aa[a]
Fr12 / 50 mL /5Np	3.63 Ab[a]
Fr12 / 50 mL /10Np	3.50 Ab[a]
Fr12 / 100 mL /5Np	5.46 Aa[b1]
Fr12 / 100 mL /10Np	5.04 Aa[b1]
Fr12 / 200 mL /5Np	4.80 Bb[a1]
Fr12 / 200 mL /10Np	5.96 Aa[b1]

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

obtained greater development of shoots and roots with prolonged frequencies (12 h). A similar trend was reported by Aka *et al*. (2020) working with myrtle (*M. communis*); they achieved longer shoots and roots, higher leaf number, and dry weight with a prolonged frequency of 8 h, compared to a short frequency of 4 h.

In terms of numbers, whether from shoots or roots, *C. palmata* is favored when the frequency is lower, *e.g.*, 6 h. These findings are consistent with those reported in other species such as carnation (*Dianthus caryophyllus* L.) (Ahmadian *et al*. 2017) and plantain (*Rasthali* AAB-Silk) (Uma *et al*. 2021). This could be explained because short immersion frequencies allow the explants to have more frequent contact with the energy molecule and bioregulators; thus, they emit new buds leaving their growth and development in the background. However, these conditions are not always the most suitable, since they would be prone to cause morphological and/or physiological disorders, considerably affecting the quality of micropropagated plant material (Pramita *et al*. 2018; Arano *et al*. 2020). Therefore, in some cases, it is preferable to sacrifice quantity for the sake of quality.

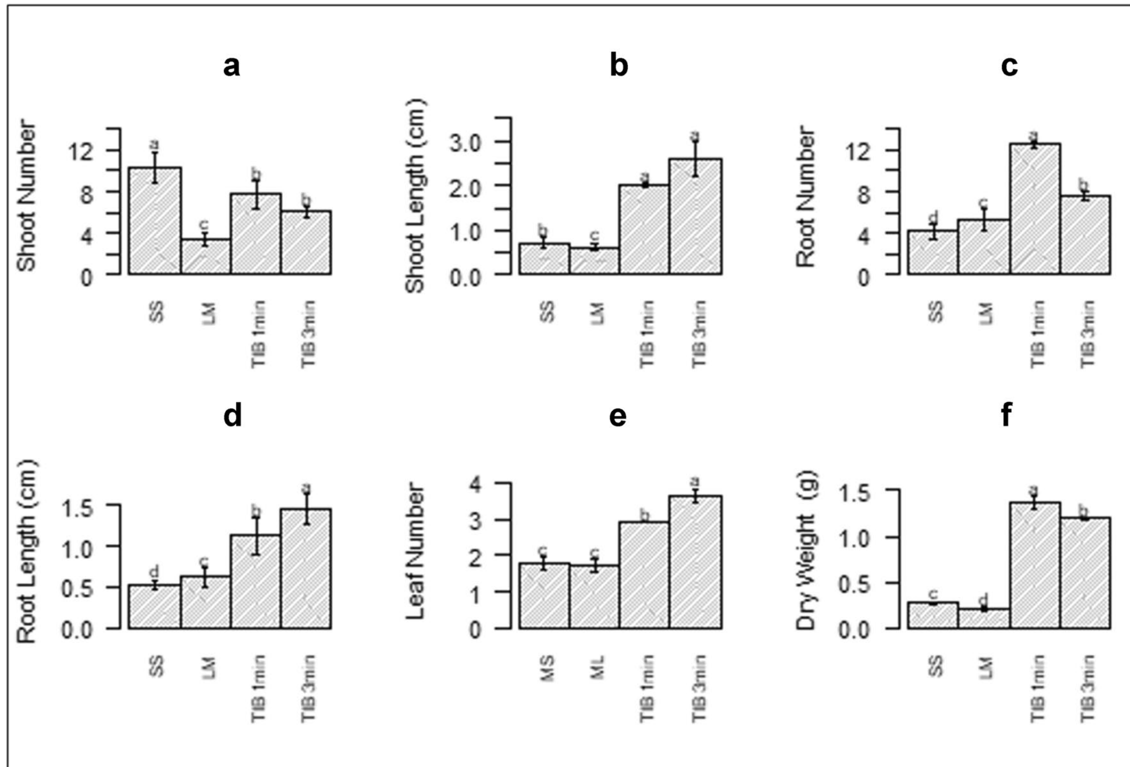

Figure 3. (*a*–*f*) Mean response and standard deviation of the six variables to three culture systems in *C. palmata* micropropagation. ***a*** Shoot number. ***b*** Shoot length (cm). ***c*** Root number. ***d*** Root length (cm). ***e*** leaf number and ***f*** Dry weight. SS, semi-solid culture medium; LM, liquid culture medium; TIB1 min, immersion time of 1 min, 200 mL of culture medium, 10 explants, and frequency of 12 h; TIB 3 min, immersion time of 3 min, 200 mL of culture medium, 10 explants, and immersion frequency 12 h. Means labeled with the same letter are not significantly different (HSD Tukey test, $\alpha = 0.05$). Note: The plotted values of the variables shoot length and dry weight were non-transformed data. Significance *letters* were obtained from transformed data.

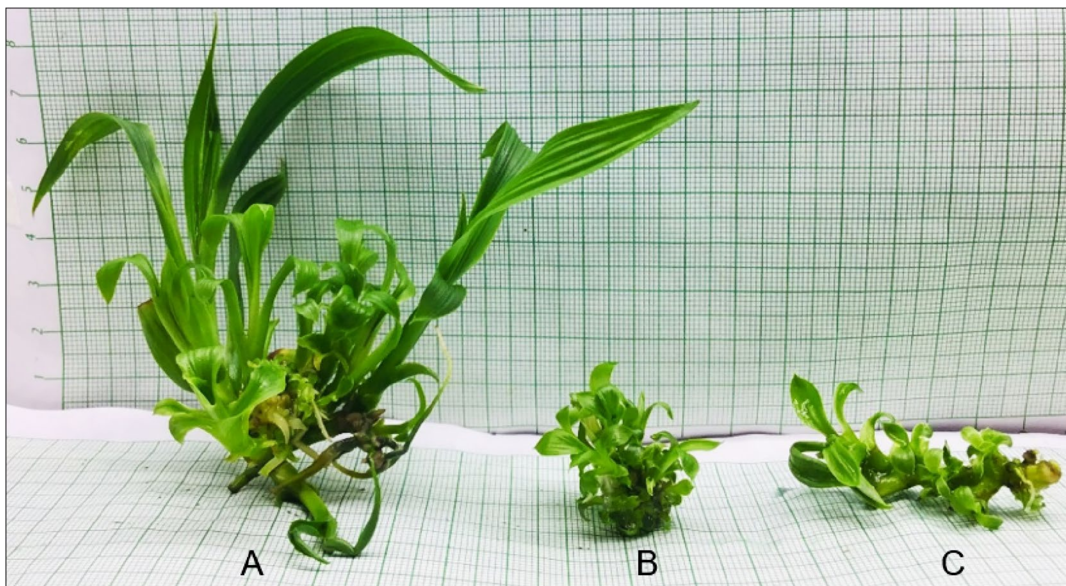

Figure 4. Differences between lengths shoots of *C. palmata* obtained from three *in vitro* culture systems. (***A***) Shoots generated by the TIB with frequency of 12 h, immersion time of 3 min in 200 mL of culture medium, and 10 explants. (***B***) Shoots generated within semi-solid medium. (***C***) Shoots generated within liquid medium. Lengths measured 60 d after the beginning of the experiment.

In this research, 100 and 200 mL of culture show to be the most suitable conditions to increase the number and length of shoots and roots in *C. palmata* compared to a volume of 50 mL, which recorded the lowest average. These results may be due to explants absorbing nutrients and bioregulators by all tissues interacting with the culture medium (Hwang *et al*. 2022). In this sense, using a low culture medium volume could cause that only a part of the explants come into contact with the nutrient medium and make it difficult to take nutrients. It would result in inefficient multiplication rates and would expose the plant material to increasing levels of stress (Ramos *et al*. 2014; Kim *et al*. 2020). Likewise, very high volumes of culture medium negatively affect the multiplication rate, probably because the plant material excretes some stimulating extracellular chemical compounds, which are diluted when large volumes are used, thus causing a decrease in the multiplication rate (Etienne and Berthouly 2002; Arano *et al*. 2020).

The present research shows that the immersion time minimally affected the micropropagation of *C. palmata*. It is evident that the number of shoots per explant is a function of a longer immersion time (Alvarenga and Salazar 2015; Esyanti *et al*. 2019). However, this time is not always optimal to achieve a plant material of excellent quality, since it could cause undesirable morphological and/or physiological disorders including genetic mutations by altering the cell cycle. However, the variables shoot length, number and length of roots, number of leaves, and dry weight of the crop are favored by a short immersion time of 1 and 3 min compared to a longer one of 5 min. Similar results were reported by Regueira *et al*. (2018) who achieved longer shoots of willow (*Salix viminalis*) with an immersion time of 1 min. Likewise, Gatica and Weber (2013) obtained higher dry weight with 1 min immersion compared to 4 min in *Humulus lupulus.*

Explant density has no significant impact on *C. palmata* micropropagation. Similar results were reported by Monja *et al*. (2021) for micropropagation of *Agave angustifolia*. They indicate that inoculation of 20 and 40 explants per TIB bioreactor did not influence the growth rate of shoots. Uma *et al*. (2021) pointed out that, in some cases, the inoculum density is not decisive in physiological and morphological terms but in economic terms, since using a low density of explants causes a waste of resources and increases the cost of the generated plantlets.

The results of this study show that the temporary immersion system is decisive to generate a plant material with better morphological characteristics such as larger shoots, adequate root development, higher number of leaves, and dry weight. However, it does not increase the number of shoots per explant compared to the semi-solid medium in *C. palmata.* In the same manner, Ghosh *et al*. (2021) showed that the temporary immersion system does not generate a greater number of shoots per explant but does favor the growth and vigor of cranberry shoots (*Vaccinium* spp. L.) compared to the semi-solid medium. Similarly, Abahmane (2020), working with date palm (*Phoenix dactylifera* L.), indicated that the use of the temporary immersion system is effective to increase the length of shoots, roots, and dry weight of shoots, but does not induce a higher number of shoots per explant. These results may be due to the fact that the TIB system influences physiological parameters such as nutrient absorption, plant bioregulators, chlorophyll synthesis, photosynthesis, stomatal regulation, and cellular respiration, which allow for better performance in the growth and development of plant material, resulting in the production of seedlings in a single type of culture medium (Silva *et al*. 2018, 2020; San José *et al*. 2020). In addition, traditional culture systems (semi-solid and liquid culture medium) are believed to have poor growth because they do not allow gas exchange, thus leading to an accumulation of volatiles such as ethylene, aromatics, and alcohols among others (Hwang *et al*. 2022).

Conclusion

The study found that frequency and immersion time were crucial factors for successful micropropagation of *C. palmata* using a BIT system. The optimal conditions were a 12-h frequency, 1-min immersion, 200 mL culture medium (10 explants), and a volume of 20 mL per explant. A volume of 50 mL was the least efficient, and 200 mL was the best for shoot, root, leaf, and dry weight development. The BIT system was more efficient than conventional systems, and liquid culture medium was the least effective. This is the first report on *C. palmata* micropropagation using a temporary immersion system.

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Declarations

Conflict of interest The authors declare no competing interests.

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