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Optimization of surface sterilization and plant growth regulator treatments for *in vitro* regeneration of *Hylocereus undatus*

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ABSTRACT

An efficient *in vitro* regeneration protocol for *Hylocereus undatus* was developed through the optimization of surface sterilization and plant growth regulator supplementation. Three sterilization treatments and different concentrations of 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA) were evaluated using stem, cotyledon, and hypocotyl explants. The sterilization treatment consisting of Phyton (1 ml L^{-1}), Novac (0.75 ml L^{-1}), Score (1 ml L^{-1}), 30% (v/v) commercial bleach, and 70% ethanol (T3) resulted in 100% contamination-free explants. Regeneration and rooting frequencies varied among the explant types and growth regulator treatments evaluated. The highest shoot induction frequency (87.5%) was observed in stem-derived explants cultured on medium supplemented with 4.0 mg l^{-1} BAP. Rooting was promoted by NAA supplementation, with the highest rooting frequency (100.0%) observed at 0.5 mg l^{-1} in stem-derived shoots. Morphological observations documented shoot emergence, shoot elongation, and root formation under the evaluated culture conditions. The integration of effective surface sterilization procedures with plant growth regulator supplementation provided a reproducible *in vitro* regeneration system for *H. undatus*. This protocol provides a foundation for future propagation and biotechnological improvement studies in pitahaya.

1. INTRODUCTION

Pitahaya (*Hylocereus undatus*) is a tropical and subtropical fruit crop of increasing global importance due to its high nutritional value, bioactive compounds, and expanding market demand [1,2]. The fruit is rich in antioxidants, vitamins, dietary fiber, and betalain pigments, which contribute to its functional and commercial value, supporting its rapid expansion in Latin America and Asia.

In Ecuador, pitahaya cultivation has become a significant agricultural activity, particularly in Amazonian and coastal regions where favorable agroecological conditions promote high productivity. However, production systems are constrained by phytosanitary problems associated with fungi, bacteria, and nematodes, which negatively affect plant health and yield [3]. These limitations are exacerbated by the widespread use of conventional vegetative propagation, which facilitates pathogen dissemination and compromises the long-term quality of planting material. The expansion of dragon fruit production in Ecuador has generated new opportunities for diversification and value creation in agricultural markets [4].

Although propagation through stem cuttings allows rapid clonal multiplication, it does not ensure the large-scale production of uniform and pathogen-free plants under controlled conditions [5,6]. Consequently, alternative propagation strategies are required to support sustainable production systems. In this context, *in vitro* micropropagation represents a reliable biotechnological approach for the rapid multiplication of genetically uniform and disease-free plant material [7]. Previous studies have demonstrated the feasibility of mass micropropagation systems for *H. undatus* under controlled conditions [8].

Despite its advantages, the success of *in vitro* culture systems depends on several interacting factors, including effective sterilization protocols, appropriate explant selection, and the optimization of plant growth regulators [9,10]. Among these, microbial contamination during the establishment phase remains one of the major limitations, as it can significantly reduce explant survival and compromise culture development. Therefore, the implementation of efficient sterilization strategies is a critical step in ensuring successful *in vitro* propagation.

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In addition, morphogenic response is strongly influenced by the balance between cytokinins and auxins, which regulate organogenesis and rhizogenesis. Cytokinins such as 6-benzylaminopurine (BAP) promote cell division and shoot induction, whereas auxins such as α-naphthaleneacetic acid (NAA) are essential for root formation and tissue differentiation [11]. The interaction between these regulators determines the efficiency of regeneration processes, and inappropriate concentrations can limit morphogenic development [12].

Explant type plays an important role in regeneration capacity. Juvenile tissues are often preferred because of their high morphogenic potential, whereas stem segments are frequently used in micropropagation because of their availability and regenerative competence. Previous studies have demonstrated that regeneration responses are influenced by explant source and plant growth regulator treatments [9,13,14]. Given these constraints, the development of optimized *in vitro* regeneration protocols for *H. undatus* is essential to enhance propagation efficiency and plant quality. Although several micropropagation protocols have been reported for *Hylocereus* species, most studies have focused primarily on shoot multiplication under specific plant growth regulator combinations. Comparatively few studies have integrated aseptic establishment, explant source evaluation, shoot induction, and root induction within a single regeneration framework. Furthermore, information remains limited for locally cultivated pitahaya germplasm in Ecuador. Therefore, the present study aimed to develop a reproducible regeneration system for the cultivar "Pitahaya roja pulpa blanca de Orellana" by evaluating surface sterilization protocols, explant source, and plant growth regulator treatments across successive stages of the regeneration process.

2. MATERIALS AND METHODS

2.1. Study Site and Experimental Conditions

The experiment was conducted between February and October 2025 in the Plant Biotechnology Unit of the Basic Sciences Laboratory at the Escuela Superior Politécnica de Chimborazo, Orellana Campus, Ecuador. *In vitro* cultures were maintained under controlled conditions at 26°C ± 2°C, with a 16/8 hour light/dark photoperiod, relative humidity of approximately 80%, and a photosynthetic photon flux density of approximately 40 µmol m^{-2} s^{-1} provided by cool-white fluorescent/LED lamps.

2.2. Plant Material and Explant Preparation

Mature fruits and stem segments were collected from healthy donor plants of *H. undatus* corresponding to the local cultivar "Pitahaya roja pulpa blanca de Orellana," cultivated in commercial pitahaya farms located in La Joya de los Sachas, Orellana Province, Ecuador. Plant material was selected from plants free of visible phytosanitary symptoms. Fruits and stem segments were obtained from representative donor plants maintained under commercial production conditions. Seeds extracted from fruits of the same cultivar were established *in vitro* to obtain juvenile explants. Hypocotyls and cotyledons were excised from germinated seedlings, whereas nodal stem segments containing visible areoles were used as direct explants.

2.3. Culture Medium

The basal medium consisted of Murashige and Skoog (MS) formulation [15], supplemented with 20 g L^{-1} sucrose and 8 g L^{-1} agar. The pH was adjusted to 5.7 ± 0.1 before sterilization. Media were autoclaved at 121°C for 20 minutes and dispensed under aseptic conditions.

2.4. Surface Sterilization

Surface disinfection was performed following standard plant tissue culture procedures [16]. Three sterilization protocols were designed and evaluated for their effectiveness in obtaining contamination-free cultures of *H. undatus*. The treatments differed in fungicide composition, commercial bleach concentration, ethanol concentration (50%, 60%, and 70% v/v), and exposure duration. The inclusion of different ethanol concentrations allowed their evaluation as components of the complete sterilization protocols.

Phyton (copper sulfate pentahydrate, 240 g L^{-1}, soluble aqueous formulation; Marketing Arm International, distributed by Ecuaquímica, Ecuador), Novac (thiophanate-methyl, 500 g L^{-1}; Agroquim, Ecuador), and Score (difenoconazole, 250 g L^{-1}, emulsifiable concentrate; Syngenta Crop Protection AG, Switzerland) were used as fungicidal agents. Commercial bleach containing 5% available chlorine was used as the primary disinfectant. These fungicides were selected because they are commonly used in horticultural production systems for the management of fungal contaminants associated with field-grown plant material.

The treatments were as follows:

T1: Explants were immersed in Phyton (1 ml L^{-1}) and Novac (0.75 ml L^{-1}) for 30 minutes, followed by 20% (v/v) commercial bleach, corresponding to approximately 1.0% available chlorine, for 5 minutes and 50% ethanol for 1 minute.

T2: Explants were immersed in Phyton (1 ml L^{-1}) and Score (1 ml L^{-1}) for 25 minutes, followed by 25% (v/v) commercial bleach, corresponding to approximately 1.25% available chlorine, for 10 minutes and 60% ethanol for 1 minute.

T3: Explants were immersed in Phyton (1 ml L^{-1}), Novac (0.75 ml L^{-1}), and Score (1 ml L^{-1}) for 60 minutes, followed by 30% (v/v) commercial bleach, corresponding to approximately 1.5% available chlorine, for 15 minutes and 70% ethanol for 1 minute.

The evaluated treatments were considered complete sterilization protocols. Because fungicide composition, commercial bleach concentration, ethanol concentration, and exposure duration varied simultaneously among treatments, the contribution of individual treatment components could not be independently assessed.

Seeds and stem segments were used as source material. After disinfection, samples were rinsed three times with sterile distilled water for 10 minutes each to remove residual chemicals before culture establishment. The presence or absence of visible contamination was assessed 15 days after culture establishment. Cultures showing no visible signs of microbial contamination at the assessment time were recorded as contamination-free.

2.5. Shoot Induction

The effect of 6-benzylaminopurine (BAP) on shoot induction was evaluated using stem, cotyledon, and hypocotyl explants cultured on MS medium supplemented with 0 (control), 1.0, 2.0, 3.0, and 4.0 mg L^{-1} BAP. Explants were cultured under the environmental conditions described above, and shoot induction was assessed 28 days after culture initiation. Explants showing at least one shoot at the time of assessment were recorded as positive for shoot induction.

2.6. Root Induction

Regenerated shoots used in the rooting experiment were obtained during the shoot induction stage from explants previously cultured

on media supplemented with different concentrations of BAP. Prior BAP exposure was not included as an experimental factor in the rooting experiment. Shoots approximately 1–2.5 cm in length were selected and transferred individually to MS medium supplemented with NAA for root induction. A control treatment without auxin (0 mg L^{-1}) and two NAA concentrations (0.3 and 0.5 mg L^{-1}) were evaluated. Rooting was assessed after 45 days, and shoots producing at least one functional root were considered successfully rooted.

2.7. Experimental Design and Statistical Analysis

A completely randomized design was used throughout the study. For the surface sterilization experiment, three sterilization treatments (T1–T3) were evaluated using seeds and stem segments. For each treatment, eight seeds and eight stem segments were assessed independently, resulting in 16 experimental units per treatment. The response was recorded as the presence or absence of visible contamination.

Shoot induction was evaluated using five concentrations of 6-benzylaminopurine (BAP; 0, 1.0, 2.0, 3.0, and 4.0 mg L^{-1}) and three explant types (stem segments, cotyledons, and hypocotyls). Eight explants were evaluated for each BAP concentration × explant-type combination, and shoot induction was recorded as a binary response (presence or absence of shoots).

Root induction was evaluated using three concentrations of NAA (0, 0.3, and 0.5 mg L^{-1}) in shoots derived from stem, cotyledon, and hypocotyl explants. Eight regenerated shoots were evaluated for each NAA concentration × explant-origin combination, resulting in 72 experimental units. Rooting was recorded as a binary response (presence or absence of roots).

In all experiments, each seed, explant, or regenerated shoot constituted an independent experimental unit and was cultured individually in a separate culture vessel.

Binary responses were expressed as proportions based on the number of successful responses relative to the total number of experimental units, and exact 95% binomial confidence intervals were calculated for the observed proportions. For the surface sterilization experiment, pairwise tests for equality of proportions were performed among sterilization treatments, with *p*-values adjusted using the Holm procedure. For shoot and root induction, pairwise comparisons among plant growth regulator treatments were performed separately within each explant type using Fisher's exact tests. Fisher's exact tests were used because extreme response frequencies (0% or 100%) could result in complete or quasi-complete separation and prevent reliable model-based estimation. For root induction, Holm adjustment was applied jointly across all nine pairwise comparisons, comprising three NAA-treatment comparisons within each of the three explant origins. Statistical significance was established at $p < 0.05$. All statistical analyses were performed in R version 4.5.1.

3. RESULTS

3.1. Effect of Sterilization Treatments on Contamination-Free Cultures

The sterilization protocols significantly influenced the frequency of contamination-free cultures (Table 1). Among the evaluated treatments, T3 achieved the highest frequency of contamination-free cultures (100.0%), whereas T2 resulted in an intermediate response (62.5%). In contrast, T1 showed the lowest frequency of contamination-free cultures (12.5%).

Pairwise tests for equality of proportions, with Holm-adjusted *p* values, revealed significant differences among sterilization treatments ($p < 0.05$), with T3 differing significantly from T1 and T2.

3.2. Effect of BAP Concentration on Shoot Induction

Shoot induction frequencies varied among BAP concentrations and explant types (Table 2). No shoot formation was observed in the control treatment, whereas shoot induction frequencies generally increased with increasing BAP concentration. The highest shoot induction frequencies were obtained at 4.0 mg L^{-1} BAP, reaching 87.5% in stem-derived explants and 75.0% in both cotyledon and hypocotyl explants. Lower BAP concentrations resulted in induction frequencies ranging from 12.5% to 62.5%.

At 3.0 and 4.0 mg L^{-1} BAP, shoot induction frequencies in stem-derived explants reached 62.5% and 87.5%, respectively. Under the evaluated treatments, the highest frequencies observed in cotyledon- and hypocotyl-derived explants were 75.0%. Pairwise comparisons using Fisher's exact tests with Holm-adjusted *p* values detected a significant difference only between the control treatment and 4.0 mg L^{-1} BAP in stem-derived explants ($p < 0.05$). No significant differences were detected among intermediate BAP concentrations or among treatments for cotyledon- and hypocotyl-derived explants after adjustment for multiple comparisons.

3.3. Root Induction

Rooting frequency increased with NAA supplementation in all explant types evaluated (Table 3). In the control treatment, rooting frequencies were 12.5%, 0.0% and 0.0% for stem-, cotyledon- and hypocotyl-

Table 1. Effect of sterilization treatments on aseptic establishment of *Hylocereus undatus*. Values are presented as the number of aseptically established units (n), establishment frequency (%), and exact binomial 95% confidence intervals (CI) estimated using the Clopper-Pearson method. Each treatment included eight seeds and eight stem segments ($N = 16$).

Treatment	Aseptic establishment (n)	Establishment (%)	CI	Group
T1	2	12.5	1.6–38.4	c
T2	10	62.5	35.4–84.8	b
T3	16	100.0	79.4–100.0	a

Different letters indicate significant differences among treatments according to Holm-adjusted pairwise comparisons ($p < 0.05$).

Table 2. Effect of BAP concentration on shoot induction of *Hylocereus undatus* explants.

BAP (mg L^{-1})	Stem			Cotyledon			Hypocotyl		
	n	%	CI	*n*	%	CI	*n*	%	CI
Control	0	0.0	0.0–36.9	0	0.0	0.0–36.9	0	0.0	0.0–36.9
1.0	2	25.0	3.2–65.1	2	25.0	3.2–65.1	1	12.5	0.3–52.7
2.0	3	37.5	8.5–75.5	3	37.5	8.5–75.5	2	25.0	3.2–65.1
3.0	5	62.5	24.5–91.5	5	62.5	24.5–91.5	4	50.0	15.7–84.3
4.0	7	87.5	47.3–99.7	6	75.0	34.9–96.8	6	75.0	34.9–96.8

Values are presented as the number of responsive explants (n), shoot induction frequency (%), and exact binomial 95% confidence intervals (CI) estimated using the Clopper-Pearson method. Eight independent explants were evaluated for each BAP concentration × explant-type combination ($N = 8$).

derived shoots, respectively. Supplementation with 0.3 mg L^{-1} NAA increased rooting frequencies to 75.0%, 62.5% and 50.0%, whereas 0.5 mg L^{-1} NAA resulted in the highest rooting frequencies, reaching 100.0%, 87.5% and 75.0% in stem-, cotyledon- and hypocotyl-derived shoots, respectively.

Pairwise comparisons evaluated using Fisher's exact tests with Holm-adjusted *p* values detected significant differences between the control treatment and 0.5 mg L^{-1} NAA for stem-derived ($p = 0.0126$), cotyledon-derived ($p = 0.0126$) and hypocotyl-derived shoots ($p = 0.0490$). No significant differences were detected between the control and 0.3 mg L^{-1} NAA or between 0.3 and 0.5 mg L^{-1} NAA within any explant type after adjustment for multiple comparisons.

3.4. Morphological Observations During the Regeneration Process

Morphological observations documented the sequential development of shoots and roots during the *in vitro* culture of *H. undatus* (Fig. 1). Shoot emergence and subsequent elongation were visually observed in the cultured explants, followed by root formation and plantlet development under the evaluated culture conditions. These observations document the morphological progression of the cultures but do not establish the cellular origin or developmental pathway of the regenerated shoots.

4. DISCUSSION

The development of efficient *in vitro* regeneration systems for *H. undatus* remains a significant challenge due to the variable morphogenic responses of explants and the limited scalability of conventional propagation methods. These constraints are particularly relevant in tropical fruit crops. Successful tissue culture depends on the interaction among contamination control, explant competence, and hormonal regulation. The results obtained in this study demonstrate that surface sterilization was important for obtaining contamination-free cultures. The 100.0% contamination-free response achieved with T3 indicates that the combination of fungicides, commercial bleach, and ethanol included in this treatment effectively prevented visible microbial contamination during culture establishment. This finding reinforces the importance of surface disinfection for obtaining contamination-free cultures and is consistent with previous studies identifying contamination as a major constraint in plant tissue culture systems [10,17].

However, because fungicide composition, disinfectant concentrations, and exposure durations varied concurrently among treatments, the individual contribution of each factor could not be determined. Consequently, T3 should be interpreted as the most effective sterilization protocol evaluated under the conditions of this study rather than as evidence supporting the effect of any individual disinfectant component. Furthermore, tissue viability and phytotoxicity were not assessed as independent response variables; therefore, no conclusions can be drawn regarding the potential phytotoxic effects associated with the sterilization treatments evaluated.

Regeneration responses varied among the explant types and plant growth regulator treatments evaluated. The highest shoot induction and rooting frequencies were observed in stem-derived explants under the experimental conditions tested. However, because the explants differed in developmental origin, ontogenic stage, physiological status, and source environment, these differences should not be interpreted as evidence of an explant-source effect. The observed responses may reflect the combined influence of pre-existing meristematic regions, endogenous hormonal balance, developmental status, and culture conditions. Previous studies have highlighted the importance of explant characteristics and developmental competence in plant tissue culture and regeneration systems [18,19]. In pitahaya specifically, the regenerative capacity of stem-derived tissues has been demonstrated in *H. undatus* micropropagation [20].

The increase in shoot induction observed across the BAP concentrations evaluated is consistent with the role of cytokinins in shoot development. Cytokinins stimulate cell division and shoot initiation through the activation of meristem-related genes and developmental signaling pathways [21]. In the present study, the highest shoot induction frequency was observed at 4.0 mg L^{-1} BAP. Previous studies have evaluated BAP-containing media for shoot regeneration and multiplication in pitahaya [6,12], although the magnitude and direction of the response may vary depending on genotype, explant source, culture conditions, and plant growth regulator combinations. However, as concentrations higher than 4.0 mg L^{-1} were not evaluated, the present results identify the most effective concentration tested rather than the absolute optimum.

Rooting frequencies increased in NAA-supplemented media, with the highest values observed at 0.5 mg L^{-1} NAA. Auxins are known to regulate adventitious root formation through the induction of cell differentiation and root primordium development [22]. The highest rooting frequencies were observed in stem-derived shoots at 0.5 mg L^{-1} NAA. However, regenerated shoots originated from cultures previously exposed to different BAP concentrations during the shoot induction stage. Therefore, a potential influence of prior cytokinin exposure on subsequent rooting responses cannot be excluded. Similar rooting responses have been reported in other *in vitro* propagation systems, highlighting the importance of plant growth regulator interactions during regeneration and rooting stages [23].

Morphological observations documented the sequential development of shoots, shoot elongation, and subsequent root formation under the evaluated culture conditions. However, the photographic evidence alone does not establish meristematic tissue identity or demonstrate that the observed shoots originated through *de novo* or adventitious organogenesis, particularly because stem explants contained pre-

Table 3. Effect of NAA concentration on root induction of regenerated *Hylocereus undatus* shoots.

NAA (mg L^{-1})	Stem			Cotyledon			Hypocotyl		
	n	%	CI	n	%	CI	n	%	CI
0.0	1	12.5	0.3–52.7	0	0.0	0.0–36.9	0	0.0	0.0–36.9
0.3	6	75.0	34.9–96.8	5	62.5	24.5–91.5	4	50.0	15.7–84.3
0.5	8	100.0	63.1–100.0	7	87.5	47.3–99.7	6	75.0	34.9–96.8

Values are presented as the number of rooted shoots (n), rooting frequency (%), and exact binomial 95% confidence intervals (CI) estimated using the Clopper-Pearson method. Eight independent regenerated shoots were evaluated for each NAA concentration × explant-type combination ($N = 8$).

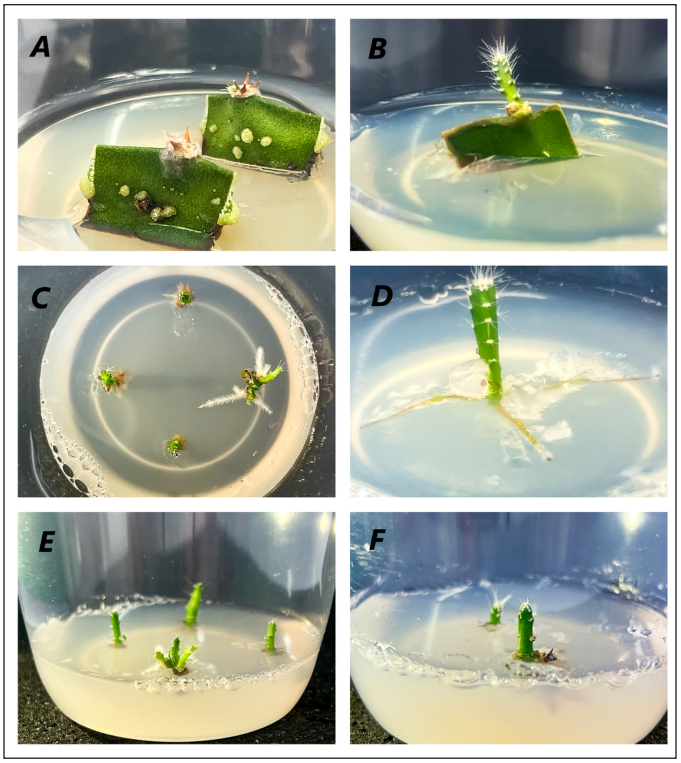


Figure 1. Morphogenic responses of *Hylocereus undatus* during *in vitro* regeneration. (A) Early morphogenic responses and formation of meristematic structures at the explant surface. (B) Emergence of adventitious shoots from areolar regions. (C) Development of multiple shoot buds and shoot differentiation. (D) Elongated shoot exhibiting adventitious root formation. (E) Proliferation of regenerated shoots under *in vitro* culture conditions. (F) Further shoot elongation and development prior to the rooting stage.

existing areoles. Although these developmental responses are consistent with the general characteristics of organogenesis described in plant tissue culture [24], the cellular origin and developmental pathway of the regenerated shoots cannot be conclusively determined from morphological observations alone. Complementary anatomical or histological analyses would be required to distinguish between shoot development from pre-existing meristematic regions and *de novo* or adventitious organogenesis.

From an applied perspective, the integration of effective surface sterilization procedures with appropriate plant growth regulator concentrations provided a useful framework for *in vitro* propagation of *H. undatus*. The combination of high aseptic establishment rates, efficient shoot induction, and enhanced rooting provides a useful framework for *in vitro* propagation and germplasm multiplication. Nevertheless, additional studies evaluating acclimatization, *ex vitro* performance, multiplication rates, and genetic stability are required before the protocol can be fully validated for commercial-scale micropropagation. Furthermore, future studies should quantify the number of shoots produced per explant to optimize multiplication efficiency and more comprehensively evaluate the propagation potential of the regeneration system, as quantitative measures of regeneration efficiency provide important criteria for protocol optimization and comparison among studies [25].

5. CONCLUSION

This study established a reproducible *in vitro* regeneration protocol for *H. undatus*, demonstrating the importance of effective surface sterilization and plant growth regulator supplementation for successful culture establishment and regeneration. The sterilization treatment consisting of Phyton, Novac, Score, 30% (v/v) commercial bleach, and 70% ethanol (T3) achieved the highest frequency of contamination-free cultures. Regeneration and rooting were successfully achieved in stem, cotyledon, and hypocotyl explants under the conditions evaluated. The highest shoot induction frequency (87.5%) was observed with 4.0 mg L^{-1} BAP in stem-derived explants, whereas the highest rooting frequency (100.0%) was observed at 0.5 mg L^{-1} NAA in stem-derived shoots. Morphological observations documented the sequential development of shoots and roots under the evaluated culture conditions. Although the protocol shows considerable potential for the propagation of *H. undatus*, further studies addressing acclimatization, multiplication rates, *ex vitro* performance, and genetic stability are required before commercial-scale application. Nevertheless, the regeneration system described here provides a useful foundation for germplasm propagation and future biotechnological improvement programs in pitahaya.

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data are available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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