



Progress towards in vitro shoot regeneration and genetic transformation in pitaya (*Hylocereus undatus*) cladode sections

Elisabeth Carmona-Martín¹ · Francisco Conde^{1,2} · Cesar Petri¹

Received: 21 April 2025 / Accepted: 17 July 2025 / Published online: 4 August 2025
© The Author(s) 2025

Abstract

The absence of effective genetic transformation methods in *Pitaya* species significantly restricts the investigation of gene function and implementation of modern breeding techniques such as gene editing. Establishing an efficient regeneration protocol is a prerequisite for genetic transformation. In this study, a procedure for adventitious shoot regeneration in *Hylocereus undatus* cv. ‘Cosmic’ is described. Various factors affecting in vitro shoot regeneration from cladode sections were evaluated, including the effects of cytokines, auxins, and light exposure. The best results were achieved on Murashige and Skoog medium supplemented with 4.5 μM N6-benzylaminopurine and 0.5 μM indole-3-butyric acid, reaching regeneration rates up to $82.1\% \pm 5.1\%$. Additionally, the effect of the antibiotic kanamycin was studied. Forty mg L^{-1} kanamycin completely inhibited shoot regeneration from non-infected explants, whereas $5.4 \pm 3.0\%$ of the explants infected with *A. tumefaciens* harboring the pBINGUSint binary plasmid regenerated at the same kanamycin concentration. Nevertheless, based on GUS assays, none of the regenerated shoots were confirmed as transgenic. The relatively high rate of shoot regeneration achieved is a critical step towards developing effective transformation techniques for this species. The development of such a method will help pitaya breeding progress more quickly by opening the door for functional genomics studies and gene editing.

Key message

An efficient adventitious shoot regeneration protocol in *H. undatus* was established as an essential preliminary tool for developing a transformation system in this species.

Keywords *Agrobacterium*-mediated transformation · Cactaceae · Dragon fruit · Organogenesis · Plant biotechnology · Tissue culture

Elisabeth Carmona-Martín and Francisco Conde contributed equally to this study.

Communicated by Ranjith Pathirana.

✉ Cesar Petri
cesar.petri@csic.es

¹ IHSM-UMA-CSIC La Mayora, Área de Mejora y Fisiología de Plantas, Grupo Mejora y Biología del Desarrollo de Frutales Tropicales, Avenida Dr. Wienberg, s/n. 29750, Algarrobo-Costa, Málaga, Spain

² IHSM-UMA-CSIC La Mayora, Unidad de Transformación Genética y Cultivo in Vitro de Plantas, Avenida Dr. Wienberg, s/n 29750, Algarrobo-Costa, Málaga, Spain

Introduction

Pitayas (*Hylocereus* spp.), also known as pitahayas, are perennial climbing cacti native to tropical areas of the Americas. Their fruit, commonly called “dragon fruit”, is a medium/large berry with bracts on the skin and a juicy pulp filled with numerous small, soft seeds. Being a tropical plant, pitaya is adapted to arid and semiarid environments, and exhibits unique physiological characteristics such as Crassulacean Acid Metabolism (CAM) pathway, metamorphosed leaves and modified stems (cladodes) with stomata to facilitate gas exchange due to their reduced leaves. Among the various *Hylocereus* species, fruits differ in mesocarp and endocarp color and/or in the color of the skin (exocarp). The most common commercially cultivated species are *H. megalanthus*, *H. monacanthus* (AKA *H. polyrhizus*), and *H.*

undatus (Ortiz-Hernández and Carrillo-Salazar 2012; Ibrahim et al. 2018).

In recent years, the nutritional properties of pitaya fruit have been extensively studied (Ibrahim et al. 2018; Chen et al. 2023). However, this crop may have also importance in the industry for bioplastics or as a natural dye for food and cosmetics (Taharuddin et al. 2023). Moreover, its high drought tolerance (Wang et al. 2021) makes it particularly attractive for cultivation in arid and semi-arid zones in the context of climate change, where water resources must be conserved.

Pitaya species currently lack efficient genetic transformation systems (Chen et al. 2023). The advancement of biotechnology tools offers substantial benefits, not only by enhancing our understanding of biological processes but also by enabling the efficient incorporation of desirable traits into cultivated genotypes through transformation or gene editing.

To develop effective genetic transformation tools in a given species, several aspects of tissue culture must first be optimized. An in vitro multiplication protocol is necessary to maintain and propagate plant material to be used as explant sources. Additionally, it is essential to develop protocols for somatic embryogenesis or shoot organogenesis, enabling the regeneration of whole plants from somatic cells. This process involves cellular reactivation or reprogramming that leads to the formation of new plantlets. Finally, it is necessary to implement a technique to stably introduce foreign genetic material into pluripotent somatic cells.

While there are some reports on pitaya micropropagation (Mohamed-Yasseen 2002; Viñas et al. 2012; Fan et al. 2013; Zambrano-Forero et al. 2015; Hua et al. 2015; Rahman et al. 2015; Qin et al. 2017; Bozkurt et al. 2022; Lee and Chang 2022; Mállap-Detquizán et al. 2022; Comlekcioglu 2024), few studies have documented adventitious shoot regeneration in dragon fruit (Infante 1992; Pelah et al. 2002; Dahanayake and Ranawake 2011; Suarez Roman et al. 2014; Rodrigues et al. 2022), and only two of these focused on *H. undatus* (Dahanayake and Ranawake 2011; Rodrigues et al. 2022). Notably, these studies did not report regeneration rates. Furthermore, to the best of our knowledge, there is currently no published manuscript providing a detailed account or experimental evidence of successful genetic transformation in this species.

Therefore, the objective of this study was to establish an efficient protocol for adventitious shoot regeneration in *H. undatus* as an indispensable first step towards the development of robust transformation systems. To this end, we explored the effects of several factors typically influencing in vitro shoot regeneration, including some that have not previously been studied in this species. Additionally, we investigated the potential to use the antibiotic kanamycin

Fig. 1 (a) Micropropagated shoots of pitaya (*H. undatus* cv. 'cosmic') of about 8 weeks old used as the source of explants. (b) Explants; cladode cross-sections of about 1–2 mm thick. Horizontal bar indicates 1 cm. (c) Adventitious shoot regeneration (blue arrows). Eventually, root regeneration was observed (red arrow). Photograph was taken at 7 weeks from the beginning of the experiment. Horizontal bar indicates 1 cm. (d) Detail of direct shoot regeneration from a cladode section. Photograph was taken at 8 weeks from the beginning of the experiment. Horizontal bar indicates 0.5 cm. (e) Detail of indirect shoot regeneration. Photograph was taken at 8 weeks from the beginning of the experiment. Horizontal bar indicates 0.5 cm. (f) Callusgenesis observed at 8 weeks from the beginning of the experiment in the different cytokinin treatments. Horizontal bar indicates 1 cm. (g) Acclimatized shoots cultured in a greenhouse. Photograph was taken at 6 weeks from the beginning of the acclimatization process

for selective shoot regeneration and conducted assays of *Agrobacterium*-mediated transformation.

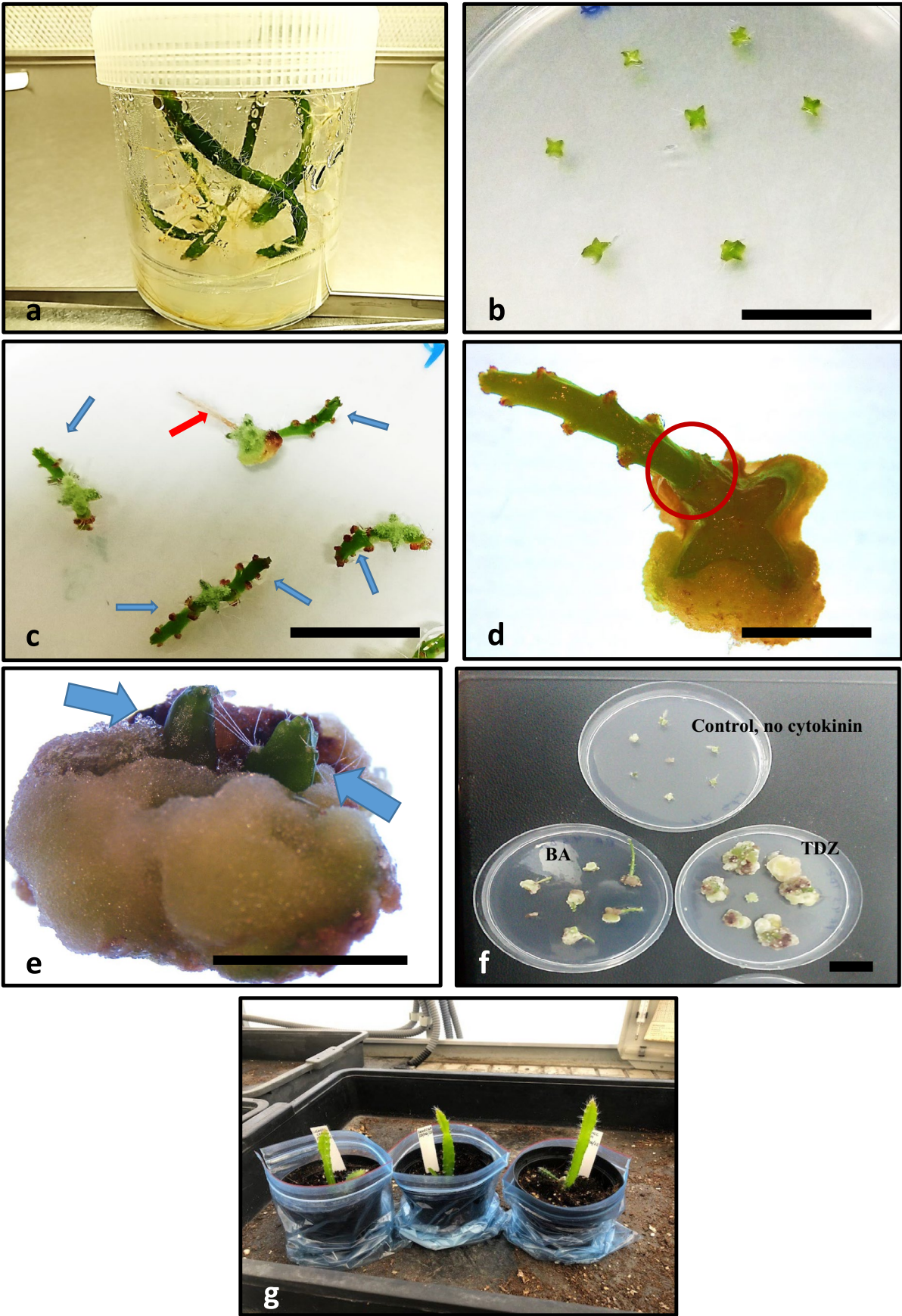
Materials and methods

Plant material and in vitro establishment

Nodal segments from adult *H. undatus* (cv. 'Cosmic') plants were collected at the experimental station of the Instituto de Hortofruticultura Subtropical y Mediterránea (IHSM) (Malaga, Spain) and used as starting material for in vitro culture establishment. For surface sterilization, the cladode nodes were first rinsed briefly with water. They were then immersed in a solution containing 20% commercial bleach (commercial bleach = 6.15% sodium hypochlorite) and 0.02% Tween-20 for 20 min, followed by three rinses with sterile distilled water under a laminar flow hood. The sterilized nodes were placed on multiplication medium (MM), consisting of Murashige and Skoog (MS) medium including MS vitamins (Murashige and Skoog 1962), supplemented with 0.2 mM adenine sulfate dihydrate, 3% (w/v) sucrose and 0.7% (w/v) plant agar (Duchefa Biochemie, Haarlem, The Netherlands. Ref: P1001). The pH was adjusted to 5.7 before sterilization in the autoclave at 121°C for 20 min. Cultures were maintained in a growth chamber under a 16/8 h light/dark photoperiod, with a light intensity of 45–50 $\mu\text{E m}^{-2} \text{s}^{-1}$ and a constant temperature of 25 ± 1 °C. Newly developed axillary shoots were subcultured onto fresh MM every eight weeks (Fig. 1a).

General regeneration strategy

Eight-week-old in vitro shoots maintained in MM were used as the source of explants; cladode cross-sections of about 0.5–1.0 mm thickness (Fig. 1b). The basal regeneration medium (BRM) consisted of MS medium including vitamins (Murashige and Skoog 1962), 3% (w/v) sucrose and 0.7% (w/v) purified agar (Sigma-Aldrich, Darmstadt, Germany).



Ref: A1296). The pH was adjusted to 5.8 before sterilization in the autoclave at 121°C for 20 min. Cultivation conditions were the same as described above, except the light intensity was reduced to 20–25 $\mu\text{E m}^{-2} \text{s}^{-1}$. Explants were remained on the same culture plates throughout the experiment. This shoot regeneration protocol was followed, with the modifications indicated below, for all of the experiments.

Regeneration was evaluated weekly, starting 3–4 weeks after the beginning of the experiment, and continued until no new adventitious bud formation was observed for two consecutive weeks (approximately 12–13 weeks from the beginning of the experiment).

Effect of cytokinins

The effect of two cytokinins at two concentrations on shoot regeneration from pitaya cladode sections was evaluated. For this experiment, explants were cultured on BRM supplemented with 0.25 μM indole-3-butyric acid (IBA) and either 4.5 or 9.0 μM N6-benzylaminopurine (BA), or 4.5 or 9.0 μM thidiazuron (TDZ). A control treatment without any cytokinin supplementation was included.

Effect of auxins

The influence of two auxins at two concentrations on shoot regeneration was examined. For this experiment, explants were cultured on BRM supplemented with 4.5 μM BA and either 0.25 or 0.5 μM IBA, or 0.25 or 0.5 μM α -naphthaleneacetic acid (NAA). A control treatment without auxin was included.

Effect of a dark period

Explants were cultured on BRM supplemented with 4.5 μM BA and 0.5 μM IBA under three light or dark treatments: a 16-h photoperiod (20–25 $\mu\text{E m}^{-2} \text{s}^{-1}$) at 25 ± 1 °C or complete darkness for 1 or 2 weeks (at 25 ± 1 °C) before transfer to a 16-h photoperiod (20–25 $\mu\text{E m}^{-2} \text{s}^{-1}$) at 25 ± 1 °C.

Effect of an auxin pulse

Explants were initially cultured on BRM supplemented with 4.5 μM BA, 0.5 μM IBA and 9.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D) for three days, then transferred to BRM with 4.5 μM BA, 0.5 μM IBA (without 2,4-D). Explants were incubated in complete darkness at 25 ± 1 °C for one week, then transferred to a 16-h photoperiod at 20–25 $\mu\text{E m}^{-2} \text{s}^{-1}$ and 25 ± 1 °C.

Shoot elongation, rooting and acclimatization

As new shoots reached 2–3 cm length, they were excised and cultured in MM for elongation and rooting. Rooted shoots were washed to remove agar residues and transferred to pots containing 60% coconut fiber, 30% commercial substrate (Turbas y Coco Mar Menor S.L., Spain) and 10% litonite. Potted plantlets were introduced into plastic bags which were sealed and maintained under shaded greenhouse conditions. Bags were opened gradually and, after 2 weeks, they were fully opened.

Regeneration dose–response curve to kanamycin

Explants were cultured on BRM supplemented with 4.5 μM BA and 0.5 μM IBA during 2 days, and then transferred to BRM with 4.5 μM BA, 0.5 μM IBA, 300 mg L⁻¹ cefotaxime sulphate (Duchefa, Haarlem, The Netherlands) and 0, 20 or 40 mg L⁻¹ of kanamycin sulphate (Duchefa, Haarlem, The Netherlands). Antibiotics were filter sterilized and added to the autoclaved medium.

Agrobacterium tumefaciens-mediated transformation

Non oncogenic *Agrobacterium tumefaciens* strain EHA105 carrying the binary plasmid p35SGUSINT (Vancanneyt et al. 1990) was used in transformation experiments. The T-DNA of the plasmid contains the Nospro-*nptII*-Noster cassette as the selectable marker gene conferring resistance to aminoglycoside antibiotics (kanamycin), and the 35Spro-*uidA*-Noster cassette as the reporter gene. The reporter gene includes an intron for plant-specific expression.

The engineered *A. tumefaciens* strain was cultured and prepared for infection as described by Wang et al. (2013). Briefly, for co-cultivation, a single colony was inoculated into 10 ml of Luria–Bertani medium (10 g L⁻¹ Bacto-tryptone, 5 g L⁻¹ Bacto-yeast extract, 1 g L⁻¹ NaCl, 1 g L⁻¹ glucose; pH 7.0) with 100 mg L⁻¹ ampicillin and 50 mg L⁻¹ kanamycin, and incubated overnight at 28°C with constant agitation (175 rpm). Overnight culture growth reached an optical density at 600 nm between 0.2 and 1.0. Cultures were centrifuged at 3,000 g for 15 min and resuspended in 50 mL of bacterial resuspension medium consisting of MS salts, 2% (w/v) sucrose and 100 μM acetosyringone. Culture in bacterial resuspension medium was shaken (175 rpm) at 25°C for 5 h before use.

Cladode sections were immersed in the bacterial suspension for 20 min, blotted briefly on sterile filter paper and placed on co-cultivation medium (BRM without antibiotics supplemented with 4.5 μM BA and 0.5 μM IBA). After 2 days, explants were washed in bacterial resuspension

Table 1 Effect of two cytokinins at two concentrations, combined with 0.25 μM IBA, on adventitious shoot regeneration from *H. undatus* cladode sections

Treatment	Regeneration rate (% $\pm$ S.E.)	No. of shoots regenerated	No. of shoots/Regenerating explant
Control (only 0.25 μM IBA)	2.0 $\pm$ 2.0	1	1.0 $\pm$ 0.1
4.5 μM BA	46.4 $\pm$ 6.7***	29	1.1 $\pm$ 0.1
9.0 μM BA	40.8 $\pm$ 7.0***	22	1.1 $\pm$ 0.1
4.5 μM TDZ	17.9 $\pm$ 5.1**	11	1.1 $\pm$ 0.1
9.0 μM TDZ	16.1 $\pm$ 4.9**	9	1.0 $\pm$ 0.1

Asterisks indicate statistical significance compared with the control (** $p\leq 0.01$, *** $p\leq 0.001$). A total of 280 explants were used in this study, with each treatment consisting of two replicates of 28 explants

Table 2 Effect of two auxins at two concentrations, combined with 4.5 μM BA, on adventitious shoot regeneration from *H. undatus* cladode sections

Treatment	Regeneration rate (% $\pm$ S.E.)	No. of shoots regenerated	No. of shoots/Regenerating explant
Control (only 4.5 μM BA)	33.9 $\pm$ 6.3	22	1.2 $\pm$ 0.1
0.25 μM IBA	46.4 $\pm$ 6.7	38	1.5 $\pm$ 0.1
0.5 μM IBA	53.6 $\pm$ 6.7*	45	1.5 $\pm$ 0.2
0.25 μM NAA	41.1 $\pm$ 6.6	34	1.5 $\pm$ 0.2
0.5 μM NAA	35.7 $\pm$ 6.4	22	1.1 $\pm$ 0.1

Asterisk indicates statistical significance compared with the control (* $p\leq 0.05$). A total of 280 explants were used in this study, with each treatment consisting of two replicates of 28 explants

medium with 300 mg L^{-1} cefotaxime, blotted briefly on sterile filter paper, and then placed on BRM with 4.5 μM BA, 0.5 μM IBA, 300 mg L^{-1} cefotaxime and 0, 20 or 40 mg L^{-1} kanamycin.

To determine stable transformation, GUS assays (Jefferson 1987) were performed 8 weeks after the infection.

Experimental design and statistical analyses

Regeneration and transformation experiments were performed in a completely randomized design. Each treatment consisted of four Petri dishes containing seven cladode sections each. All experiments were repeated twice, resulting in 56 explants per treatment. To test for differences among more than two independent groups, data were transformed as needed to fit to a Normal distribution and ANOVA was performed. Mean comparisons were conducted using Student's t-test (for number of shoots per regenerating explant) or Pearson's Chi-square test (for regeneration percentages).

Results

Nodes, collected from mature plants (cv. 'Cosmic') cultivated in the field, were established in MM and they developed new axillary shoots in 3–4 weeks. The new in vitro axillary shoots were excised and sub-cultured at 8-week intervals onto fresh MM. As they were subcultured, some them were used as the source of cladode cross-sections used in the regeneration and transformation experiments.

First adventitious shoots started appearing from cladode sections after 3–4 weeks of culture. Both, direct and indirect regeneration was observed (Fig. 1c, d, e). Eventually, regeneration of adventitious roots was also detected (Fig. 1c). Additional buds did not appear after 12–13 weeks of culture.

The addition of cytokinins to BRM significantly improved regeneration rates compared with the cytokinin-free control (Table 1). Statistical differences in the regeneration rates between the two cytokinins tested were also detected. BA, significantly (9.0 μM BA; $p\leq 0.01$ and 4.5 μM BA; $p\leq 0.05$) enhanced shoot regeneration compared with the TDZ treatments. BA favored predominantly direct shoot formation (Fig. 1d), whereas TDZ promoted indirect organogenesis (Fig. 1e) and callus formation (Fig. 1f).

Among the auxin treatments, only 0.50 μM IBA (combined with 4.5 μM BA) produced a significant increase ($p\leq 0.05$) in shoot regeneration frequency relative to the auxin-free control (Table 2). Neither IBA at 0.25 μM nor NAA at 0.25 or 0.50 μM enhanced regeneration (Table 2).

Across all plant growth regulators treatments, the average number of shoots per regenerating explant did not differ significantly (Tables 1, 2). The best combination (4.5 μM BA + 0.5 μM IBA) yielded 53.6 $\pm$ 6.7% regeneration with an average of 1.5 shoots per regenerating explant (Tables 1, 2).

Twenty-five regenerated shoots were randomly selected for elongation, rooting, and acclimatization. All shoots elongated and produced roots on MM within 4–5 weeks. After that, they were successfully acclimatized to greenhouse conditions (Fig. 1g).

A pre-culture in darkness for one or two weeks did not improve overall regeneration compared with continuous light (Fig. 2). However, a two-week dark period reduced regeneration significantly ($p\leq 0.05$) relative to a one-week dark treatment (Fig. 2). The average shoot number per responsive explant was unaffected among light treatments.

A three-day pulse with 9.0 μM 2,4-D strongly induced callus but almost completely suppressed shoot regeneration (Fig. 3).

Regarding the effect of antibiotics, kanamycin at 20 mg L^{-1} significantly decreased regeneration in non-infected explants ($p\leq 0.001$), and 40 mg L^{-1} inhibited it (Fig. 4a). Cefotaxime, at 300 mg L^{-1} , did not affect

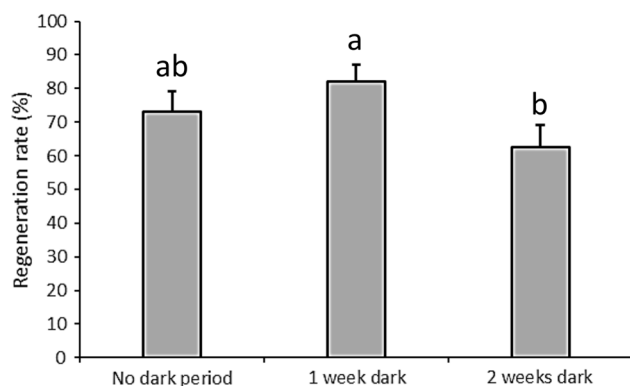


Fig. 2 Effect of different dark incubation regimes on adventitious shoot regeneration from *H. undatus* cladode sections. Different letters indicate statistically significant differences among treatments ($p < 0.05$). A total of 168 explants were used in this study, with each treatment consisting of two replicates of 28 explants

regeneration (Fig. 4a). In the *Agrobacterium*-inoculated explants, when kanamycin was not added to the medium, regeneration was comparable to non-inoculated explants (Fig. 4a). At 20 mg L^{-1} kanamycin, infected explants regenerated at a significantly higher rate than non-infected ones ($p \leq 0.01$). Infected explants ($5.4 \pm 3.0\%$) produced shoots even at 40 mg L^{-1} kanamycin (Fig. 4a). Histochemical GUS assays detected no activity in non-infected controls (Fig. 4b) and only occasional blue spots on a few infected explants (Fig. 4c, d). None of the regenerated shoots expressed GUS. Based on explant-level GUS staining, the apparent transformation efficiency was 5.6%.

Discussion

At least two different cellular strategies are available to plants to initiate the regeneration process. One method involves reactivation of undifferentiated cells, while the

other involves reprogramming differentiated somatic cells (Ikeuchi et al. 2016). In these processes, plant growth regulators play a crucial role, among which auxins and cytokinins are key. During in vitro plant organogenesis, the ratio of auxin-to-cytokinin, and their concentration, is a critical factor that initiates in vitro developmental events. A comparatively high auxin-to-cytokinin ratio encourages root regeneration, the opposite is generally required for shoot regeneration, while similar levels of auxin and cytokinin result in callus (Zhao et al. 2008; Ikeuchi et al. 2016).

Consistent with these principles, adding cytokinins was essential for shoot induction from pitaya cladode sections (Table 1). Adenine or phenylurea are the common precursors of cytokinins. Phenylurea derivatives, such as TDZ, are synthetic cytokinins, while BA is a natural adenine derivative (Savelieva et al. 2018). In our study, BA proved significantly more effective than TDZ. Adventitious meristems may theoretically arise through two distinct pathways: (i) Direct organogenesis, wherein meristems originate directly from differentiated cells within a freshly excised segment of whole-plant tissue, without the intermediary formation of undifferentiated cellular masses; or (ii) indirect organogenesis, involving the development of meristems from dedifferentiated, unspecialized, and unorganized cells derived from callus tissues. We observed both, direct and indirect organogenesis (Fig. 1). However, when BRM was supplemented with BA, the majority of the shoots appeared as direct regeneration. This fact is generally preferred when genetic stability is desired and somaclonal variation is to be avoided (Bairu et al. 2011). Our observations agree with previous results on *H. undatus* where TDZ, at $9.0 \mu\text{M}$, stimulated indirect shoot regeneration (Rodrigues et al. 2022) whereas BA, at $11.0 \mu\text{M}$, induced direct shoot organogenesis (Dahanayake and Ranawake 2011).

The best regeneration was achieved with $4.5 \mu\text{M}$ BA plus $0.5 \mu\text{M}$ IBA; this combination was therefore used to test

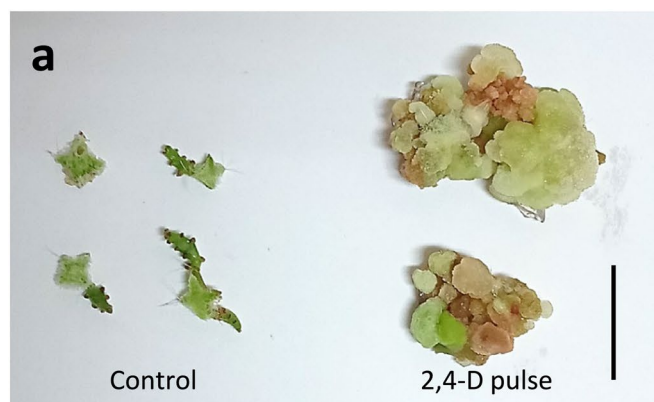
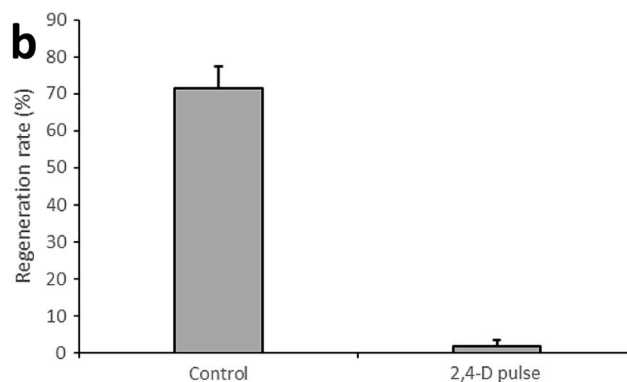
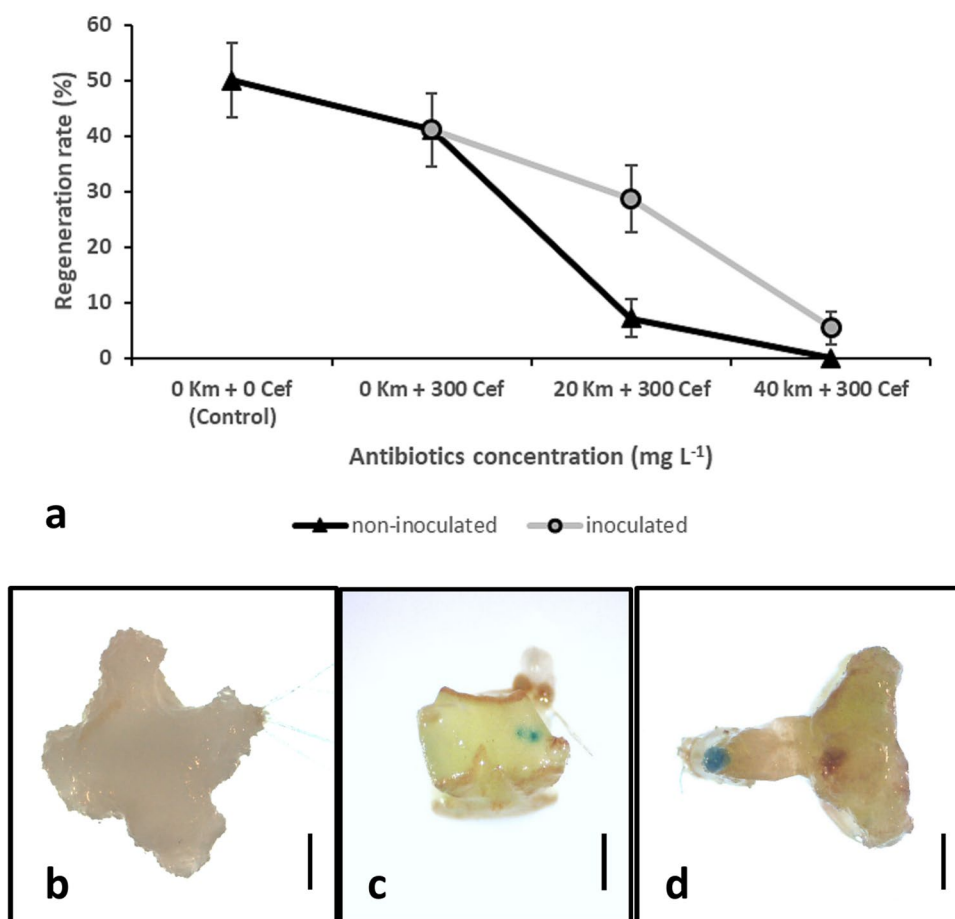


Fig. 3 Effect of a 2,4-D pulse ($9.0 \mu\text{M}$), during the first 3 days of culture, on shoot regeneration from *H. undatus* cladode sections. (a) Effect on callogenesis. Photograph taken at 8 weeks from the begin-



ning of the experiment. Vertical bar indicates 1.0 cm. (b) Effect on regeneration rates. A total of 112 explants were employed in this study, with each treatment consisting of two replicates of 28 explants

Fig. 4 (a) Effect of the antibiotics kanamycin (Km) and cefotaxime (Cef) on shoot regeneration from *H. undatus* cladode sections, non-infected or infected *Agrobacterium tumefaciens* carrying the binary plasmid pBINGUSint. A total of 224 explants were used in this study. (b) Histochemical GUS detection on non-infected (control) or (c, d) infected explants with *A. tumefaciens* carrying the binary plasmid pBINGUSint. GUS assay was carried out 8 weeks after infection. Vertical bars indicate 1 mm



two factors not previously evaluated in *H. undatus*: dark pre-culture and an auxin pulse. These factors have been identified as key determinants in adventitious shoot regeneration in other species (Pérez-Tornero et al. 2000; Petri et al. 2005; Petri and Scorza 2010). However, neither treatment improved regeneration here. A three-day exposure to 9.0 μ M 2,4-D almost completely inhibited shoot formation while stimulating callogenesis, in agreement with other reports where 2,4-D induced non-organogenic calli in pitaya (Lopes et al. 2016; Rodrigues et al. 2022). In the model plant *Arabidopsis*, shoot regeneration requires an auxin-rich induction medium followed by a cytokinin-rich medium. This two-steps process provokes the expression of different genes to achieve reprogramming differentiated somatic cells for subsequent shoot organogenesis (Che et al. 2007). In *Prunus* spp. a higher auxin dose at the first steps of the in vitro culture process enhances adventitious shoot organogenesis (Petri et al. 2005, 2008; Petri and Scorza 2010; Carmona-Martin and Petri 2020). *H. undatus*, however, did not respond similarly, suggesting species-specific hormonal requirements. The poorer performance after a two-week dark treatment may likewise reflect an unfavorable shift in the endogenous cytokinin-to-auxin balance, as darkness can

raise indole-3-acetic acid (Lopez-Carbonell et al. 1992). The increase in auxin levels during the dark period at the early stage of the regeneration process may be comparable to the exogenous addition of 2,4-D during the 3 days assayed in the current study. Longer periods, over 2 weeks, in dark may reduce even more shoot organogenesis from pitaya cladode sections.

The average number of shoots per regenerating explant (about 1.5) is modest compared with the up to 18 shoots per explant reported by Dahanayake and Ranawake (2011), using 5 mm *H. undatus* stem sections. Notably, regeneration rates were not clearly explained within the manuscript. Future work should test additional combinations, including additional BA and NAA treatments, and explore alternative plant growth regulators.

Cefotaxime is a broad-spectrum cephalosporin that is frequently applied in plant transformation experiments for the elimination of *Agrobacterium* after co-cultivation. The concentration tested in our study did not affect shoot regeneration from pitaya cladode sections suggesting it could serve as an effective tool to control *Agrobacterium* overgrowth. Kanamycin is an aminoglycoside antibiotic commonly used as the selection agent for *nptII*-transformed cells/tissues in

plant tissue culture. Here, the detoxifying effect of *nptII* was clearly notable in terms of adventitious regeneration, significantly increasing the rates on infected explants when 20 mg L⁻¹ kanamycin was applied and allowing shoot regeneration at the inhibitory concentration of 40 mg L⁻¹. However, these results did not correlate with the detection of the reporter maker gene (*uidA*). GUS events (spots) in the surface of the explants were infrequent and none of the regenerated shoots showed activity of the reporter gene.

Several factors are known to affect GUS detection. Luth and Moore (1999) described that only 11.9% of transformed grapefruit plants clearly showed GUS activity. In other reports, the expression was different among tissues, with more blue spots in roots than in green tissues (Gutoranov et al. 2001; Motioike et al. 2002). This non-uniform gene expression could be associated with gene silencing processes, its position in the chromosome or post-transcriptional silencing (Iocco et al. 2001), with the promoter driven the gene also being very important (Gittins et al. 2001). All of these results collectively demonstrate that X-glu staining patterns do not always accurately reflect the gene's specificity of expression. Under our experimental conditions, GUS detection was very poor. Molecular tissue analyses (PCR, DNA blots or DNA sequencing) could probably have shown more precise transformation efficiencies.

Rooting and acclimatization of pitaya plantlets were previously described as highly efficient steps, reaching rates of 90–100% (Mohamed-Yasseen 2002; Viñas et al. 2012; Fan et al. 2013; Hua et al. 2015; Rahman et al. 2015; Qin et al. 2017; Bozkurt et al. 2022; Lee and Chang 2022; Mál-lap-Detquizán et al. 2022; Comlekcioglu 2024). The good performance of *Hylocereus* spp. in these stages may be explained by their natural ability to produce adventitious roots and their adaptation to drought stress and poor soil conditions (Ortiz-Hernández and Carrillo-Salazar 2012; Shah et al. 2023). As expected, we likewise achieved high success in rooting and acclimatization of the plantlets to ex vitro conditions.

Conclusions

Here, we describe an efficient shoot regeneration procedure for *H. undatus* (cv. 'Cosmic'). This system might be transferable, with more or less efficiency, to other genotypes. Although the regeneration rates achieved were relatively high (up to 82.0%), the number of buds per regenerating explant remained low (around 1.5). To successfully integrate the regeneration protocol with a transformation system, it may be necessary to enhance the number of regeneration

events per explant in order to increase the likelihood of recovering transformed shoots. Further experiments will focus on optimizing the procedure by evaluating additional factors such as the effect of ethylene inhibitors and polyamines, nanoparticles (Kim et al. 2017), fungi-associated biostimulants (Li et al. 2019; Faize et al. 2021; Essalimi et al. 2022) or the use of small molecule inhibitors to improve in vitro plant cell reprogramming (Carneros et al. 2024).

In addition, the inhibitory curve of a selective agent (kanamycin) has been established and, to the best of our knowledge, susceptibility of pitaya tissues to *A. tumefaciens* infection has been shown for the first time. The procedure seems promising and could lead to an efficient transformation method for this species. Elucidating such a technique paves the way for functional genomics studies and molecular breeding with desirable traits (through gene editing or transformation), which will help accelerate pitaya breeding efforts.

Acknowledgements Authors acknowledge José I. Hormaza Urroz and Ruth Aranda Nebot for providing the initial plant material. We appreciate the support of Chris Dardick, who kindly revised the manuscript for English language and style.

Authors contribution All authors contributed to the study conception, design of the experiments, data collection and analysis. The first draft of the manuscript was written by CP and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

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

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Bairu MW, Aremu AO, van Staden J (2011) Somaclonal variation in plants: causes and detection methods. *Plant Growth Regul* 63:147–173. <https://doi.org/10.1007/s10725-010-9554-x>
- Bozkurt T, İnan S, Dündar İ, Özdemir SK (2022) Effect of different plant growth regulators on micropropagation of some pitaya varieties. *J Trop Life Sci* 12:183–190. <https://doi.org/10.11594/jtls.12.02.04>
- Carmona-Martin E, Petri C (2020) Adventitious regeneration from mature seed-derived tissues of *Prunus cerasifera* and *Prunus insititia*. *Sci Hortic (Amsterdam)* 259. <https://doi.org/10.1016/j.scienta.2019.108746>
- Carneros E, Berenguer E, Pérez-Pérez Y et al (2024) Small molecule inhibitors of human LRRK2 enhance in vitro embryogenesis and microcallus formation for plant regeneration of crop and model species. *J Plant Physiol* 303:154334. <https://doi.org/10.1016/j.jplph.2024.154334>
- Che P, Lall S, Howell SH (2007) Developmental steps in acquiring competence for shoot development in *Arabidopsis* tissue culture. *Planta* 226:1183–1194. <https://doi.org/10.1007/s00425-007-0565-4>
- Chen J, Sabir IA, Qin Y (2023) From challenges to opportunities: unveiling the secrets of pitaya through omics studies. *Sci Hortic (Amsterdam)* 321:112357. <https://doi.org/10.1016/j.scienta.2023.112357>
- Comlekcioglu S (2024) In vitro propagation and rooting strategies for *Hylocereus undatus* and *Hylocereus polyrhizus*. *Plant Biotechnol Rep* 18:829–838. <https://doi.org/10.1007/s11816-024-00935-z>
- Dahanayake N, Ranawake AL (2011) Regeneration of dragon fruit (*Hylocereus undatus*) plantlets from leaf and stem explants. 14:85–89
- Essalimi B, Esserti S, Rifai LA et al (2022) Enhancement of plant growth, acclimatization, salt stress tolerance and *verticillium* wilt disease resistance using plant growth-promoting rhizobacteria (PGPR) associated with plum trees (*Prunus domestica*). *Sci Hortic (Amsterdam)* 291:110621. <https://doi.org/10.1016/j.scienta.2021.110621>
- Faize M, Faize L, Burgos L et al (2021) Application of *Ascophyllum nodosum*-based soluble extract on micropropagation and regeneration of *Nicotiana benthamiana* and *Prunus domestica*. *Plants* 10:1354. <https://doi.org/10.3390/plants10071354>
- Fan QJ, Zheng SC, Yan FX et al (2013) Efficient regeneration of dragon fruit (*Hylocereus undatus*) and an assessment of the genetic fidelity of in vitro-derived plants using ISSR markers. *J Hortic Sci Biotechnol* 88:631–637. <https://doi.org/10.1080/14620316.2013.11513017>
- Gittins JR, Hiles ER, Pellny TK et al (2001) The *Brassica napus* extA promoter: a novel alternative promoter to CaMV 35S for directing transgene expression to young stem tissues and load bearing regions of transgenic apple trees (*Malus pumila* Mill.). *Mol Breed* 7:51–62. <https://doi.org/10.1023/A:1009637706570>
- Gutoranov GP, Tsvetkov IJ, Colova-tsolova VM, Atanassov AI (2001) Genetically engineered grapevines carrying GFLV coat protein and antifreeze genes. *Agric Conspec Sci* 66:71–76
- Hua Q, Chen P, Liu W et al (2015) A protocol for rapid in vitro propagation of genetically diverse pitaya. *Plant Cell Tissue Organ Cult* 120:741–745. <https://doi.org/10.1007/s11240-014-0643-9>
- Ibrahim SRM, Mohamed GA, Khedr AIM et al (2018) Genus *Hylocereus*: beneficial phytochemicals, nutritional importance, and biological relevance—a review. *J Food Biochem* 42:1–29. <https://doi.org/10.1111/jfbc.12491>
- Ikeuchi M, Ogawa Y, Iwase A, Sugimoto K (2016) Plant regeneration: cellular origins and molecular mechanisms. *Development* 143:1442–1451. <https://doi.org/10.1242/dev.134668>
- Infante R (1992) In vitro axillary shoot proliferation and somatic embryogenesis of yellow pitaya *Mediocalyx coccineus* (Salm-Dyck). *Plant Cell Tissue Organ Cult* 31:155–159. <https://doi.org/10.1007/BF00037700>
- Iocco P, Franks T, Thomas MR (2001) Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. *Transgenic Res* 10:105–112. <https://doi.org/10.1023/A:1008989610340>
- Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol Biol Rep* 5:387–405. <https://doi.org/10.1007/BF02667740>
- Kim DH, Gopal J, Sivanesan I (2017) Nanomaterials in plant tissue culture: the disclosed and undisclosed. *RSC Adv* 7:36492–36505. <https://doi.org/10.1039/c7ra07025j>
- Lee Y-C, Chang J-C (2022) Development of an improved micropropagation protocol for red-fleshed pitaya ‘Da Hong’ with and without activated charcoal and plant growth regulator combinations. *Horticulturae* 8:104. <https://doi.org/10.3390/horticulturae8020104>
- Li ZT, Janisiewicz WJ, Liu Z et al (2019) Exposure *in vitro* to an environmentally isolated strain TC09 of *Cladosporium sphaerospermum* triggers plant growth promotion, early flowering, and fruit yield increase. *Front Plant Sci* 9:1–19. <https://doi.org/10.3389/fpls.2018.01959>
- Lopes CA, De Maria Gomes Dias G, Pio LAS, et al (2016) Indução de calos, potencial embriogênico e estabilidade genética em pitaya vermelha. *Rev Bras Ciencias Agrar* 11:21–25. <https://doi.org/10.5039/agraria.v11i1a5355>
- Lopez-Carbonell M, Alegre L, Prinsen E, van Onckelen H (1992) Diurnal fluctuations of endogenous IAA content in aralia leaves. *Biol Plant* 34:223–227. <https://doi.org/10.1007/BF02925873>
- Luth D, Moore G (1999) Transgenic grapefruit plants obtained by *Agrobacterium tumefaciens*-mediated transformation. *Plant Cell Tissue Organ Cult* 57:219–222. <https://doi.org/10.1023/A:1006387900496>
- Mállap-Detquizán G, Vilca-Valqui NC, Meléndez-Mori JB, et al (2022) In vitro multiplication of yellow dragon fruit (*Hylocereus megalanthus*) from seedlings obtained in vitro. *Agron Mesoam* 33:45472. <https://doi.org/10.15517/am.v33i1.45472>
- Mohamed-Yasseen Y (2002) Micropropagation of pitaya (*Hylocereus undatus* Britton et Rose). *In Vitro Cell Dev Biol-Plant* 38:427–429. <https://doi.org/10.1079/IVP2002312>
- Motioike SY, Skirvin RM, Norton MA, Otterbacher AG (2002) Development of methods to genetically transform American grape (*Vitis × labruscana* L. H. Bailey). *J Hortic Sci Biotechnol* 77:691–696. <https://doi.org/10.1080/14620316.2002.11511558>
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15:473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Ortiz-Hernández YD, Carrillo-Salazar JA (2012) Pitahaya (*Hylocereus* spp.): a short review. *Comun Sci* 3:220–237
- Pelah D, Kaushik RA, Mizrahi Y, Sitrit Y (2002) Organogenesis in the vine cactus *Selenicereus megalanthus* using thidiazuron. *Plant Cell Tissue Organ Cult* 71:81–84. <https://doi.org/10.1023/A:1016585108094>
- Pérez-Tornero O, Egea J, Vanoostende A, Burgos L (2000) Assessment of factors affecting adventitious shoot regeneration from in vitro cultured leaves of apricot. *Plant Sci*. [https://doi.org/10.1016/S0168-9452\(00\)00303-4](https://doi.org/10.1016/S0168-9452(00)00303-4)
- Petri C, Scorza R (2010) Factors affecting adventitious regeneration from in vitro leaf explants of “Improved French” plum, the most important dried plum cultivar in the USA. *Ann Appl Biol* 156:79–89. <https://doi.org/10.1111/j.1744-7348.2009.00364.x>
- Petri C, Alburquerque N, Pérez-Tornero O, Burgos L (2005) Auxin pulses and a synergistic interaction between polyamines and ethylene inhibitors improve adventitious regeneration from apricot leaves and *Agrobacterium*-mediated transformation of leaf tissues. *Plant Cell Tissue Organ Cult* 82:105–111

- Petri C, Webb K, Hily JM et al (2008) High transformation efficiency in plum (*Prunus domestica* L.): a new tool for functional genomics studies in *Prunus* spp. *Mol Breed* 22:581–591
- Qin J, Wang Y, He G et al (2017) High-efficiency micropropagation of dormant buds in spine base of red pitaya (*Hylocereus polyrhizus*) for industrial breeding. *Int J Agric Biol* 19:193–198. <https://doi.org/10.17957/IJAB/15.0264>
- Rahman Z, Othman A, Meon Z et al (2015) Establishment of organogenesis protocol for genetic modification of ‘Yellow Pitaya’ *Selenicereus megalanthus* (Cactaceae). *Int J Plant Soil Sci* 7:102–108. <https://doi.org/10.9734/ijpss/2015/16725>
- Rodrigues MA, da Silveira FA, Moreira RA et al (2022) Regeneration of pitaya by indirect organogenesis evaluated by scanning electron microscopy and flow cytometry. *Pesqui Agropecu Bras* 57:e02312. <https://doi.org/10.1590/S1678-3921.pab2022.v57.02312>
- Savelieva EM, Oslovsky VE, Karlov DS et al (2018) Cytokinin activity of N6-benzyladenine derivatives assayed by interaction with the receptors in planta, in vitro, and in silico. *Phytochemistry* 149:161–177. <https://doi.org/10.1016/j.phytochem.2018.02.008>
- Shah K, Chen J, Chen J, Qin Y (2023) Pitaya nutrition, biology, and biotechnology: a review. *Int J Mol Sci* 24:1–28. <https://doi.org/10.3390/ijms241813986>
- Suarez Roman RS, Caetano CM, Ramirez H, Morales Osorio JG (2014) Multiplication of *Selenicereus megalanthus* (yellow pitahaya) and *Hylocereus polyrhizus* (red dragon fruit) by somatic organogenesis. *Acta Agronómica* 63:272–281
- Taharuddin NH, Jumaidin R, Mansor MR et al (2023) Unlocking the potential of lignocellulosic biomass dragon fruit (*Hylocereus polyrhizus*) in bioplastics, biocomposites and various commercial applications. *Polymers* 15:2654. <https://doi.org/10.3390/polym15122654>
- Vancanneyt G, Schmidt R, O’Connor-Sanchez A et al (1990) Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Mol Gen Genet* 220:245–250. <https://doi.org/10.1007/BF00260489>
- Viñas M, Fernández-Brenes M, Azofeifa A, Jiménez VM (2012) In vitro propagation of purple pitahaya (*Hylocereus costaricensis* [F.A.C. Weber] Britton & Rose) cv. Cebra. *In Vitro Cell Dev Biol* 48:469–477. <https://doi.org/10.1007/s11627-012-9439-y>
- Wang H, Petri C, Burgos L, Albuquerque N (2013) Phosphomannose isomerase as a selectable marker for transgenic plum (*Prunus domestica* L.). *Plant Cell Tissue Organ Cult* 113:189–197. <https://doi.org/10.1007/s11240-012-0259-x>
- Wang A, Ma C, Ma H et al (2021) Physiological and proteomic responses of pitaya to PEG-induced drought stress. *Agric* 11:632. <https://doi.org/10.3390/agriculture11070632>
- Zambrano-Forero CJ, Rios-Osorio JA, Marcela D, et al (2015) Evaluación de reguladores de crecimiento en la propagación in vitro de *Hylocereus megalanthus* (pitahaya amarilla). *RevistasUtEduCo* 76–87
- Zhao XY, Su YH, Cheng ZJ, Zhang XS (2008) Cell fate switch during in vitro plant organogenesis. *J Integr Plant Biol* 50:816–824

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.