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Contrasting roles of gibberellin and cytokinin in the *in vitro* development and acclimatization of pitaya (*Selenicereus undatus* (Haw.) D. R. Hunt)[1]

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ABSTRACT

The use of plant growth regulators is a widely adopted strategy to optimize germination and propagation under *in vitro* conditions; however, their application can also cause morpho-physiological changes in plants that need to be investigated. The study aimed to evaluate the effect of gibberellic acid (GA) and 6-benzyladenine (BA) supplementation on the *in vitro* germination, structure, and shoot proliferation of *S. undatus,* as well as to test different substrates during the acclimatization of the obtained plantlets. Seeds were inoculated in MS medium without plant growth regulators (MS0) and in MS medium supplemented with GA (1.0 and 3.0 mg L^{-1}) and BA (1.0 and 2.0 mg L^{-1}). Seeds were also germinated under *ex vitro* conditions as a control. *In vitro* culture conditions accelerated germination and initial development of *S. undatus*. However, GA supplementation did not influence seed germination speed. Greater hypocotyl and epicotyl lengths were obtained in plants grown in a medium supplemented with 1 mg L^{-1} GA. Regardless of the concentration, BA led to the emergence of multiple axillary shoots, altering seedling architecture. Furthermore, BA and GA acted antagonistically on the morphoanatomy of the hypocotyls, increasing and decreasing the thickness of this organ, respectively. The treatments supplemented with BA regenerated twice the number of plantlets per seed. The commercial substrate allowed a higher survival rate (~85%) of the plantlets compared to sand (~30%), and it has been recommended for acclimating *S. undatus* plantlets obtained through *in vitro* propagation.

Keywords: dragon fruits, hypocotyl anatomy, phytohormones, plant development, micropropagation.

INTRODUCTION

Cactaceae of the genus *Selenicereus* represent a growing niche in the exotic fruit market.[1] Popularly known as pitahaya, pitaya, or dragon fruit, these species have high potential as ornamental crops, as well as a source of bioactive, nutritional, and functional compounds that promote health benefits, with potential use in reducing the risk of chronic and degenerative diseases.[2-5] These crops have fruits with a strong flavor, which can be consumed naturally or processed, and have high value, both in the commercialization of seedlings and in the sale of their fruits.[6,7]

Pitaya is native to the tropical regions of Mexico, Central America, and South America.[8] In Brazil, it can be found in areas of the Atlantic Forest, Cerrado, and Pantanal biomes under the canopy of trees, shrubs, and rocks.[9] Pitaya has a succulent, photosynthetic stem (cladode), fasciculated roots, large hermaphrodite flowers that are bell-shaped, and large fleshy fruits with numerous seeds arranged in the pulp.[3,10] In addition, this species has prolonged resistance to drought.[11,12]

The propagation of *Selenicereus* species, formerly *Hylocereus* spp., is commonly performed by seeds or vegetative cuttings.[13,14] However, seed propagation is inadvisable due to the long juvenile period.[15] On the other hand, vegetative propagation using cuttings may be inefficient due to phytosanitary problems, such as diseases caused by *Fusarium,*[16] *Colletotrichum,*[17] and *Erwinia*[18] species; it may generate losses of up to 80% and increase production costs by up to 50%, due to the need for constant management, reducing the potential of the crop.[19]

Alternatively, *in vitro* germination and micropropagation can minimize propagation time and eliminate damage caused by pathogens in the pitaya. *In vitro* cultivation also allows for uniform germination. Micropropagation, in turn, enables the propagation of better-quality plantlets, as this technique allows for producing healthy plantlets on a large scale from a small amount of propagation material.[20] Furthermore, *in vitro* regeneration enables the study of cellular processes during morphogenetic expression and regulatory mechanisms through the structural, molecular, biochemical, and physiological study of the underlying developmental processes.[21,22] In recent years, there has been a growing number of species for which micropropagation protocols have been established.[23,24] Despite the increasing number of *in vitro* propagation protocols available for cacti, *in vitro* development systems are still incipient for *Selenicereus undatus*.

Factors such as the type of explant and exogenous supplementation of plant growth regulators are decisive in the success of micropropagation.[25,22] Cytokinins and gibberellins stand out as plant growth regulators active in *in vitro* propagation processes. The modulation induced by cytokinins can result in the formation of multiple shoots and an increase in the number of plants *in vitro*.[26,27] In turn, gibberellins play an essential role in seed germination, plant growth, leaf expansion, stem elongation, and floral initiation,[28] the latter being desirable in the induction of the juvenile phase in seed-propagated Pitayas.

The coordinated action of cytokinins and gibberellins plays a significant role in the formation and maintenance of meristematic cells, which is essential for the establishment of the plant body. While cytokinin levels are highly correlated with the identity of meristematic cells and control their division activity,[29] gibberellin modulates the differentiation of plant tissues.[30,31] Therefore, the balance between the activity of cytokinin and gibberellin, which act antagonistically, is a key factor in modulating the length, width, and thickness of plant organs. Anatomical studies reinforce that cytokinins and gibberellins have a well-regulated control over the size and shape of plant organs.[32,33] Therefore, supplementation of plant growth regulators in the culture medium can promote changes in plantlet morphology and size.

The current research aimed to evaluate the effect of 6-benzyladenine (BA) and gibberellic acid (GA) supplementation on the *in vitro* germination and propagation of *S. undatus* seeds. We also assessed the effects of BA and GA on the morphoanatomy of the seedlings to understand how they modulate, in part, the morphological pattern of the species, and we tested different substrates for the acclimatization of *in vitro S. undatus* plantlets.

MATERIAL AND METHODS

In vitro and ex vitro germination

Seeds were obtained from ripe fruits of five parent plants of *S. undatus* derived from an open pollination orchard at the Federal University of Jataí. The seeds were washed in running water and subjected to disinfestation with a 1% chlorine dioxide solution for 5 min, 70% alcohol for 5 min, sodium hypochlorite (2.5%) for 30 min, and then rinsed three times using autoclaved deionized water in a laminar flow chamber. After disinfestation, the seeds

were inoculated into test tubes containing 15 mL of MS medium[34] supplemented with gibberellic acid (GA) (1 and 3 mg L^{-1}) and 6-benzyladenine (BA) (1 and 2 mg L^{-1}). In the control treatment, no plant growth regulators were added (MS0). Three explants (seeds) were inoculated into each test tube. The tubes were kept in a growth room at 26 ± 1 °C and a photoperiod of 16 hours. Disinfected seeds were also sown in a rigid black plastic seedling tray with 162 cells containing washed sand (without plant growth regulators) in a greenhouse (*ex vitro* treatment). The intermittent nebulization system was activated for irrigation for two minutes at one-hour intervals.

Germination counting began on the first day and was performed daily until the 16th day after *in vitro* inoculation (DAI) and *ex vitro* sowing (DAS). Seeds that presented primary root protrusion were considered germinated. After 30 DAI / DAS, the germination speed index in percentage (GSI%)[35], the distribution of the relative frequency of germination, and the germination percentage were calculated.

The experiment followed a completely randomized design with six treatments (five under *in vitro* conditions with different plant growth regulators) and the *ex vitro* treatment. The treatments were composed of 60 replicates. Under *in vitro* conditions, each replicate consisted of a test tube containing three seeds, totaling 300 tubes. Under *ex vitro* conditions, the replicates (n = 60) were seedling tray cells with three seeds each.

Seedling evaluation

S. undatus seedlings obtained after 60 DAI under *in vitro* conditions and after 60 DAS in the *ex vitro* treatment were evaluated for the following morphometric parameters: epicotyl and hypocotyl length, number of axillary shoots, and root length. The evaluations were carried out based on photographic records of 50 seedlings per treatment using ImageJ 1.43a software (National Institutes of Health, Bethesda, USA). The images were obtained in the laminar flow during the transfer of the seedlings to the elongation culture medium. Therefore, this evaluation was non-destructive.

Microscopic and micromorphometric analyses

For microscopical analysis, the median region of hypocotyls was collected from three seedlings of each treatment after 60 DAI / DAS. The samples were fixed in a solution of formalin, acetic acid, and 70% ethyl alcohol (FAA 70%). Subsequently, the samples were dehydrated in an increasing ethanol series and embedded in methacrylate (Historesin, Leica Instruments, Germany). Cross sections with 5 µm thickness were obtained with a rotary microtome (RM2125, Leica, Germany) and stained with 0.05% toluidine blue (pH 3.2)[36] and mounted in acrylic varnish. Images were captured using a light microscope (Olympus AX70TRF, Olympus Optical, Japan) with a digital camera attached (Spot Insight color 3.2.0, Diagnostic Instruments Inc., USA).

For the micromorphometric analyses of the hypocotyl, 15 images were obtained per treatment. Then, the thickness of the cortex and vascular cylinder, and hypocotyl were measured with five measurements of each parameter per section using the ImageJ 1.43a software (National Institutes of Health, Bethesda, USA). The data obtained were subjected to analysis of variance, and the means were compared using the Tukey test at a 5% significance level.

Elongation and individualization

After 60 DAI / DAS, 50 seedlings of each *in vitro* treatment were transferred to test tubes with MS medium without plant growth regulators, except for the plants obtained *in vitro* in the BA treatments, which were transferred to test tubes containing MS culture medium supplemented with 2 mg L^{-1} of GA for shoot elongation. After 45 days of cultivation, all seedlings and axillary shoots with lengths greater than 0.5 cm obtained in all treatments were individualized and transferred to MS medium supplemented with 1 mg L^{-1} of indolebutyric acid (IBA). After 45 days in this last culture medium, the number of plantlets obtained in each treatment was directly counted, and the data obtained were subjected to descriptive analysis.

Acclimatization

After the elongation and individualization phase, 30 plantlets from each treatment were removed from the test tubes, washed to remove excess culture medium, and 15 plantlets were transferred to 1L plastic bags containing commercial substrate (Bioplant®), and 15 plantlets were transferred to plastic bags of the same capacity containing washed sand. The plantlets were maintained covered with transparent plastic bags. After seven days, the plastic bag covers were removed, and the plants were taken to the greenhouse. The plants were irrigated daily in the morning and kept in a greenhouse with natural sunlight. Plants that survived in the greenhouse for more than 15 days were considered acclimatized.

Statistical analysis

The data were tested for normality and homogeneity using the Shapiro-Wilk and Bartlett tests. The data were then subjected to analysis of variance (ANOVA), and the means, when significant by the F test, were compared by the Tukey test at the 5% probability level, using the statistical program R Studio, version 4.3.2.[37] There was no data transformation.

RESULTS AND DISCUSSION

In vitro cultivation accelerates the germination process and development of S. undatus

The *in vitro* culture conditions accelerated germination compared to the *ex vitro* environment. In the *ex vitro* condition, the seeds began to germinate on the second day after sowing, presenting 1.9% relative germination (Figure 1A). In this environment, the maximum germination was observed on the fourth day after sowing, presenting 37.7% relative germination (Figure 1A). In the *in vitro* environment, the *S. undatus* seeds germinated two days after inoculation in culture medium. However, at this stage, all treatments presented relative germination percentages higher than 20% (Figure 1A). The GSI was higher in all *in vitro* treatments than in the *ex vitro* treatment (Figure 1B). However, there was no difference between the treatments supplemented with plant growth regulators (Figure 1B).

The germination percentage of *S. undatus*, was also greater under *in vitro* conditions compared to *ex vitro* treatment, although there were no differences regarding supplementation of plant growth regulators (Figure 1C). The germination process is critical for plant establishment and growth. In environments that provide control of light, temperature, and humidity, such as the *in vitro* environment, germination can be stimulated and accelerated. Several studies have demonstrated the role of plant tissue culture techniques in reducing germination time to obtain uniform seedlings with phytosanitary quality, compared to conventional propagation methods.[38-41] Currently, there is no manual, as well as the Rules for Seed Analysis – RAS[42] for the germination of seeds of pitaya species, and the results obtained here may be of interest to guide further studies on seed germination for this group of plants and their application in *in vitro* cultivation.

Exogenous supplementation of GA and BA in the culture medium did not increase the speed or percentage of *in vitro* seed germination of *S. undatus* (Figure 1B, C). The same was observed in *M. zehntneri* (cactus), where these plant growth regulators in the culture medium did not promote improvements in the germination processes.[43] Gibberellins and cytokinins are frequently reported as germination promoters of *S. undatus*.[43-46] In a study carried out by Anagha et al. (2024)[46] with the same species, increasing doses of GA and BA promoted increased germination, differing from the results obtained in the present study. According to Mascot-Gómez et al.,[44] the genotype, light, mucilage covering the seeds, and GA supplementation directly influence cactus germination. Therefore, it is possible that, within the same species, there is heterogeneity in morphogenic responses to the cultivation conditions to which the different genotypes of *S. undatus* are subjected. The absence of a significant effect on germination by the plant growth regulators confirms that the *S. undatus* seeds did not present physiological dormancy, requiring only more controlled conditions to optimize germination.

GA and BA balance alter the morphology and architecture of S. undatus

Morphological changes were observed in the plants when grown in culture media supplemented with BA and GA (Figure 2A–F). Plants obtained through *in vitro* germination in the presence of GA showed significantly greater epicotyl and hypocotyl lengths when compared to the other treatments (Figure 2G, H), evidencing that GA supplementation in the culture medium may have increased the cellular concentration of gibberellin[47] and, consequently, promoted cell elongation.[48] Gibberellins are vital regulators in determining the height and length of plant organs, and several studies have shown the elongation of plant organs in response to GA application.[49-51] Gibberellins drive plant cell expansion, which is induced by the degradation of the growth repressor protein called DELLA.[28] This protein directly reduces cell division and expansion by inhibiting the transcription of xyloglucan endotransglycosylase, α- and β-expansins.[52] These components are essential for microtubule orientation and the direction of cell expansion.[53,54] Furthermore, gibberellins and prefoldin proteins may be closely related since these proteins also act in microtubule orientation and the direction of cell expansion. The continuous balance between gibberellin perception and DELLA protein degradation governs the responses promoted by this plant hormone.[54]

Treatments supplemented with BA resulted in shorter

epicotyl and hypocotyl lengths, although no significant differences were observed concerning the *ex vitro* treatment (Figure 2G, H). However, in the presence of BA, the plants produced a more significant amount of axillary buds (Figure 2A-F, I), and the use of 2 mg L^{-1} of BA induced significantly greater bud proliferation than the treatment with 1 mg L^{-1} of BA (Figure 2I), altering the branching and architecture of *S. undatus*. Cytokinin signaling is necessary to maintain and form meristems in plants. BA supplementation in the culture medium overcame apical dominance and the inhibitory effect of auxin on the activity of axillary meristems, triggering cytokinin signaling and, consequently, the activation of genes essential for the formation and growth of axillary meristems, such as *WUS* and *STM*.[55,56]

The MS medium without BA and GA supplementation provided greater root length, differing statistically from treatments with plant growth regulators. The use of BA or GA inhibited the root development of plants obtained *in vitro*, with the minor root lengths observed in treatments supplemented with BA (Figure 2J). Cytokinin is a known negative regulator of root development. At high concentrations, cytokinins induced shoot formation and inhibited root development.[57] This duality of cytokinins in plant body establishment was also characterized in *Dietes bicolor*, in which BA supplementation in the culture medium promoted axillary bud growth and greater shoot development and inhibited root formation.[58] The same was reported in *Adenium obesum,* in which exogenous application of BA promoted a significant inhibition of root formation.[33]

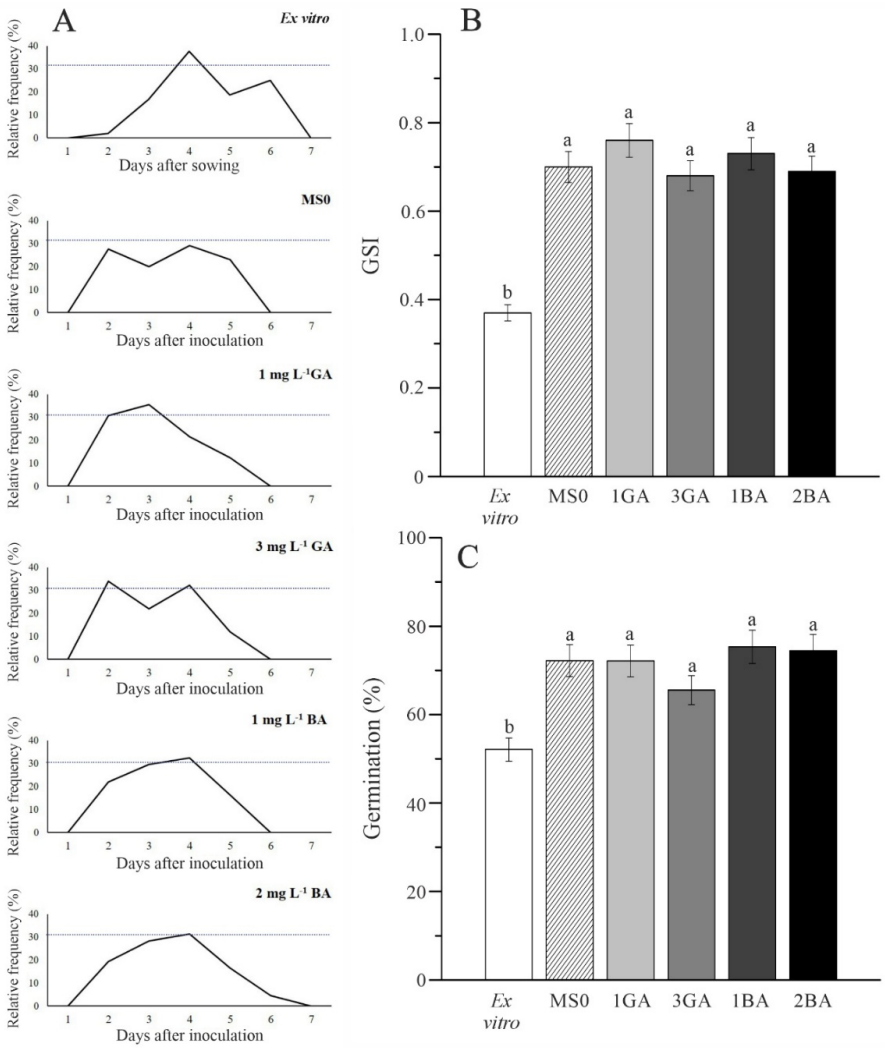

Figure 1. *Ex vitro* and *in vitro* germination of pitaya seeds. Distribution of the relative frequency of germination (A). Germination speed index (B). Germination percentage (C). Means followed by the same letter in each parameter evaluated do not differ statistically by Tukey's test at 5% significance. Error bars indicate the standard error. *Abbreviations:* 1BA (1 mg L^{-1} 6-benzyladenine), 2BA (2 mg L^{-1} 6-benzyladenine), 1GA (1 mg L^{-1} gibberellic acid), 3GA (3 mg L^{-1} gibberellic acid), MS0 (MS medium without plant growth regulator).

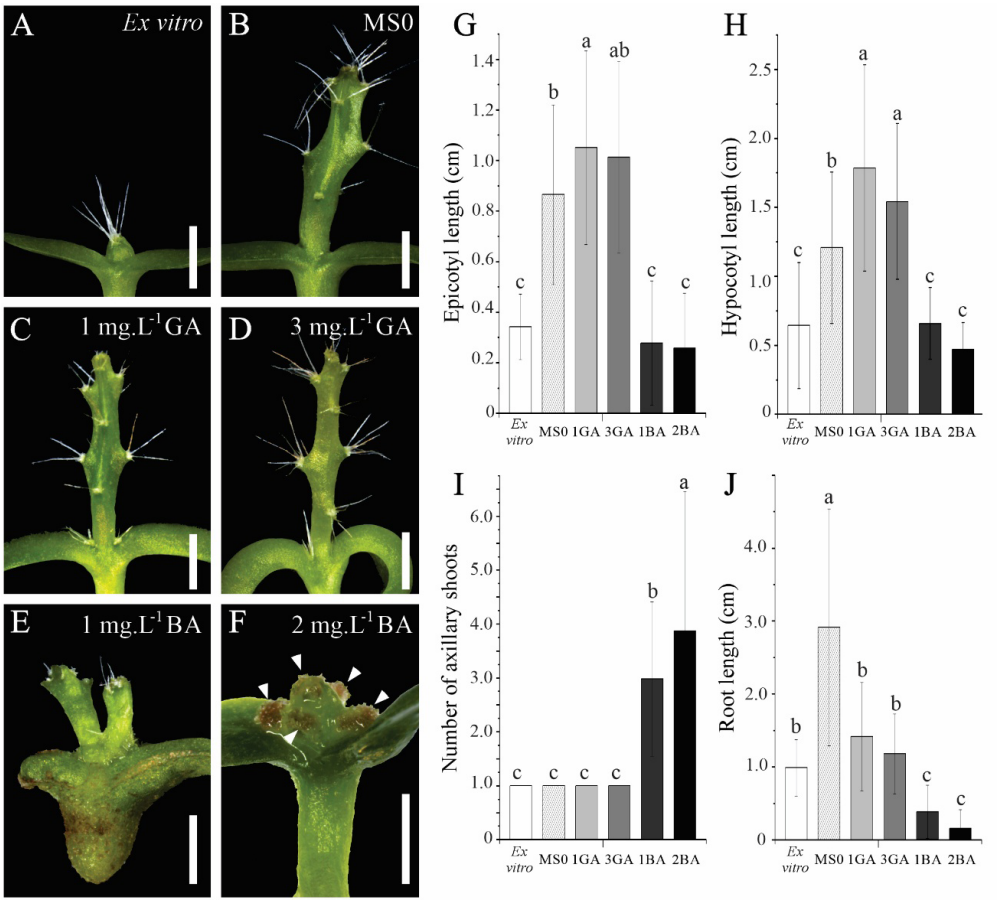

Figure 2. Morphology and growth parameters of pitaya seedlings. The morphological aspect of the epicotyl of pitaya seedlings after 60 days of culture (A-F). Morphometric parameters of pitaya seedlings after 60 days of culture (G-J). Epicotyl length (G). Hypocotyl length (H). Number of axillary shoots (I). Root length (J). Means followed by the same letter in each parameter evaluated do not differ statistically by Tukey's test at 5% significance. Error bars indicate the standard error. Abbreviations: 1BA (1 mg L^{-1} 6-benzyladenine), 2BA (2 mg L^{-1} 6-benzyladenine), 1GA (1 mg L^{-1} gibberellic acid), 3GA (3 mg L^{-1} gibberellic acid), MS0 (MS medium without plant growth regulator), (►) axillary shoots. Scale bars = 1 cm.

Gibberellins, in turn, have a controversial role in root system formation and elongation. GA supplementation in *Araucaria heterophylla* L.[59] and *Mentha arvensis* L.[60] seedlings significantly increased root length. Leilah, Khan[61] also reported an increase in root length, diameter, and fresh weight after exogenous GA application in *Beta vulgaris* L. On the other hand, GA negatively regulate root growth, resulting in shorter and thinner roots in *Medicago truncatula* Gaertn.[62] In *Adenium obesum*[33] and *Dietes bicolor,*[58] GA supplementation also negatively regulated root development, as observed here. These contrasting results suggest that the effects of gibberellins on root system architecture are species-dependent.[58]

GA and BA alter hypocotyl development and thickness of S. undatus

The hypocotyl of *S. undatus ex vitro* was composed of uniseriate epidermis, seven to nine layers of cortical parenchyma with the presence of idioblasts and eustele consisting of four vascular bundles organized concentrically, with the beginning of the vascular cambium (Figure 3A). In the absence of plant growth regulators (MS0), the anatomical structure of the hypocotyl was not altered (Figure 3B), except for the diameter of the vascular system, which was slightly larger in comparison to the *ex vitro* treatment (Figure 3H).

Supplementation with BA and GA in the culture medium promoted antagonistic effects on the anatomical structure of the hypocotyl of *S. undatus* (Figure 3C-F). While BA, at a concentration of 1 mg L^{-1}, promoted an increase in the thickness of the cortex and vascular cylinder and, consequently, in the width of the hypocotyl, the plants that grew in the presence of GA presented thinner hypocotyls, due to the smaller dimensions of the tissues that compose

this organ (Figure 3G-I). Antagonistic effects between cytokinin and gibberellin have been reported in different plant development processes,[63-66] and they seem to be associated with regulating cell proliferation and expansion phases, respectively.[65,67] Considering that cytokinin and gibberellin act antagonistically to adjust the balance between morphogenesis and cell differentiation,[68] our data indicate that BA application may have extended the morphogenetic activity, providing an increase in the number of cell layers, as well as cell volume and, consequently, an increase in the hypocotyl width of *S. undatus*. On the other hand, GA accelerated the onset of the differentiation phase, resulting in the formation of longer but thinner hypocotyls. Cell division activity is directly related to the growth of lateral organs. Therefore, the time spent growing in this phase plays a crucial role in the final size of the plant organ.[67,69]

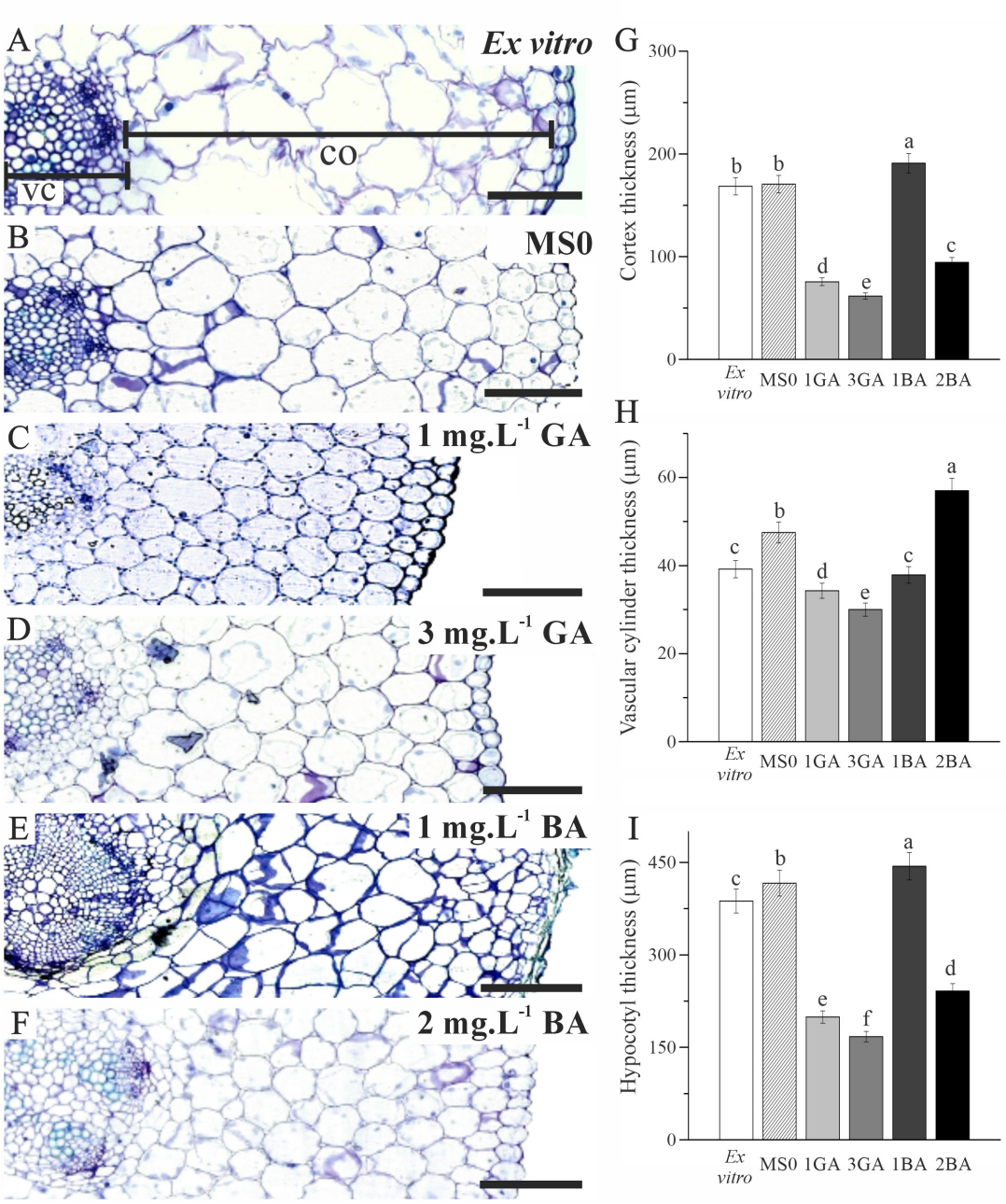

Figure 3. Micromorphometric characterization of pitaya seedlings hypocotyls after 60 days of culture. Cross sections of the hypocotyl (A-F). Cortex thickness (G). Vascular system diameter (H). Hypocotyl diameter (I). Means followed by the same letter in each parameter evaluated do not differ statistically by Tukey's test at 5% significance. Error bars indicate the standard error. Abbreviations: 1BA (1 mg L^{-1} 6-benzyladenine), 2BA (2 mg L^{-1} 6-benzyladenine), 1GA (1 mg L^{-1} gibberellic acid), 3GA (3 mg L^{-1} gibberellic acid), co cortex, MS0 (MS medium without plant growth regulator), vc vascular cylinder. Scale bar = 200 µm.

BA supplementation at a concentration of 1 mg L^{-1} also promoted the differentiation of lateral meristems such as vascular cambium and phellogen (Figure 3E). In addition, parenchyma cells were hypertrophied and in the process of division (Figure 3E), which provided a significant increase in cortex thickness and, consequently, in the thickness of the hypocotyl (Figure 3J). Cytokinins are necessary to determine the identity of cambial cells and control their periclinal division activity.[70] Since vascular cambium formation also depends on the interdependent processes of cell proliferation and differentiation,[71] BA application apparently enabled the proliferation of cambial cells, partly explaining the increase in vascular cylinder diameter and hypocotyl circumference of *S. undatus*. Interestingly, these results were not observed at the highest BA concentration studied (2 mg L^{-1}) (Figure 3F), although this treatment provided the formation of a greater number of axillary buds (Figure 2I). This behavior demonstrates the compensatory effect that cytokinins have on plant development, since high concentrations of cytokinins induce greater formation and activation of axillary buds, but, with smaller organs.[72] The correct regulation of cell proliferation and expansion mechanisms is essential to determine the final size and shape of the plant body.[73] However, the mechanisms that coordinate morphogenesis and differentiation during plant development are not fully understood.

BA induces shoot proliferation in S. undatus

Explants grown in culture media containing BA (1 and 2 mg L^{-1}) provided more plantlets per seed. After elongation and individualization of axillary buds, the concentration of 2 mg L^{-1} BA resulted in the formation of twice the number of plantlets obtained in the *ex vitro* treatment (Figure 4), evidencing the potential of this cytokinin for the establishment of micropropagation systems of *S. undatus*. BA is a cytokinin frequently recommended for the *in vitro* regeneration of *Selenicereus* spp. formerly genus *Hylocereus*. For example, in *Hylocereus polyrhizus*, 3 mg L^{-1} BA was recommended to induce a more significant number of axillary shoots.[74] According to Trivellini *et al*.[75] and Lee & Chang,[76] the use of BA was sufficient to induce the formation and activation of axillary buds of *S. undatus* and in the 'Da Hong' variety of Pitaya, with no need to associate it with other plant growth regulators to promote the *in vitro* multiplication of this species.

Acclimatization

The use of commercial substrate proved to be more efficient for the acclimatization of pitaya plantlets *in vitro*, providing mortality rates lower than 20% in all treatments with plant growth regulators. The transfer of plantlets established *in vitro* for sand promoted a high mortality rate (Figure 5). Most commercial substrates have physical and chemical characteristics that are favorable for the initial development of plants. These results partly agree with data reported by Santos et al.,[77] who observed that rooting of *S. undatus* cuttings was favored in substrates whose composition provided greater nutrient availability. However, the association with organic matter may provide a more significant initial growth of *S. undatus*.[78] The commercial substrate generally provides the best formation of high-quality seedlings, guaranteeing their vigor and health and reducing the production cycle.[79]

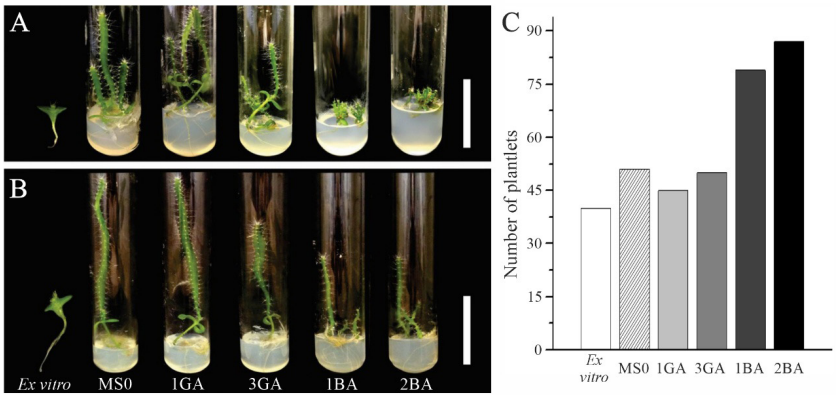

Figure 4. Shoot elongation of pitaya plantlets. The morphological aspect of pitaya plantlets at 30 days of culture in shoot elongation medium (A). Pitaya plantlets at 30 days after individualization (B). Number of plantlets after elongation and individualization of the axillary shoots (C). Abbreviations: 1BA (1 mg L^{-1} 6-benzyladenine), 2BA (2 mg L^{-1} 6-benzyladenine), 1GA (1 mg L^{-1} gibberellic acid), 3GA (3 mg L^{-1} gibberellic acid), MS0 (MS medium without plant growth regulator). Scale bars = 3 cm.

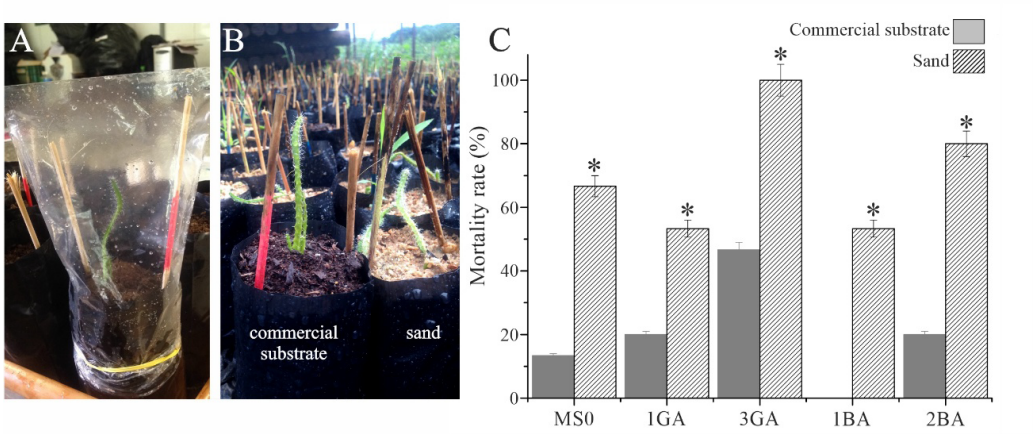

Figure 5. Acclimatization of pitaya plantlets. Regenerated plants after transfer from *in vitro* conditions to soil (A). The plants were maintained covered with plastic bags for 7 days. Acclimatized regenerated plants in the commercial substrate and in the sand (B). Mortality rate (C). Means with an asterisk (*) were significantly different according to the F test ($P \leq 0.05$). Error bars represent the standard deviation. Abbreviations: 1BA (1 mg L_{-1} 6-benzyladenine), 2BA (2 mg L_{-1} 6-benzyladenine), 1GA (1 mg L_{-1} gibberellic acid), 3GA (3 mg L_{-1} gibberellic acid).

CONCLUSIONS

The *in vitro* cultivation accelerated germination and seedling development of *S. undatus*, although BA and GA supplementation did not influence *in vitro* germination. The use of BA provided the regeneration of more plants per germinated seed. In addition, we describe the effect of GA and BA supplementation on the structure and architecture of seedlings, contributing to the understanding of how these plant growth regulators can determine the morphoanatomy of *S. undatus* organs.

DATA AVAILABILITY STATEMENT

All datasets supporting the results of this study were used in this article.

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Writing – review & editing: Diego Ismael Rocha, Givanildo Zildo da Silva, Maurecilne Lemes da Silva, Otalício Damásio da Costa Júnior.

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