Standardization of a regeneration protocol in yellow pitahaya *(Selenicereus megalanthus* (K. Schum. ex Vaupel) Moran)

Estandarización de un protocolo de regeneración en pitahaya amarilla (*Selenicereus megalanthus* (K. Schum. ex Vaupel) Moran)

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Abstract

A protocol for *in vitro* regeneration in yellow pitahaya by using indirect organogenesis pathway from axillary meristem grown on MS medium was standardized. The medium was supplemented with three concentrations of 2,4-D (2.26, 3.26 and 4.26 pM) or 2,4-D + 6- BAP to 2.21 mM, or with three concentrations of TDZ (200, 300 and 400 pM) or TDZ + 6- BAP, a total of 12 treatments. Growing conditions evaluated included the use of light (photoperiod of 16/8) and darkness (0/24). The 2.4-D induced calli but regeneration efficiency for the species was nil. In turn, the TDZ or TDZ + BAP were more efficient in inducing compact calli, green-purple, with capacity of regeneration following indirect organogenesis. With 300pM of TDZ more efficiency of response was observed, because the number of buds formed by regeneration point was the highest. Histological analysis confirmed the route of regeneration in *S. megalanthus*, showing primary structures characteristic of the formation of shoots from *calli*.

Key words: Indirect organogenesis, *in vitro* culture, regenerating calli, thidiazuron.

Resumen

A partir de meristemos axilares (aréolas) cultivados en medio MS (Murashige y Skoog) se estandarizó un protocolo de regeneración in vitro vía organogénesis indirecta en pitahaya amarilla (*Selenicereus megalanthus*). Se ensayaron tres concentraciones de 2,4-D (2.26, 3.26 y 4.26 pM) y 2,4-D suplementado con 6-BAP al 2.21 pM; y tres concentraciones de TDZ (200, 300 y 400 pM) o TDZ con 6-BAP, para un total de 12 tratamientos. Las condiciones de crecimiento evaluadas incluyeron el uso de luz (fotoperiodo de 16/8) y oscuridad (0/24). El 2,4-D indujo callo, pero la eficiencia en regeneración para la especie fue nula. A su vez, el TDZ o TDZ suplementado con BAP se mostró más eficiente en la inducción de callos compactos, de color verde-morado, con capacidad de regeneración vía organogénesis indirecta. Con el TDZ a 300 pM se observó mayor eficiencia de respuesta, ya que el número de brotes formados por punto de regeneración fue el más alto. Un análisis histológico confirmó la vía de regeneración en *S. megalanthus*, evidenciando estructuras primordiales características de la formación de brotes a partir de callos.

Palabras clave: Callo regenerante, cultivo *in vitro*, organogénesis indirecta, thidiazuron.

Introduction

The cactaceae are a family with approximately 1,600 species, distributed in native form from Argentina to Canada. The species that comprise it are characterized by the efficient use of water – five to ten times better than in other conventional crops – relative to the CAM (Crassulacean acid metabolism) photosynthetic pathway (Wallace and Gibson 2002).

Several species of the genus *Hylocereus, Selenicereus, Cereus, Leptocereus, Escontria, Myrtilloactus, Stenocereus* and *Opuntia* are known as pitahaya, pitaya, pitajaya, chalice flower, dragon fruit, among others, and around 35 have potential as a crop. The edible part is the fruit consumed in fresh or processed; besides, its high content of soluble solids gives it a great commercial and agro-industrial potential. In some species from the peel or the pulp pectin and dyes are extracted (Esquivel, 2004).

Selenicereus megalanthus is known in Colombia as yellow pitahaya because of the color of its exocarp. It presents numerous bracts, from which 10 to 15 spines arise; the endocarp is white (primary difference from the other species grown in other countries) and numerous seeds. It is distributed in Peru, Bolivia, Ecuador, Colombia and Venezuela (Britton and Rose 1920).

Yellow pitahayas are propagated by cuttings (cladodes) and naturally by seeds spread by birds and other animals. For culture purposes, propagation sexual is not recommended as the seedlings require too much care and take between 4 and 6 years to reach reproductive age (Suárez-Roman 2011).

Tissue culture is a suitable method for the propagation of the species. According Roca and Mrogisnki (1991) this technique consists in isolating a portion of plant (explant) and artificially provide the physical and chemical conditions for the cells to express their intrinsic or induced potential. This method requires the adoption of strict aseptic procedures to keep cultures free of contamination. The tissue culture technique has been described in different cactaceae (Pérez-Molphe-Balch *et al.*, 1998; Malda *et al.*, 1999; Bhau 1999; Giusti *et al.*, 2002; Mohamed-Yasseen, 2002; Pérez-Molphe-Balch and Davila-Figueroa 2002; Rubluo *et al.*, 2002; Pelah *et al.*, 2002; Medeiros *et al.*, 2006; Ferreira Gomes *et al.*, 2006; Angulo-Bejarano and Paredes-Lopez 2011).

Materials and methods

Location of the study area

The research was conducted between August 2010 and June 2012 in the laboratory of plant tissue culture, greenhouse and germplasm work collection of yellow dragon fruit of the Universidad Nacional de Colombia at Palmira (UNAL-P), at 3° 32' 5" N and 76° 17' 44" O, 1001 m.a.s.l., with an annual average precipitation of 1,000 mm and 24 °C.

Plant material

It consisted of a genotype of yellow pitahaya, from the collection of the Universidad Nacional at Palmira (UNAL-P), with outstanding morpho-agronomic characteristics such as fruit weight, pulp:peel relation, soluble solids (°Brix), carbohydrates, ether extract, energy and pH, among others.

Type of explants

The explants used corresponded to areas of meristematic tissue (lateral areoles that form vegetative and reproductive structures) obtained from *in vitro* seedlings grown from seeds germinated in Petri dishes with moistened sterile filter paper, and from disinfected adult plants.

Disinfection of plant material

Stems from mature plants were cut in 'V' in 60 cm long cuttings, washed for 10 min with the detergent Tween-20 at 1% in sterile distilled water, followed by disinfection with the fungicide Captan® in a concentration of 0.5 g/l spray-applied. The cuttings were planted in a greenhouse with an average temperature of 30 °C in plastic pots containing a substrate of peat and sand in a ratio 2:1, previously sterilized by autoclaving for 20 min at 121 °C and 103 kPa. The irrigation with water without fertilizers was made at intervals of 3 days. To obtain the explants, the plantlets were kept in a greenhouse until the development of new shoots.

Induction of indirect organogenesis

Starting from the previously in vitro grown shoots, areolas were used as explants to obtain calli, with which different combinations and concentrations of plant growth regulators were tested in order to induce in vitro regeneration via indirect organogenesis of yellow pitahaya. As regulators were used: an auxin (2, 4-dichlorophenoxyacetic acid 2,4-D) and two cytokinins (6-benzylaminopurine 6-BAP and Thidiazuron TDZ) on MS medium (Murashige and Skoog, 1962) with a total of 12 treatments (T1 - T12) (Table 1) plus a control (13). In Petri dishes 25 ml of sterile medium was poured and in each of them, prior to solidification of the medium, six explants were plated. The treatments, with five replicates were subjected to light (photoperiod 16/8h) and dark (0/24) conditions for 21 days after planting. After this time, materials exposed to light continued in the same condition and those in darkness were exposed to a 16/8h photoperiod.

Calli maintenance, proliferation and rooting of shoots

Calli obtained from darkness induction treatments (T8 - 12) were used. To release the pressure from the phytoregulators that induced calli, explants were planted on MS medium for 30 days and then the planting of these structures was tested in three media compositions (MS1 - MS3) for 30 days (Table 2). For rooting of shoots, the MS medium containing 5.3 mM NAA was used, as recommended by Pelah *et al.*, 2002.

Histological analysis of the calli

Callus samples where shoots were developed were fixed in FAA, stored in 75% ethyl alcohol and then were dehydrated in a graded series of alcohols, rinsed in butyl

Table 1. Combinations and concentration of growth regulators for in vitro regeneration of yellow pitahaya via indirect organogenesis.

Products	**Treatments (T) (pM)**					
	T1	**T2**	**T3**	**T4**	**T5**	**T6**
2,4-D	2.26	3.26	4.26	T1 + BAPa	T2 + BAP	T3 + BAP
	T7	**T8**	**T9**	**T10**	**T11**	**T12**
Thidiazuron (TDZ)	200	300	400	T7 + BAP	T8 + BAP	T9 + BAP

a. The BAP was applied in equal dosages of 2.21 pM, combined with the initial doses of 2,4-D (T1 –T3) y TDZ (T7 – T9)
b. Concentrations of 2,4D according to Angulo-Bejarano and Paredes-Lôpez (2011) and TDZ from Pelah *et al.* (2002).

Table 2. Compositions of calli maintenance and shoot proliferation media in yellow pitahaya after 30 days in MS media.

Media	**Growth regulator**	**Reference**
MS (MS1)	BAP 0.5 µM	Angulo-Bejarano and Paredes-Lôpez (2011)
1/2MS (MS2)	0.05 mg/l de GA + 0.04 mg/l de BAP	Roca 1984
MS (MS3)	—	Pelah *et al.*, 2002

alcohol and embedded in paraffin (Paraplast). The cuts were made in microtome and were carried to dry before staining with Safranin and Fast Green (Roth, 1964).

Statistical analysis

An analysis of variance and a complete randomized design with five replicates per treatment was used. Each repetition consisted of six explants, in a factorial arrangement 2 x 6 with two controlled factors: regulators (2, 4-D, TDZ and BAP, the first two alone or combined with BAP) and concentrations (2,4-D: 2.26 pM, 3.26 pM and 4.26 pM; TDZ 200 pM, 300 pM and 400 pM, the three concentrations of 2,4-D + 2.21 pM BAP; the three concentrations of TDZ + 2.21 pM BAP (Table 1). Duncan's multiple range test was performed, with a significance level of 0.05 and a simple linear correlation. The program Statistical Analysis System SAS ® version 9.0 was used. Response variables evaluated were the percentage of survival or surviving explants producing calli and callus formation percentage or calli per explant formed in each treatment.

Results and discussion

Indirect organogenesis

In treatments T1 - T6 in both light and dark, when 2,4-D and 2,4-D plus BAP were used at different concentrations, between 70 and 100% of callus formation was observed, however, these had no regeneration capacity into new plant organs. Conversely, when TDZ and TDZ plus BAP (T7 - T12) were used, regenerating callus formation (Table 3) was observed.

According to Bhau (1999), Llamoca-Zarate *et al.* (1999) Medeiros *et al.* (2006), Angulo-Bejarano and Paredes-Lopez (2011), 2, 4-dichlorophenoxyacetic acid (2,4-D) is a herbicide that acts as auxin and promotes callus formation in plant tissues; it has been used in some species of cacti, with different concentrations or with other growth regulators acting in different explants or tissues. These authors agree with Zhao *et al.* (2005) that callus formation occurs when the same concentration of auxin and cytokinin is added to the culture medium; therefore, the relationship among growth regulators is the critical factor that triggers the reactions of in vitro development.

In this paper, with yellow pitahaya a favorable response to different concentrations of 2, 4-D or 2, 4-D + BAP was not observed as it did not induce the development of regenerative calli under both light and dark conditions. The calli formed, not regenerating, presented variable characteristics in color and texture.

It is possible that 2,4-D, even though

Table 3. Treatments with TDZ tested under light (16/8) and dark (0/24) conditions, percentages of explants that formed calli and regenerating calli and induction efficiency, 45 days after sowing in yellow pitahaya.

Treatment (no.)	Condition		Calli (%)	
	Light	Darkness	Produced	Regenerants
7	X	—	100	75
	—	X	0	0
8	X	—	100	55
	—	X	96	66.6
9	X	—	96	80
	—	X	96	75
10	X	—	100	70
	—	X	100	91
11	X	—	100	83
	—	X	96	66.6
12	X	—	96	86
	—	X	100	70

it acts similarly to an auxin, does not have the property of inducing regeneration in yellow dragon fruit, due to an unknown factor that might be intrinsic to this species. By contrast, BAP combined with TDZ was efficient for regeneration in *S. megalanthus*, under both light and dark. Although it is a cytokinin that promotes the formation shoots on different cacti (Pérez-Molphe-Balch *et al.*, 1998; Pérez-Molphe-Balch and Davila-Figueroa, 2002; Davila-Figueroa *et al.*, 2005) it showed no effect for yellow pitahaya when it was combined with 2,4-D. Treatment T8 (300 μM TDZ), among treatments with TDZ and TDZ + BAP, was the one that most favored shoot production, therefore there was not an enhanced response by the use of both regulators, or a pronounced synergistic effect.

The plant regulator TDZ is structurally different to auxin and cytokinin, but can 'imitate' its effects on the growth and differentiation of cultured explants. In vitro it has been shown that TDZ has a high level of activity at concentrations as low as 10 pM (Preece *et al.*, 1991a; Murthy *et al.*, 1998). Exposing plant tissue to TDZ for a relatively short time is sufficient to stimulate regeneration (Visser *et al.*, 1995; Hutchinson and Saxena 1996; Murthy *et al.*, 1998).

In this paper, TDZ was the best performing phytoregulator on formation callus for yellow dragon fruit, being able, alone or in combination with BAP, to induce regeneration from explants in the presence of both light and darkness. The concentrations used induced regenerating calli formation, which shows that the species is sensitive to TDZ, particularly in its action on organogenic differentiation. Unlike the calli formed by supplementation with 2,4-D, these were presented compact, of colors green and purple (Photo 1c).

This is the first work where the induction of indirect organogenesis is achieved in *S. megalanthus* using as explants lateral areolas from stems and as phytoregulator TDZ or a combination of BAP + TDZ in light and dark conditions. Regeneration efficiency, corresponding to the number of regenerating calli per treatment averaged 1.3 for light treatments, while in darkness was 1.4, both with TDZ.

Characteristics of the calli

The calli under light and dark treatments had similar appearances. However, those experiencing light with TDZ and BAP + TDZ, in addition to revealing regenerating callus, presented non-regenerative portions, possibly due to the light. Furthermore and according to the regulator, different responses were observed in the tissue tested, as described below: (1) in treatments 1-3 (2,4-D) callus formation without ability to form shoots (non-regenerative) was evidenced, of light green to white color in light and white in darkness (Photo 1a) and of spongy texture in both lighting conditions.

According to Angulo-Bejarano and Paredes-Lopez (2011) the light green-to-yellow calli have visible reduction in the production of chlorophyll, while the brown areas can result from phenolic oxidation. Similarly, in the treatments 4-6 (2,4-D + BAP) callus formation was observed, also without regeneration capacity, with light-green to white and spongy for the ones under light and white and spongy for the ones kept in the dark (Photo 1b).

Combinations in treatments 7-9 (TDZ) exhibited regenerating calli; however, as mentioned before, those subjected to light presented regenerating, compact green-purple parts, with non-viable white spongy parts (Photo 1c); those under darkness revealed green-purple color, without non-regenerating part. Callus treatments 10-12 (BAP + TDZ) were also viable. When kept in light conditions these showed a green-purple compact viable regenerating part and a white fluffy, non-viable fragment. Callus in the dark were regenerating, green, with compact texture (Photo 1d). Therefore, treatments where regeneration occurred coincided with those that achieved compact calluses, purple-green, conditions found in treatments with

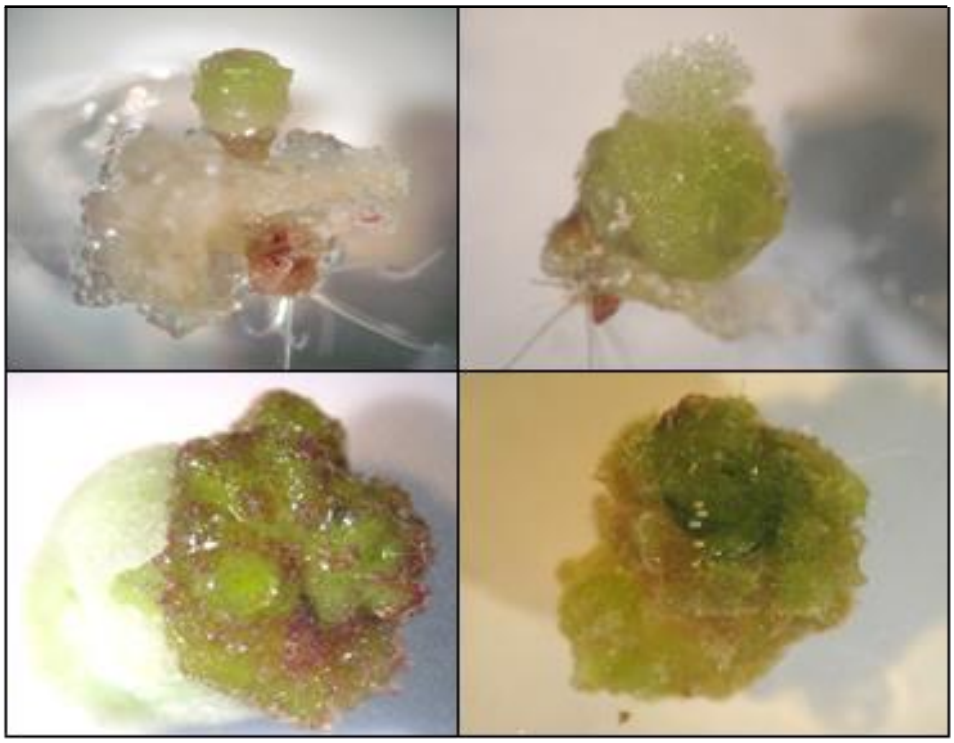

Photo1. Formation of unviable calli in treatments with 2,4-D under light (a) and dark (b), and viable in treatments with TDZ plus BAP under light (c) and darkness (d) conditions.

TDZ and BAP + TDZ, in light or dark conditions.

In controls exposed to light, in C1, shoot formation and white non-organogenic callus was observed, while in C2, the formation of a shoot, a non-organogenic callus and a root was presented. Furthermore, in each of the dark controls, C3 and C4, the formation of a non-organogenic callus and a root was observed. This shows that, for a successful regeneration, the medium should be supplemented with a growth regulator, as it is not only the presence of callus, but also, it must be competent, with tissue capable of giving rise to specific organs, so that it is possible to know the morphogenic route that should be taken.

According to Murthy *et al.* (1998) low concentrations of TDZ form green compact nodular callus. Pelah *et al.* (2002) when working with cotyledons and hypocotyls of yellow dragon fruit, with TDZ concentrations of 100, 200 and 440 mM, observed the formation of yellowish green organogenic callus in the proximal base of the cotyledons. In this paper, with different explants from Pelah *et al.* (2002) consisting of lateral areolas from mature stem shoots of yellow dragon fruit, indirect organogenesis was also observed. Green and green-purple calli, of compact consistency, obtained from TDZ and BAP + TDZ showed regeneration. These two studies in *S. megalanthus* organogenesis are the only reported to date. Despite the commercial importance of this species, there are no materials propagated by phytosanitary standards, therefore this can be a propagation path to meet the requirements of the productive sector.

Calli maintenance and shoot proliferation

For this purpose, the calli originated in treatments T8 - T12 in darkness were used. MS3, in which no regulator was added, had an average of 0.13 and 0.23 shoots/callus more than in MS1 and MS2, respectively (Figure 1). This is possible because these calli came from a medium with a high concentration of growth regulator, being

this an acclimatization response to the new conditions and a late expression of the effect of the previous treatment. In some cases this is useful for shoot induction, because of the change of level of growth regulators in the explant. No differences (P > 0.05) between MS1 and MS2, or between MS1 and MS3 were found. However, between MS2 and MS3 did occur significant differences (P < 0.05) (Figure 1). Similar results were obtained by Pelah *et al.* (2002) in *S. megalanthus*, who found the best shoot production and elongation with MS medium without regulators vs. MS medium supplemented with different concentrations of BAP and GA. In this work, also with *S. megalanthus* using the same medium without regulators, an average of a shoot/explant was found; while Angulo-Bejarano and Paredes-Lopez (2011) in *Opuntia ficus-indica*, found two shoots/explant using medium MS1.

In the present study, the highest averages in the production and elongation of shoots per explant (in parentheses) were observed in T12 (1124) and T8 (1,123), followed by treatments T11 (1099) and T10 (1097). By contrast, the lowest values were found in the T9 (0862). Pelah *et al.* (2002) in *S. megalanthus* found the greatest number of explants with at least one shoot and the greatest number of shoots, when the proximal section of cotyledons was cultured in medium with TZD at a concentration of 200 µM, achieving 80% of explants with shoots. In this work it was observed that the areolas grown in 300 µM TDZ had an average of 1,123 shoots/areola.

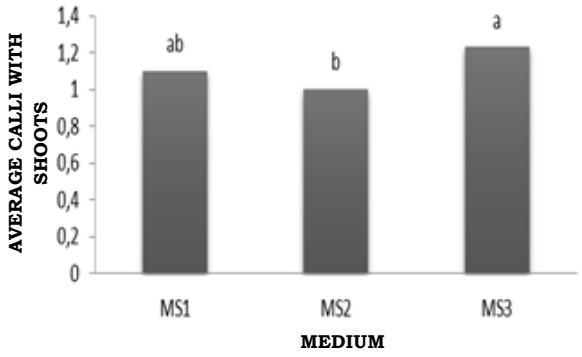

Figure 1. Average calli with shoots in yellow pitahaya, in three media, after 30 days of growth.

The highest average of shoots occurred in the treatment T8 (1,876) followed by treatments T12 (1,549) and T10 (1,336), which formed a group. Treatments T11 (1.2398) and T9 (0934) formed another group. Bhau (1999) in *Coryphantha elephantidens* obtained the formation of 1.7 shoots/callus when 2, 4-D was used at a concentration of 2.2 mM and 4.6 mM Kn, four weeks after planting. Therefore, the efficiency of TDZ treatments was higher in this study, particularly in treatment T8, which produced more shoots / callus (1.9 shoots) in lower phytoregulator concentrations (TDZ 300 µM).

The number of shoots/callus and per treatment varied from 1-4, within and among treatments; for example, in the treatment T8 (TDZ -300 µM) some calli showed 1 shoot (Photo 2a), while others showed four shoots (Photo 2b), with a higher average than the other treatments (Photo 2c). After treatment T8, T12 (400 µM TDZ + 2.21 µM BAP) and T10 treatments (200 µM TDZ + 2.21 µM BAP) appear. The difference in the number of shoots between treatments T12 and T10 (Photo 2d) is due mainly to a concentration effect from the phytoregulator TDZ, which was 400 µM in the first and 200 µM in the second, and not from the synergy of the growth regulators TDZ and BAP. The efficiency of the response presented by the treatment T8 shows that TDZ is only capable of inducing regeneration at a concentration of 300 µM and that not always there is a positive TDZ-BAP synergy effect.

Effect of the type of explant

In cactaceae different structures can be used as explants. Bhau (1999) achieved in *Coryphantha elephantidens* regeneration from explants of roots. Llamoca-Zarate *et al.* (1999) obtained calli from cotyledons and hypocotyls from *Opuntia ficus-indica*. Pelah *et al.* (2002) used cotyledons and hypocotyls of *S. megalanthus* and with the proximal portion of the cotyledon obtained effective regeneration. For regeneration of *O. ficus-*

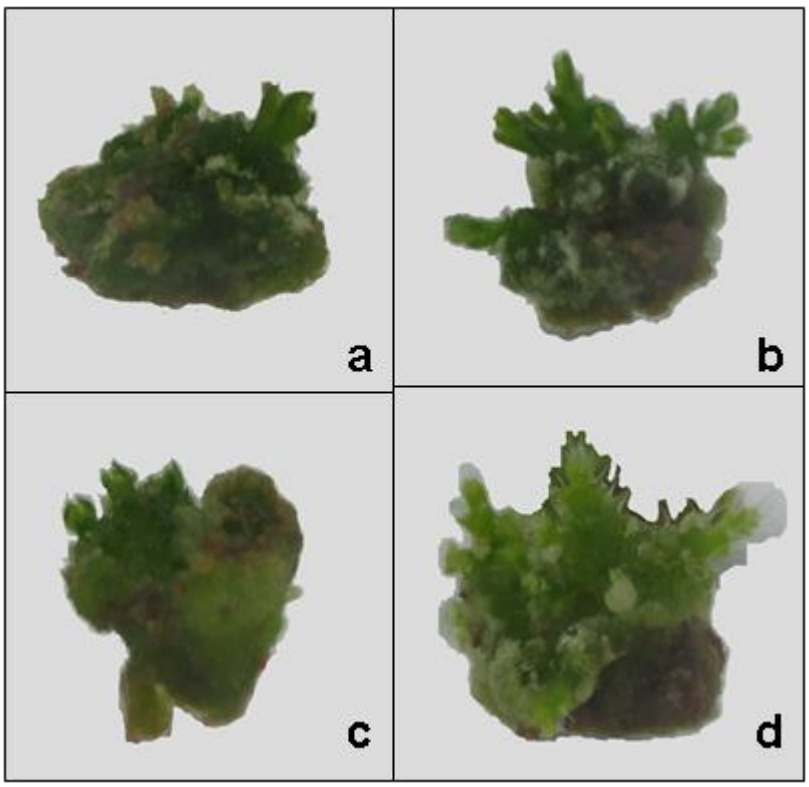

Photo 2. Average of regenerating calli and shoots of yellow pitahaya, in different treatments. **a**: callus with one shoot; **b**: callus with four shoots-T8; **c**: callus with three shoots-T9; **d**: callus with four shoots-T10.

indica through somatic embryogenesis, Ferreira Gomes *et al.* (2006) induced apex shoot culture in MS solution. In the variety White Thornless of the same species, Bejarano and Paredes-Lopez (2011) achieved a protocol for regeneration by indirect organogenesis, from cladodes.

The explant evaluated in this work, lateral areola of the stem, proved to be a good alternative for regeneration and propagation of *S. megalanthus* as it is a meristematic area is that presents in abundance; also proved to be effective when subjected to different treatments with various phytoregulators (TDZ or TDZ + BAP) in three concentrations and the combination of both factors (Table 4).

Histologic analysis of the regenerating calli

The conventional protocol applied for histological analysis of regenerating calli did not reveal a good definition of the primary structures of yellow pitahaya, because the fixation and dehydration processes were not enough to counter the high water content, making it difficult to cut and stain. On the contrary, with a protocol developed in this work, the base tissues and shoot primordia in regenerating calli were evidenced; the key consisted of a oven drying step at 60 °C, between the cut in microtome and staining with Safranin and Fast Green.

In the Photo 3 can be distinguished callus cells, disorganized parenchymal cells with large spaces in its interior (Pa), cells of the tunica (Tú), apical meristem (Ma), shoot primordia (Br) and epidermis (Ep). Parenchymal cells are evidence that the disorganized tissue in the base (callus) could give rise to differentiated structures (shoots), confirming regeneration via indirect organogenesis; and also demonstrates the role of the regulators in cell differentiation process.

Table 4. Average calli and regenerating shoots of yellow pitahaya produced in the different concentrations of BAP + TDZ and TDZ.

Phytoregulador (µM)	Concentration (no.)	Regenerating calli (no©	Shoots
TDZ	200	2.23	0
300	1.87	1.9	
400	2.00	0.9	
TDZ/BAP	200/2.21	2.00	1.3
	300/2.21	2.50	1.2
	400/2.21	2.17	1.5

Regeneration protocol in *Selenicereus megalanthus*

This protocol uses as explant the lateral areolas of young stems, preferentially collected from plants developed by seed germination.

Organogenesis in yellow pitahaya from meristems of shoots obtained from plantlets grown from seeds

The procedure consists of separating the seeds from the pulp starting from ripe fruits and follow these steps:

1. Wash with Tween 20 solution (0.1%) for 10 min in a beaker,
2. Wash with distilled sterile water and afterwards with alcohol (70%) for 30 s,
3. Washing with sterile distilled water and subsequently with sodium hypochlorite (2%) for 10 min,
4. Washing with sterile distilled water for 30 seconds,
5. Sow seeds of yellow pitahaya in Petri dishes with filter paper with moistened water as germination medium at an average temperature of 26 °C and relative humidity between 70% and 80%,
6. In vitro propagation of cotyledonary leaves and hypocotyls for shoot formation (Suarez Roman, 2011), from which the areolas will be taken as explants,
7. Transfer of the explants to a callus induction medium consisting of MS solution supplemented with TDZ in a concentration of 200 µM, and distributed in Petri dishes at 25

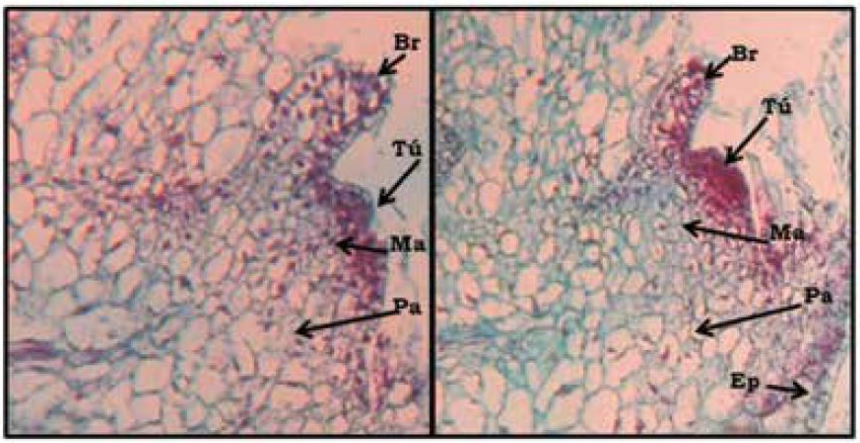

Figure 1. Tissues and shoot primordia obtained from regenerating callus. In a and b, tunic (Tú), apical meristem (Ma), bud primordia (Br), parenchymal cells (Pa) and epidermis (Ep). Staining with Safranin and Fast Green; 40x.

ml/dish. The explants are kept 20 days in the dark at 26 °C, and 25 days in light (photoperiod 16/8) at 26 °C,
8. Planting of formed calli on MS medium without growth regulators, distributed and stored in vials (30 ml/vial) for 60 days till shoot formation in 16/8 photoperiod at 26 °C,
9. Planting of the shoots resulting from regenerating calli on MS medium supplemented with 5.3 µM of NAA (Pelah *et al.* 2002), divided into vials (30 ml/vial) in photoperiod 16/8 to 26 °C, till root production, and
10. Transfer of plantlets to a substrate in pots to start the acclimatizaton phase.

Organogenesis of yellow pitahaya from shoot meristems of adult plants

For this case it is required:

1. Collect in field mature yellow pitahaya stems, making cuts in 'V' to obtain cuttings 60 cm long,
2. Transport the cuttings to a greenhouse,
3. Disinfect the cuttings in Tween-20 detergent solution (1%) in sterile distilled water for 10 min,
4. Disinfect cuttings by spraying the fungicide Captan® in a concentration 0.5 g/l,
5. Keep the cuttings in a greenhouse with an average temperature of 30 °C, in plastic pots with sand-peat substrate 2:1, previously autoclaved for 20 min at 121 °C and a pressure of 103 kPa,
6. Apply irrigation to each plant in pots on three day intervals. The plants remain in a greenhouse for 1 to 4 months until the development of new shoots,
7. Disinfect by immersion those young stems (shoots) of yellow pitahaya developed from the stakes grown in the greenhouse; this requires a sequence of alternating washes in Tween 20 (1%) for 10 min, distilled water for 1 min, ethyl alcohol (70%) for 1 min, distilled water for 1 min, sodium hypochlorite (3%) for 10 min and finally distilled water for 1 min,
8. Make cuts in 'V' to obtain the explants – lateral areolas that correspond to meristematic zones – in each young shoot grown in greenhouse,
9. Plant explants in vials of 30 ml capacity with MS medium and addition of fungicide Vitavax diluted in distilled water at a concentration of 7.4 µM. These explants must remain for three weeks in the medium with the fungicide, in a growth room, in a culture laboratory in vitro with photoperiod 16/8 at 26 °C,
10. Plant the same explants into new vials with 30 ml of MS for a period of one month in the same environmental conditions mentioned above,
11. Transfer explants to a callus induction medium consisting of MS medium supplemented with TDZ at a concentration of 200 µM. The explants are maintained for twenty days in darkness (0/24) at 26 °C, and twenty five days in 16/8 photoperiod at 26 °C,
12. Plant calli formed in MS medium without growth regulators, for sixty days for shoot formation, in 16/8 photoperiod at 26 °C,
13. Plant shoots resulting from regenerating calli on MS medium supplemented with NAA, as proposed by Pelah *et al.* (2002), 16/8 photoperiod at 26 °C, till the production of roots and,
14. Transfer the plantlets to a substrate in pots for acclimatization.

Conclusions

- The route of regeneration observed, indirect organogenesis, is the first step to establish tests of genetic

transformation for pitahaya by biotechnological strategies.

- For the first time an indirect organogenesis protocol was standardized in yellow pitahaya. The explant evaluated (areola), a meristematic area present in large quantities in the vegetative plant structure, showed an effective response to stimuli.
- In contrast to 2,4-D, TDZ was effective in the regeneration via indirect organogenesis in yellow pitahaya, with the best performance at a concentration of 300 µM.

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