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Mariane Aparecida Rodrigues⁽¹⁾ ,
Flávia Aparecida da Silveira⁽¹⁾ ,
Renata Amato Moreira⁽¹⁾ ,
Marlúcia Souza Pádua⁽²⁾ ,
José Eduardo Brasil Pereira Pinto⁽¹⁾ ,
Leila Aparecida Salles Pio⁽¹⁾ ,
Dalílhia Nazaré dos Santos⁽³⁾ ,
Júlio Sílvio de Sousa Bueno Filho⁽⁴⁾ ,
Letícia Alves Carvalho Reis⁽⁵⁾ 


⁽¹⁾ Universidade Federal de Lavras,
Departamento de Agricultura, Caixa Postal
3037, CEP 37200-900 Lavras, MG, Brazil.
E-mail: marianerodriguesst@gmail.com,
flaviasilveirax@yahoo.com.br,
renata_amato@hotmail.com,
jeduardo@ufla.br,
leilapio.ufla@gmail.com

⁽²⁾ Universidade Estadual de Minas Gerais,
Avenida Paraná, nº 3.001, Jardim Belvedere
I, CEP 35501-170 Divinópolis, MG, Brazil.
E-mail: marluciabio@yahoo.com.br

⁽³⁾ Empresa Mato-Grossense de Pesquisa,
Assistência e Extensão Rural, Laboratório
de Biotecnologia, Avenida Miguel Sutil,
Distrito Dom Aquino, CEP 78015-100 Várzea
Grande, MT, Brazil.
E-mail: dalilhians@gmail.com

⁽⁴⁾ Universidade Federal de Lavras,
Departamento de Ciências Exatas, Caixa
Postal 3037, CEP 37200-900 Lavras, MG,
Brazil. E-mail: jssbueno@ufla.br

⁽⁵⁾ University of Guelph, Department of Plant
Agriculture, Crop Science Building, 50 Stone
Road East, N1G 2W1 Guelph, ON, Canada.
E-mail: lreis@uoguelph.ca

 Corresponding author

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Regeneration of pitaya by indirect organogenesis evaluated by scanning electron microscopy and flow cytometry

Abstract – The objective of this work was to evaluate the induction of indirect organogenesis by concentrations of dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ) in pitaya (*Hylocereus undatus*) explants, using scanning electron microscopy and the flow cytometry technique. The treatments consisted of the concentrations of 0, 2.0, and 4.0 mg L⁻¹ 2,4-D and TDZ and of the combinations of these regulators. Percentages of callus coverage at 45 and 60 days were evaluated. The explants subjected to the treatments were analyzed by flow cytometry and scanning electron microscopy. All treatments induced endoreduplication, and there was no somaclonal variation. Under the combination of 2.0 mg L⁻¹ TDZ and 4.0 mg L⁻¹ 2,4-D, calluses were formed in 95% of the explants, but were smaller than those produced with 2,4-D separately. The concentration of 2.0 mg L⁻¹ TDZ induces the indirect organogenesis in pitaya explants, confirmed by the presence of conducting vessels through scanning electron microscopy.

Index terms: *Hylocereus undatus*, dragon fruit, endoreduplication, micropropagation.

Regeneração de pitaya por organogênese indireta avaliada por microscopia eletrônica de varredura e citometria de fluxo

Resumo – O objetivo deste trabalho foi avaliar a indução de organogênese indireta por concentrações de ácido diclorofenoxiacético (2,4-D) e tidiazurom (TDZ) em explantes de pitaya (*Hylocereus undatus*), por meio de microscopia eletrônica de varredura e da técnica de citometria de fluxo. Os tratamentos consistiram das concentrações de 0, 2,0 e 4,0 mg L⁻¹ de 2,4-D e TDZ e das combinações desses reguladores. Avaliaram-se as percentagens de cobertura de calos aos 45 e 60 dias. Os explantes submetidos aos tratamentos foram analisados por citometria de fluxo e microscopia eletrônica de varredura. Todos os tratamentos induziram endoreduplicação, e não houve variação somaclonal. Na combinação de 2,0 mg L⁻¹ TDZ e 4,0 mg L⁻¹ 2,4-D, calos foram formados em 95% dos explantes, mas foram menores do que os produzidos com 2,4-D separadamente. A concentração de 2,0 mg L⁻¹ de TDZ induz organogênese indireta em explantes de pitaya, comprovada pela presença de vasos condutores por meio da microscopia eletrônica de varredura.

Termos para indexação: *Hylocereus undatus*, pitaya, endoreduplicação, micropropagação.



Introduction

Pitaya (*Hylocereus undatus* (Haw.) Britton & Rose), cactaceae, has attracted worldwide attention, since it presents precocity of production, rusticity, exoticism, and high economic return. Pitaya shows attributes such as a sweet and mild taste, consistent pulp, and abundant seed, which generated expressive receptivity in consumer markets, and high value per kilogram of fruit, which has aroused the interest of producers (Lopes et al., 2016).

Pitaya sexual propagation can be done by seed, and vegetative propagation is usually performed by cuttings (Menezes et al., 2012). Seed propagation shows the disadvantages of low viability (Thinesh & Eran, 2015), high genetic variability, slow initial growth, and high juvenility period. Contrastingly, the vegetative propagation maintains the genetic characteristics of the material and shows low juvenility period. In the propagation by cuttings, there is a high risk of pest and disease. Using tissue culture techniques, it is possible to obtain healthy plants and seedling production on a large scale from a small amount of propagating material (Barrueto Cid & Teixeira, 2014).

Through micropropagation, the organogenesis technique can be used. Organogenesis technique is the differentiation of organs and buds of the explant itself, named direct organogenesis, or from the callus formation, named indirect organogenesis (George & Debergh, 2008). Organogenesis depends on several internal and external factors, including the interaction of the explant source with the culture medium and the environment (George et al., 2008), and even with plant growth regulators (PGR). There are specific organogenesis protocols with PGRs for each plant material of interest, and they require tests and changes of protocols.

One of the most used PGR in the micropropagation is cytokinin. Cytokinins are growth regulators mainly involved in the process of cell division, with effects on tissue differentiation, cell elongation, growth, senescence, apical dominance, organelle development, enzymatic activity, stomatal opening, and fruit development (Cruvinel et al., 2019). Different cytokinins have been used in the micropropagation protocols of pitaya species, such as benzylaminopurine (BA) (Qing et al., 2017; Pedda Kasim et al., 2019). However, TDZ and 2,4-D are PGRs with potential to be used with the organogenesis technique.

Thidiazuron (TDZ) was tested with cacti from yellow pitaya by Nunez et al. (2014) and Pelah et al. (2002). TDZ generates a rapid division, stimulating the bud organogenesis and acting in a way that its physiological effect can alter oxidative stress in cells (Guo et al., 2017). It was also seen that the growth regulators producing the most callous tissue in pitaya are TDZ, NAA, and 2,4-D (Zambrano-Forero, 2015). The growth regulator 2,4-D is the most frequently used auxin in callus induction; pitaya explants responded positively to its presence. Different auxin concentrations added to the culture medium influenced the formation of calluses (Lopes et al., 2016). The better growth of calluses in a medium supplemented with auxins, especially 2,4-D, has provided satisfactory results in organogenesis (Bonfill et al., 2002). There is little information on pitaya micropropagation techniques by organogenesis using TDZ and/or 2,4-D; therefore, the development of this technique could contribute to future expansion of pitaya seedlings production.

Efficient techniques to assess the presence of regenerative calluses and genetic stability are needed. One technique that has been used to confirm the presence of regeneration by organogenesis is the scanning electron microscopy (Fernando et al., 2007). Scanning electron microscopy technique allows of the study of fully fresh samples by the routinely used optical microscopy, as it has higher resolution that helps better understand the uneven surface sample microstructure (Vlašínová et al., 2017). Flow cytometry has also been used, to verify the genetic stability of regenerated plant material (Silva & Carvalho, 2014). It is an efficient and reliable technique that can estimate the plant nuclear DNA index (Loureiro et al., 2021).

The objective of this work was to evaluate the induction of indirect organogenesis by concentrations of dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ) in pitaya explants, using scanning electron microscopy and the flow cytometry technique.

Materials and Methods

The experiment was carried out at the Tissue Culture Laboratory of the Universidade Federal de Lavras (UFLA). The nodal segments used came from pitaya seed collected from four-year-old fruit from the UFLA Fruit Sector.

Apical and median cladode segments of seedlings obtained from pitaya seed germination *in vitro* were used as explant sources. The explants were grown in MS medium supplemented with gelled L2 vitamins, following the method by Phillips & Collins (1979), plus 25 g L⁻¹ sucrose, 6 g L⁻¹ agar, and 100 mg L⁻¹ glutamine, and the medium pH was adjusted to 5.7±0.1 before autoclaving. The treatments consisted of concentrations 0, 2.0, and 4.0 mg L⁻¹ of dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ), besides combinations 2.0×4.0 mg L⁻¹ and 4.0×2.0 mg L⁻¹ of these regulators. Also, 200 mL vials containing 35 mL of medium were used. The plants were left for 60 days in a growth room, at 25±1°C, without light.

At 45 and 60 days, the callus coverage percentages were evaluated. The data were subjected to the variance analysis by the mixed linear model with the aid of RStudio software (RStudio Team, 2012), in which the random effect was the plant; and the fixed effects were the segment types (apical and median region), and the auxin and cytokines concentrations (0.0, 2.0, 4.0 mg L⁻¹ and their combinations).

For subsequent analyses, only the most visually discrepant treatments were chosen, as follows: control treatment without the addition of a PGR; treatment with the addition of 2.0 and 4.0 mg L⁻¹ of TDZ, with yellow calluses and plant regeneration; and the 4.0 mg treatment of 2,4-D, which showed friable calluses.

Representative samples of the calluses whose treatments had friable or compact characteristics, and with cladodes were analyzed by flow cytometry and scanning electron microscopy (SEM), at 60 days of culture. In the analysis by SEM, the callus samples were fixed in the Karnovsky's solution (2.5% glutaraldehyde, and 2.5% paraformaldehyde), in a 0.05 mol L⁻¹ cacodylate buffer, pH 7.0, at 4°C for 24 hours. The calluses were placed in 30% glycerol for 30 min, and then washed three times (10 min) in 0.05 mol L⁻¹ cacodylate buffer and fixed in 1% osmium tetroxide for two hours. The samples were dehydrated once at increasing acetone gradients (25, 50, 75, and 90%) for 10 min each, and twice at 100% acetone for 10 min. The samples were then taken to the critical point apparatus using liquid CO₂ for complete drying. Subsequently, the samples were mounted on aluminum stubs, covered with gold, using a sputter coating device SCD 050 gold evaporator (SCD 050, Balzers, Schaan, Liechtenstein), and observed under

a LEO EVO 40XVP, SEM (Carl Zeiss, Oberkochen, Alemanha) (Akhtar et al., 2018).

Flow cytometric analyses are performed to verify the genetic stability of materials, as well as the occurrence of tissue endoreduplication. It is a technique that can estimate the nuclear DNA index of plants. These analyses are made by means of a comparison with nuclei belonging to a reference standard whose DNA index is previously known (Loureiro et al., 2021). Usually, for dragon fruit, *Pisum sativum* (pea) is the species used as an internal reference standard (Dolezel & Bartos, 2005). Thus, 20-30 mg of callus and cladode tissue, together with the same amount of *P. sativum* leaf tissue were ground in a Petri dish containing 1 mL of cold Marie buffer for the nuclei release. The nuclei suspension was aspirated through two layers of gauze with a plastic pipette and filtered through a 50 µm mesh. The nuclei were stained by the addition of 25 µL of a 1 mg mL⁻¹ propidium iodide solution for each sample. In each sample, 10,000 nuclei were analyzed using a logarithmic scale. The analysis was performed using the FACSCalibur cytometer (Becton Dickinson, San José, CA, USA), the histograms were obtained with Cell Quest software and statistically analyzed using the WinMDI 2.8 software.

Nuclear DNA (pg) content of the plants was estimated using the ratio of fluorescence intensities of G1 nuclei (nuclei that are in the G1 phase of interphase) of the reference standard (*P. sativum*), and G1 nuclei of the sample, by multiplying this ratio by the amount of DNA in the reference standard (9.09 pg). Three replicates and one explant/structure per replicate were used.

Results and Discussion

No significance was observed in any isolated factor, segment, and growth regulator, for the production of calluses, except for the interaction between 2,4-D and the portion of the apical and median explant. This fact can be observed in the interaction model graph (Figure 1) in the varying and controlling curves by segment and auxin 2,4-D, using a mixed linear model, where the variance between the explant donor plants was the source of the experimental error.

The interaction between 2,4-D and median segments showed a higher percentage of callus induction with increasing 2,4-D concentration (Figure 1). This is probably occurred because the median explant may

have more meristematic tissue (areolas) than apical segments, by containing intermediate structures between spines and leaves, and a greater vascular pattern. Even if at the apical segments, the endogenous auxin concentration is higher, as it is produced there, it can be translocated to basal regions in a polar way (Hu et al., 2017). This may be related to the breakdown of the apical dominance, since the explants were inoculated in a horizontal position, and might be inhibiting this auxin translocation, favoring greater callus induction in the middle segments. When studying inoculation position and region of inoculated segments in *Hyptis marrubioides*, Botrel et al. (2015) obtained larger number of buds in the lateral buds of horizontally arranged segments. Venturieri & Venturieri (2004) also evaluated the area covered by calluses on the explant, to characterize their production in relation to the plant growth regulator (PGR) used. The highest percentage of callus coverage on the explant was auxin 2,4-D. However, it did not regenerate seedlings, as happened for cytokine TDZ, in which organ regeneration was observed.

In the control treatment, root emission was observed in all replicates in both explants. However, there was no formation of aerial part (Figure 2), which may have occurred because of the position in which

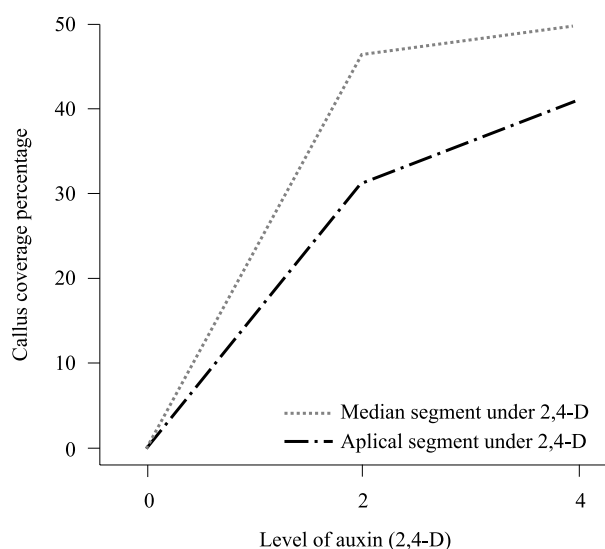


Figure 1. Callus induction percentage in apical and median segments, in pitaya (*Hylocereus undatus*) subjected to culture media at different concentrations of 2,4-D auxin (0.0, 2.0, and 4.0 mg L⁻¹).

the explants were positioned in the culture medium. Since the explants were placed horizontally, in contact with the culture medium, the part that was in contact with the medium may have been more responsive to rooting, possibly increasing the absorption of water and nutrients.

The use of 2.0 mg L⁻¹ of TDZ resulted in the formation of light yellowish calluses and peculiar structures appeared which resembled a cactus miniature, that was chlorophyllous due to its cultivation in the absence of light, originating from small calluses (Figure 2). These calluses regenerated from the basal part of the explants, but it was initially difficult to know where the new structure originated from, which led to SEM (Figure 3). These structures appeared in about 50% of the explants, 78% of which came from median segments; according to Pickens et al. (2006), the rate of regeneration depends on the type of explant (Table 1).

Suarez Román et al. (2014) obtained a protocol of regeneration of yellow pitaya (*Selenicereus megalanthus*) by indirect organogenesis, using the TDZ growth regulator in purplish green calluses. Pelah et al. (2002) also used the same regulator, in segments of parts close to cotyledons, obtaining the maximum regeneration of shoots in yellow pitaya.

In the presence of 4.0 mg L⁻¹ of TDZ, there was a small formation of light calluses that showed peculiar structures like those observed with half the concentration, but smaller and more discrete (Figure 2), these calluses appeared in about 50% of the explants, 78% of which came from median segments, just as it was with half of this concentration.

In the treatments with the addition of 2.0 mg L⁻¹ of 2,4-D (Figure 2) and in the 4.0 mg L⁻¹, pinkish, purplish and whitish calluses were formed in 73% of the explants, including all medians, and in 100% of the explants, respectively (Table 1). Lopes et al. (2016) noted this color, when developing a growth curve of pitaya (*H. undatus*) calluses, grown in the presence of the 2,4-D growth regulator and glutamine; these authors attributed this fact to the presence of anthocyanins. However, when betalains (class of nitrogen compounds positioned in the cell vacuoles) are present, more specifically betacyanins – a red-violet color group of betalains in plants – there is no presence of anthocyanin and vice versa (Gandía-Herrero et al., 2016; Kluge & Preczenhak, 2016; Ribeiro et al., 2017). This pigment has an antioxidant function (Cai et al.,

2003), and it was observed that when this pigmentation was present, there was no regeneration.

In the combination of growth regulators 2.0 mg L^{-1} TDZ and 4.0 mg L^{-1} 2,4-D, callus formation occurred in 95% of the explants, including all medians, and

they were dark pink and reddish (Figure 2 F), but smaller than calluses produced with 2,4-D separately. However, in the combination of 2.0 mg L^{-1} of 2,4-D and 4.0 mg L^{-1} of TDZ (Figure 2 G), whitish or and less red calluses than in the previous treatment were

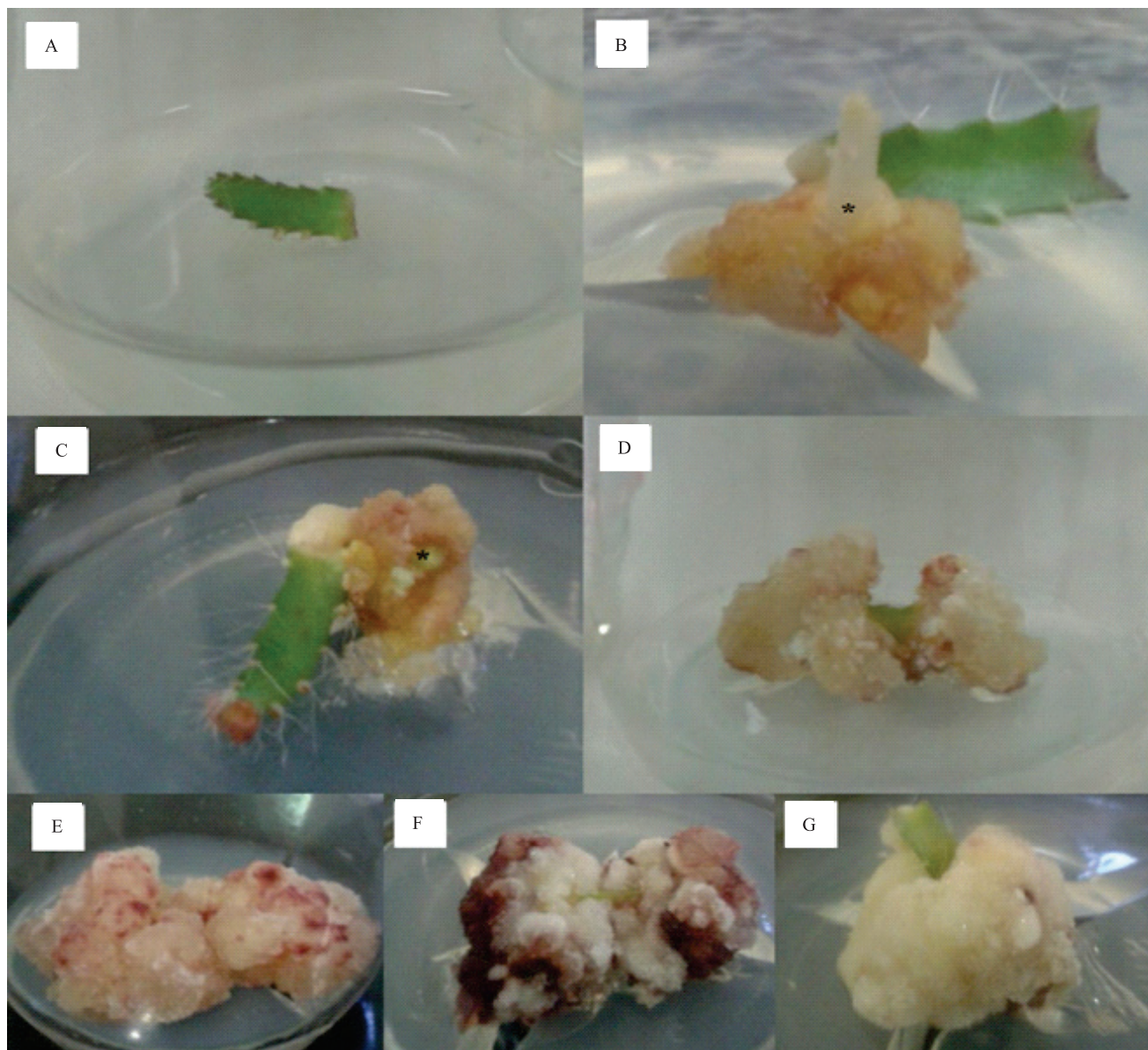


Figure 2. Callus and cladode induction of pitaya (*Hylocereus undatus*), at 60 days of cultivation, subjected to different concentrations of growth regulators: A, control treatment; B, explant inoculated in the culture medium containing 2.0 mg L^{-1} TDZ with callus (asterisk) and cladode; C, calluses induced in the treatment 4.0 mg L^{-1} TDZ, with very discrete and small cladode (asterisk); D, explant inoculated in the culture medium containing 2.0 mg L^{-1} of 2,4-D, with a friable appearance and mixed color with pinkish and whitish pigments; E, explant inoculated in the culture medium containing 4.0 mg L^{-1} of 2,4-D, forming pink, purplish, and friable calluses; F, calluses induced on the treatment with 2.0 mg L^{-1} of TDZ and 4.0 mg L^{-1} of 2,4-D, with dark pink and reddish color; G, calluses induced in the treatment 2.0 mg L^{-1} of 2,4-D and 4.0 mg L^{-1} of TDZ, with sugary and white appearance. Photos by Mariane Aparecida Rodrigues.

formed, and calluses were found in 100% of the segments (Table 1).

Explants of the control treatment (medium-free PGRs) observed by SEM showed paracytic stomata, an

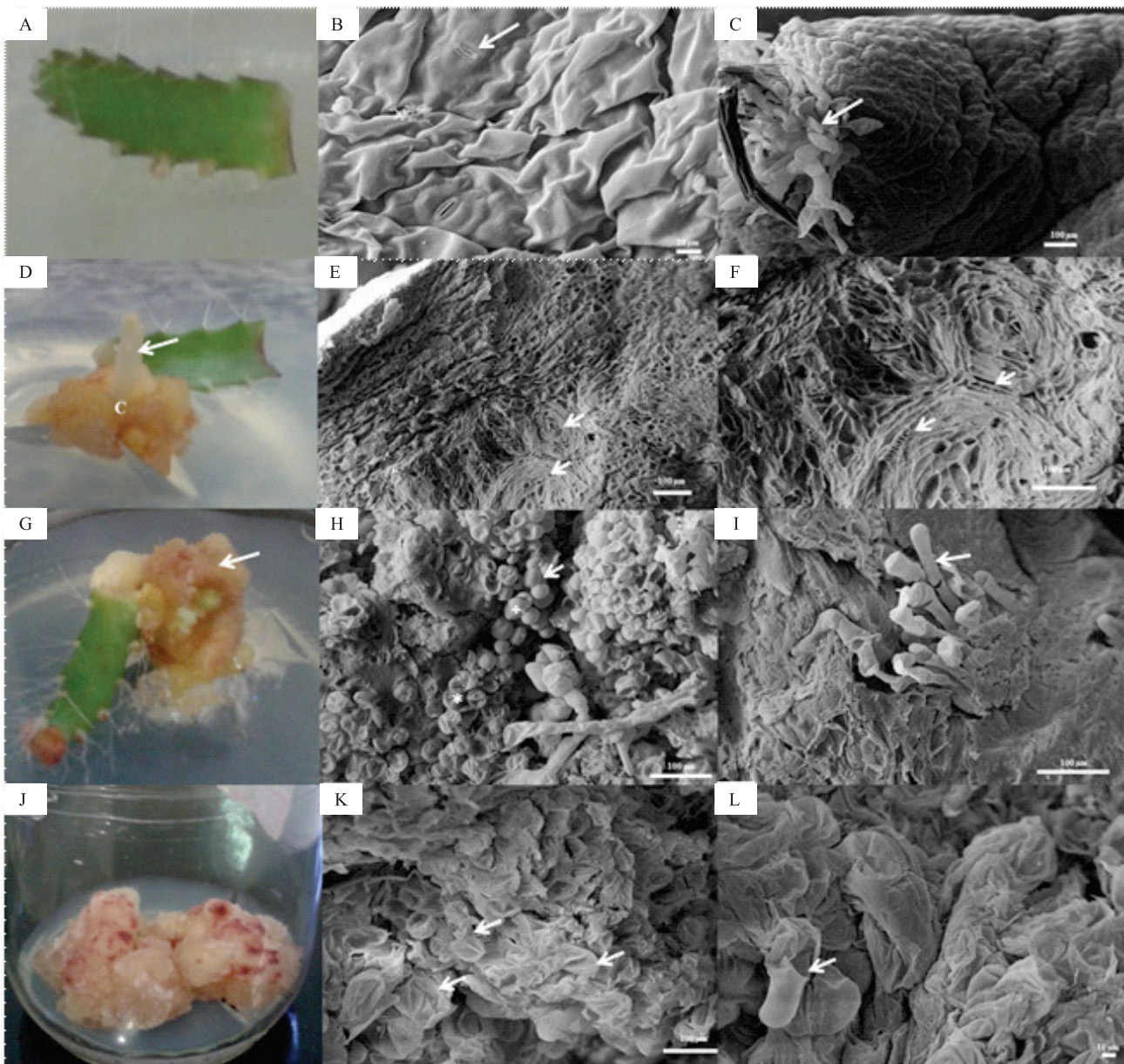


Figure 3. Morphological and scanning electron microscopy analyses of in vitro cultures of pitaya (*Hylocereus undatus*) explants subjected to different concentrations of plant growth regulators. A, control treatment of explant (cladode or leaf segments); B, stomata (arrows); C, it is assumed that this is a region with a high concentration of meristematic cells (areolas), with a high multiplication capacity rather than a lack of growth regulator stimulus, without multiplied (arrows) and organized tissue; D, explant inoculated in the culture medium containing 2.0 mg L⁻¹ of TDZ, with callus formation (c) and achlorophyllous structures without root (arrow); E, electromicrograph of pro-cladodes showing cellular structures similar to conducting vessels (arrows); F, cellular organization similar to vessel elements (arrows); G, calluses induced in the treatment 4.0 mg L⁻¹ of TDZ (arrows); H, calluses with isodiametric and small cells (arrows), cells with ruptured cell wall (+), and withered cells (asterisk); I, elongated cells with a broken cell wall are supposed to be areolas (arrows); J, calluses induced in the treatment 4.0 mg L⁻¹ of 2,4-D (arrows); K, withered cells (arrows); L, elongated and ruptured cells (arrow). Photos by Mariane Aparecida Rodrigues.

epicuticular striated epidermis, and organized tissue (Figure 3). Thorns were also present, surrounded by long tapered cells resembling trichomes. The existence of a tissue used to identify cactaceae that has a thorn-forming meristem known as areolas, which has trichomes, leaves and flowers was observed by Elias et al. (2015). However, these cells also resembled calluses, as if this region had suffered a differentiation and hyperplasia, but for lack of stimulation of a PGR, these cells had not multiplied.

The treatment with 2.0 mg L⁻¹ of TDZ generated calluses that developed structures similar to cladodes (common in Cactaceae), which are unrooted chlorophylls (Figure 3). The SEM made it possible to visualize the formation of conductive vessels, showing characteristics of indirect organogenesis that is evident by unipolar structures at the place where the vascular system is attached to the initial explant tissue (George & Debergh, 2008).

When the 4.0 mg L⁻¹ of 2,4-D was used, the calluses showed a similar color and appearance to those generated by half of this concentration (Figure 3). However, the images showed calluses with many ruptured and withered cells of the cell wall, showing poor characteristics, but also revealing clusters of cells with embryogenic potential. This treatment also showed elongated cells, with a ruptured cell wall that resembled the structures similar to axillary meristematic trichomes found in the control.

In callus cultivation there is a great heterogeneity, and only a limited number of cells has embryogenic potential due to the genetic potential. Thus, only the

parts with interesting attributes are subcultured. Usually, these embryogenic cells are small and isodiametric, a feature that allows of intense division and formation of cell clusters that give rise to somatic embryos (Angelo et al., 2013; Silva et al., 2014).

In the present study, the addition of 4.0 mg L⁻¹ of 2,4-D resulted in pinkish and whitish calluses with translucent points. This is a characteristic of calluses with elongated shape cells that are poor in cytoplasmic organelles, without embryogenic capacity, and that does not show organogenic capacity, generating withered, elongated, and ruptured cells, all unfeasible characteristics for regeneration (Figueiredo et al., 2007).

The DNA content showed no statistical difference, as it did not vary with the presence of PGR and treatments that regenerated cladodes by indirect organogenesis. There was no evidence of somaclonal variation, a condition that is stimulated when the explants are subjected to PGR concentrations. This fact illustrates that, for these treatments, pitaya shows genetic stability (Figure 4).

The micropropagated plant used as a control (Figure 4), as well as the yellowish-colored calluses obtained from culture medium supplemented with 2.0 and 4.0 mg L⁻¹ TDZ, obtained four peaks, that is, four levels of ploidy (Figure 4), while promoted calluses with 4.0 mg L⁻¹ of 2,4-D obtained five peaks (five ploidy levels). In a study on pitaya calluses, the authors observed that, by the addition of 2,4-D and glutamine, yellow calluses showed six peaks, while calluses with purple color and the micropropagated plant showed four peaks (Lopes et al., 2016).

The analyses by flow cytometry showed the presence of endoreduplication, a phenomenon that often occurs during the differentiation of cells that are highly specialized morphologically, as is the case of the pitaya cladodes, described as a modified stem with water storage function. This endoreduplication phenomenon is characterized by cells with several ploidies within a tissue. Besides, endoreduplication was also observed in stem segments of in vitro cultivated pitayas by Menezes et al. (2012).

In the present study, the absence of differences verified for the amount of DNA suggests that the plant material shows genetic stability, even when undergoing stress through dedifferentiation.

Tabela 1. Callus coverage in pitaya (*Hylocereus undatus*).

Treatments	Coverage (%)		
	Apical	Median	Grand total
Control (0 mg L ⁻¹)	0	0	0
TDZ (2 mg L ⁻¹)	22	78	50
2,4-D (2 mg L ⁻¹)	44	100	73
TDZ (4 mg L ⁻¹)	22	78	50
2,4-D (4 mg L ⁻¹)	89	100	100
TDZ (2 mg L ⁻¹) x 2,4-D (4 mg L ⁻¹)	44	100	95
2,4-D (2 mg L ⁻¹) x TDZ (4 mg L ⁻¹)	100	100	100

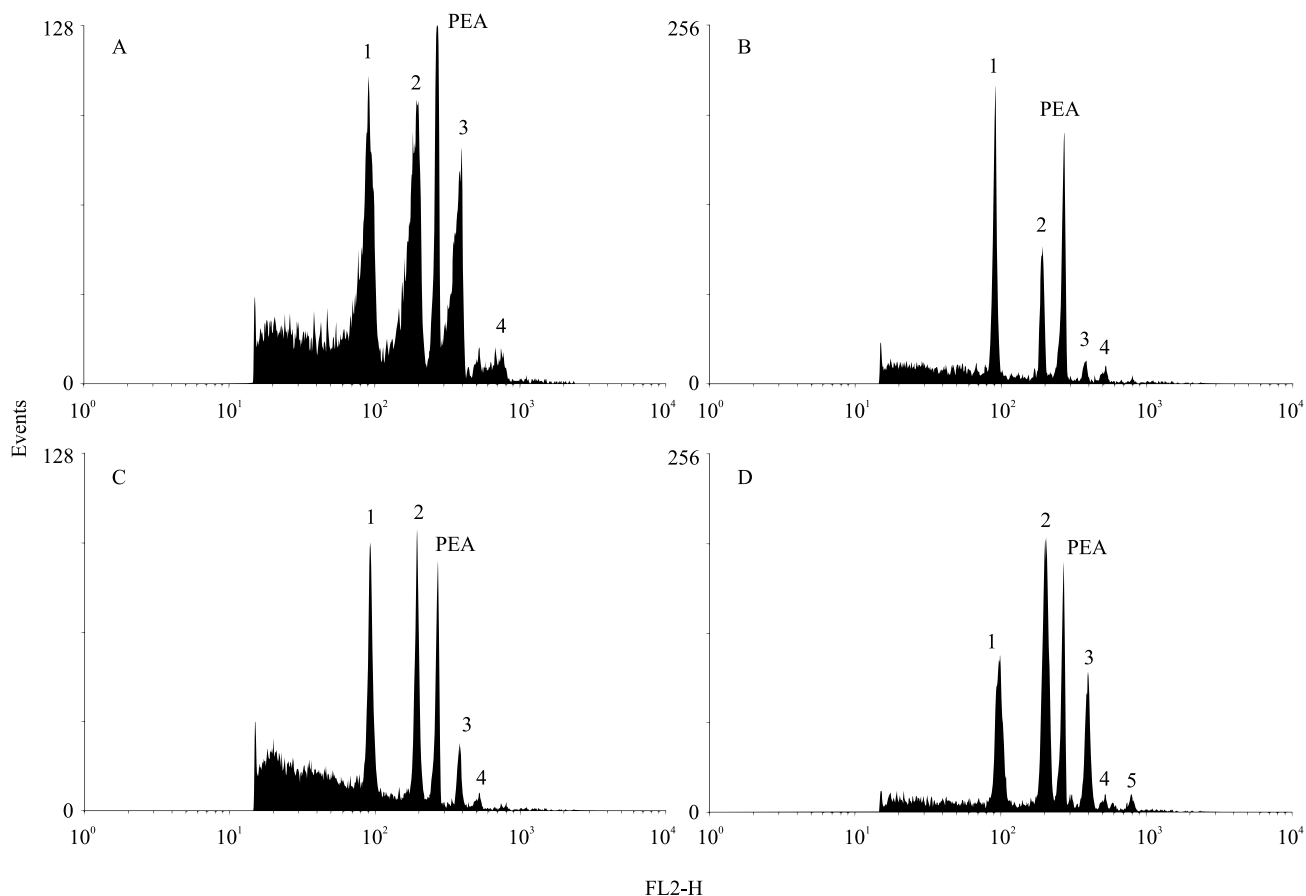


Figure 4. Flow cytometry histograms statistically analyzed through the WinMDI 2.8 software for in vitro cultured explants of pitaya (*Hylocereus undatus*) subjected to different concentrations of plant growth regulators: A, histogram of the control treatment showing four peaks; B, calluses and pro-cladodes obtained in the treatment at 2.0 mg L⁻¹ of TDZ, showing 4.0 peaks; C, calluses and pro-cladodes obtained in the treatment at 4.0 g L⁻¹ of TDZ, showing four peaks; D, calluses obtained in the treatment at 4.0 mg L⁻¹ of 2,4-D, showing five peaks.

Conclusions

1. The thidiazuron concentration of 2.00 mg L⁻¹ induces indirect organogenesis in pitaya (*Hylocereus undatus*) explants, which is confirmed by the presence of conductive vessels, using scanning electron microscopy.

2. All studied treatments induce endoreduplication without somaclonal variation.

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