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Establishment of callus and cell suspension cultures of *Ariocarpus retusus* (Scheidweiler)

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Abstract. *Ariocarpus retusus* is an endemic species of Mexico subject to special protection by the Mexican government due to its vulnerability to habitat disturbance. Previous studies have reported secondary metabolites in *A. retusus* with interesting biological activity. However, the use of these secondary metabolites has limitations. The aim of this study was to establish protocols for obtaining friable callus and cell suspension cultures that allow the subsequent development of strategies for obtaining secondary metabolites. Seedlings were obtained from seeds which were exposed to different concentrations of auxins and cytokinins to induce callogenesis. The callus generated was subcultured in Murashige and Skoog (MS) medium with modifications in the content of ammonium nitrate and potassium nitrate, and incubated in different photoperiod conditions (16:8 h light: dark and continuous darkness). Likewise, cell suspensions were established and characterized by friable callus. The MS medium supplemented with 2 mg L^{-1} of 2,4-D, 2 mg L^{-1} of BAP, and 3 mg L^{-1} of KIN allowed the development of vigorous callus. Callus friability was enhanced by decreasing ammonium nitrate (410 mg L^{-1}) and increasing potassium nitrate (2850 mg L^{-1}). The absence of light during incubation induced friable callus. The addition of myo-inositol (3 mg L^{-1}) significantly ($p = 0.000$) influenced the increase in biomass of cell suspensions.

Keywords: *ammonium nitrate; Cactaceae; callogenesis; potassium nitrate; myo-inositol.*

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Introduction

Ariocarpus is a genus of the Cactaceae family with a high vulnerability due to its sensitivity to habitat disturbance (Arroyo-Cosultchi *et al.*, 2014). Therefore, since 1992, the species of this genus have been listed in Appendix I of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), which includes all species in danger of extinction that are or may be affected by trade; they are also classified as species subject to special protection in the Mexican Official Standard NOM-059-SEMARNAT-2010. *Ariocarpus retusus* Scheidweiler, known as peyote cimarrón or chaute, is endemic to Mexico and is distributed in the states of Coahuila, Nuevo León, San Luis Potosí, Zacatecas, and Tamaulipas (Aguilar-Morales *et al.,* 2011).

A. retusus has been the subject of phytochemical studies that report the presence of important compounds such as isoprenoids and saponins; phenolic derivatives such as phenols, phenolic acids, flavonoids, anthocyanins, and a third group of metabolites made up of nitrogenous compounds, including alkaloids, which report biological activity of interest (Rodríguez *et al.,* 2010). However, obtaining these secondary metabolites presents certain limitations, including slow plant

growth, low concentration of metabolites in plant tissues, and overexploitation of wild plants (Ochoa-Villarreal *et al.,* 2015).

Plant cell suspension culture is a tool that enables us to determine the different aspects of *in vitro* culture, such as its metabolic, biochemical, and physiological behavior; as well as control and optimize culture conditions to produce biomass and secondary metabolites using different elicitors (Moscatiello *et al.,* 2013). The development of this strategy initially requires protocols that allow the obtaining of friable callus, which can be used to establish cell suspensions later. At the same time, it is necessary to know the dynamics of cell growth in these systems in order to determine the subculture and/or elicitation times (Trejo-Tapia and Rodríguez-Monroy, 2007; George *et al.,* 2008). This study describes the protocols for obtaining friable callus and establishing cells in suspension, as well as the growth kinetics, morphology, and viability of *A. retusus* suspension-cultured cells.

Materials and Methods

Plant material and in vitro culture

Seeds were used as plant material, which were donated by the Botanical Garden of the Faculty of Higher Studies of Cuautitlán, National Autonomous University of Mexico (FESC-UNAM). Seeds were germinated *in vitro* to obtain seedlings, which were used as a source of explants. They were washed with liquid detergent as well as running water and then disinfected for 20 min in a 1.8% sodium hypochlorite solution. After disinfection, the seeds were rinsed three times with sterile distilled water. Establishment was performed in a laminar flow hood on 50% MS basal medium (Murashige and Skoog, 1962), supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar, adjusted to pH 5.8, autoclaved at 121 °C for 15 min at 1.3 kg cm^2, and seeds were incubated at 25 ± 2 °C under a 16:8 h light-dark photoperiod.

Callus induction

Complete seedlings (radicle, hypocotyl, cotyledons, and epicotyl) from *in vitro* germinated seeds were used as explants for callus induction in experiments 1 and 2. The selected seedlings were 40 days after germination. The MS medium was supplemented with different doses of auxins and cytokinins, 30 g L^{-1} sucrose, and 6 g L^{-1} agar, adjusted to pH 5.8, and autoclaved at 121 °C for 15 min at 1.3 kg cm^2. The experimental unit was one seedling per container. The treatments tested are shown in detail below in experiments 1 and 2.

Experiment 1

Three different concentrations of the auxin 2,4-D (2,4-dichlorophenoxyacetic acid) and a fixed concentration of the cytokinin BAP (6-benzylaminopurine) were tested (table 1); with two different photoperiods: a) 16:8 h light-dark and b) continuous darkness, resulting in six treatments and a control (table 2) with three replicates per treatment in a completely randomized design. To access callus induction, calli size was recorded qualitatively using an ordinal scale (no callus, small callus: ≤ 0.5 cm^3, medium callus: 0.6 – 1.0 cm^3, large callus: > 1.0 cm^3), 45 days after the experiment was established.

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Table 1. Concentrations of plant growth regulators in experiment 1: Callus induction.

Dose	2,4-D	BAP
D1	1.0	0.2
D2	2.0	0.2
D3	3.0	0.2

Compounds in mg L^{-1}, ***2,4-D:*** *2,4-dichlorophenoxyacetic acid,* ***BAP:*** *6-benzylaminopurine.*

Table 2. Treatments tested in experiment 1: Callus induction.

Treatment	Dose†	Photoperiod (h)
T1	D1	16:8
T2	D2	16:8
T3	D3	16:8
T4	D1	Darkness
T5	D2	Darkness
T6	D3	Darkness
CTRL	No plant growth regulators	16:8

†*Concentrations from table 1.* ***CTRL:*** *Control.*

Experiment 2

Due to the low response of the genotypes to callus induction with the doses tested in experiment 1, different combinations of auxins and cytokinins were tested, using 2,4-D, BAP, NAA (1-naphthaleneacetic acid), KIN (6-furfurylaminopurine), and IBA (indole-3-butyric acid) (table 3), with a 16:8 h light-dark photoperiod, and five replicates per treatment in a completely randomized design. Calli size was recorded qualitatively using an ordinal scale (no callus, small callus: ≤ 0.5 cm^3, medium callus: 0.6 – 1.0 cm^3, large callus: > 1.0 cm^3).

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Table 3. Treatments of experiment 2: Callus induction.

Treatment \ Plant growth regulator	2,4-D	BAP	NAA	KIN	IBA
T1	3.0	2.00	2.0	0.0	0.0
T2	2.0	2.00	0.0	3.0	0.0
T3	0.5	0.02	0.0	0.0	0.5
CTRL	0.0	0.00	0.0	0.0	0.0

The data was taken 45 days after the *Compounds in mg L^{-1}.* ***CTRL:*** *Control,* ***2,4-D:*** *2,4-dichlorophenoxyacetic acid,* ***BAP:*** *6-benzylaminopurine,* ***NAA:*** *1-naphthaleneacetic acid,* ***KIN:*** *6-furfurylaminopurine,* ***IBA:*** *indole-3-butyric acid.*

Callus friability

In order to obtain friable callus appropriate for the establishment of cell suspensions, a genotype from the treatment with the best response to callus induction in experiment 2 (T2) was selected, and subsequently subcultured. Using callus from the subcultures, different concentrations of ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) were tested (table 4). The MS medium was supplemented with 30 g L^{-1} of sucrose, 6 g L^{-1} of agar, adjusted to pH 5.8, autoclaved at 121 °C for 15 min at 1.3 kg cm^2, and a 16:8 h light-dark and continuous darkness photoperiod, with three replicates per treatment, in a completely randomized design. The experimental unit was one large callus per container. Callus friability was qualitatively evaluated according to its consistency: compact (hard, non-crumbly, and dark green color), friable (easily crumbly and yellow color), medium friability (intermediate consistency between friable and compact, but closer to friable), and low friability (intermediate consistency between friable and compact, but closer to compact). The data was taken 45 days after the establishment of the experiment.

Table 4. Treatments tested in the callus friability experiment.

Treatment	NH_4NO_3	KNO_3	Photoperiod (h)
T1	820	0950	16:8
T2	820	1900	16:8
T3	410	1900	16:8
T4	410	2850	16:8
T5	820	0950	Darkness
T6	820	1900	Darkness
T7	410	1900	Darkness
T8	410	2850	Darkness

Compounds in mg L^{-1}. ***NH_4NO_3:*** *ammonium nitrate,* ***KNO_3:*** *potassium nitrate.*

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Establishment of cell suspensions culture

Cell suspensions were established from subcultures of callus from the treatment with the best response in the friability experiment (T8), using 125 mL Erlenmeyer flasks with 30 mL of liquid medium, under permanent orbital shaking at 110 rpm. In order to increase biomass and decrease oxidation, MSC medium (MS medium, 5 mg L^{-1} KIN, and 0.02 mg L^{-1} NAA) and MS1 medium (MS medium, 2 mg L^{-1} 2,4-D, and 0.05 mg L^{-1} KIN) with different concentrations of ascorbic acid, hydrolyzed casein, and myo-inositol were tested (table 5). All media were supplemented with 30 g L^{-1} sucrose, pH adjusted to 5.8, and autoclaved for 15 min at 1.3 kg cm^2 at 121 °C. After 28 days, the samples were filtered under a vacuum (medium pore filter paper). The filter papers were previously weighed on an analytical balance, both fresh and dry, to subtract the weight of each filter paper from the final measurement. Once the samples had been filtered, they were weighed fresh and placed in a convection oven at 60 °C for 24 h. The weight of each filter paper was then determined. Subsequently, the dry weight of the samples was determined.

Table 5. Treatments for the establishment of cell suspensions.

Treatment	Medium	Ascorbic acid	Hydrolyzed casein	Myo-inositol
1	MSC	10	0	1
2	MSC	0	1	1
3	MSC	0	0	1
4	MSC	10	1	1
5	MS1	0	0	3

Compounds in mg L^{-1}.

Growth kinetics of cell suspensions culture

Suspension cells were subcultured in the liquid medium with the best yield from the establishment of cell suspension experiments (MS1 medium) to obtain sufficient biomass to set cell growth kinetics.

In order to determine cell growth kinetics, 2 g of subcultured cells were inoculated into 125 mL Erlenmeyer flasks. The entire contents of three Erlenmeyer flasks were taken randomly every three days for 28 days. The samples were processed separately, filtered under vacuum, weighed with an analytical balance, and placed in a convection oven at 60 °C for 24 h, after which the dry weight of the samples was determined. Cell growth was measured in grams of dry biomass per liter of culture (g L^{-1}).

Morphology and viability of cells in cell suspensions culture

Cell morphology of cell suspensions was examined by visualization of single cells and cellular aggregates under a light microscope every 3 days for 28 days.

To determine cell viability, differential staining with Evans blue and acetocarmine was performed according to the method of Gupta and Durzan (1987) with modifications. For this purpose, 100 µL of cell suspension was placed in an Eppendorf® tube with 100 µL of acetocarmine. Then the tubes

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were placed in a water bath for 5 min to remove the excess dye, followed by two rinses with warm water. Next, three drops of Evans blue at 0.05 M were added, left to incubate for 5 min, and then the observations were made under a light microscope.

Statistical analysis

The results obtained for the qualitative variables (callus size and friability) were analyzed using contingency tables with Pearson's Chi-square test (equation 1). The quantitative variable (dry weight) was analyzed by analysis of variance (ANOVA) and comparison of means with Tukey's test (0.05). All analyses were carried out using the Statistical Package for the Social Sciences (SPSS®) v.21.

Equation 1. Chi-square formula

$$x^2 = \sum \frac{(O - E)^2}{E}$$

Where:
O = observed values
E= expected values

RESULTS AND DISCUSSION

Callus induction (experiments 1 and 2)

Experiment 1

The response of explants to treatments was visibly diverse since not all treatments stimulated callus induction (Figure 1), T3 (3 mg L^{-1} of 2,4-D, 0.2 mg L^{-1} of BAP, and 16:8 h light-dark) and T4 (1 mg L^{-1} of 2,4-D, 0.2 mg L^{-1} of BAP, and continuous darkness) did not induce callus. The treatment that visibly induced a greater response in callus generation was T6 (3 mg L^{-1} of 2,4-D, 0.2 mg L^{-1} of BAP, and continuous darkness) with 66.6% callus generation, of which 33.3% were large and 33.3% medium; followed by T1 (1 mg L^{-1} of 2,4-D, 0.2 mg L^{-1} of BAP, and 16:8 h light-dark), and T2 (2 mg L^{-1} of 2,4-D, 0.2 mg L^{-1} of BAP, and 16:8 h light-dark) which presented 66.6% medium callus generation. Likewise, T5 (2 mg L^{-1} of 2,4-D, 0.2 mg L^{-1} of BAP, and continuous darkness) had a callus induction response of 66.6%, generating medium and small calli. The control treatment (no plant growth regulators and 16:8 h light-dark) showed 33.3% of small callus induction (Figure 2).

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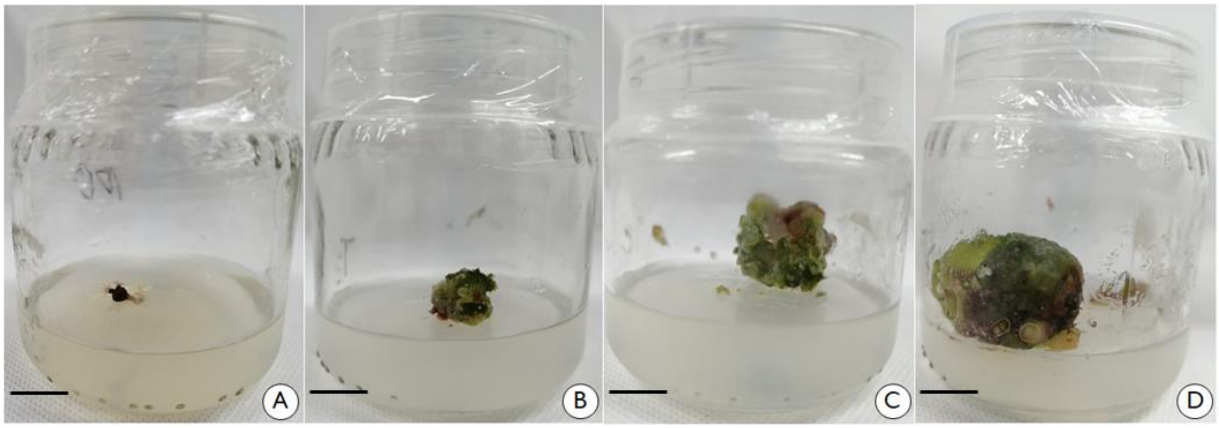

Figure 1. Callus obtained from seeds of *Ariocarpus retusus* with the treatments tested in Experiment **1. A)** Explant without callus**, B)** small callus, **C)** medium callus, **D)** large callus. Scale bars equals 1 cm.

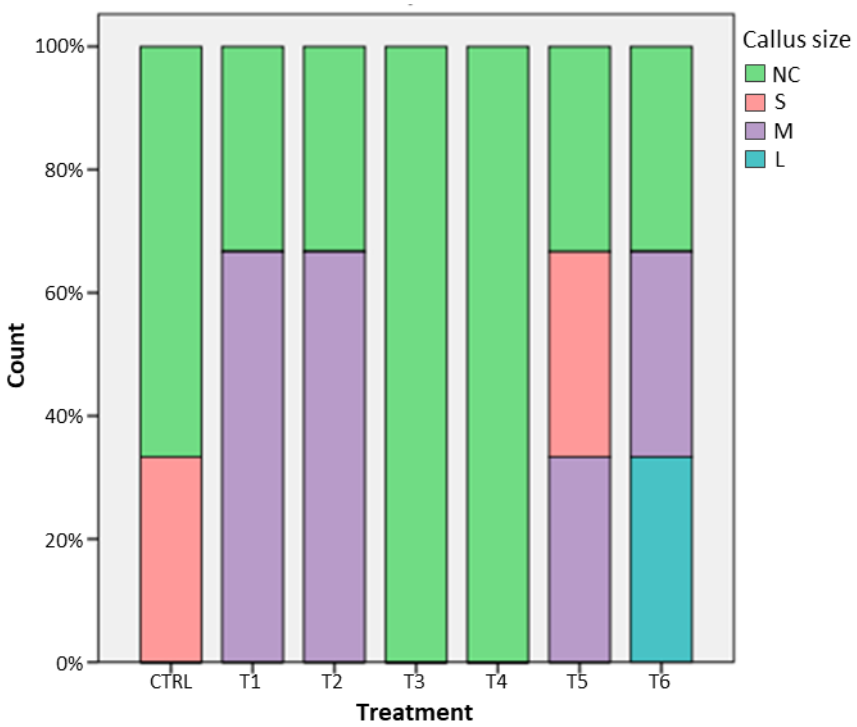

Figure 2. Callus induction in the experiment 1. Percentage of response presented according to callus size by treatment, 45 days after experimentation. The graph indicates the type of response. **NC**: no callus, **S:** small callus, **M:** medium callus, and **L:** large callus, according to treatment. Each bar indicates the treatment used (table 2).

The analysis of the data by Pearson's Chi-square showed a p-value of 0.342, which indicates that there is no relationship between treatments and calli size. This could be due to the fact that the plant material used came from seeds, which would represent different genotypes in each experimental unit.

Experiment 2

The treatment that presented 100% induction was T2 (2 mg L^{-1} of 2,4-D, 2 mg L^{-1} of BAP, and 3 mg L^{-1} of KIN). The size of the callus obtained in this treatment was 60% for large callus and 40% for small callus. This was followed by T1 (3 mg L^{-1} of 2,4-D, 2 mg L^{-1} of BAP, and 2 mg L^{-1} of ANA)

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and T3 (0.5 mg L^{-1} of 2,4-D, 0.02 mg L^{-1} of BAP, and 0.5 mg L^{-1} of IBA) with 40% induction to large callus and 40% induction to medium callus, respectively. The control treatment without plant growth regulators had a 20% induction of large callus (Figure 3).

The Pearson's Chi-square analysis of the data reported a p-value of 0.028, which indicates that there is a relationship between treatments and calli size.

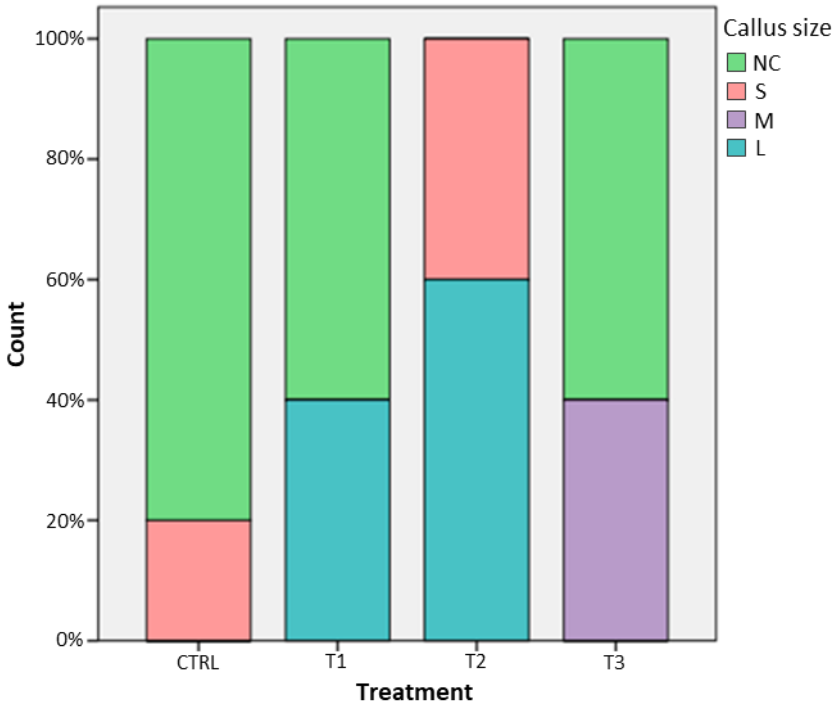

Figure 3. Callus induction in experiment 2. Percentage of response presented according to callus size by treatment, 45 days after experimentation. The graph indicates the type of response. **NC**: no callus, **S:** small callus, **M:** medium callus, **L:** large callus, according to treatment. Each bar indicates the treatment used (table 3).

In these experiments, the relationship between 2,4-D concentration and callus formation was demonstrated. 2,4-D at moderate concentrations can promote callus growth. However, it can also inhibit the process of callogenesis when the dose is too high, which coincides with what was observed at T3 in experiment 1 and T1 in experiment 2 with the highest concentration of 2,4-D (3 mg L^{-1}), where callus induction was total and partially inhibited, respectively. Abass *et al*. (2017) mentioned that 2,4-D at high concentrations has a genotoxic effect, causing adverse effects on callus development. On the other hand, experiment 2 showed a better response to callus induction in treatments with higher amounts of cytokinins in relation to auxins. These data contrast with that reported by Robles-Martínez *et al*. (2016), who found that the combination of 3 mg L^{-1} of 2,4-D and 0.5 mg L^{-1} of BAP produced 70% callus induction in *Opuntia streptacantha* and 100% in *O. megacantha* and *O. ficus-indica* on day 15 of culture. However, studies by Poljuha *et al*. (2003) reported callus induction of *Mammillaria gracilis* on MS basal medium without plant growth regulators, which coincides with what was observed in the performed control treatments (without plant growth regulators), where callus formation was also observed in both experiments.

It is important to note that, in general, cacti show a particularly different response among species to the stimulus of plant growth regulators. Arellano-Perusquía *et al.* (2013), Rubluo *et al*. (2002), and Clayton *et al.* (1990) found contrasting results, where the explants of the different species of

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cacti studied were independent of the concentrations and types of the plant growth regulators added to the medium. These responses could be due to differences in the endogenous levels of auxins and cytokinins, considering that these vary according to the species (Touati *et al.,* 2015), and even among genotypes in a particular species (George *et al.,* 2008), which is the case in the present study.

However, it should also be considered that other factors could modify the response of explants under *in vitro* culture conditions, such as genetic and epigenetic factors. Molinier et al. (2006) demonstrate that epigenetic variations respond to external stressors in the organism (Molinier et al., 2006). In this regard, it should be noted that explants develop under artificial conditions, and fluctuations in the *in vitro* microenvironment impose stress on plant cells (De Klerk, 2007), which causes these epigenetic variations (Ghosh *et al.,* 2021).

Likewise, cell dedifferentiation is necessary for plant cells to recover their totipotent capacity, which involves erasing epigenetic marks (Neelakandan and Wang, 2012). Thus, epigenetic reprogramming of plant cells cultured *in vitro* could be responsible for differences in regeneration capacity among genotypes (Smulders and De Klerk, 2011). Berdasco *et al.* (2008) reported that in callus and cell suspensions of *Arabidopsis thaliana*, cell dedifferentiation led to hypermethylation of the promoters of several genes, which is associated with silencing of linked genes in the genotypes worked on, and therefore changes in their gene expression.

Callus friability

The Pearson's Chi-square analysis of the data for this experiment showed a p-value of 0.012, which indicates that there is a relationship between the variables treatment and callus friability. The results obtained indicated a greater friability in the callus of the treatments where ammonium nitrate (NH_4NO_3) was decreased in greater proportion and potassium nitrate (KNO_3) was increased. At the same time, T8 (410 mg L^{-1} of NH_4NO_3, 2850 mg L^{-1} of KNO_3, and continuous darkness) was the treatment with the best friability results, with 66.6% of friable callus and 33.3% of medium friability callus; followed by T7 (410 mg L^{-1} of NH_4NO_3, 1900 mg L^{-1} of KNO_3, and continuous darkness), with 66.6% of medium friability callus and 33.3% of friable callus (Figure 4). This coincides with that reported by Flores-Hernández *et al.* (2003) in *Opuntia* spp. calli, which presented higher friability when grown in media where NH_4NO_3 (410 mg L^{-1}) was decreased, and KNO_3 (1900 mg L^{-1}) was increased. With reference to the photoperiod, the differences found were conspicuous in that the treatments with photoperiod (16:8 h light: dark) presented green colorations and visibly greater growth, but low friability in their majority (Figure 5A), while the treatments in continuous darkness were white and friable (Figure 5B). Concerning the aforementioned, Reuveni and Evenor (2007) mentioned that light quality, quantity, and photoperiod have a strong influence on the morphogenesis and growth of plant cells, tissue, and organ cultures. Sidiqque and Islam (2015) suggested that under light, callus cells have photosynthetic pigments that make them autotrophic in nature. Thus, callus could produce carbohydrates and other metabolites needed to grow vigorously under light conditions rather than dark conditions. George mentions that callogenesis can be induced under darkness, continuous light, or regular photoperiod conditions, depending on the species (George *et al.,* 2008).

On the other hand, brown colorations were observed in callus, regardless of the treatments used, which could be the result of oxidative degradation of phenolic compounds caused by the action of polyphenol oxidase and peroxidase enzymes (Lei *et al*., 2004), probably caused by mechanical manipulation at the time of callus subculture, since the cuts suffered by the explant can trigger oxidation processes (Van Staden *et al*., 2006).

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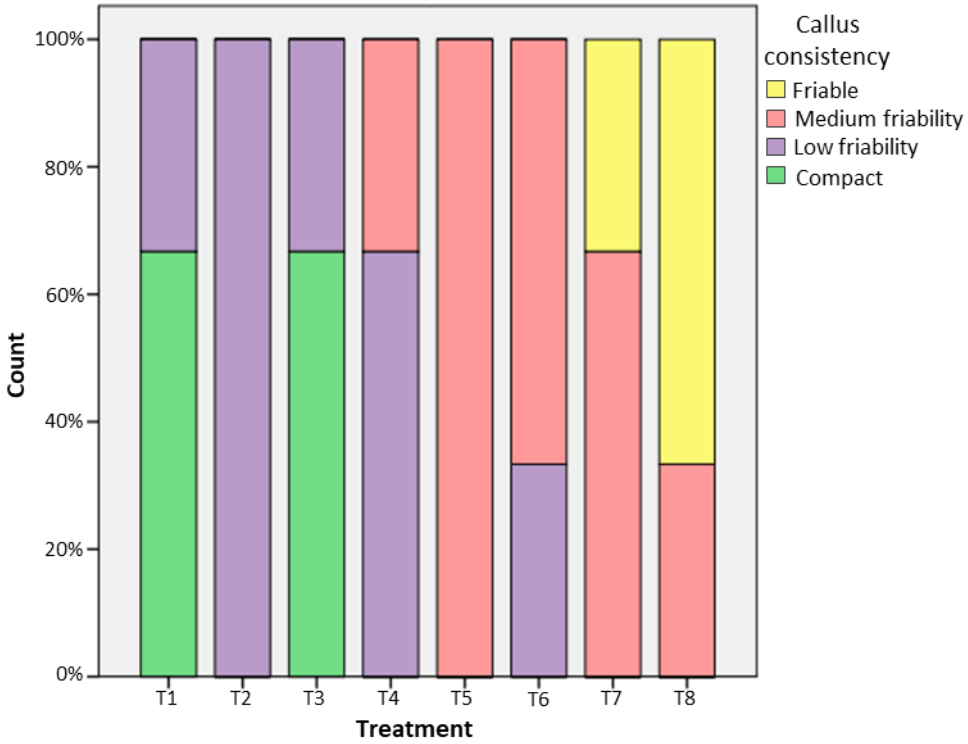

Figure 4. Callus friability. Percentage of callus according to its consistency. Friable, medium friability, low friability, and compact, depending on the treatment, 45 days after of experimentation. Each bar indicates the treatment used (table 4).

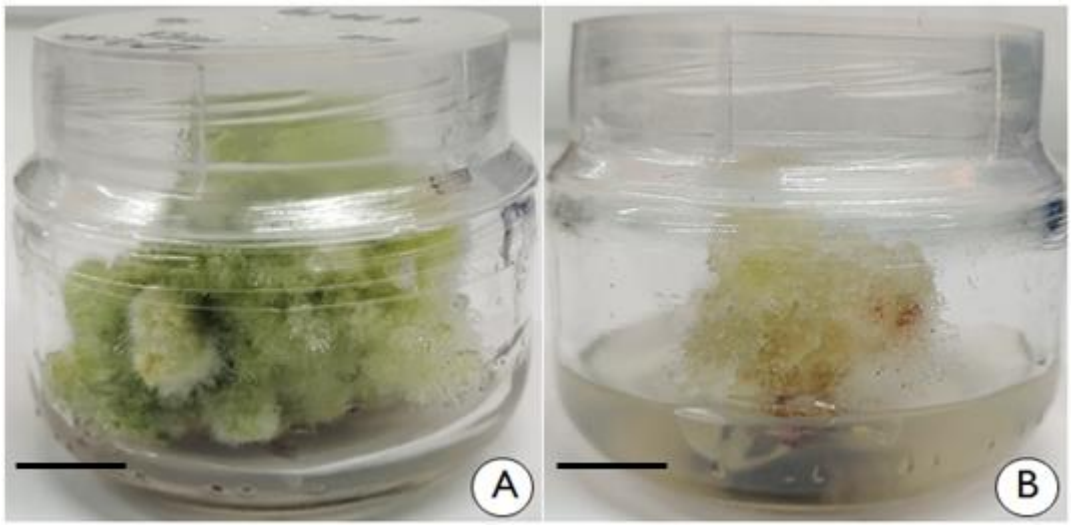

Figure 5. *Ariocarpus retusus* calli 45 days after of incubation with treatments for friability. **A)** T4 callus (410 mg L^{-1} of ammonium nitrate, 2850 mg L^{-1} of potassium nitrate, and 16:8 h light-dark), **B)** T8 callus (410 mg L^{-1} of ammonium nitrate, 2850 mg L^{-1} of potassium nitrate, and continuous darkness). Scale bars equals 1 cm.

Establishment of cell suspensions culture

Dry weight was measured to determine the increase in cell biomass in the cell suspensions evaluated. ANOVA showed highly significant differences (0.000) among treatments. The T5 (MS supplemented with 2 mg L^{-1} of 2,4-D, 0.05 mg L^{-1} of KIN, and 3 mg L^{-1} of myo-inositol) accumulated more biomass, reaching an average of 1.2 g L^{-1} dry weight (Figure 6). This could be the effect of

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myo-inositol, which generates several derivatives after phosphorylation; such as phosphatidylinositols that act as a membrane structural lipid molecules involved in signal transduction and activation of plant growth regulator receptors (Sheard *et al.,* 2010; Gillaspy, 2011; Valluru and Van den Ende, 2011).

Given that T5 showed superior yields, cell suspension cultures were established from this treatment and the respective growth curve was elaborated.

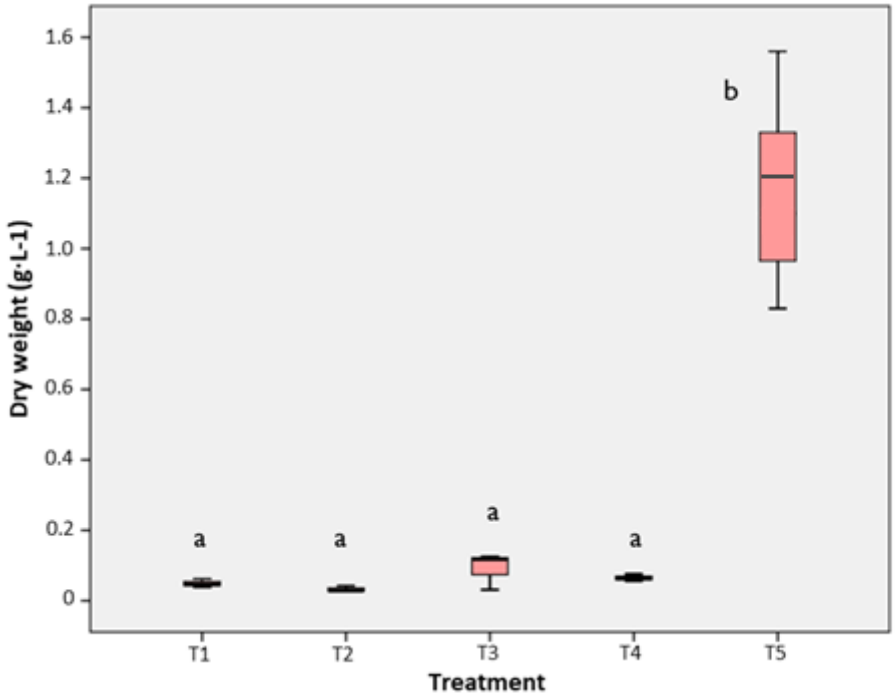

Figure 6. Biomass (dry weight) of *Ariocarpus retusus* cell suspensions with different treatments (table 5). Boxplots with different letter are significantly different (<0.05). The bars indicate the standard error.

Growth kinetics of cell suspensions culture

The growth curve did not present a lag phase, which may be because the suspensions were initiated with an inoculum of previously subcultured cells, indicating that no time was required for cell adaptation. Nover *et al.* (1982) mentioned that the lag phase does not occur or is very short when cell suspensions are subcultured. The exponential phase lasted until day 23, when the maximum amount of biomass production (dry weight) was an average of 3.29 g L^{-1} (Figure 7). Gonçalves and Romano (2018) mentioned that this phase is when the production of secondary metabolites increases. On the other hand, the duration of the exponential phase depends directly on the availability of nutrients in the culture medium. Once cell division begins, they will remain active until they are consumed, so the use of low cell densities at the beginning of the culture or the use of high concentrations of nutrients in the medium will prolong the exponential phase (Szabados *et al.,* 1991).

Apparently, there was no stationary phase in the cell growth curve, since from day 23 onwards there was a decline in dry weight, indicating that the cell death phase had begun due to the depletion of nutrients in the medium, which causes quick cell lysis and death (Caballero and Cardona, 2006).

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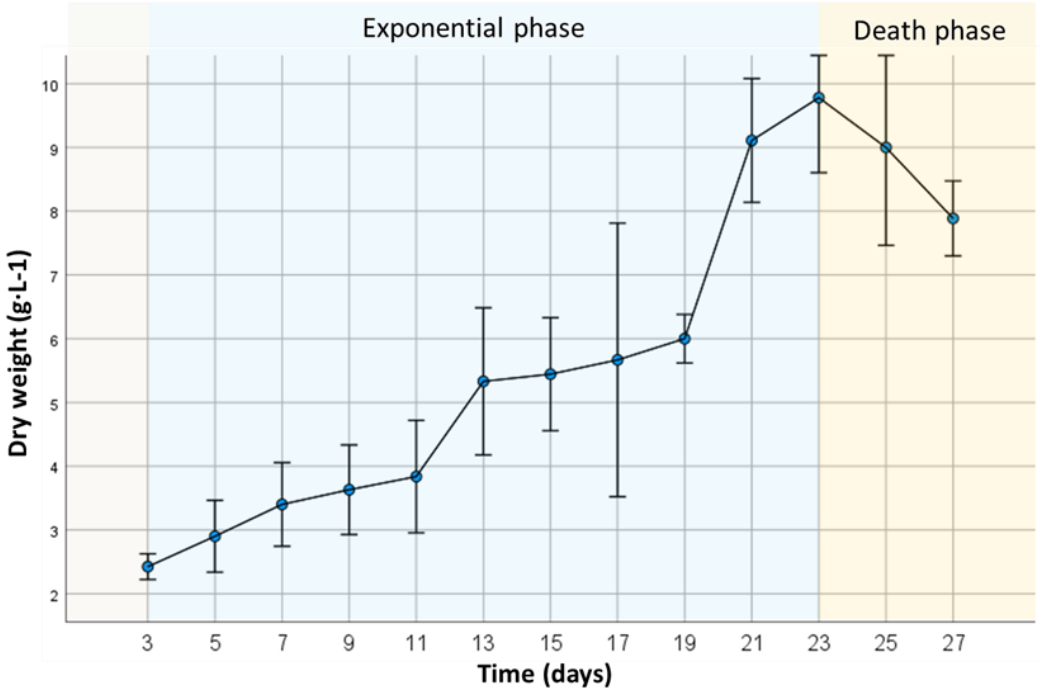

Figure 7. Growth curve of cells in suspension of *Ariocarpus retusus*. The bars indicate the standard error.

Morphology and viability of the cells in suspension culture

Microscopic analysis of cells in suspension culture revealed round and elongated cells with thick cell walls, most of them apparently viable, with bright yellowish inclusions (Figure 8). Small aggregates of cells in suspension were visualized (Figures 8A-8F). Arias *et al.* (2009) and Mustafa *et al.* (2011) mentioned that the formation of cell aggregates is very characteristic of plant cells in suspension, which could be because when cells divide, they do not completely separate or subsequently associate. Actively dividing cells were also observed, showing the septum in the equatorial region (Figures 8B-8F).

The cells mostly did not absorb the Evans blue dye, demonstrating the integrity of the cell membrane, as the color did not penetrate the cell, so it assumed that they were viable cells (Herrera and Rodriguez, 2008; Shigaki and Bhattacharyya, 1999). On the other hand, Evans blue and acetocarmine staining is also a protocol used to observe the onset of cell differentiation (as observed in Figure 8C-F) (Gupta and Durzan, 1987) that leads the cell to perform specialized functions, such as differential metabolite production (Portillo *et al.,* 2012) and regeneration processes (organogenesis and somatic embryogenesis), which have been previously reported in *Ariocarpus* species (Stuppy and Nagl, 1992; Olguín, 1994; Moebius-Goldammer *et al.*, 2003).

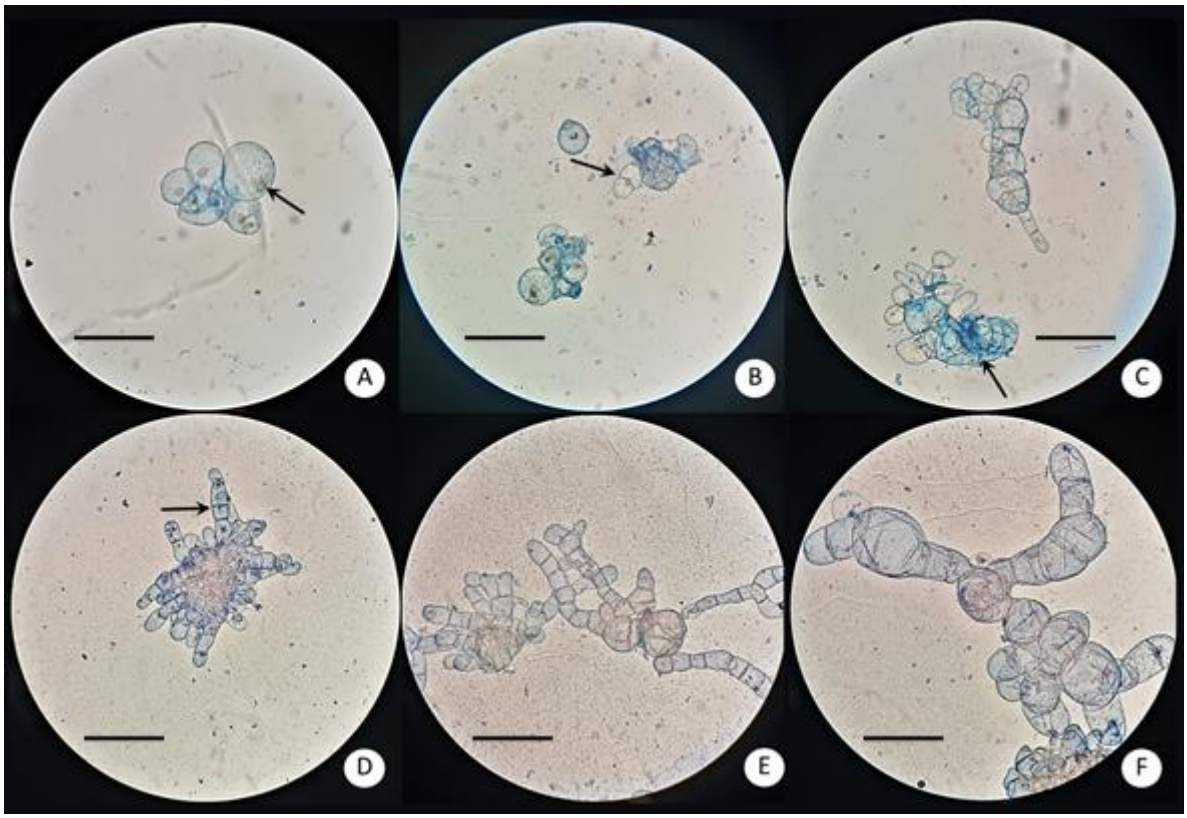

Figure 8. Microscopic visualization (10X) of cells in the suspension culture in exponential phase of *Ariocarpus retusus* with Evans blue and acetocarmine staining. **A)** Aggregate of round cells with yellowish inclusions. **B)** Cells in cell division. **C)** Cells with rupture of the cell membrane. **D)** Septum in the equatorial region of the dividing cell. **E)** Aggregate of elongated cells with active cell division. **F)** Cell aggregate in the final stage of the exponential phase. Scale bars equal 500 µm.

Conclusions

The use of 2,4-D at moderate concentrations (2 mg L^{-1}) allowed the obtaining of a vigorous callus of *Ariocarpus retusus,* and with 3 mg L^{-1}, the callogenesis process was inhibited. Callus friability was favored when ammonium nitrate (NH_4NO_3) was decreased in greater proportion and potassium nitrate (KNO_3) was increased. The absence of light during incubation was determinant in the friability of the callus, showing greater friability in the callus grown in continuous darkness. The addition of myo-inositol significantly (0.000) influenced the increase in biomass of the cell suspensions. The recording of the cell growth curve will provide insight into the behavior of *A. retusus* cells in suspension and thus allow estimation of subculture and/or elicitation times for metabolite production.

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It is not applicable.

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Declaration of competing interest

The authors declare that they have no competing interests.

CONSENT FOR PUBLICATION

It is not applicable.

AVAILABILITY OF SUPPORTING DATA

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contribution statement (CRediT)

Conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing-original draft preparation, Ilse Fiedler; resources, writing-review and editing, Ilse Fiedler, Liberato Portillo, and Fernando Santacruz-Ruvalcaba; visualization, Liberato Portillo and Fernando Santacruz-Ruvalcaba; supervision, Liberato Portillo. All the authors read and approved the manuscript.

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