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Research Article

Obtaining and Propagation in vitro of Plants of Beaucarnea Recurvata Lem

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Abstract

Beucarnea recurvata or "Elephant's Leg," is an ornamental plant with enormous potential, both in green areas and in decorative gardening. In order to promote seed germination and in vitro release of new shoots, tissues of newly germinated seedlings were planted. The seeds, once disinfested, were placed for 0, 24 and 48 h in H2O2 (hydrogen peroxide); incubating under light and dark conditions. From the germinated seedlings, tissues were taken from three regions of the stem, grown in a medium with the inorganic salts of Murashige and Skoog (1962), (MS) at 100%, supplemented with 0.40 mg. L-1 thiamine, 100 mg. L-1 of myo inositol, 3% sucrose, pH 5.7 +-0.1 and 7.0 gr. L of agar, evaluating the effect of cytokinins. Benzyladenine (BA), kinetin (KIN), and 2-isopenteniladenine (2iP); at 3.0 mg.L-1 and 10 mg.L-1 combining the effect with indoleacetic acid (AIA), indolbutyric acid (AIB) and naphthaleneacetic acid (ANA), at 0.1 and 0.3 mg·L-1. The effect of hydrogen peroxide during 48 and 24 h, inhibited the presence of contaminating organisms such as fungi and bacteria, so germination was promoted 100% at 32 days. The best response for the emission of new shoots was achieved by culturing the stem tissues of the Intermediate and basal region, with the medium MS (1962), and 10 mg·L-1 of BA with 0.3 mg·L-1 AIA, successfully obtaining up to 10 new shoots of 3.0 cm in length at 12 weeks of culture.

Key Words: asepsis; H2 O2; in vitro propagation; growth regulators

Introduction

The species Beaucarnea recurvata Lem. known as "elephant's foot", originally from Mexico, it has taken importance as an ornamental, for its colorful bearing in gardens and collections of succulent plants, because of this they are over-collected extracting them from many regions of our country illegally. The reduction of their natural populations, coupled with their slow growth, puts these species in danger of extinction (Semarnat, 2002). The use of in vitro culture of plant tissues, represents an excellent option for the rescue of multiplication and conservation of important species, this biotechnological tool allows to have procedures to carry out its efficient and massive propagation, of It is proposed to have an in vitro protocol as a viable method of propagation of elephant foot, being able to benefit its reincorporation into different protected areas as well as its conservation. In this research was raised as an objective, to establish a methodological process in vitro to promote both seed germination as well as obtaining new sprouts or shoots of B. recurvata Lem. This species usually appears within the family of Liliaceae, Nolinaceae and other authors cite it within the Agavaceae, families in which several studies have been reported where favorable results were obtained with in vitro propagation techniques. The proliferation of axillary buds is the most reported method in micropropagation in Agavaceae, which is based on the phenomenon called apical dominance, which is the regulatory control of growth over the remaining meristematic regions (Rodríguez et al., 1996), if the apical bud is cut, the source of inhibitory hormones is interrupted, beginning to grow one or more axillary buds that will give formation to a new outbreak. It has been observed that cytokinin's, mainly benzyladenine (BA), is very effective in removing apical dominance (Pierik, 1990), in some cases the use of a high concentration of cytokinins and low concentration of auxins is favorable. Regeneration of adventitious branches from intact dicotyledonous stems is relatively rare; however, in culture media it is possible to induce the formation of adventitious shoots in separate parts of plants with high multiplication rates (Harmann and Kester, 1981)

Methods And Materials

The research was carried out in the Plant Tissue Culture Laboratory of the Department of Plant science of the Autonomous Chapingo University, México. The seeds of B. recurvata Lem. They were provided by the PLANTEC COMPANY nursery, located in Amacuzac, Morelos, from trees in San Luis Potosi. Mexico.

The research was divided into two parts, the first: to establish the conditions for the establishment and in vitro germination of seeds and the second: to develop a methodology to promote the obtaining of news sprouts or shoots from sections of stem tissue.

Seed disinfection.

Initially, the seeds were washed vigorously with commercial detergent powder plus a few drops of tween 80 (surfact agentante) dissolved in water; they were then placed in a 70% ethanol solution (v / v) for 3min and finally immersed in sodium hypochlorite (6% active chlorine) at 10% and 20% for 15min, respectively.

For the treatments of desinfestation of the seeds, hydrogen peroxide (H_2O_2) (3% v / v) was used, for different stirring times (0, 24 and 48 h.)

Culture medium.

The culture medium used were the inorganic salts of Murashige and Skoog (1962) at 100 %, supplemented with myo-inositol 100 mg·L-1, thiamine-HCL 0.40 mg · L-1 and sucrose 3 %, adjusting the pH to 5.7± 1; finally incorporated as a gelling agar 7 g ·L.1. The medium was distributed in test tubes, 20 ml in each one, and then Sterilize in autoclave a 121 °C y 1.5 kg·cm-2 pressure for 15 min.

Planting and incubation conditions

The isolation and establishment of explants in vitro, was done inside a laminar flow hood; after the time of agitation under hydrogen peroxide, the seeds were rinsed with sterile water three times, placing one seed per tube. The experiments were kept in an incubation area with controlled environment at a temperature of 25 ± 2 ° C, in different lighting conditions depending on the treatment: 16 light h with a light intensity of 3000 lux, and darkness.

Statistical analysis

The percentages of contamination and in vitro in vitro were evaluated. Data collection for germination was performed at eight days after establishment (DDE), so on until 48 DDE after germination of seeds grown in vitro no longer occurred. The length of emitted shoots, the number and length of the roots emitted were evaluated, the data were taken eight days after germination.

Treatments were distributed using a completely randomized experimental design with factorial arrangement 3 X 2. In the first of them the seeds were exposed to different stirring times (0, 24, 48 h) in hydrogen peroxide (3 % V / V) (Factor TA) and then lighting conditions of 16 h light and darkness, (LU Factor) were placed. The total treatments generated were 6 which are indicated in Table 1

Treatment	Stirring in hydrogen peroxide (H_2O_2) 3% (v/v),	Lighting conditions during incubation
1	0 h	16 h light
2	0 h	Dark
3	24 h	16 h light
4	24 h	Dark
5	48 h	16 h light
6	48 h	Dark

Table 1: Procedures for in vitro seed establishment and germination.

The experimental unit was to place one seed per tube, establishing 15 repetitions per treatment. Frequency tables were obtained under the chi-square test for the variable contamination and germination.

Establishment of stem of seedlings obtained in vitro.

Within the laminar flow chamber, from the seedlings obtained from in vitro germination, three different regions of the thickened stem zone were isolated and cultivated; Making cross-sections located in: basal zone, intermediate and the apical or terminal zone, which were placed in the culture medium maintaining their normal polarity.

Culture medium

In all treatments the culture medium included: A basic medium including inorganic salts of MS (1962) 100%, myo-inositol (100 mg·L-1), thiamine-HCL (0.40 mg·L-1), adenine sulfate (80 mg·L-1), sucrose (3%) and agar (7g. L-1), with different growth regulator types and concentrations. The cytokinins used were: benzyladenine (BA), kinetine (KIN) and 2-isopentyladenine (2iP); auxins: indoleacetic acid (AIA), indolebutyric acid (AIB) and nahthalenacetic acid (ANA).

Incubation Conditions

The experiments were held in a room with a controlled environment at a temperature of 25 ± 2 ° C, under a light intensity of 3000 lux, photoperiod 16h light and 8 h darkness.

Statistical analysis

The variables evaluated were: number and length of outbreaks, The experimental design was a completely random with factorial design 3 X 9, where the first refers to the type of explant used (basal, intermediate or terminal), in different combinations of cytokinin-auxin; thus, in a design of treatments of complete factorial type 27 treatments were generated, as shown in table 2.

Trat.	Explant	Citocinin-auxin Concentratión ($mg \cdot L^{-1}$).	Trats.	Explant	Citocinin-auxin Concentratión ($mg \cdot L^{-1}$).
1	B	BA (10) - AIA (0.3)	15	I	2ip (3.0) - AIA (0.1)
2	B	BA (10) - AIB (0.3)	16	I	KIN (10) - ANA (0.3)
3	B	BA (10) - AIB (0.3)	17	I	KIN (3.0) - ANA (0.1)
4	B	BA (3.0) - AIA 0.1)	18	I	----
5	B	2ip (10) - AIA (0.3)	19	T	BA (10) - AIA (0.3)
6	B	2ip (3) - AIA (0.1)	20	T	BA (10) - AIB (0.3)
7	B	KIN (10) - ANA (0.3)	21	T	BA (10) - AIB (0.3)
8	B	KIN (3.0) - ANA 0.1)	22	T	BA (3.0) - AIA 0.1)
9	B	----	23	T	2ip (10) - AIA (0.3)
10	I	BA (10) - AIA (0.3)	24	T	2ip (3.0) - AIA (0.1)
11	I	BA (10) - AIB (0.3)	25	T	KIN (10) - ANA (0.3)
12	I	BA (10) - AIB (0.3)	26	T	KIN (3.0) - ANA (0.1)
13	I	BA (3.0) -AIA (0.1)	27	T	----
14	I	2ip (10) – AIA (0.3)			

Table 2: Distribución de tratamientos, para promover las respuestas hacia la brotación in vitro de de B. recurvata Lem.

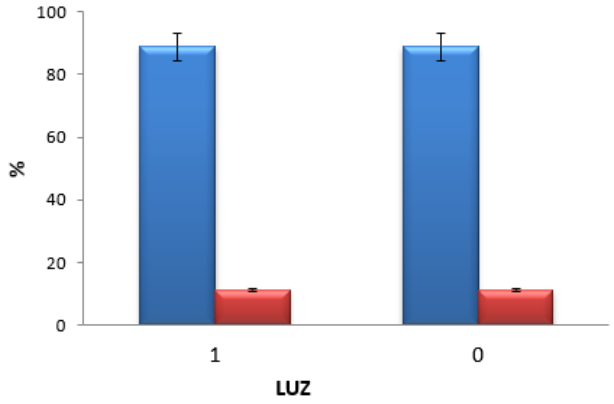
The different stem sequences were taken as experimental units, establishing five repetitions for each treatment. For the quantitative variables, an analysis of variance was performed using the Statical Analysis System (SAS) package, and the comparison of Tukey's means (α=0.05). For the qualitative variables, frequency tables were obtained under the chi-square test.

Results and Discussion

Establishment and in vitro germination of seeds

Light and dark effect

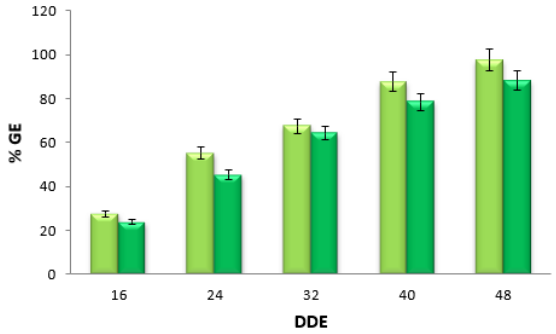
The presence and absence of light during the experimental incubation period did not influence promoting the presence of either fungi and bacteria in vitro, since the process for their control and cleaning was similar in both cases. (Figure 1).



1: Light 0: Darkness I 5% error bars

Figure 1: Percentage of clean seeds of Beaucarnea recurvata Lem. in light and dark conditions. Blue=clean, Red=Contamination percentage,

The percentages of seed germination under the light and dark condition, at the end of the experimental period, and in the data collection, was not statistically different for both conditions, reaching similar percentages (Figure 2 and 5); however, during the incubation time the trend was slightly higher with the presence of light.



I 5% error bars

Figure 2: Percentage of in vitro germination of Beaucarnea recurvata Lem. seeds in the presence of light and dark. Ligh=green with ligh, Dark= green dark.

Agitación in hydrogen peroxide (H_2O_2)

The seeds stirred in H2O2 in the presence and absence of light at the beginning, presented a higher percentage of contamination (30 %), while after 24 h of stirring only 3.3 % were contaminated and those that were stirred for 48 h, were completely cleaned, free of the presence of contaminating organisms (Figure 3). In most cases, the contamination was mainly due to the presence of fungi.

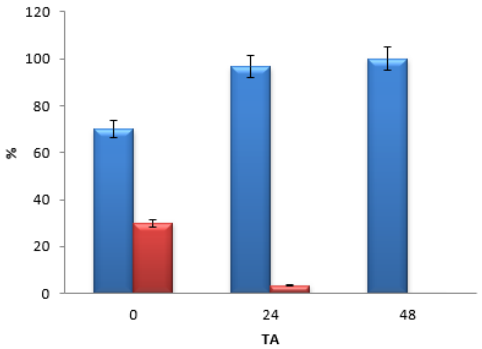
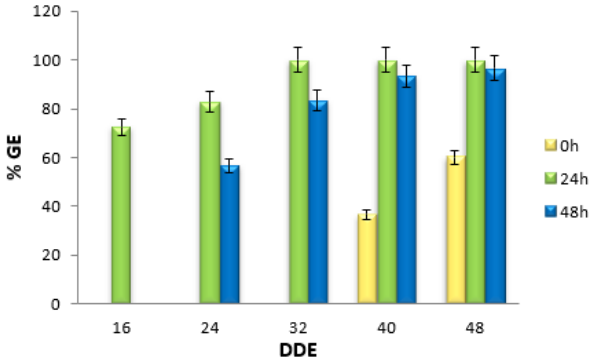


Figure 3: Percentage of contaminated and clean seeds of Beaucarnea recurvata Lem. in 0, 24 and 48 h of stirring in H_2O_2. Blue=clean, Red=contaminants presence.

The seeds of the treatments of 24 and 48 h in agitation in H_2O_2 showed a percentage of germination of 100 and 96.67, respectively; while the seeds that were not stirred in the presence of H_2O_2 78.26 % did not germinate; so the real percentage for this treatment was 59.9 (Figure 4); it should be noted that seeds with agitation of 24 h, germinated from 16 DDE; however, while seeds that were 48 h in stirring began to germinate a week later, but reaching almost the same percentage at 48 DDE.



I 5% error bars

Figure 4: Percentage of germination of Beaucarnea recurvata Lem. According to the stirring time in H_2O_2

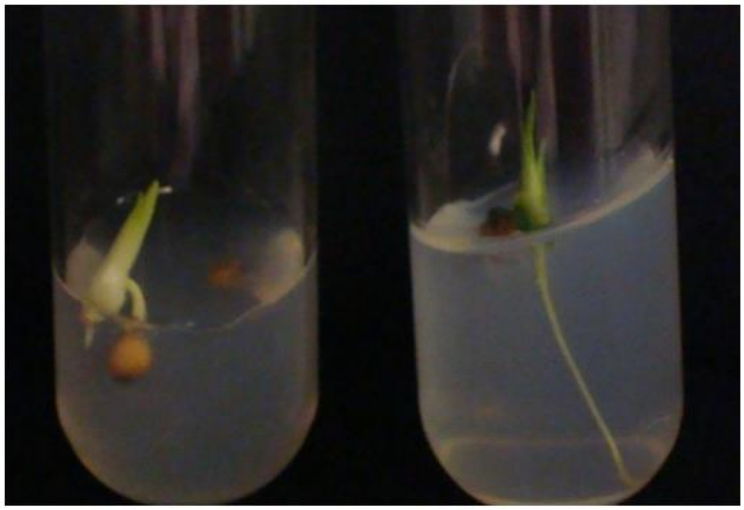


Figure 5: In vitro germination of Beaucarnea recurvata Lem seeds.

Stem tissue responses of germinated seedlings in vitro.

Type of explant

A greater number of outbreaks was obtained in the intermediate segment (I); however, there are no statistically significant differences between Intermediate (I) and Basal (B), but there are differences with respect to Terminal (Figure 6, 7 and 8). While the terminal stem tissue was the one that presented a greater length of shoots compared to the basal and intermediate showing significant difference according to the comparison de means (Table 3).

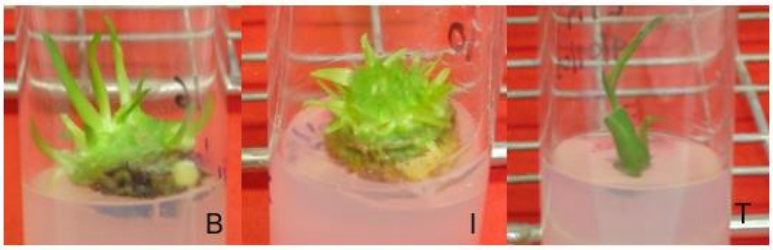


Figure 6: Promotion of sprouts of basal region (b) intermediate (i) and terminal (t) at 30 DDE in medium MS (1962) added with 10 mg· L-1 BA plus 0.3 mg· AIA L-1.

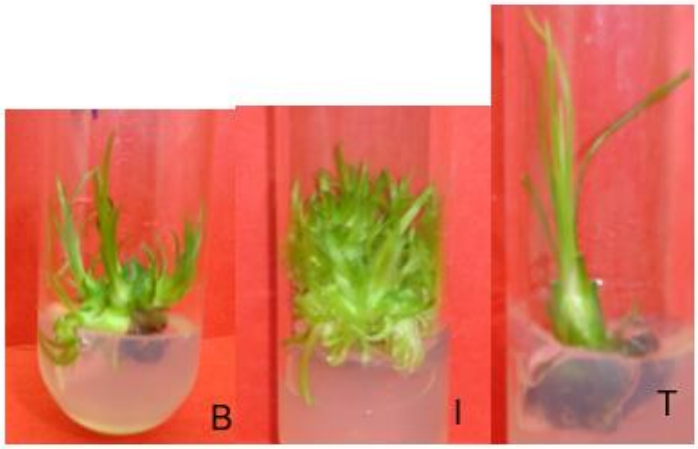


Figure 7: Sprouts obtained in basal region (B) intermediate (I) and terminal (T) at 60 DDE in medium MS (1962) added with 10 mg·L-1 BA plus 0.3 mg·L-1 AIA.

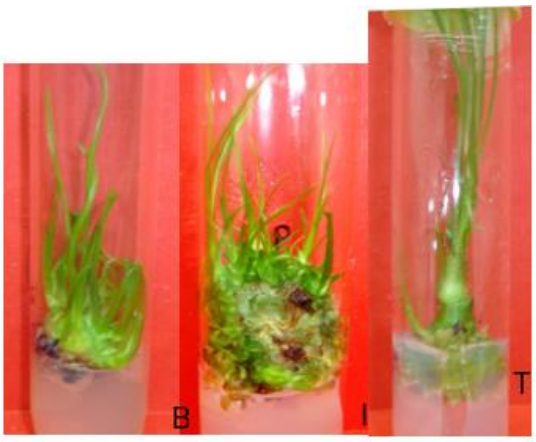


Figure 8: Promotion of sprouts in vitro of basal region (B) intermediate (I) and terminal (T) at 90 DDE in medium MS (1962) added with 10 mg·L-1 BA plus 0.3 mg·L-1AIA.

Figure 10: Plantlet of Beaucarnea recurvata establishment in soil.

Cytokinin-auxin effect.

The medium with the cytokinin-auxin 1 combination (10 mg·L-1 BA- 0.3 mg·L-1 AIA) was the one with the highest number of outbreaks 80 days after its establishment (Figure 8). The longer shoots were obtained in the medium with a combination of 10 mg·L-1 2ip - 0.3 mg·L-1 AIA; however, the results showed no significant differences between the combinations of growth regulators (Table 3).

CUADRO 3. Number and lenght of sprouts obtained according to the type or region of explants and cytokinin -auxin combination in the culture medium.

FACTOR Level	Sprouts number	Length of sprouts or shoots
Explant		
-Tejido Basal	4.68 a[z]	1.41 b
-Tejido Intermedio	6.45 a	1.27 b
-Tejido Terminal	1.23 b	3.26 a
Citocinin-auxin		
-1	10.46 a	1.6308 a
-2	4.46 bc	1.4889 a
-3	8.50 ba	1.9667 a
-4	2.09 dc	2.8778 a
-5	4.64 bc	3.1500 a
-6	0.80 dc	2.8333 a
-7	0.45 dc	2.8400 a
-8	1.33 dc	2.5200 a
-9	0.23 d	0.9333 a

z Values with the same letter within factor in each column are equal according to Tukey's test with a $P \leq 0.05$.

Results And Discussion

Establishment and in vitro germination of seeds

The works reported in Beaucarnea recurvata Lem. By Atta-Alla (2003) showed that the best responses towards germination was under light conditions, on the other hand Benítez et al. (2004) in four species of Mammilaria (Cactáceae). They showed that light was not a factor that conditioned germination, other results reported in Tillandsia eizii were between 76 and 88% seed germination (Kimberly et al., 2003). Hartman and Kester (1988) and Salisbury and Ross (1992), reported that in some species seed germination is stimulated by light; however, in other plant species germination may be inhibited; Salysbury and Ross (1992) reported that the seeds of many plant species whose light-responding seeds have not been domesticated.

Effect of hydrogen peroxide ($H_2 O_2$)

Flores (2005a) in Nolina parviflora, reported having immersed the seeds for 24 h in stirring in 3% diluted hydrogen peroxide, which did not show the presence of contaminating organisms such as fungi or bacteria, other similar cases have been reported for Abies religiosa (Alvarez et al., 2008.). In Pinus palustris Mill. and P maximartinezii (Rzedowski) the use of H2O2 in the disinfestation process also proved to be very effective, other similar results were reported to control harmful organisms, it was when commercial fungicides were used in the soaking (Barnett and McGilvary 2001, Ojeda et al., 2006). Hydrogen peroxide (H_2O_2), is described as an inorganic compound, which acts by oxidizing the components of the membrane and enzymes of microorganisms (Mateos, 2002 cited by Flores 2005a), because of that it has been widely used as a desinfestant for its high oxidizing power (Tom, 1998).

The results reported with seed germination by Osorio and Mata (2005). in Beaucarnea gracilis and B. recurvata, were between 89.8 and 95.3 % after 30 days of culture, in an MS culture medium, added with 2 mg·L-1 adenine, and 100 mg· L-1 of myo-inositol, while Flores et al (2005a), reported percentage of seed germination in an 87.5 in Nolina parviflora (H.B.K.) Hemls., when stirred for 24 h in (H_2 0_2) and placed at 20°C. In Pinus palustris Mill. by immersing seed in 30% hydrogen peroxide for one hour, 93% germination was achieved (Barnett and McGilvary, 2001). In pine seeds, it has been found that H_2O_2 increases germination, because it softens its testa or deck and increases permeability to water and oxygen (Barnett and McGilvary, 2001), which are important conditions, to which the seed must be exposed, to carry out its germination. The humidity or the soaking in water of the seeds, is important so that the imbibition is carried out and consequently to that a whole physiological process that will allow to activate the germination; On the other hand, oxygen is essential for the process of respiration of the seed (embryo), since the absorption of this is proportional to the metabolic activity that is taking place, so that a rapid and uniform germination takes place (Hartman and Kester, 1988).

In vitro culture of stem tissues.

The associated responses to promote the emission of new shoots from tissues sown in vitro of the terminal segment of the stem, showed a lower emission of new shoots, possibly to which in this region is observed high meristematic activity and synthesis of compounds associated with the metabolism of plant growth associated with the activity concentrated in the apical region of the stem, since the tissues taken from the intermediate region differentiated more new shoots, in addition the central part of the stem, by competition with the rest of the cells of this zone are in a constant cell division, and the growth that allows secondary thickening in stems in monocots (Stevenson, 1980); The responses observed in terms of the emission of new shoots, were more successful, when stem tissues taken from the basal and intermediate regions were used, the above can be interpreted by which synthesis of growth regulators is presented in the apical regions and their displacement towards the basal regions of the stem, in this way the stem tissues cultured from these regions can promote more in vitro the issuance of new shoots. Also recognizing the participation of growth regulators such as auxins incorporated in vitro or in the culture medium. The variable length of shoots was observed much more evidently in tissues cultured in vitro of the terminal or apical region. This is explained by the greater meristematic activity of cells for the growth of stems coupled with the nutritional conditions of the culture medium and the conditions of incubation light and temperature, jointly stimulating a greater length of the shoots.

In species such as Nolina parviflora (H.B.K.), Yucca valida, Y. aloifolia, Y. filamentosa and Agave parrasana Berger. Similar cases are reported where the source of explant used, and the region of the stem used is important to promote direct organogenesis, the above has been reported by: Flores, 2005b; Arce and Rodriguez, 2006; Santacruz et al., 1999; Atta-Alla and Van, 1997. Similarly, Nava (1988), in Agave tequilana Weber "Azul", observed that the best region for obtaining shoots were segments of the center of the stem. It is well known that monocots lack secondary tissue or cambium, associated with cell division Cronquist (1981); however, in some taxa such as Agavaceae and specifically in B. recurvata, it has been shown that if they present a meristem that allows secondary thickening of the stem (Nava, 1988)

Effect of combining: cytokinins-auxins

In B. recurvata have reported the responses to sprouting with the use of the cytokinin Benzyladenine (BA), or using the combination of different cytokinins-auxins; such is the case of Osorio and Mata (2005) to obtain 11 outbreaks per explant in B. recurvata and 8 in B. gracilis in an MS medium supplemented with 22.2 µM of BA. Likewise, Atta-Alla (2003) obtained on average 7.8 sprouts in B. recurvata in DM medium supplemented with 0.4 mg·L-1 of ANA and 4.0 mg·L-1 of BA. In several species of the genus Yucca, a response to sprouting has been found with the combination of a cytokinin-auxins; however, not all respond in the same way, such is the case of Y. valida, where they used 10-20 µM of BA with 5 µM of AIA Arce and Rodríguez, (2006), or Y. aloifolia, which responded successfully in a medium with 9.0 mg·L-1 of 2ip plus 0.1 mg·L-1 ANA as opposed to Y. filamentosa var. Variegated with 6.0 mg·L-1 BA plus 0.1 mg. L-1 ANA. Based on the above, we can conclude that the different species respond variably to the application of growth regulators in the culture medium; recognizing that the effect of cytokinins is basically to stimulate cell growth and differentiation, since auxin complement stimulates cell division and differentiation (Pierik, 1990).

There was no statistical difference in spout length using cytokinin-auxin combination culture medium; similarly this effect was reported in Nolina parviflora (H.B.K.) Helms., did not report mainly differences for this variable, observing that the concentrations used both auxins and cytokinins, had no influence on these responses (Flores, 2005b), unlike Yucca aloifolia, where a longer shoot length was obtained when 4.4 µM of BA was incorporated into the medium, observing that this variable decreases as the concentration of cytokinins increases (Atta-Alla and Van, 1997). Finally, growth regulators, both types and concentrations, not only intervene within

the cultured tissues but also depending on the type of plant used, but also on the species, its stage of development, and the type and concentration of regulators used, as well as the interactions between different environmental factors such as light and temperature (Salisbury and Ross 1992).

Conclusions

The best disinfestation process for the establishment of in vitro seeds of B. recurvata Lem. was the application of hydrogen peroxide, under stirring for 24 and 48 hours; We can conclude that germination is not conditioned by the effect of darkness or presence of light; The periods of agitation with the effect of hydrogen peroxide for 24 hours, accelerated the times to activate seed germination. And finally, the best explants used to promote the emission of new sprouts were those isolated and sown from the intermediate and then basal region of the stems of the seeds newly germinated in vitro in the media with the inorganic salts of the MS medium and the combination of growth regulators10 mg· L-1 BA plus 0.3 mg·L-1 AIA

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