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In vitro induction of callogenesis and organogenesis in explants of *Krameria erecta* Willd

Inducción *in vitro* de callogénesis y organogénesis en explantes de *Krameria erecta* Willd

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ABSTRACT

The objective of the present investigation was to evaluate *in vitro* the germination and induction of callogenesis and organogenesis in explants of *Krameria erecta*. The culture medium used was WPM/50 with half the salts and made up of vitamins, sucrose and indolebutyric acid (IAB), in concentrations (0, 0.5, 1.0, 1.5 and 2 mgL^{-1}). The explants were incubated in a controlled environment of 25 °C and photoperiod of 16 light hours. The experimental design was completely randomized with a 5x3 factorial arrangement with an ANDEVA ($P < 0.05$) and the Tukey mean test. *In vitro* germination did not present significant differences, showing from 79 to 83%. There were significant differences in seedling height, the concentrations of 1.5 and 2.0 mgL^{1} of AIB were higher with 11.60 to 11.65 cm. The height and width of callus in axillary bud and leaf presented significant differences in concentrations of 1.0 and 2 mgL^{1} of AIB. Regarding the number of root in the leaf it was from 0.50 to 2.50, showing significant differences in the concentrations, in the axillary bud there was no root; therefore, there were no significant differences. In contamination, there were no significant differences presented below 12.50%. According to the disinfection with alcohol and sodium hypochlorite (NaClO), there was no oxidation in seeds, but in axillary bud and leaves.

Keywords: tissue culture, indolebutyric acid, explants and *Krameria erecta.*

RESUMEN

El objetivo de la presente investigación fue evaluar *in vitro* la germinación e inducción de callogénesis y organogénesis en explantes de *Krameria erecta*. El medio de cultivo utilizado fue WPM/50 a la mitad de sales y conformado de vitaminas, sacarosa y ácido indolbutirico (IAB), en concentraciones (0, 0.5, 1.0, 1.5 y 2 mgL^{-1}). Los explantes fueron incubados en ambiente controlado de 25°C y foto periodo de 16 horas luz. El diseño experimental fue completamente aleatorizado con un arreglo factorial de 5x3 con un ANDEVA ($P<0.05$) y la prueba de media de Tukey. La germinación *in vitro no* presento diferencias significativas mostrando de un 79 a 83%. Se presentaron diferencias significativas en altura de plántulas, las concentraciones de 1.5 y 2.0 mgL^{1} de AIB fueron superiores con 11.60 a 11.65 cm. La altura y ancho de callo en yema axilar y hoja se presentaron diferencias significativas en concentraciones de 1.0 y 2 mgL^{-1} de AIB. En lo que respecta al número de raíz en hoja fue de 0.50 a 2.50, mostrándose diferencias significativas en las concentraciones, en yema axilar no se presentó raíz por lo tanto no se presentaron diferencias significativas. En contaminación no se presentaron diferencias significativas presentado abajo del 12.50% de acuerdo la desinfección con alcohol e hipoclorito de sodio (NaClO) no se presentó oxidación en semillas, pero si en yema axilar y hojas.

Palabras clave: cultivo de tejido, ácido indolbutirico, explantes y *Krameria erecta*

INTRODUCTION

The Krameriaceae family have 17 species in a single genus *Krameria erecta* W. (Dominguez *et al.,* 1987; Simpson *et al.,* 2004). It is distributed geographically in northern Mexico, mainly in Sonora, Sinaloa, Chihuahua, Durango, Coahuila, Baja California, and Zacatecas and in California states, Utah and Nevada in the United States of America (Simpson and Salywon, 1999).

It is a forage shrub with economic importance in the arid and semi-arid zones of northwestern Mexico (Mc-Caughey-Espinoza *et al.,* 2019). Its importance in the field of medicine is because it has high antiproliferative activity in cancer cells and a high content of flavonoids. Furthermore, it has hepatoprotective, antioxidant and anti-inflammatory effects, and has five times more antioxidant activity than ascorbic acid (Carini *et al*., 2002; Jiménez-Estrada *et al.,* 2013; Morán-Palacio *et al.,* 2014). It is worth mentioning that the tincture of the root is from this plant extracted, which is used by Seris, an important ethnic group in Sonora State.

In the last 20 years, the work of *in vitro* propagation of some forest species has gradually increased; however, to date no studies have been carried out that report the *in vitro* micropropagation of *Krameria erecta* Willd.

The distribution of *K. erecta* is limited in natural ecosystems, by factors such as over grazing, change of land use, low rainfall, presence of insects, etc. In its natural habitat, it can find from zero to 70 plants in 2,500 square meters. The presence of new individuals is practically non-existent.

With the application of biotechnology, the natural ecosystems still present in our environment can be preserved. One of the strategies used to counteract the problems present in the propagation of forest species and their extinction, is *in vitro* cultivation of vegetable crops (Delgado *et al.,* 2008).

To lessen the situation of the low populations that exist in the pastures of Sonora state, and for being an appetizing plant for herbivorous and medicinal animals; the purpose of this research is to establish a methodology for the germination and induction of *in vitro* callogenesis and organogenesis in axillary buds and leaf of *Krameria erecta* W.

MATERIAL AND METHODS

The collection region of *Krameria erecta* Willd was made between the coordinates 29° 03'21.30 ”of North Latitude and 110° 45'12.22” of West Longitude (Las Cruces ranch). During the month of September 2019, the useful parts of the plant were the stem, leaf and seed explants, generally only 20% can be physiologically mature and viable seeds. The material used was scissors, ziplot bags and an ice chest. The research was carried out in the Biotechnology laboratory of the Horticultural Engineering Education Program of the Sonora State University (UES).

Culture medium

The culture medium used was Woody Plant Medium (WPM/50) (Trigiano and Gray, 2011). This medium was used at half its concentration, consisting mainly of mineral salts and vitamins, such as: thiamine, myo-inositol, sucrose and agar. The phytohormone used was indolbutyric acid, at concentrations of 0, 0.5, 1.0, 1.5, and 2.0 mgL^{-1}. Antioxidants were not to the medium applied.

Laminar flow chamber

The laminar flow chamber (Brand Edge Gard Hood), was previously disinfected before performing the cultures, using 99% alcohol and vickor, and then for 30 min a 40 W fluorescent light lamp was left on, which provided an irradiation of 8-10 W m^{-2}. Test tubes and clear glass Gerber flasks were used. Glass Petri dishes, clamps and scalpel were also used; all the material was previously sterilized in a sterilmatic model autoclave, at a temperature of 120 °C and a pressure of 15 kg/cm^2 for 15 minutes.

Explant culture

The seeds were previously soaked with deionized water, for 2 hours before sowing. The explants (leaves, stems, axillary and apical buds, and seeds without pericarp, were imbibed for 3 hours in deionized water), then they were disinfected with 70% ethyl alcohol for 3 minutes; and subsequently in 15% sodium hypochlorite (NaClO) (CLOROX®) for 12 min, adding a drop of tween 20; 3 rinses were performed with deionized water. Subsequently, they were placed in an antioxidant solution of ascorbic acid (150 mg/L) and citric acid (100 mg/L) for 5 minutes, and were subsequently seeded.

Incubation

Gerber-type glass jars were used for sowing. The explants were previously planted. The incubation room was subsequently placed under controlled conditions, maintaining a temperature of 25±1 °C, and a photoperiod of 16 light hours with 8 hours of darkness and 70-75% relative humidity.

Parameters evaluated

The evaluation was carried out by observation and measurement in the different explants, beginning on the third day of their incubation, and subsequently, measurements were observed and made every seven days for 2 months.

The parameters evaluated were:

• Germination percentage (%), using the ISTA (2019) criterion.

• Callus measurements present in the explants; a Mitutoyo Absolute Model CD-6CSX Vernier Serial No. 06401649 6" was used

• Seedling height (cm)

- Callus width and height (mm)
- Number of roots
- Contamination and oxidation

Statistical analysis

The experimental design used in this research work was completely randomized, with a factorial arrangement of 5x3 (five concentrations and three explants), with 10 repetitions. An analysis of variance (ANDEVA) was performed, with a significance level of $P < 0.05$, and a comparison of Tukey means. For the analysis of the data, the JMP statistical package, version 9.0.1 (Statistical Analysis System, [SAS Institute Inc.], 2011) was used.

RESULTS AND DISCUSSION

In vitro seed germination

In vitro germination started on the 4th and ended on the 15th day. There were no significant differences in the percentage of germination between AIB treatments, used in this study with a germination percentage of 79.0 to 83.0%. These results indicate that for the germination of the *Krameria erecta* seed the use of any type of phytohormone is not required in the culture medium; as their germination percentage is not significant, see table 1 and figure 1.

Regarding the percentage of germinated normal seedlings, significant differences were presented ($P \leq 0.05$), between AIB concentrations (0.5, 1.0, 1.5 and 2 mgL^{-1}), with respect to the control (without phytohormone); presenting for the control 71.25% of normal seedlings, and between 31.25 to 32.0% with the use of AIB, see table 1. It is important mentioning that significant differences were also ($P \leq 0.05$), when evaluating the percentage of abnormal seedlings of *Krameria erecta* observed, with respect to the treatments, presenting 11.5% of abnormal seedlings in the control; while in the AIB treatments, there were from 49.75 to 51.0% of abnormal seedlings.

Table 1. *In vitro* germination and seedling height of *Krameria erecta* Willd.

Treatments AIB mgL^{-1}	Sprouted seeds (%)	Normal Seedlings (%)	Abnormal seedlings (%)	Not germinated (%)	Seedlings Height (cm)
0	82.75±1.49^{a}	71.25±^{a}	11.50±1.29^{b}	8.50±1.00^{a}	10.07±0.21^{b}
0.5	81.50±1.49^{a}	31.25±^{b}	50.25±2.75^{a}	9.75±0.50^{a}	10.42±0.21^{b}
1.0	83.00±1.49^{a}	32.00±^{b}	51.00±2.44^{a}	9.75±0.95^{a}	10.52±0.21^{b}
1.5	79.00±1.49^{a}	31.75±^{b}	49.75±2.50^{a}	10.50±1.29^{a}	11.65±0.21^{a}
2.0	82.00±1.49^{a}	31.50±^{b}	50.50±3.10^{a}	10.25±0.95^{a}	11.60±0.21^{a}

Means with equal letters within the same column indicate that there are no significant differences ($P < 0.05$). The data presented are the average of 10 repetitions with three samples each bottle per treatment.

When analyzing the percentage of non-germinated seeds, there were no significant differences between the treatments. It presented from 8.5 to 10.5% of seeds that did not germinate, see table 1. These results show that the use of auxins stimulated negative geotropism in germination, when presenting above 50% of abnormal seedlings.

Golubov *et al.*, (2007), mentions that germination is to the growth of the embryo linked. The germination of normal seedlings was less than 32.00%, when presenting a well-defined radicle and the sprouting of its seminal leaves. According to AOSA (2005), it indicates that seedlings that presented abnormal growth do not develop, as they present deficiencies in their root system (lack of radicle, negative phototropism, or other malformations).

Figure 1. *In vitro* germination of *Krameria erecta*

The germination percentage in *Krameria erecta* was similar to that obtained by Golubov *et al.,* (2007), they obtained percentages of *in vitro* germination greater than 80% in *B. recurvata* seeds, which were stored (in dehydration with silica gel), for six years, at 6 ° C, with low relative humidity.

Seedling height

The height of the *Krameria erecta* seedlings showed significant differences (P≤0.05) between the evaluated treatments, see table 1. The seedlings with the treatments of 1.5 to 2.0 mgL^{-1} of AIB presented 11.60 to 11.66 cm in height, without having between these significant differences; while 0, 0.5 and 1.0 mgL^{-1} of AIB, did not present significant differences, showing 10.07 to 10.52 cm. The seedlings presented their own characteristics and were morphologically similar to the plants present in their wild habitat.

Callus and root induction

According to the explants used in this investigation, it was shown that there are significant differences (P≤0.05) regarding callus height, from 0.58 to 0.67 mm high, see Table 2 and Figure 2; except for the AIB zero-mgL^{-1} treatment (control), there was no callus. Regarding the width of the callus, there were also significant differences (P≤0.05), according to the

statistical analysis, indicating 0.76 to 0.96 mm in the 0.5, 1.0, 1.5 and 2.0 mgL^{-1} treatments of AIB.

In axillary bud, there were significant differences (P≤0.05) between treatments, except in 1.5 and 2.0 mgL^{-1} of AIB. It presents heights in buds from 0.93 to 0.96 mm regarding the width of the callus in the axillary bud, there were significant differences (P≤0.05) between the treatments of 1.0, 1.5 and 2.0 mgL^{-1} of AIB, with respect to 0.5 and 0 mgL^{-1} of AIB. The zero mgL^{-1} treatment of AIB (control) did not present callus, see table 2. Despite the significant difference that exists between the treatments, it is worth mentioning that the application of auxin (AIB) is effective for the induction of callus in leaf and axillary bud of southern cosahui (*Krameria erecta*). It has an initial white color and turning to light green, with a firm consistency; therefore, it can point out that calluses are viable for the induction of being an embryogenic callus.

Table 2. Callus and root induction in explants of *Krameria erecta* Willd.

Treatment AIB mgL^{-1}	Callus height (mm)		Callus width (mm)		Number of root	
	Leaf	Axillary bud	Leaf	Axillary bud	Leaf	Axillary bud
0	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{a}
0.5	0.58±0.00^{c}	0.45±0.00^{b}	0.76±0.01^{c}	0.56±0.01^{b}	0.50±0.57^{b}	0.00±0.00^{a}
1.0	0.65±0.01^{b}	0.46±0.00^{a}	0.86±0.02^{b}	0.58±0.00^{a}	1.25±0.95^{b}	0.00±0.00^{a}
1.5	0.67±0.00^{a}	0.47±0.00^{a}	0.96±0.03^{a}	0.59±0.00^{a}	2.50±0.57^{a}	0.00±0.00^{a}
2.0	0.67±0.00^{a}	0.47±0.00^{a}	0.93±0.03^{a}	0.59±0.00^{a}	2.00±0.81^{a}	0.00±0.00^{a}

Means with equal letters within the same column indicate that there are no significant differences (P <0.05). The data presented are the average of 10 repetitions with three samples each bottle per treatment.

The percentage in relation to the presence of callus in the explants, in the leaves presented up to 80%, while in the control it presented 0%. Callus growth occurred 30 days after incubation and its proliferation after four weeks of incubation. The percentage of calluses in axillary buds was up to 60%, except for the control that did not present. Callus induction in axillary buds occurred after 6 weeks of incubation.

The results indicate that the best callus induction response in leaves or axillary buds was when applying the concentration of 1.0 to 2.0 presented. It is important to note that the ideal explant for callus production was the leaves, according to the applied statistical analysis.

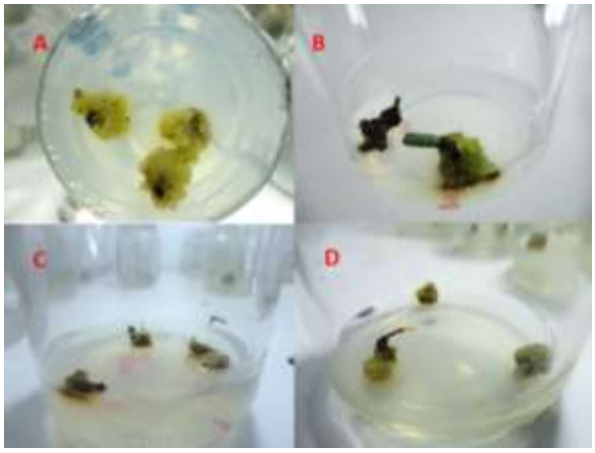

Figure 2- A: callus induction in leaves, B: callus induction in axillary bud, C: root in leaf and D: callus and root in leaf

For cell growth, auxins must induce this induction according to the genes that encode protein factors (Córdova *et al.,* 2014); in this way, the cells expand by the pressure of the water inside the vacuole, to continue growing until there is resistance from the cell wall (Azcón-Bieto y Talón 2008).

According to Smith y Atkins (2002); Arellano *et al.,* (2008), callus induction occurs as cells grow and store nutrients, and with the presence of auxins and cytosines they cause cyclin accumulation and stimulation at the transcriptional level, triggering the activation of kinases; and also promote the new cell cycle and callus formation. Callus induction is independent of the type of explant and phytohormone used (Howell *et al*., 2003; Shiram *et al*., 2008; Rodríguez *et al.,* 2014).

In this study, callogenesis production was with the use of auxins in axillary buds and leaves of *Krameria erecta,* achieved; therefore, there is a stimulation in mitosis, promoting the formation of callus tissue (Smith 2012).

Regarding the number of roots present in the leaves, significant differences were observed between the treatments, showing roots with the connotations of 0.5, 1.0, 1.5 and 2.0 mgL^{-1} of AIB, except in the treatment of zero mgL^{-1} of AIB (control), who had no root induction. While in axillary bud there were no significant differences between the treatments (0, 0.5, 1.0, 1.5 and 2.0 mgL^{-1} of AIB), since no roots were present, see table 2. The percentage of root present in the leaf was 30%, showing in leaves with presence of callus.

Until nowadays there are no works referring to this shrub or other shrubs, therefore, the discussion of the information is complicated by the scarce information that was collected; However, there are other studies of other species and different genera, such as *Oryza sativa* (Pérez *et al.,* 2009) and *Eucalyptus globulus* (Gómez *et al.,* 2006), etc. With the in vitro culture method, it is possible to obtain plants of uniform quality, according to a selected genotype (Levitus *et al.,* 2010). Phytohormones are not specialized to induce

mutations, since in high concentrations it accelerates the processes of cell division, and possibly generates genetic variations (George *et al*., 2008).

Contamination

The explants, due to their origin (wild area), bring some phytopathogens, which can cause problems at the time of planting and develop for their proliferation. The contamination occurred in all the concentrations and explants used. Environmental bacteria and fungi (biotic and abiotic) appeared, indicating this contamination from the third day of incubation. When analyzing the percentage of contamination present in the seeds of *Krameria erecta*, according to the concentration and soaking time that was handled in this investigation, there were no significant differences between the treatments evaluated, since a contamination rate of 7.25 to 8.75 % was presented.

It is important to note that the seedlings developed in vitro were free of contamination for more than 3 months; therefore, the disinfection method for *Krameria erecta* seeds was adequate. There were also no significant differences regarding the contamination present in the axillary bud and leaf explants, showing from 11.25 to 12.50% contamination, see table 3. We can point out that the disinfection treatment of the explants was effective, when using ethanol at 70% for 3 minutes, and then for 12 minutes in 15% commercial sodium hypochlorite. The death of microorganisms is due to the combination of sodium hypochlorite with the proteins of cell membranes and enzymes (Flores *et al.,* 2008).

Table 3.-Contamination and oxidation in explants of *Krameria* erecta Willd.

Treatment AIB mgL^{-1}	Contamination (%)			Oxidation (%)		
	Seeds	Leaf	Apical bud	Seeds	Leaf	Apical bud
0	8.75±1.08^{a}	11.25±2.50^{a}	11.25±2.50^{a}	0.00±0.00^{a}	28.7±2.5^{a}	26.2±7.50^{a}
0.5	8.75±1.08^{a}	12.50±2.88^{a}	12.50±2.88^{a}	0.00±0.00^{a}	28.7±4.7^{a}	30.0±7.07^{a}
1.0	7.25±1.08^{a}	11.25±2.50^{a}	11.25±2.50^{a}	0.00±0.00^{a}	28.7±2.5^{a}	26.2±7.50^{a}
1.5	8.00±1.08^{a}	11.25±2.50^{a}	12.50±2.88^{a}	0.00±0.00^{a}	30.0±4.0^{a}	26.2±7.50^{a}
2.0	7.75±1.08^{a}	11.25±2.50^{a}	12.50±2.88^{a}	0.00±0.00^{a}	28.7±2.5^{a}	26.2±7.50^{a}

Means with equal letters within the same column indicate that there are no significant differences (P <0.05). The data presented are the average of 10 repetitions with three samples each bottle per treatment.

The physiological stage of the plant tissue has a great influence on the disinfection process. Growing plant material is easier than mature plant material disinfected; its wax and lignin deposits are greater and are a barrier to the action of the disinfecting agent on endogenous microorganisms (López *et al*., 2010; Pedroza *et al.*, 2007). Among the most common contaminants *in vitro* are bacteria, these are difficult to detect and cause serious problems (Orlikowska *et al.,* 2017). Many microorganisms need time to adapt to new

conditions, prior to manifesting their presence, this generally occurs in the multiplication phase (Hernández y González, 2010).

Oxidation

Regarding the oxidation rate (darkening), in the explants in general, there were no significant differences when analyzing this variable in the different explants (axillary bud and leaf); showing in leaves 28.7 to 30.0% oxidation, while in the axillary bud there was a 26.2 to 30.0%. Regarding the seeds, they did not present oxidation, see table 3. According to the low percentage of oxidation present, it can be attributed to the mixture of antioxidants used (ascorbic acid + citric acid). In this work, were favorable results obtained.

According to Vatanpour-Azghandi *et al*., (2002); Tang y Newton, (2004); Gratão *et al.*, (2005); Pompeu *et al*., (2008, when the enzyme is in contact with the cell, damage, stress or senescence occurs, resulting in the death of the explant cells. There are some factors or substances that can trigger oxidative and nitrosative stress, such as: light intensity, cuts, herbicides, senescence, pathogens, heavy metals, injuries or abrasive substances (Mittler *et al*., 2004; Pompeu *et al*., 2008). The explants of woody species present this oxidation reaction Azofeifa (2009).

In *in vitro* tissue culture, oxidation is mainly caused by the disinfecting agent during explant asepsis, cuts, composition of the culture medium, volume and quality of the culture bottle (Tabiyeh *et al.*, 2006; Van Staden *et al*., 2006; Abdelwahd *et al.,* 2008). Explant darkening or oxidative stress has been linked to other physiological, morphological, epigenetic, and genetic disorders that cultured explants present, such as recalcitrance, hyperhydricity, somaclonal variation, and habituation (Cassells y Curry 2001; Van Staden *et al.,* 2006).

Therefore, we can point out that the oxidation in the *K. erecta* explants used in this study could be due to various factors, such as those mentioned above; as well as the time of exposure to the temperature present in the flow chamber.

CONCLUSIONS

From 79 to 83% *in vitro* germination in *Krameria erecta* seeds occurred. The concentrations of 0.5, 1.0, 1.5 and 2.0 1.5 mgL^{-1} of AIB that were used in the seedlings showed negative phototropism, except in the zero concentration. The in vitro seedlings presented heights of 10.07 to 11.65 cm. When using the concentration of 1.5 mgL^{-1} of AIB they presented calluses and leaf root. The aseptic conditions handled were ideal, since there was no contamination above 15% in the different explants used. Oxidation occurred from 26.2 to 30%, only in axillary buds and leaves.

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