Abanico Agroforestal. January-December 2020; 2:1-14. http://dx.doi.org/10.37114/abaagrof/2020.4
Original Article. **Received: 06/12/2019. Accepted: 20/04/2020. Published: 25/04/2020.**

In vitro germination and induction of callus and root in *Bursera laxiflora* S. Watson

Germinación *in vitro* e inducción de callo y raíz en *Bursera laxiflora* S. Watson

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ABSTRACT

Torote prieto (*Bursera laxiflora* S. Watson) is an endemic, forest and medicinal species of the arid and semi-arid areas of Sonora State. In the present study the germination and induction of callus and root of tight torote was evaluated from *in vitro* cultivated seedlings used as a medium of WPM growth in half of mineral salts, vitamins and indolbutyric acid (AIB) at different concentrations (0, 0.5, 1.0, 1.5 and 2.0 mgL^{-1}). When using 1.5 and 2.0 mgL^{-1} of AIB, 70% germination occurred. The contamination was 5.0 to 27.5% and the height of the seedlings was 6.67 to 10.1 cm. The calluses presented heights of 2.4 to 3.64 mm in leaf, 2.45 to 3.55 mm in stem and in apical bud of 2.49 to 3.42 mm. The width of the calluses in leaf was of 2.20 to 2.78 mm, apical bud was 2.13 to 2.22 mm and stem 2.11-to 2.28 mm. Root induction occurred in concentrations of 0.5 to 2.0 mgL^{-1} of AIB in stem and leaf explants except in apical bud. The application of AIB stimulates the induction of callus and root in Torote prieto.

Keywords: *Bursera laxiflora*, micropropagation, explants, calluses and root.

RESUMEN

El Torote prieto (*Bursera laxiflora* S. Watson) es una especie endémica, forestal y medicinal de las zonas áridas y semiáridas del estado de Sonora. En el presente estudio se evaluó la germinación e inducción de callo y raíz de torote prieto a partir de plántulas cultivadas *in vitro*. Se utilizó como medio de crecimiento WPM al 50% de sales minerales, vitaminas y ácido indolbutírico (AIB) a diferentes concentraciones (0, 0.5, 1.0, 1.5 y 2.0 mgL^{-1}). Al utilizar 1.5 y 2.0 mgL^{-1} de AIB se presentó un 70% de germinación. La contaminación fue de 5.0 a 27.5% y la altura de las plántulas fue de 6.67 a 10.1 cm. Los callos presentaron alturas de 2.4 a 3.64 mm en hoja, 2.45 a 3.55 mm en tallo y en yema apical de 2.49 a 3.42 mm. El ancho de los callos en hoja fue de 2.20 a 2.78 mm, yema apical 2.13 a 2.22 mm y tallo 2.11 a 2.28 mm. La inducción de raíz se presentó en las concentraciones de 0.5 a 2.0 mgL^{-1} de AIB en los explantes de tallo y hoja excepto en yema apical. La aplicación de AIB estimula la inducción de callo y raíz en torote prieto.

Palabras clave: *Bursera laxiflora*, micropropagación, explantes, callos y raíz.

INTRODUCTION

In vitro cryopreservation and regeneration of plants is used to conserve and micropropagate specific plant material, in order to carry out *ex situ* conservation and allow the development of clonal or micropropagation forestry Martínez *et al.,* 2003). These techniques offer a series of advantages such as the possibility of producing a high number of homogeneous plants and of a very high phytosanitary quality, in a shorter period and in reduced spaces (Sharry *et al.,* 2015).

In vitro culture constitutes a propagation route with satisfactory results in the multiplication coefficients and due to the possibilities of forest plantation success. The main advances in *in vitro* tissue culture have allowed the multiplication of interest species, through organogenesis and somatic embryogenesis (Daquinta *et al.,* 2000; Barbón *et al*., 2011). Timber and non-timber plants represent a forest genetic resource of socio-economic, agroforestry and scientific (medicinal) importance. Forest genetic resources are essential to maintain the different ecosystems present; however, they are subject to the gradual pressures of climate change and unsustainable use (Yanchuk, 2002).

The genus *Bursera Jacquin* ex L. (Burseraceae) is in different regions of Mexico diversified and has 82 taxa registered (Rzedowski *et al.,* 2005). The torote prieto has medicinal properties, coupled with the benefits that this species brings to natural ecosystems, which are exploited continuously, implying the low population and regeneration of this species in its natural habitat. However, there are few works related to the *in vitro* propagation of forest species in northwestern Mexico, which constitutes a problem for the conservation of the forest genetic resource.

Therefore, it is feasible to create viable mechanisms to offset the propagation problems of species of forest interest, using biotechnology in plant tissues that allow cloning species without changing the natural environment of the species' habitat; obtaining plants free of pathogens and in less time (Rebolledo-Camacho *et al.,* 2006; Delgado *et al.*, 2008).
In recent years, the use of biotechnology for the *in vitro* propagation of forest species has gradually increased; however, there are no studies related to obtaining *in vitro* plants of torote prieto from sterile seedlings.

The objective of this work was to evaluate the germination and induction of callus and root of torote prieto from *in vitro* cultivated seedlings, used as a growth medium Woody *Plant Medium* (WPM/50) at half the concentration of mineral salts, vitamins and indolebutyric acid at different concentrations.

MATERIAL AND METHODS

This research was carried out in the tissue culture laboratory of the Department of Scientific and Technological Research of the University of Sonora (DICTUS).

Collection site

The plant material was collected at Rancho Bella Vista, which is located at 29° 10´02.83 "North Latitude and 110° 58´47.48" West Longitude, at 277 meters above sea level. It has an average annual rainfall of 330 mm and average temperature of 24 °C (SAGARPA, 2010) Mature seeds were collected from vigorous plants of torote prieto (*Bursera Laxiflora*), in the month of September 2019.

Preparation of culture medium

Woody Plant Medium (WPM/50) (Trigiano and Gray, 2011) was used as the culture medium. This medium was used at half its concentration of salts, consisting of sucrose, agar and vitamins, such as thiamine and myo-inositol. The phytohormone used was indole butyric acid at different concentrations, (0, 0.5, 1.0, 1.5 and 2.0 mgL^{-1}), the pH was adjusted to 5.7 with NaOH, at a concentration of 0.1 N. The laminar flow chamber was sterilized with 99% ethyl alcohol, 25 ml of WPM medium was used per glass bottle, Gerber type. Finally, sterilization was carried out in the sterilmatic model autoclave, at a temperature of 120 °C and with a pressure of $15/cm^2$ for 15 minutes.

Seed germination

The seeds were disinfected with ethyl alcohol (70%) for 3 minutes, and sodium hypochlorite (NaClO) (CLOROX® 15% active chlorine) for 12 minutes, and adding a drop of Tween 20. Applying three rinses with deionized water, and subsequently placing them in a mixture of citric and ascorbic acid for 5 minutes, then sow them in the *WPM culture medium* (Trigiano and Gray, 2011).

Obtaining explants

After germination, seedlings were obtained that presented all the morphological characteristics of the wild plant. These plants were subcultured using explants (apical bud, stem and leaves), using 50% of the *WPM medium*, added with AIB. For the seeding of the explants obtained from the seedlings, they were passed to the laminar flow chamber (Edge Gard Hood brand). Petri dishes, scalpel and tweezers previously sterilized were used. The cuts and obtaining of leaf segment, apical buds and stem (explants) did not need to be disinfected, being an aseptic material.

Crop condition

The culture was kept in the growth room under controlled conditions, at a temperature of 25 °C, with a photo period of 16 hours of light; presenting a luminous intensity of 30 $\mu mol.m^2.s^{-1}$ and 8 hours of darkness, with a temperature of 25±2 °C.

Variables to evaluate

The evaluation was by direct observation, starting on the third day after planting the explants, to subsequently carry out the evaluations every seventh day. The evaluation was until 30 days after sowing. The variables that were evaluated were germination percentage (%), seedling height (cm), contamination percentage (%), callus height (%), callus width (cm) and number of roots. According to ISTA (2019), the germination percentage was carried out. For the measurements of calluses present in the explants, a Mitutoyo Absolute model CD-6CSX Serial Nº. 06401649 6 was used.

Statistical analysis

A completely randomized design and a 5x3 factorial arrangement with 10 replications were in this research work used. An analysis of variance (ANDEVA) was carried out, with a significance level of $P < 0.05$, and a comparison of means by Tukey. For data analysis, the JMP statistical package version 9.0.1 (Statistical Analysis System, SAS Institute Inc., 2011) was used.

RESULTS AND DISCUSSION

In vitro germination of the seeds of torote prieto (*Bursera laxiflora*) began on the ninth day and concluded its evaluation 21 days after sowing. There were no significant differences ($P \geq 0.05$) when using the concentrations of 2, 1.5, 1.0 and 0.5 mgL^{-1} of AIB, showing 45 to 70% germination; except the control (without treatment), with 30% germination in the culture medium used (WPM/50) at different concentrations of AIB (0, 0.5, 1.0,1.5 and 2.0 mgL^{-1}). There were no significant differences ($P \geq 0.05$) in the percentage of non-germinated seeds, showing 22.5 to 42.5%.

It is important to note that in this study, there were no abnormal seedlings; therefore, there are no significant differences, see Figure 1 and Table 1 Kameswara *et al.,* (2007). It emphasizes that germination determines the growth of the embryo, the protrusion of the radicle and testa.

Regarding this study, the seedlings were considered to have normal growth since they presented roots, shoots, and organs important for their development. When presenting these organs, the seedlings show deficiencies that will affect the quality of the seedlings (AOSA, 2005).

Figure 1. *In vitro* germination of *Bursera laxiflora* S. Watson

The germination percentage obtained in this study for the seeds of torote prieto is very favorable, because this tree species is very important for the state of SonoraM. However, the results of the germination percentage obtained in this study do not coincide with those obtained by (Pinta *et al.,* 2017), who used the MS culture medium (Murashige y Skoog (1962); these researchers reported 11.11% germination *Bursera graveolens* (KUNTH), with the treatment of mechanical scarification and 0.5 mg/L of gibberellic acid (AG^3).

Similar results have been reported by (Bonfil-Sanders *et al.,* 2008), who obtained from 30 to 60% germination in situ in seeds of *Bursera bicolor*, *Bursera copallifera* and *Bursera glabrifolia*, stored for six months in refrigeration at 5 °C. Therefore, the results show that the propagation of some *Bruceras* species, including *Burcera laxiflora*, present difficulties for their germination, as they do not reach 80% germination values.

Recently, Mero *et al.,* (2017), evaluated the effect of auxinic growth regulators at different concentrations, for the regeneration of plant tissue in *Bursera graveolens* cuttings, obtaining shoot and callus formation, except in roots, when using 800 ppm AIB 60 days after planting. Unfortunately, there is little information in the literature regarding the germination of *Burseras*; the reports show results with low germination percentage (Andrés-Hernández y Espinosa-Organista, 2002). Similarly (Ray y Brown, 1995; Ortiz-Pulido y Rico-Gray, 2006), reported percentages of germination under natural conditions, lower than those obtained at the laboratory level.

Due to the previously described on the difficulty that this woody presents for its propagation naturally and/or artificially, *in vitro* tissue culture techniques allow new techniques for the propagation of slow growing cultures and the cryopreservation of tissues (Engelmann, 2000; Dixit *et al.,* 2004; Wang *et al.,* 2005). The low germination percentage of the seeds of this species is because they are not in a position to germinate quickly, after being the seeds collected; need a rest period, of a transitory nature, of approximately six months (Morillo *et al.,* 2017).

Table 1. *In vitro* germination, contamination and seedling height of seeds of torote prieto (*Bursera laxiflora* S. Watson)

Treatment AIB mgL^{-1}	Sprouted seeds (%)	No seeds Sprouts (%)	Contamination (%)	Height of seedlings (cm)
0	30.0±24.49[b]	42.5±17.07[a]	27.5±22.17[a]	6.67±4.45[a]
0.5	45.0±12.90[a]	32.5±22.17[a]	22.5±12.58[a]	9.05±0.61[a]
1.0	67.5±9.57[a]	22.5±9.57[a]	10.0±0.00[a]	10.01±0.14[a]
1.5	70.0±8.16[a]	22.5±5.00[a]	7.5±5.00[a]	10.01±0.25[a]
2.0	70.0±8.16[a]	25.0±5.77[a]	5.0±5.77[a]	10.21±0.24[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.

Seedling height

There were no significant differences (P≥0.05) in the seedling height variable, with respect to the concentrations that were in this work managed, showing heights of 6.67 to 10.21 cm respectively. The seedlings of torote prieto (*Bursera laxiflora*) showed good growth and adequate development. No published research evaluating the *in vitro* micropropagation of *Burseras* was found, see Table 1 and Figure 2.

Contamination

The contamination of the seeds was from 5 to 27.5%, caused mainly by environmental fungi and bacteria. They did not present significant differences (P≥0.05) in this variable when using the different concentrations of WPM/50, see table 1. The contamination of the seeds of this investigation are lower than those obtained by Pinta *et al.,* (2017),, who reported up to 93% *in vitro* contamination in *Bursera graveolens* explants (Kunth).

In vitro cultures in general have two fundamental characteristics: asepsis (absence of fungal and bacterial contaminating microorganisms), and control of factors that disturb growth, such as the environmental conditions of culture. Therefore, a correct detection of these sources is required and the type of microorganism must be identified. These are important aspects for the success of the crops, in order to prevent the primary contamination in the *in vitro* cultures that come from the donor plant (George *et al.,* 2008; Levitus *et al.,* 2010; Sharry *et al.,* 2015).

Figure 2. Obtaining explants, stems with callus and sprouts

To minimize the problem of contamination, disinfectants and/or mixtures of fungicides and bactericides have been to explants applied (Das *et al.,* 2010; Jayakrishna *et al.*, 2011). Similarly, Pérez-Alonso *et al.,* (2015), mention the importance of collecting plant material according to the time of year, influence of temperatures, rainfall and high rates of contamination, which would be vital for the reduction of pollution.

Callus and root in *Bursera laxiflora*

The percentage of callus present in the explants according to the treatments used with AIB, showed significant differences with respect to the zero treatment (control), without mgL^{-1} of AIB. The highest average callus formation was presented when using the 1.5 mgL^{-1} AIB treatment, in the three explants (leaf, apical bud and stem), obtaining 80% of calluses, followed by this, it is treatment 2.0 mgL^{-1} of AIB, with 78% callus, 1.0 mgL^{-1} of AIB, with 77%; and finally the 0.5 mgL^{-1} of AIB, with 7.2%. The use of AIB positively stimulates callus growth after 30 days.

Table 2 shows the induction of callus present in leaves, apical bud and stem at different concentrations of indolebutyric acid (AIB). Significant differences (P≤0.05) were with respect to the height and width of the callus presented. At the concentration of 2.0 mgL^{-1} of AIB, higher height values were presented in 3.64 mm leaf and 3.55 mm stem.
However, there were no significant differences (P≥0.05) in the values of apical bud at the concentrations of 1.5 and 2.0 mgL^{-1} of AIB with 3.42 and 2.72 mm, respectively. A similar behavior at callus height was observed in callus width in explants. At the 2.0 mgL^{-1} concentration of AIB, greater widths of leaf (2.78 mm) and stem (2.68 mm) were presented. In the apical bud, there were no significant differences in the concentrations of 0.5 and 2.0 mgL^{-1} of AIB with 2.21 mm and 2.22 mm, respectively.

Table 2 Callus induction in explants of torote prieto (*Bursera laxiflora* S. Watson)

Treatment AIB mgL^{-1}	Height of callus (mm)			Width of callus (mm)		
	Leaf	Stem	Apical bud	Leaf	Stem	Apical bud
0	0.00±0.00^{d}	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}
0.5	2.40±0.08^{c}	2.45±0.16^{c}	2.49±0.19^{b}	2.20±0.08^{c}	2.11±0.60^{b}	2.13±0.03^{b}
1.0	2.69±0.14^{bc}	2.62±0.12^{c}	2.73±0.26^{b}	2.36±0.08b^{c}	2.13±0.04^{b}	2.13±0.04^{b}
1.5	3.09±0.23^{b}	3.06±0.17^{b}	3.42±0.49^{a}	2.53±0.08^{ab}	2.27±0.03^{a}	2.21±0.01^{a}
2.0	3.64±0.31^{a}	3.55±0.21^{a}	2.72±0.19^{a}	2.78±0.24^{a}	2.28±0.02^{a}	2.22±0.01^{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.

It is important to note that at low concentrations of AIB (0.5 and 1.0 mgL^{-1}), in the apical bud, calluses with lower heights and width were induced. Stem also presented significant differences (P≥0.05), with respect to the concentrations evaluated; the best being the dose of 2.0 and 1.0 mgL^{-1}, in both measurements (height and callus width), see table 2 and figure 3. Regarding the control (without AIB), there was no callus height and width. This indicates that *Bursera laxiflora* explants (leaf, apical bud and stem) require a phytohormone that stimulates the induction of callogenesis. The calluses obtained regardless of the concentration and explant in this study, presented a firm consistency and a creamy white color, turning to a light green.

According to the presence of the root number in the explants (leaf, apical bud and stem) of *Bursera laxiflora*, there were significant differences (P≤0.05) in the stem, at a concentration of 2.0 mgL^{-1} of AIB (2.7 cm). At the concentration of 0.5 mgL^{-1} of AIB, the lowest values of stem and leaf were presented (1.00 and 0.25 cm), respectively. Regarding the apical bud, roots did not appear in lower concentrations (0.5 and 1.0 mgL^{-1} of AIB). In explants without AIB (control), there was no root growth in the different stem, leaf and apical bud explants, see Table 3 and Figure 3.

Table 3. Root induction in explants of torote prieto (*Bursera laxiflora* S. Watson)

Treatment mgL^{-1} AIB	Explant type (cm)		
	Leaf	Stem	Apical bud
0	0.00±0.00^{c}	0.00±0.00^{a}	0.00±0.00^{a}
0.5	1.00±0.81b^{c}	0.25±0.50^{a}	0.00±0.00^{a}
1.0	1.75±0.50^{ab}	0.50±0.57^{a}	0.00±0.00^{a}
1.5	1.25±0.57^{a}	0.50±0.57^{a}	0.25±0.50^{a}
2.0	2.7±0.50^{a}	0.50±0.57^{a}	0.25±0.50^{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 explants evaluated in this experiment did not present direct embryogenesis; however, they produced callus tissue without the embryogenic tissue, gradually developing more proembryogenic mass over the course of the incubation time. These results are similar to

those obtained by Kryvenki *et al.,* (2008), who obtained callus in all treatments, using Murashige and Skoog (MS) semi-solid in *Stevia rebaudiana* Bert as culture medium, except in the control (without culture).

Figure 3. Callus and root on stem

In relation to the percentage of calluses present in the explants used in this work (apical bud, stem and leaf), they were higher than those obtained by Rodríguez *et al.,* (2014),, they used Murashige and Skoog (MS) as culture medium, supplemented with 0.5 mg L^{-1} of naphthalenacetic acid (ANA), in hypocotyl explants (62, 62, 74 and 64%).

According to Smith (2012), the type of callus present is an important indicator of the morphogenic path to follow, regardless of the callus color; these indicate that organogenic calluses come from nodular calluses that are green in color or have an oxidized appearance, such as phenolics (Bandyopadhyay *et al.,*1999; Ainsley *et al.*, 2000). Larson *et al.,* (2006)), have reported the change in appearance of calluses as they are cultivated over time. In this regard, Shiram *et al.,* (2008) point out those high concentrations of auxin or cytokinins stimulate callus production and that its appearance is related to the type of hormone used during its induction.

Several investigations have reported that auxins/cytokinin favor the induction of calluses in *Pinus strobus* L., as well as in hybrids of *Eucalyptus granáis* and *Eucalyptus urophylla* or only auxins originating caulogenic calluses (Tang y Newton, 2005; Hajari *et al.,* 2006). In other cases, such as in *Eucaliptus nitens*, *E. globulus*, and *E. camaldulensis*, induction by auxins has generated embryogenic calluses Bandyopadhyay *et al.,* 1999; Gopalakrishnan *et al.*, 2010).

CONCLUSIONS

In vitro germination of the seed of torote prieto (*Bursera laxiflora* S. Watson), can be promoted with the application of indolburtyric acid (AIB), in concentrations from 1.5 or 2.0 mgL^{-1}; using WPM/50 culture medium. The *in vitro* germinated plants produced shoots and roots, presenting morphological characteristics of the wild plant. The use of indole burtyric acid at concentrations of 1.0, 1.5 and 2.0 mgL^{-1} promote the formation of reliable callogenesis with a white color, turning to light green and organogenesis in explants of

torote prieto (*Bursera laxiflora* S. Watson). These results indicate that it is possible to stimulate the development of calluses and roots for the conservation of the torote prieto (*Bursera laxiflora* S. Watson), as it is an endemic species in Sonora state.

ACKNOWLEDGEMENT

To Yeimi C. Mc Caughey Espinoza, for her great support in the collection of seeds, for carrying out this research work.

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