Production of callus and roots from lateral meristems of *Loeselia mexicana*

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Abstract

Background: *Loeselia mexicana*, known as Mexican false calico, or *espinosilla* in Spanish, is a widely distributed secondary forest plant with a significant number of medicinal and cosmetic uses. This species is threatened by the lack of regulation over collection methods and changes in land use. *In vitro* culture could be used to preserve the species by shoot induction, callus production and cell-suspension to obtain secondary metabolites; this would reduce the need to affect wild populations.
Hypothesis: A combination of cytokinins and auxins can induce structural development in the plant, promoting the formation of shoots, roots or callus *in vitro*. By applying this combination to *L. mexicana* stem segments, we expected to observe new shoots or callus.
Study site and dates: "El Teuhtli" volcano, Xochimilco; from June 2015 to February 2016.
Methods: Distal stems cuttings were used as explants. They were disinfected with 1 % soap, 0.6 sodium hypochlorite and 70 % ethanol, and rinsed with distilled water. Two different times of disinfection with ethanol were tested. The distal stem segments were then planted in solid MS medium with 3, 5 or 7 mg L^{-1} KIN combined with 3 mg L^{-1} NAA, and 2 % AC.
Results: A favorable response was observed in the treatment with 5 mg L^{-1} KIN and 3 mg L^{-1} NAA, which produced green callus with root in five weeks. Furthermore, a lower explant mortality rate was achieved, 46.66 % in three weeks, with a shorter disinfection time.
Conclusions: Disinfection time is important for this species, and callus production is possible.
Keywords: callus, espinosilla, medicinal, meristems, organogenesis.

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Lizeth Aguirre-Alberto collected material, performed the treatments, and wrote the article.
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Resumen

Antecedentes: *Loeselia mexicana*, conocida como espinosilla, es una planta de vegetación secundaria con diversos usos medicinales y cosméticos. Esta especie podría estar amenazada debido a la falta de regulación en la colecta y por cambios en el uso del suelo. El cultivo *in vitro* es una alternativa para la preservación de la especie por inducción de brote, producción de callo, y células en suspensión para la obtención de metabolitos secundarios; esto reduce la necesidad de intervenir en las poblaciones silvestres.
Hipótesis: La combinación de citocininas y auxinas puede inducir el desarrollo de las estructuras en la planta, promoviendo la formación *in vitro* de brotes, raíz o callo. Al aplicar esta combinación a segmentos de tallo de *L. mexicana*, se esperaba observar nuevos brotes o callo.
Sitio de estudio y fechas: Volcán "El Teuhtli", Xochimilco, de junio de 2015 a febrero de 2016.
Métodos: Se cortó la parte distal del tallo como explante. Se desinfectó con jabón al 1 %, cloro al 10 %, etanol a 70 % y se enjuagó con agua destilada. Se probaron dos tiempos con el etanol. Se sembró en MS sólido con 3, 5, o 7 mgL^{-1} de KIN combinado con 3 mgL^{-1} de ANA, y 2 % de CA.
Resultados: En la combinación con 5 mgL^{-1} KIN y 3 mgL^{-1} ANA se observó una respuesta favorable al producir callo verde con raíz en cinco semanas. Además, con un menor tiempo de desinfección se logró una menor tasa de mortalidad de los esquejes, el 46.66 % en tres semanas.
Conclusiones: El tiempo de desinfección es importante con esta especie, y es posible la producción de callo.
Palabras clave: callo; espinosilla; medicinal; meristemos; organogénesis.

Loeselia mexicana (Polemoniaceae) is a monocotyledonous, perennial, bushy angiosperm, with ramified and woody stems (CONABIO 2009), not entirely lignified, according to Rzedowski & Calderón (1995), with red to rosy 1 cm long flowers. It grows in generally undisturbed pine-oak areas. It is widely distributed from the south of the United States to western and southwestern Mexico (Arizaga 2007), and it is considered native to Mexico. It is locally known as "espinosilla" (prickly), and it has several medicinal uses in the treatment of "scares" (*sustos*) and anxiety (Navarro-García *et al.* 2007, Herrera-Ruiz *et al.* 2011, Guzmán-Gutiérrez *et al.* 2014), diarrhea (Rojas *et al.* 1999, Salud-Pérez *et al.* 2005, Palombo 2006), postpartum fever, some respiratory ailments (CONABIO 2009), skin diseases (BDMTM 2009), and kidney inflammation (FAO 2002); it is also used as an antifungal agent (Navarro-García *et al.* 2011). This plant also has cosmetic uses in hair and skin care, for which the aerial part is ground or consumed as an infusion. Thus, this species has great industrial and commercial potential (SEDEREC 2012) and has been classified as a non-wood forest product (FAO 2002).

Mexico loses more than two hectares of wild vegetation per minute (Reyes-Santiago *et al.* 2011), while 99 % of medicinal plants are of wild origin (Muñetón-Pérez 2009). The irrational exploitation of vegetation and the destruction of natural habitats threaten many wild species and the resources associated with them (RECNAT 1997). In the case of *L. mexicana*, if collection methods and changes in land use are not regulated, it could soon become an endangered species.

An alternative method of plant propagation is tissue culture from sprouts, or callus and root induction from explants such as lateral meristems of shoots, leaves and flowers, among others (Cardoza 2008). Auxins and cytokinins are the most widely used plant growth regulators (PGR); in combination, they promote organogenesis and can induce the development of lateral shoots, callus or roots. If callus is produced, secondary organogenesis can then occur, or the callus can be used to produce secondary metabolites. Plant micropropagation is another alternative for the preservation of wild species of medicinal and commercial importance (Rout *et al.* 2000).

Since there are no known studies on the culture of *L. mexicana*, this study focused on the development of a protocol for the *in vitro* production of callus from lateral buds of *L. mexicana*, and on the determination of adequate disinfection times, and the concentration and ratio of kinetin (KIN) and naphthaleneacetic acid (NAA) that can induce the growth of shoots or callus in culture.

Materials and methods

Study area. Northwestern slope of the "El Teuhtli" volcano, located at 19° 13' 44" N, 99° 2' 55" W, between the municipalities of Tláhuac, Xochimilco and Milpa Alta in Mexico City.

Biological material. Distal and green parts of the stems of *Loeselia mexicana* were collected every two months between June 2015 and February 2016. They were wrapped in Kraft-type paper and plastic bags and refrigerated at 7 °C for a maximum of two days after collection.

Culture media. Solid MS (Murashige & Skoog 1962) was supplemented with 30 g L^{-1} (3 %) sucrose and 0.4 % Phytagel, and in treatments 4 and 5 20 gL^{-1} Activated Carbon (AC). In all the treatments, KIN was added in concentrations of 3, 5, or 7 mg L^{-1}, as well as 3 mg L^{-1} of NAA; five repetitions per treatment were performed. The pH was adjusted to 5.8 and sterilization was performed in an autoclave at 120 atm for 20 min. Five assays or treatments were performed.

Disinfection of biological material. After removing the leaves, the stems were cut in 5 cm segments and disinfected in a sterile area using a) 0.1 % Soap for 10 min, b) 0.6 % Sodium hypochlorite for 15 or 10 min, and c) 70 % Ethanol for 10 or 5 minutes. The stem segments were rinsed with distilled water for 2 min before the application of each disinfectant. The washing time was adjusted based on the results obtained (Figure 1).

Planting and incubation. Three 1 cm cuttings were planted in each jar and incubated in a growth chamber at 25 °C with a photoperiod of 16 h of light and 35 µmol m^{-2} s^{-1} of light intensity. The

STAGES

DONOR PLANT

EXPLANTS: lateral meristems (1 cm long)

DISINFECTION

EXP 1 (min)	EXP 2	EXP 3	EXP 4	EXP 5
Soap 1% 10 Chlorine 10% 10 Ethanol 70% 10 Destilled water 10	Soap 10 Chlorine 15 Ethanol 10 Water 2	Soap 10 Chlorine 15 Ethanol 10 Water 2	Soap 10 Chlorine 15 Ethanol 10 Water 2 AC 2%	Soap 10 Chlorine 10 Ethanol 5 Water 2 AC 2%

CALLUS INDUCTION

MS Medium

1 (mg L^{-1})	2	3
KIN 3 NAA 3	KIN 5 NAA 3	KIN 7 NAA 3

INCUBATION

25 ºC
16 hrs light
8 hrs dark
Intensity light 35
$\mu mol\ m^{-2}\ s^{-1}$

Response
(week 5)

ROOT 6,66%	GREEN CALLUS WITH ROOT 6.66%	Brown callus 13.33%

SUBCULTURE
(week 11)

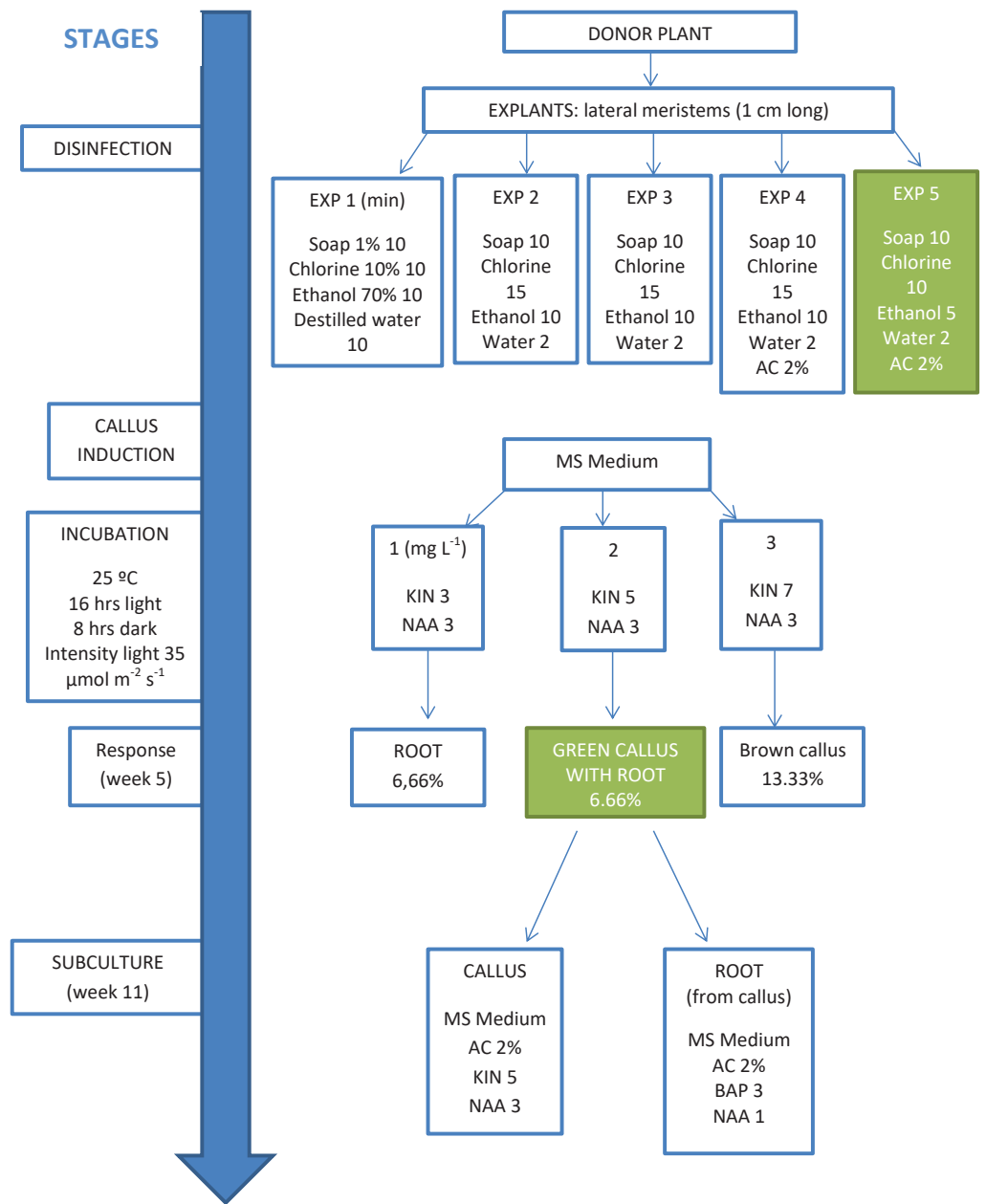| CALLUS MS Medium AC 2% KIN 5 NAA 3	ROOT (from callus) MS Medium AC 2% BAP 3 NAA 1

Figure 1. Protocol for callus induction using lateral meristems from shoots of *Loeselia mexicana* (Green: favorable response).

number of initial buds per cutting, the total mortality rate (% of dead explants and % of contaminated explants) and the survival rate were recorded at the fifth week of incubation, together with the presence of callus and roots.

Replanting. The explants that showed a response were replanted on week 11 in the same type of medium they were incubated in. After one month, the obtained callus was replanted with 3 or 7 mg L^{-1} KIN combined with 3 mg L^{-1} NAA. Before replanting, the callus was segmented into units of approximately 0.5 cm in length. The roots produced from callus were cultured on solid MS medium with 2 % AC and 3 mg L^{-1} BAP, combined with 1 mg L^{-1} NAA to increase root production.

Statistical analysis. To determine the adequate disinfection time, the total mortality rate was compared with the survival rate using a chi-square test ($P < 0.05$). A Z-test was used to find differences between the mortality rate values. The values were adjusted to avoid errors.

Results

In the study site, between June and August, most of the species population was found in a vegetative state; flowering was observed from September to October, and capsules with seeds were found from November to February.

After one week, some explants turned yellowish and died, while others were contaminated. Half of the treatments had a mortality rate of 100 % at week 3, which was associated with disinfection times. This association is shown in Table 1. In treatments 1 to 3, without AC, the mortality rate was 100 %. In treatment 3, the mortality rate was 55.55 % at week 10, but it later reached 75.75 % (not shown in Table 1). In treatments 4 and 5 (the latter used a shorter disinfection time), with AC, the survival rate was 26.66 % and 53.34 % respectively, while the contamination rate was 35.56 % and 23.33 %.

The results of the chi-square test indicated significant differences between the treatments shown in Table 1 ($\chi^2 = 127.54$; $P = 1.30 \times 10^{-26}$); treatments 3 and 5 are statistically the same ($Z = -1.26$), even though AC was used in treatment 5. When the type and time of disinfection required to obtain aseptic cultures with acceptable survival rates were determined, all following inductions were carried using those parameters. Table 2 shows the response obtained at week 5. In treatment 3, which used a combination of 3 mg L^{-1} KIN and 3 mg L^{-1} NAA, a brown protuberance and white villosity were observed in one cutting (Figure 2a); this represents 6.6 % of the total cuttings planted. At week 14 (Figure 3a), this cutting developed a considerable amount of root tissue as well as a small amount of brown callus without shoots. At week 20, this cutting was replanted using another concentration of PGR and it didn't show any further response.

In the treatment with 5 mg KIN and 3 mg L^{-1} NAA, green-colored protuberances were observed in one cutting at week five (Figure 2b); represents 6.6 % of the total number of cuttings planted using this combination of growth regulators. Three weeks after replanting, the presence of thin roots was observed, as well as more green-colored callus. Roots grew up to 4 cm, approximately (Figure 4). The first roots that appeared in the callus with this treatment —which was replanted in MS medium with BAP 3 mg L^{-1} and NAA 1 mg L^{-1}— produced more roots (Figure 5); however, no shoots were produced.

In the treatment with 7 mg L^{-1} KIN and 3 mg L^{-1} ANA (Figure 2c), brown callus was observed on two cuttings, also at week 5; this represents 13.3 % of the total number of cuttings planted using this combination of growth regulators. At week 11, one of the calluses developed a root of approximately 0.5 cm; the other callus showed no response. The responsive callus was replanted twice in MS medium with another concentration of PGR, 5 mg L^{-1} KIN and 3 mg L^{-1} NAA, to induce another type of response; it developed green calluses five months after replanting. The callus obtained with 5 mg L^{-1} KIN and 3 mg L^{-1} NAA was divided into two 0.5 cm units and

Table 1. Survival Rate (SR) and Mortality Rate (MR) of *L. mexicana* during three weeks of incubation and for different washing times per solution.

EXP	DISINFECTANT (min)				CONTAMINATION (%)	DEATH (%)	TOTAL MR	SR
	Soap	Chlorine	Ethanol	Distilled water				
1	10	15	10	10	13.33	86.67	100	0
2	10	15	10	2	60	40	100	0
3	10	15	10	2	13.33	42.22	55.55	44.45
4	10	15	10	2	35.56	37.78	73.34	26.66
5	10	10	5	2	23.33	23.33	46.66	53.34

EXP = treatment

Table 2. Total nodes and planted explants per treatment, and response per combination, week 5.

EXP	NO. OF EXPLANTS	NO. OF NODES	CALLUS			RESPONSE (%)		
			K:A 1	K:A 2	K:A 3	K:A 1	K:A 2	K:A 3
1	45	83	0	0	0	0	0	0
2	45	99	0	0	0	0	0	0
3	45	106	1	1	2	6.66	6.66	13.33
4	45	169	0	0	0	0	0	0
5	30	156	0	0	0	0	0	0

EXP = treatment; K:A1 = 3 mgL^{-1} KIN : 3 mgL^{-1} NAA; K:A2 = 5 mgL^{-1} : 3 mgL^{-1}; K:A3 = 7 mgL^{-1} : 3 mgL^{-1}.

replanted in the same medium. After a month, both had increased in volume, and it was possible to divide them into eight parts, which were subcultured. All the calluses obtained in these treatments were subcultured in MS with 5 mgL^{-1} KIN and 3 mgL^{-1} ANA and continued to increase in volume and developed roots (27 units), although some were oxidized on the surface.

Discussion

The washing time was decisive in achieving greater explant survival rate since it allowed to obtain aseptic *in vitro* cultures. In the present study, it was observed that reducing the time of exposition to the proposed disinfectants reduced the mortality rate while also controlling contamination (Table 1, treatments 3 and 5). Gamborg & Phillips (1995) recommend reducing the washing time or the concentration of chlorine and ethanol because of their reactivity. Iliev *et al.* (2010) recommend removing the stem's epidermis for better disinfection. Using a short disinfection time yielded similar results to those obtained *in vitro* with another bushy species (Wang *et al*. 2016), *Cerasus humilis*; however, they washed the explants with water first, and then with 75 % ethanol for 1 min and with 1 % sodium hypochlorite for 4 min.
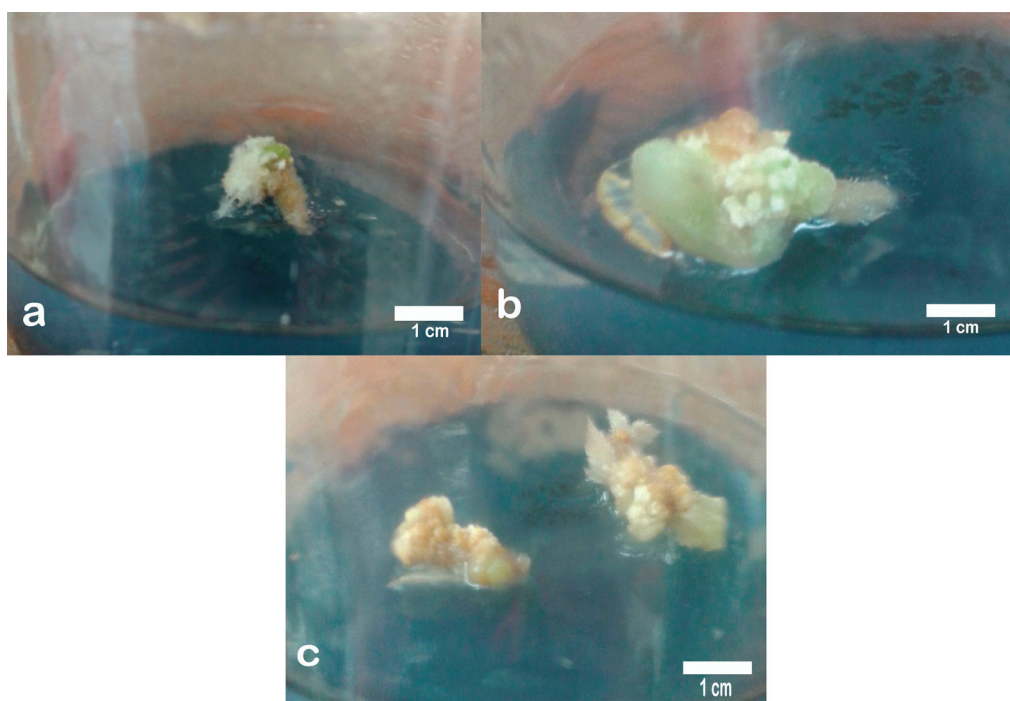

Figure 2. Callus of *L. mexicana* after five weeks in culture on solid MS medium with KIN:NAA. Combinations: a) 3:3, b) 5:3, c) 7:3 mg L^{-1}.
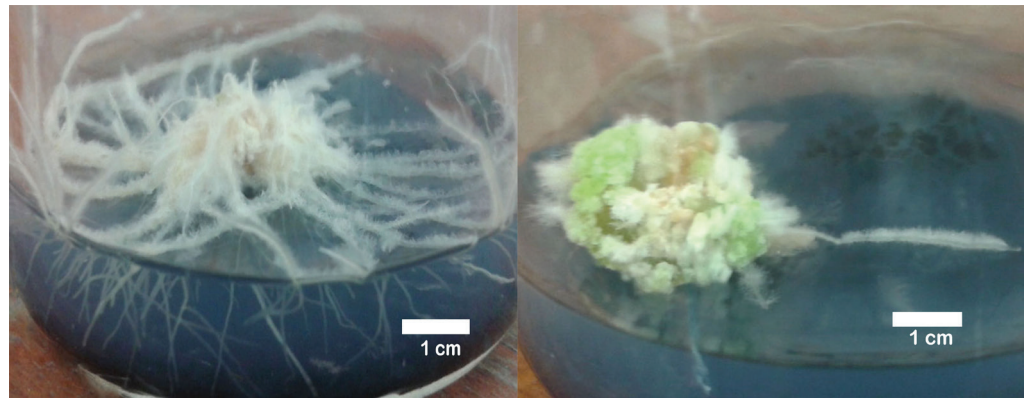
Figure 3. Callus of *L. mexicana* four weeks after subculture (week 15 after cutting) on solid MS medium with KIN:NAA: a) 3:3, b) 5:3, c) 7:3 mg L^{-1}, and AC 2 %.

Belukurava & Kuchuk (2014) report that young explants with woody or almost lignified stems secrete oxidized polyphenols when they are cut, which cause a delay in growth and, eventually, death. This effect is greater in lignified plants, and thus it is better to use explants with only primary growth, since they generally show a better response to *de novo* organogenesis (Olmos *et al*. 2010). This effect is the likely cause of the high mortality rate observed here in the first treatments with *L. mexicana*. In the treatments without AC, the explants suffered oxidation. For this reason, it was decided to use AC, mainly to avoid stem oxidation, and for its ability to remove inhibitory and toxic substances from the culture medium, even those released by the explant during incubation (Azofeifa 2009). In addition to serving as phenol adsorbent, AC contributed, together with a shorter disinfection time, to prolong the life of the planted cuttings (Table 1, Treatment 5).

Other recommended alternatives to control or prevent death by oxidation in herbaceous plants involve using an antioxidant solution, such as ascorbic acid combined with citric acid, in the culture medium (Çördük & Aki 2011); reducing the concentration of sucrose in the culture medium (Dong *et al*. 2016); or reducing to a minimum the disturbance of explants during the cutting process (Iliev *et al*. 2010). We recommend exploring the use of an anti-oxidant solution with and without AC, since the treatments performed in the present study did not show a significative difference in the mortality rate between treatments 3 and 5. We also recommend checking the oxidation percentage of explants, or to minimize the use of substances reducing the concentration of sucrose in the MS medium.

We determined that the brown and green protuberances that appeared in axillary stem buds were callus (Figures 2, 3) because they appeared in the nodal and internodal segments. Callus developed roots without the need to apply an additional PGR, which means that the number of substances and stages needed to form roots and achieve indirect organogenesis is not very large. Although the callus can be subjected to a redifferentiation process to obtain shoots and, afterwards, the regeneration of the plant through indirect organogenesis (Roca & Mroginski 1991), it is possible to produce more callus and subject it to a metabolite extraction process (Razavi *et al.* 2017). If the material is friable, cell suspensions can be obtained (Tang *et al.* 2010, Jin *et al.* 2017). There are even reports of other species, such as *Catharanthus roseus*, of which the transformed roots can be subjected to the same type of treatment used for cell suspension cultures (Tang *et al.* 2010). For these reasons, obtaining callus from lateral meristems of *L. mexicana* is an opportunity to verify whether the callus contains the secondary metabolites of medicinal interest found in the aerial part of the plant (leaves and stem), as well as certain coumarines (Navarro-García 2007, Herrera-Ruiz *et al.* 2011, Navarro-García *et al.* 2011), flavones and sesquiterpene lactones (Pérez *et al.* 2005).

Regarding the response of the explants to the used PGR, Loyola-Vargas & Vázquez-Flota (2006), as well as Rout *et al*. (2000), mention that a low auxin concentration, combined with
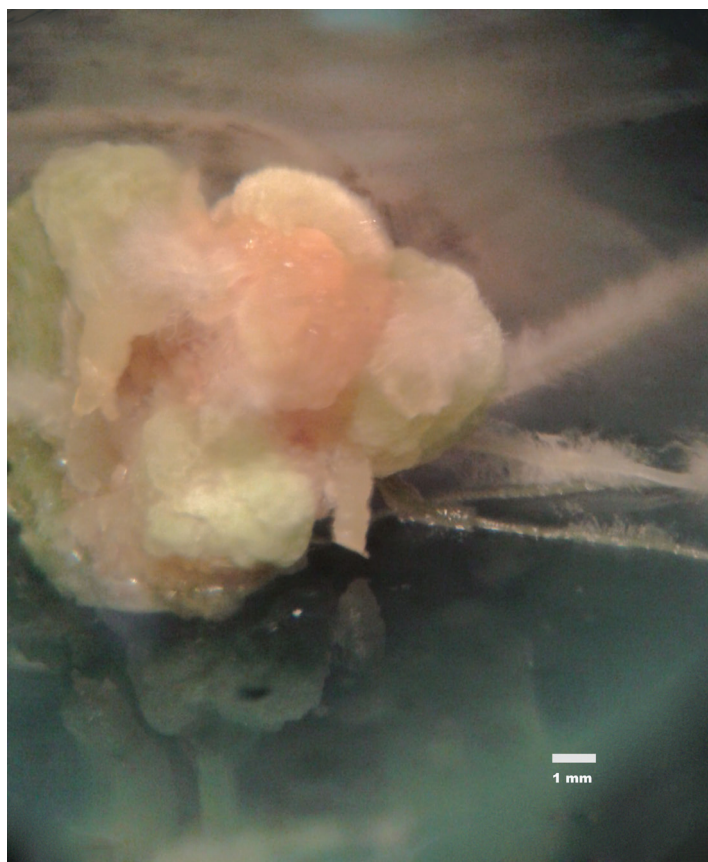
1 mm

Figure 4. Callus 7 weeks after subculture with KIN:NAA: 5:3 mg L-1. Root hairs were observed (20X).

high concentrations of cytokinins, induces the development of shoots, while a high concentration of auxins, combined with a low concentration of cytokinins, induces the formation of roots. In the present study, a concentration of growth regulators of 3 mg L^{-1} (13 µM) KIN and 3 mg L^{-1} (16 µM) NAA caused the explants to develop a large number of roots (Figure 3a). In contrast, a low amount of the auxin NAA —3 mg L^{-1} (16 µM)— and a higher concentration of KIN —5 mg L^{-1} (23 µM)— resulted in the production of as much callus as roots in the culture medium (Figure 4).

The response rate to callus induction in treatment 3, considering all the treatments and cultured explants, was between 2.22 % and 4.44 %, with a response time of 5 weeks, which means that the *in vitro* culture of this species could be performed in a relatively short time. It is necessary to test other explant types with the same or other combinations of KIN and NAA to see if it is possible to produce callus like the one obtained with 5 mgL^{-1} KIN and 3 mgL^{-1} NAA in an even shorter period or to increase the response efficiency of the explants. An alternative could be to use other cytokinins such as BAP to try to induce the growth of shoots from callus, as Wang *et al*. (2016) did with bushy plants; it would be necessary to check whether the plants produce mainly callus.

Auxins are usually used for the generation roots *in vitro* root (Olmos *et al*. 2010). In the present work, we tested a combination of BAP and NAA in roots obtained from callus; after five weeks, the main root did not show any root hairs or villosities, as seen in Figure 5. This combination of PGR is used to promote the growth of buds from roots in herbaceous plants (Trejo-Tapia *et al*. 2012). Although the roots obtained here were thin and fragile, they could be candidates for a protocol to produce transformed roots and to obtain metabolites. Even though the biological material was collected from plants in two different developmental stages, the influence of climate on explant survival *in vitro* was discarded because, beginning with the time
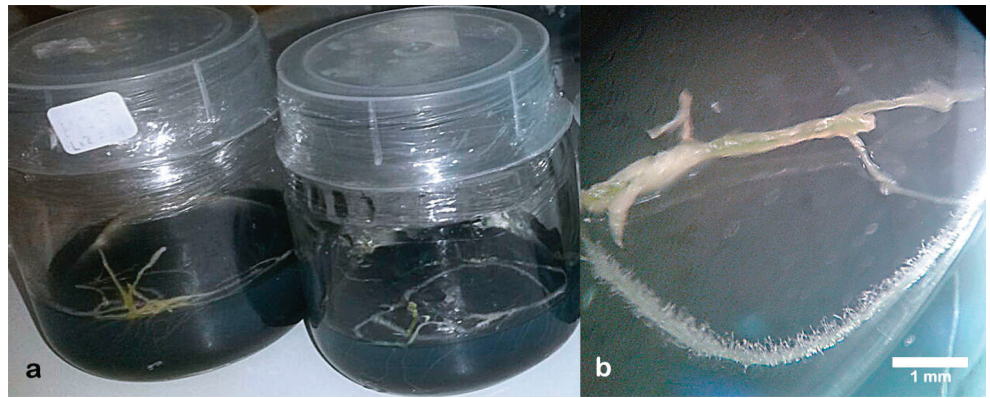
Figure 5. Roots from callus cultured on solid MS medium with BAP:NAA. a) After five weeks with KIN:NAA 5:3 mg L^{-1}. b) Only new roots had root hairs (20X).

of storage, all explants were kept under certain conditions and exposed to exogenous PGR in a controlled way. Even so, we recommend keeping a stock or donor plant under controlled conditions (Olmos *et al*. 2010), such as a greenhouse. Keeping a sample of this species under controlled conditions, when attempting callus culture or *in vitro* propagation, could help establish an standard of the characteristics of the plant that have commercial interest without the need to intervene or disturb the wild population.

Based on our results, the time and type of disinfection were essential to achieve a longer survival time for the used explants. In this case, the best treatment was 10 min of soap, 10 min of 10 % chlorine, 5 min of 70 % ethanol, and 2 min of distilled water, with the addition of AC. The combination of PGR that showed the best response (in terms of production of callus and root) was 5 mg L^{-1} KIN and 3 mg L^{-1} NAA for five weeks. This shows that *in vitro* propagation from lateral buds of *L. mexicana* may be possible using callus induction. This research will serve as the basis for the further study of the best way to propagate *L. mexicana in vitro* or obtain friable callus, and even of the possible production of cell suspensions to search for secondary metabolites or induce shoots.

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