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Original Article

Linalol and Linalyl Acetate Quantitation in the Essential Oil of Somatic Embryos of *Bursera linanoe* R.

Horacio Larqué[1,*], Eugenio Sánchez-Arreola[1], Gregorio Arellano-Ostoa[2], Luis R. Hernández[2], Horacio Bach[3], Abelardo Chávez-Montes[4]

[1]Department of Chemical Biological Sciences, University of Americas Puebla (UDLAP), Ex Hacienda Santa Catarina Mártir s/n, Cholula, Puebla, MÉXICO.
[2]Genetic Resources and Productivity-Fruit Growing Program, Postgraduate College (COLPOS), Highway México-Texcoco, Montecillos, Texcoco, Estado de México, MÉXICO.
[3]Department of Medicine, Division of Infectious Diseases, University of British Columbia (UBC), Vancouver, CANADA.
[4]Department of Chemistry, Autonomous University of Nuevo León (UANL), Av. de los Rectores s/n, Cd. Universitaria, San Nicolás de los Garza, Nuevo León, MÉXICO.

ABSTRACT

Introduction: Mexican artisans use the linaloe tree for the production of handcrafts. The extracted oil from the endangered Mexican tree *Bursera linanoe* which main components are the terpenes linalol and linalyl acetate is protected because of its excessive exploitation. An alternative to mitigate the deforestation of the linaloe tree is the use of somatic embryos, which can be cultured *in vitro*. **Objectives:** In this study, we quantify the yield of linaloe oil extracted from three different linaloe embryogenic lines (LEL) B5, 54, and 2011 cultured in different micropropagation systems. **Materials and Methods:** Using a microwave technique extraction coupled with a Cleavenger device, it was possible to obtain the essential oil from the somatic embryos of *B. linanoe*. **Results:** This study suggest that the tested clones have different proliferation responses, depending on the propagation system culture. The LEL B5 harvested in a permanent immersion system showed the highest growth index (GI= 4.23) as well as the highest concentration of linalol and linalyl acetate with 0.1439 mg/mL and 0.5391 mg/mL respectively, while the LEL 54 harvested in a permanent immersion system and semi-solid, produced the lowest concentration of linalol and linalyl acetate with 0.0090 mg/mL and 0.0923 mg/mL respectively. **Conclusion:** The proliferation and oil yield varied according to the LEL's. However, the highest production of the biomass was measured under the TI system type RITA®.

Keywords: *Bursera linanoe* R., Linaloe oil, Extraction, Secondary metabolites, Microwaving, Bioreactors.

Correspondence
Dr. Horacio Larqué
Department of Chemical Biological Sciences, University of Americas Puebla (UDLAP), Ex Hacienda Santa Catarina Mártir s/n, Cholula-72810, Puebla, MÉXICO.
Email id: horaciolarque@hotmail.com
ORCID ID 0000-0002-9029-2187

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INTRODUCTION

Humans have been attracted to fragrances associated with plants, traceable back to the beginning of recorded history. Some centuries ago, it was discovered that the active principle responsible for the smell of plants could be separated by applying heat. These compounds contained an aqueous and an oiled phase, which were collected by condensation on a cold surface. The oily part was defined as the essential oil. The production and use of these oils are mainly oriented to medicinal uses, although it is well known that in the perfumery industry are highly valued.

In Mexico, one of the many tree species with an economic significance is the *Bursera linanoe*.[1] This tree has an industrial importance because it is an economic source for the inhabitants of the southern regions of Puebla, Morelos, and Northern Guerrero.[2] Mexican artisans located in the town of Olinalá Guerrero, produce Olinalá boxes and chests, which are lacquered handcraft decorated with aboriginal symbols. The internal surface of the box is coated with the oil, offering a special aroma.[3]

As a result of an excessive logging for the extraction of the oil, this tree has been classified as a threatened species and its use has become restricted. The protection of threatened species results in an urgent need to propose new alternatives for their propagation, as well as the development of new extraction methodologies. On the other hand, despite the high costs of production of essences generated through plant material and the demand that exists worldwide, it is estimated that the global use of the linalol essence exceeds 1,000 metric tons per year.[4]

Somatic embryogenesis, or organogenesis, is a reliable and feasible technique because plant cells, unlike animal cells, can regenerate a complete individual (totipotentiality) from any

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plant material.[5] Traditional embryonic cultures are prepared in semi-solid medium, but the nutrient absorption originated by osmosis leads to a decrease in cell proliferation.[6] Therefore, new biotechnological tools have been developed as in the case of liquid culture media, such as the Automated Temporary Immersion Vessel (RITA®).[6-8] This technique, which includes induction, maintenance, proliferation, and maturation of the explants, is frequently performed in Murashige and Skoog (MS) culture medium.[9]

Essential oils are extracted mainly by using the steam trapping technique in which the plant material, such as stems and fruits, are used. This technique requires a large amount of biomass from the embryogenic callus or plant material to obtain a significant sample of essential oil.[10] In this study, we propose the use of three Linaloe Embryogenic Lines (LEL) cultured in three different micropropagation conditions such as Semi-Solid (SS), Permanent Immersion (PI) and Temporary Immersion (TI) systems, for the production and extraction of the linaloe oil. In addition, we propose a new extraction technique based on the principle of microwaving with a Clevenger apparatus modification. The advantages of this technique are that the extraction of the essential oil is performed in less time and with a lower amount of both, biomass and without organic solvents.

MATERIALS AND METHODS

Plant Material

Samples were collected following the published protocols of Arellano-Ostoa *et al*.[5] identified by an expert taxonomist and deposited at the Benemérita Universidad Autonoma de Puebla herbarium. The Linaloe Embryogenic Lines (LEL) used in this study were B5, 54 and 2011. The somatic embryos of *B. linanoe* used in this study have been maintained in *in vitro* conditions for more than six years. They were generated from young leaves and cotyledons of different collections of adult trees selected in the seasonally dry tropical forest (18°03'52.8"N longitude and 98°59'54.9"W latitude), extending through the states of Puebla, Guerrero, and Morelos in México.

Explant material and culture conditions

Proembryogenic masses and secondary somatic embryos were used as explants, which were transferred to the nutrient medium MS with some modifications; supplemented with 3% (w/v) sucrose, 0.027 µM 1-naphthaleneacetic acid (ANA) and 0.022 µM benzyladenine (BA); and solidified with 0.6% (w/v) agar (Merck®) (5). The pH of the nutrient medium was adjusted to 5.7 ± 1.0 before autoclaving for 20 min (1.5 psi at 120°C). The cultures were maintained in a growth chamber under LED type lamps (5 W) that provided a photosynthetic active radiation of 40 µmol m^{-2} s^{-1} and 16 hr photoperiod at 25 ± 2°C. Proliferating somatic embryos were subcultured every 30 days. These lines have been maintained for 6 years by secondary embryogenesis, with sequential subculture at 5-week intervals.

Seedling of embryogenic lines in three propagation systems

Embryogenic clusters (1.5 g) comprising globular, heart, torpedo and cotyledonal shaped embryos of the green-looking proembryogenic masses, were dissected from each of the stock embryogenic lines (Figure 1). They were used as explants for embryogenic proliferation experiments and for the obtention of the essential oil that contains linalol (1) and linalyl acetate (2). During the selection, the necrotic material was avoided. The explants were replanted in a laminar flow hood under sterile conditions in three different micropropagation systems SS, PI and TI (Figure 1a, b and c). In all of the three systems, the MS medium was used. In the SS system, 20 mL of the medium supplemented with 0.6% of agar was dispensed. In the case of PI, 75 mL of MS was added to a 250 mL flask and shaken at 250 rpm for 5 weeks. In the case of the TI, 200 mL of MS was added to the bioreactor RITA® (CIRAD, Montpellier, France) using 6 immersions per day for 1 min.[11] All systems were incubated in the same conditions of light and temperature as previously described. All embryogenic cultures were sub-cultured every five weeks and

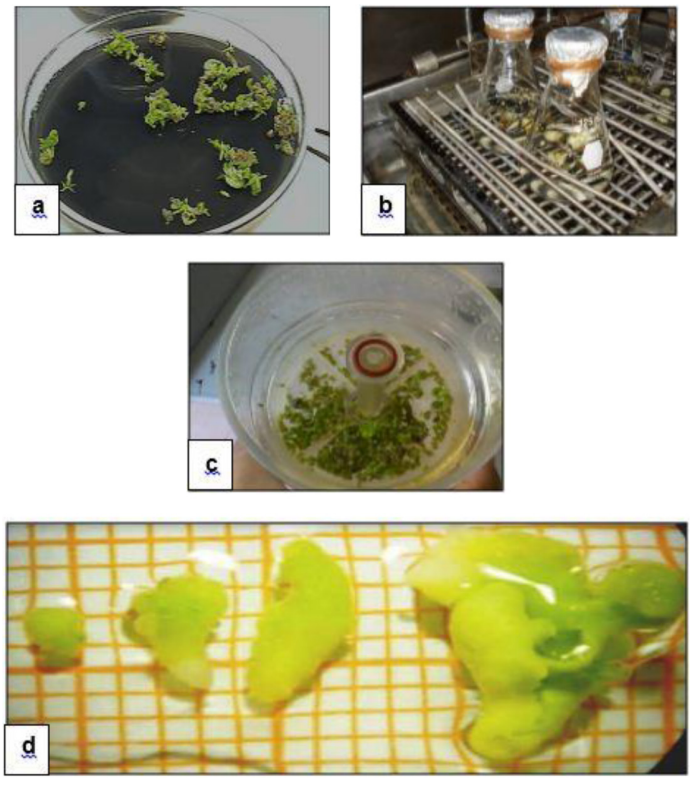

Figure 1: Production of somatic embryos of linaloe in three micropropagation systems.
a) Semi Solid (SS), b) Permanent Immersion (PI) and c) Temporary Immersion (TI); all of them after 5 weeks of culture. d) Somatic embryos of linaloe in different stages of development (from left to right), globular, heart, torpedo and cotyledon present in the embryogenic masses used as explant.

the experiments were repeated twice. After this period, the fresh weight (mg) of the biomass obtained for each LEL for the three systems of propagation were recorded.

The growth index was calculated as:

$$\text{Growth index (GI)} = \text{Final biomass weight} - \text{Initial inoculum weight/Initial inoculum weight}$$

Quantification of linalol and linalyl acetate

For the quantitation of the essential oil extracted from the somatic embryos a calibration curve was established for each chemical specie, linalol and linalyl acetate. Serial dilutions of (-) Linalol (Fluka®) were prepared in a range of 0.01-0.1 mg/mL using methanol (HPLC grade, Sigma-Aldrich®). An HPLC (Perkin Elmer® Model 250) was used to measure the concentrations of linalol using a C_{18} reverse phase column (4.6 × 250 mm, XTerra, Waters®) and an isocratic mobile phase of MeOH/H_2O (7:3). Each sample was injected by triplicate until linearity was acquired. Linalyl acetate standard was prepared according to published protocols.[12] In brief, 4 g of linalol were dissolved in 4 mL of dried pyridine (in NaOH pellets) and 3.67 mL of acetic anhydride (Ac_2O) (Figure 2). Subsequently, the reaction was placed in a steam bath for 4 hr, afterwards in a separatory funnel with 1 g of ice, and then extracted with 200 mL of ethyl acetate (EtOAc) twice. The aqueous phase was removed and the organic phase was washed with 40 mL of distilled H_2O, followed by washes (×5) with 40 mL of 10% HCl until the pyridine residues were removed. Finally, it was sequentially washed with 40 mL of distilled H_2O (×5), 40 mL of a saturated solution of $NaHCO_3$ (×5), and again with 40 mL of distilled H_2O (×5). To remove the remaining water, the reaction was dried with anhydrous Na_2SO_4. The organic phase was placed on a rotary evaporator under reduced pressure in order to evaporate the EtOAc. To verify the product of the reaction, a small amount of it was analyzed by thin layer chromatography (TLC) using Silica gel 60 F_{254} (Merck®) run in a mobile phase of Hex/$CHCl_3$ (2:1). Finally, the purity of the compound and its identification were established according to the spectroscopic data in comparison with those previously reported in the literature. ^{1}H-NMR and ^{13}C-NMR experiments were recorded in a Bruker® Avance III, GC-MS/MS in a Varian® 3800 and FT-IR in a Cary 630 from Agilent Technologies®.

For the purification of the reaction mixture, a silica gel (60-230 mesh, RBM®) chromatography column was placed, and a mobile phase of Hex/$CHCl_3$ (9:1) was used for separation. A total of 40 fractions (100 mL each) were collected and evaporated under reduced pressure. In parallel, TLC was run to reveal the fractions in which the product of the reaction had already been separated. Once the product was obtained, a standard solution of 1 mg/mL of linalyl acetate in methanol was prepared and then 10 serial dilutions were prepared with the lowest concentration 0.1 mg/mL. The same HPLC separation methodology mentioned above was used and samples were analyzed by triplicate.

Oil extraction

The extraction of oils was carried out in an oven microwave (Daewoo®) coupled to Clevenger type equipment with a refrigerant. For each extraction, 30 g of somatic embryos from each clone and each micropropagation systems were placed in a pre-sterilized 500 mL flat bottom ball flask with 100 mL of distilled water and extracted at 1200 W for 15 min for two cycles. The oil extracted was located into the Clevenger type apparatus, where it was separated by densities differences.

Analysis of the essential oil by HPLC

The oil samples obtained from the extraction were injected by triplicate (3µL each injection) into the HPLC under the same conditions in which the calibration curves were constructed to integrate and obtain the average of the area under the curve of each chemical compound, same methodology was run for the linalyl acetate obtained from the acetylation reaction (Figure 3).

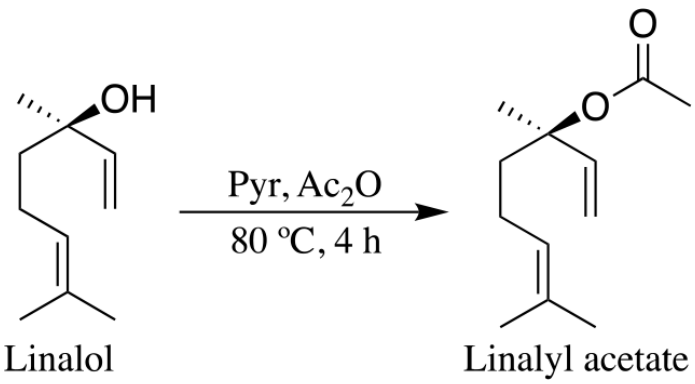

Figure 2: Reaction of the acetylation of linalol for the obtention of linalyl acetate.

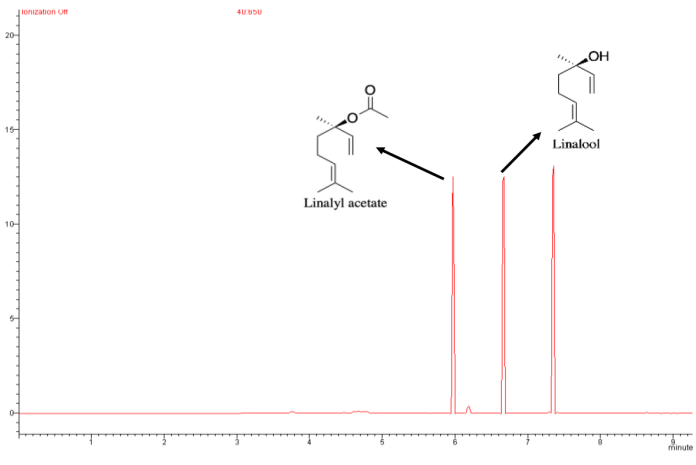

Figure 3: HPLC chromatography with a refractive index detector (RID). Chromatogram of the reference standard linalol and the acetylated derived linalyl acetate, used for the quantitative analysis.

Statistical analysis

The influence of the main experimental factors (3 LEL and 3 propagation system) on the concentrations of linalol and linalyl acetate was analyzed by a two-way ANOVA ($P < 0.05$) to examine the influence of each of the two main factors and their interaction. When statistical differences were observed, a Tukey test ($P < 0.05$) was used to separate the means. The software GraphPad Prism v. 6.00 (CA, USA) was used.

RESULTS AND DISCUSSION

Propagation of the embryogenic lines

All of the three embryo micropropagation systems used in this study showed that LEL's were able to grow (data not shown). When comparing the biomass of the different clones propagated by the HGI (Highest Growth Index) calculated by the formula above described, the clone LEL B5 produced 3.5 times its weight, while LEL 54, and LEL 2011 only 1.3 times (Table 1).

Linalyl acetate, structural determination

Yellowish oil; IR ν_{max} (film) 3457, 3090, 2972, 2931, 2860, 1739, 1645 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD) δ 5.89 (1H, dd, *J* = 16.8, 10.9, H-5), 5.29 (1H*cis*,dd, *J* = 10.9, 1.3, H-4), 5.28 (1H*trans*, dd, *J* = 17.1, 0.9, H-4), 5.20 (1H, dd, *J* = 16.8, 1.33, H-3), 2.19 (3H, s, H-13), 1.94 (2H, dt, *J* = 7.43, 7.13, H-2), 1.86 (3H, s, H-8), 1.70 (3H, s, H-9), 1.55 (2H, t, *J* = 7.43, H-2), 1.24 (3H, s, H-3). ^{13}C-NMR (125.75 MHz, CD_3OD) δ 170.2 (C-12), 143.1 (C-5), 131.3 (C-7), 124.7 (C-3), 82.9 (C-6), 39.5 (C-1), 25.4 (C-10), 24.6 (C-8), 22.7 (C-2), 21.4 (C-13), 18.6 (C-9). EI-MS (positive ion): *m/z* (%) = 196 [M + 3] 121 (23), 93 (100), 80 (33), 69 (45), 55 (16), 43 (61), 41 (57), 27 (12). $C_{12}H_{20}O_2$.

Based on its spectroscopic data and those previously published structures by Williams *et al.*[13] we definitely obtained 2, which was identified as linalyl acetate, a secondary metabolite synthesized by the plant and a constituent of the linalol oil.

Effect of the propagation system on the metabolite amount

For the quantitation of linalol, the LEL B5 propagated in all the three systems was statistically significant superior compared to the other two LEL's, with the highest production of 1 in the liquid systems (PI and TI) with a total amount of 0.1439 and 0.1390 mg/mL respectively, followed by SS with 0.0490 mg/mL. This confirms that LEL B5 multiplied in a liquid medium is optimal for the production of linalol. On the other hand, we report the LEL 2011 with the lowest amount of 1 in all the systems. In addition, significant differences were observed in the total content of linalyl acetate; again, being the LEL B5 with the highest production of 2 propagated in PI with 0.5391 mg/mL, followed by LEL 2011 in SS and finally LEL B5 in TI, quantifying 0.4374 and 0.3335 mg/mL respectively, in sum the LEL with the lowest amount of 2 was LEL 54 (Table 2) (Figure 4 A and B). In this study we found that the higher proliferation observed in a TI system such as RITA® can be explained in three ways during the growing period, (i) a constant separation of the proembryogenic masses was observed to be derived from the agitation of the medium in the flask, (ii) a constant gas exchange that undergoes the vegetal material; and (iii) the total contact of the medium with the embryogenic calli, which results in an ideal environment for the proliferation,[6,14] This shows that the conditions of the RITA® system favor the development of somatic embryos, as well as the diffusion of nutrients towards the explant, ensuring a complete impregnation of the medium to the LEL's.[15] The positive effect of the TI on the micropropagation of several species has been indicated, and our results align to a previously published study referring to the high biomass yields obtained when the same system was used.[6,16-17] Although the experimental conditions in the TI method were the same for all of the LEL's, the differences observed in the biomass yield as well as the content of linalol and linalyl acetate might be attributed to the genetic variation of the embryos, the type of carbohydrates and growth factors used, which have a direct effect to the biomass yield.[1,15] On the other hand, these differences can also be attributed to the age and useful life of the bioreactor itself. For example, as a result of the continuous sterilization of the bioreactors, the polycarbonate gradually becomes opaque; this prevents the incidence of the light to be uniformly distributed to all plant material, a situation that affects the cell growth. Differences in the linaloe oil yields observed among the

Table 1: Growth index of somatic embryos multiplied in Temporary Immersion.

Embryogenic line	Growth index (g)	Initial weight (g)	Final weight (g)
B5	4.23 ± (0.14)	1.20 ± (0.74)	5.16 ± (0.01)
54	2.14 ± (0.98)	1.78 ± (0.51)	5.37 ± (0.64)
2011	2.29 ± (0.02)	1.76 ± (0.54)	5.39 ± (0.83)

The data are shown as the mean (±) of the Standard Deviation (SD) of 10 clones by triplicate. Final weight was obtained after 5 weeks of growth.

Table 2: Total yield of linalol and linalyl acetate in the three propagation systems correlated with the three LEL's.

Embryogenic line	Propagation system	Linalol (mg/mL)	Linalyl acetate (mg/mL)
54	SS	0.0414 ± 0.0002 ^{D}	0.0923 ± 0.0002 ^{F}
B5		0.0490 ± 0.0003 ^{C}	0.0959 ± 0.0028 ^{F}
2011		0.0205 ± 0.0003 ^{F}	0.4374 ± 0.0044 ^{B}
54	PI	0.0090 ± 0.0004 ^{I}	0.1314 ± 0.0087 $^{E F}$
B5		0.1439 ± 0.0004 ^{A}	0.5391 ± 0.0100 ^{A}
2011		0.0191 ± 0.0002 ^{G}	0.2212 ± 0.0068 ^{D}
54	TI	0.0307 ± 0.0003 ^{E}	0.1583 ± 0.0222 ^{E}
B5		0.1390 ± 0.0002 ^{B}	0.3335 ± 0.0094 ^{C}
2011		0.0172 ± 0.0003 ^{H}	0.1509 ± 0.0061 ^{E}

Data are presented as the mean (±) of the Standard Deviation (SD) (n = 3). Different superscript letters within the same column indicate significant differences between them. Tukey method, ANOVA ($P \leq 0.05$).

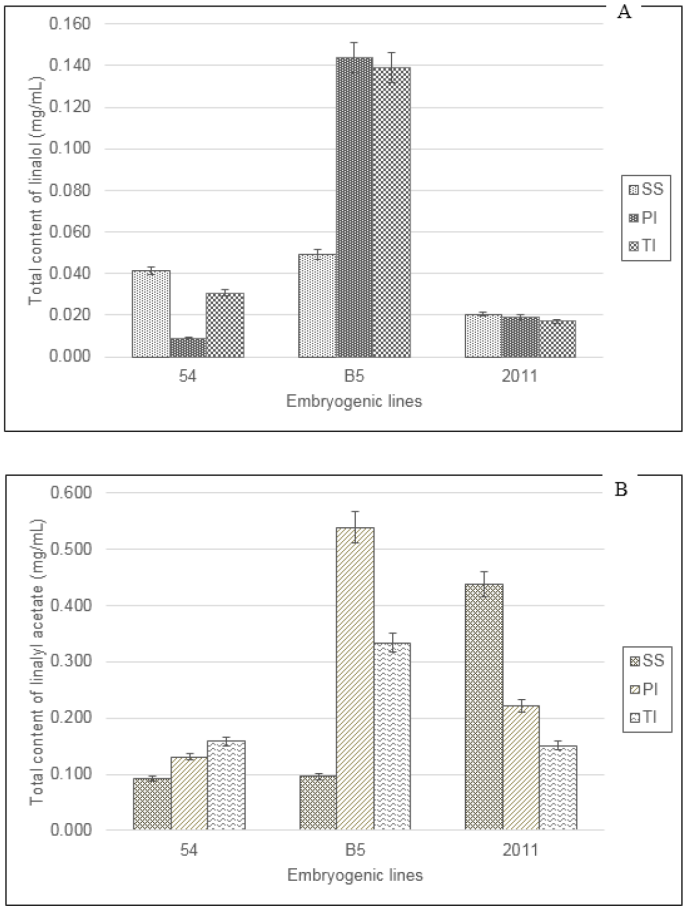

Figure 4: A) Quantity of the terpene linalol in three different embryogenic lines in fresh weight of *Bursera linanoe* cultured in three different micropropagation systems. B) Quantity of the derivative linalyl acetate in three different embryogenic lines in fresh weight of *Bursera linanoe* cultured in three different micropropagation systems. Values are means (±) standard deviation ($P \leq 0.05$).

different culturing systems are most likely due to the fact that in liquid systems the plant material is permanently or temporarily immersed in the liquid, causing a diffusion of the metabolites into the liquid medium. In the case of the TI, a gas exchange may produce losses of compounds such as terpenes (linalol and linalyl acetate) when the explants are not exposed to the medium.[18-19] In addition, the marked difference in the yield of the oil in LEL B5 between the liquid and SS systems may be due to the microenvironment generated within the flask of the SS system. This micro environment can cause a decrease in the biosynthesis of the linalol. Another cause of the different yield can be the result of the variation in the synthesis of secondary metabolites reported among individuals of the same species.[18] Regarding to the yield of linalyl acetate, the PI system showed the highest production of the metabolite as compared to the other two systems, whereas LEL B5 yielded the highest concentration of linalol followed by LEL 2011 and LEL 54. It is noteworthy that the SS is the system that produced the lowest concentration of linalol and linalyl acetate. This is explained by the fact that the terpenes linalol and linalyl acetate have a low vapor pressure, which causes them to evaporate with time.[20-21] Another alternative for the production of linalol has been reported using *Escherichia coli*. In this report, a heterologous expression of the linalol synthase in *E. coli* yielded approximately 85 mg/mL of linalol after a fermentation time of 96 hr.[22] Although this yield is higher than the yield, we report here using the LEL's, the downstream purification steps in the bacterial culture have not been indicated, which will reduce considerably the yield because of the volatile nature of the compounds. Finally, linalyl acetate was not produced by the bacteria, suggesting that the fragrance of the oil produced may have a lower quality, compared to the LEL's proved in this study.

In this study, we evaluated the extraction of linalol and linalyl acetate from embryogenic masses of three LEL's as an alternative to the extraction of the compounds from the endangered tree.

CONCLUSION

The proliferation and oil yield varied according to the LEL's. However, the highest production of the biomass was measured under the TI system type RITA®. Overall, the LEL B5 showed the highest production of both linalol and linalyl acetate using both liquid systems (PI y TI), suggesting that this condition can be used for the operation of bioreactors for the production of both linalol and linalyl acetate at an industrial scale. On the other hand, the extraction by microwave is a good technique of continuous extraction, since it reduces the amount of plant material used and the solvents. Moreover, the somatic embryos that showed a better propagation yield was the LEL B5, thus, probably to the juvenile leaves that were used for the obtention of the somatic embryos. In addition, somatic embryogenesis is an excellent technique for the propagation and protection of threatened species; however, further research is required to bring it to an industrial scale.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LEL: Linaloe Embryogenic Line; **MS:** Murashige and Skoog; **SS:** Semi Solid; **PI:** Permanent Immersion; **TI:** Temporary Immersion; **ANA:** 1-naphthaleneacetic acid; **HGI:** Highest Growth Index; **BA:** Benzyladenine.

SUMMARY

The terpenes linalol and linalyl acetate, which are the principal components in the essential oil of *Bursera linanoe* R. were obtained *via* microwave extraction from the somatic embryos of three different cell lines, also, cultivated in three different micropropagation systems. Our results shows that the cell line B5 cultivates in PI was the one with the highest amount of the mono terpenes linalol and its acetyl derivative linalol acetate.

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