

Lippia graveolens (Lamiales: Verbenaceae) and *Oryganum vulgare* (Lamiales: Lamiaceae) obtained by means of the *in vitro* propagation technique

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ABSTRACT

Objective: The objective of this study was to establish the method of propagation of *Oryganum vulgare* and *Lippia graveolens* employing a plant tissue culture technique that decreased the phenolization percentages and increased the multiplication coefficients.

Design/methodology/approach: The *in vitro* germination percentage was evaluated in both MS and MS medium + activated carbon. Microcuttings (small shoots) of both species were established in base medium added with different antioxidant agents to decrease the phenolization of explants; the treatments were arranged in a completely randomized block design. For the propagation phase, a completely randomized factorial design was used, where the auxin/cytokinin phytohormones, type of explants (axillary buds and leaves), and the species (*Lippia graveolens* and *Oryganum vulgare*) were considered as factors.

Results: Maximum germination (63.3% ± 12.5) was obtained on day 15 in both culture media for *L. graveolens* and *O. vulgare*. The use of antioxidant agents mainly activated carbon, increased the *in vitro* establishment and activation of vegetative buds in both species by up to 90%. There were significant differences in the variables evaluated regarding the treatments, the explant, and the species in the multiplication phase. The combination 1.0/ 0.5 mg L⁻¹ BA/AIB induces callus formation for both species. When used as leaf explants, callus formation was potentiated.

Study Limitations/Implications: The results presented are advances from a long-term experiment.

Findings/conclusions: The germination of *L. graveolens* seeds can be achieved in MS medium after 15 days. Microcuttings of both *L. graveolens* and *O. vulgare* were successfully established in MS basal medium enriched with 1 g L⁻¹ charcoal that showed low oxidation percentages and induced up to 90% the production of shoots

in the explants. The mixture of 1.0/0.5 mg L⁻¹ BA/AIB induces callus formation for both species; when this medium is in contact with leaves as an explant, its formation is potentiated, achieving diameters up to 15 mm. In order to achieve the induction of shoots and roots, buds should be established in MS medium enriched with 0.5 mg L⁻¹ IBA for both species; this mixture increased the multiplication coefficients.

Keywords: Micropropagation, Mexican oregano, phenolization, multiplication coefficient, plant growth regulators.

INTRODUCTION

The most common oregans in Mexico are *Oryganum vulgare*, better known as common oregano or Greek oregano; it is a herbaceous species of the Lamiaceae family native from Europe (Arcila-Lozano *et al.*, 2004) and *Lippia graveolens*, better known as Mexican oregano (Clarenc *et al.*, 2020). The latter is a species that is distributed in the semi-arid climates of Mexico, mainly in the states of Hidalgo, Veracruz, Durango, Yucatán, Puebla, Coahuila, Oaxaca, San Luis Potosí, Querétaro, Guerrero (CONAFOR, 2011; Castellano-Hernández *et al.*, 2013; Cortés-Chitala *et al.*, 2021), in the vast majority of these areas this species is a wild resource in areas with a high degree of economic and social marginalization, making it necessary to establish adequate management of this resource (Castillo *et al.*, 2017; Díaz-De León *et al.*, 2020). Around 90% of *L. graveolens* production in Coahuila is obtained from wild areas (CONAFOR, 2009), causing natural regeneration and conservation of genetic variability of the species scarcity. An alternative for the conservation of wild oregans is the cultivation of plant tissues.

In vitro propagation studies of various oregans have been carried out, mainly via direct organogenesis (García-Pérez *et al.*, 2012) using MS medium (Murashige and Skoog, 1962) combined with the auxin cytosine complex. Sánchez-Pérez *et al.* (2021) reported the *in vitro* propagation of *L. graveolens* using axillary buds enriched with 2.5 mg L⁻¹ of 6-benzyl aminopurine and evaluated different luminosity intensities to make the *in vitro* photosynthetic process more efficient. Well (2014) reported that the micropropagation of this species from vegetative explants (microcuttings) presents a high percentage of phenolization, which prevents cultivation. Castellano-Hernández *et al.* (2013) reported the generation of shoots of *L. graveolens* from axillary buds of plants germinated *in vitro* with cytokinins and the development of shoots with the addition of auxins. Therefore, the objective of the present work was to establish the propagation method of *Oryganum vulgare* and *Lippia graveolens* employing plant tissue culture technique or micropropagation that decreases the phenolization percentages and increases the multiplication coefficients.

MATERIALS AND METHODS

Selection of plant material

The seeds of *O. vulgare* were purchased from the company HortaFlor and mother plants from the Botanical Garden of Medicinal Plants of the Institute for Research and Training, Agriculture, Agriculture and Forestry of Mexico (ICAMEX) to validate their origin.

The seeds of *L. graveolens* were collected in the community of Higuerrillas, municipality of Peñamiller, Querétaro. In addition, two specimens were selected as the mother plant.

For both species, the seeds were kept refrigerated at 10 °C until their establishment *in vitro*. The mother plants were kept under greenhouse conditions at the Lerma Autonomous Metropolitan University and the mother plant in a greenhouse of the Biotechnology Laboratory of the Protectora de Bosques del Estado de México (PROBOSQUE), where all the *in vitro* study was carried out.

***In vitro* establishment**

The seeds and microcuttings were used as explants. The seeds were disinfected following the micropropagation process of *Thymus piperella* (Sáez *et al.*, 1994), with some modifications. The seeds were disinfected by soaking them in constant agitation for five minutes with two drops of commercial liquid soap; subsequently rinsed until the soap was removed with distilled water, followed by 30% v/v commercial sodium hypochlorite solution for 20 minutes and rinsed again with distilled water (three times). Afterward, they were placed in a laminar flow hood in a 70% ethanol solution for 30 seconds and rinsed with sterilized distilled water.

A completely randomized block design was established to evaluate the seed germination of both species, ten repetitions were used for each treatment, and each repetition contained five seeds. Two treatments of base culture medium were established: T1. 100% MS culture medium (Murashige and Skoog, 1962), with the whole mineral salts. T2. 100% MS culture medium plus 1 g L⁻¹ of activated carbon. Both treatments were enriched with 30 g L⁻¹ of sucrose, 7 g L⁻¹ of bacteriological agar, and the pH was adjusted to 5.7±0.1. The treatments were placed in Gerber-type flasks with 20 milliliters of medium each; they were sterilized in an autoclave at 120 °C for 15 minutes. Germination percentage was evaluated at 5, 10, and 15 days.

The disinfection of vegetative explants was carried out as Chanbe (2008) for common oregano microcuttings, changing the ascorbic acid by citric acid in the same concentration. As this type of species presents a high degree of oxidation due to the release of phenols at the cutting time, a completely randomized design was established with five treatments with ten repetitions per treatment to minimize phenolization. For the treatments, MS was used as the basal medium at 100% of its concentration enriched with 30 g L⁻¹ of sucrose, 7 g L⁻¹ of bacteriological agar supplemented with: O1 control; O2 with 2.5% lemon juice; O3 with 1 mg L⁻¹ of citric acid; O4 with 1 g L⁻¹ of activated carbon and O5 with 5% lemon juice. The oxidation percentage and the shoot regeneration percentage were evaluated at 30 days.

Multiplication and rooting stage

For the multiplication stage, a factorial design with five repetitions (five explants per repetition) was used, where the effect of the cytokinin-auxin complex for the stimulation of morphogenetic responses was evaluated. Factor A was the type of explant (axillary bud and leaf), and factor B was the components of the culture medium. For the latter, 6-benzyl amino purine (BAP) was used in concentrations 0, 0.5, and 1 mg L⁻¹ combined with indol butyric acid at 0, 0.1, and 0.5 mg L⁻¹ in MS base medium with 30 g L⁻¹ of sucrose, 7 g L⁻¹ of bacteriological agar. After 30 days of establishment, the following variables were

evaluated: % oxidation, % of callus formation, callus diameter, % of shoot formation, shoot length, number and length of roots, and multiplication coefficient.

Statistic analysis

The variables for each phase were evaluated employing an analysis of variance. A Tukey test was performed for the variables that presented significant differences ($P < 0.05$) (Steel and Torrie, 1980). The SAS software for Windows 10 was used.

RESULTS AND DISCUSSION

In vitro establishment

Regarding the *in vitro* establishment of seeds, *O. vulgare* did not germinate in any treatment. For *L. graveolens*, there were no significant statistical differences for the three evaluation dates, obtaining germination percentages of 30.0 ± 7.8 and $26.76 \pm 8.3\%$ in T1 and T2, respectively, at five days. Ten days after sowing, the trend was similar, with 56.67 ± 10.0 and $50.0 \pm 11.4\%$ germinated seeds. Maximum germination occurred 15 days after sowing with $63.3 \pm 12.5\%$ for both treatments. These results were similar to those reported by Castellano-Hernández *et al.* (2013) for *L. graveolens*; they observed *in vitro* germination of seeds in MS medium to use them as initial micropropagation material, material that was subcultured in MS medium plus 0.5 mg L^{-1} of BA to activate $83.3 \pm 0.4\%$ of buds. Regarding the microcuttings established as initial explants, there were significant statistical differences ($P < 0.001$) between treatments, species, and treatment \times species interaction in, both % of oxidation and % of explants that formed shoots.

The lowest percentage of oxidation was observed in the O4 treatment ($20.5\% \pm 2.92$), O1 showed $42.5\% \pm 8.68$, the O2 and O3 treatments showed $46.0\% \pm 3.28$ and $51.0\% \pm 3.3$; respectively, and the highest oxidation was observed in O5 treatment ($90.0\% \pm 3.55$). The *L. graveolens* microcuttings showed a lower percentage of oxidation ($42.80\% \pm 4.76$) than *O. vulgare* ($57.20\% \pm 3.78$).

The treatment effect on the species is shown in Figure 1; it can be observed that treatment O4 ($13.0 \pm 1.6\%$) and O1 ($10.9 \pm 0.4\%$) had a significant effect on *L. graveolens*. On the other hand, *O. vulgare* showed less response to O4 treatment ($28.00 \pm 1.7\%$) and O1, where the percentage of oxidation was $78.00\% \pm 2.9$. Thus, indicating that microcuttings as the initial explant of Mexican oregano responded well to antioxidant agents. The addition of citric acid reduced the oxidation percentages, however, when it is used in high doses it is counterproductive.

The formation of shoots was inversely proportional to oxidation; when there was more oxidation, there was less presence of shoots. The presence of activated carbon (O4) considerably increased the presence of shoots ($90.5\% \pm 3.52$). *Lippia graveolens* regenerated more shoots than *O. vulgare* ($66.24\% \pm 6.49$ and $31.72\% \pm 7.92$, respectively).

The results of Bueno (2014) showed the contrary because he added 2% ascorbic acid to the MS base culture medium and considerably reduced the phenolization of buds used as initial explants.

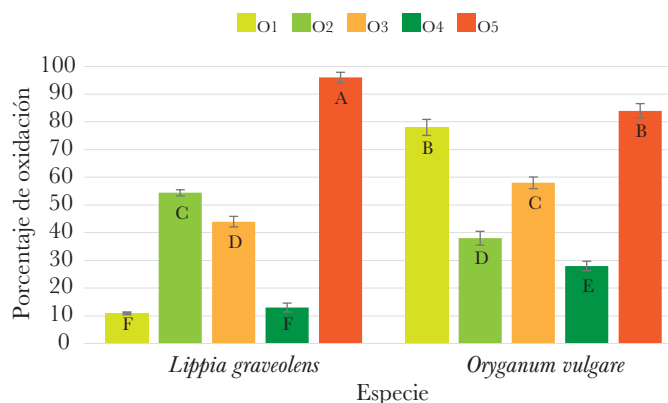


Figure 1. Effect of antioxidant agents in the culture medium for establishing microcuttings as an initial explant in *Lippia graveolens* and *Oryganum vulgare*. O1: control; O2: 2.5% lemon juice; O3: 1 mg L⁻¹ of citric acid; O4: 1 g L⁻¹ of activated carbon; O5: 5% lemon juice. Different letters present statistically significant differences P ≤ 0.05.

Multiplication and rooting stage

For percentage of oxidation, percentage of callus formation, callus diameter, and percentage of shoot formation, there were significant statistical differences between the treatments, the species, and the explant (P < 0.0001) except for the percentage of callus formation referring to the type of explant (P = 0.7022) used (Table 1).

The treatments consisting of 0.5/0.5 and 1.0/0.5 mg L⁻¹ BA/AIB presented the lowest oxidation percentages with 12.5 and 12.4% oxidation compared to the treatment without growth regulators that presented the highest percentage of oxidation, 32.9%. The 1.0/0.5 mg L⁻¹ BA/AIB treatment was isolated entirely from the other treatments, causing callus formation in 69% of the explants, and the treatment without regulators presented only 7% of the explants. In the explants, the leaf presented a high degree of oxidation compared to the bud; both explants formed callus, but the diameter of the callus was more significant in the leaf than the bud, as shown in Table 2, the more significant number of sprouts were presented with oregano buds.

The *in vitro* behavior of *L. graveolens* was better than that of *O. vulgare*, from both the leaf and the bud had the best values for callus formation, the Mexican oregano leaf formed a

Table 1. Mean squares for the *in vitro* multiplication stage of *Lippia graveolens* and *Oryganum vulgare*.

	Oxidation	Callus formation	Callus diameter (mm)	Shoots formation
Treatment(T)	3068.3 *	2852.48 *	521.7 *	316.79 *
Species (SP)	194245 *	12520.06 *	4843.66 *	1355.3 *
Explant (E)	348668 *	30.81 NS	919.23 *	126271 *
T*SP	3003.2 *	1381.96 *	251.3 *	405.89 *
T*E	3212.4 *	187.70 *	49.62 *	121.11 *
SP*E	3212.4 *	295.23 *	830.09 *	0 NS
CV	39.00	25.64	35.50	34.70

* Significant at 0.05. NS. Not significant at 0.05.

Table 2. Percentages of oxidation, callus formation and diameter, and bud formation in *Lippia graveolens* and *Oryganum vulgare*.

		Oxidation (%)	Callus formation (%)	Callus diameter (mm)	Shoots formation (%)
Species	<i>Oryganum vulgare</i>	42.01 ^A	1.9 ^B	0.03 ^B	57.2 ^B
	<i>Lippia graveolens</i>	7.88 ^B	88.7 ^A	5.4 ^A	66.2 ^A
Explant	Leaves	53.1 ^A	56 ^A	5.1 ^A	1 ^B
	Bud	5.5 ^B	56 ^A	2.6 ^B	91.4 ^A
<i>L. graveolens</i>	Leaves	24.8 ^B	91.1 ^A	8.4 ^A	0 ^B
	Bud	6.3 ^C	87.6 ^A	4.1 ^B	94.6 ^A
<i>O. vulgare</i>	Leaves	95.6 ^A	5.6 ^B	0.1 ^C	2.2 ^B
	Bud	14.4 ^C	0 ^A	0 ^C	85.6 ^A

Different letters represent statistically significant differences $P \leq 0.05$.

large callus diameter (8.4 mm) (Figure 2), suggesting that the suspension cell culture of this species may be very viable to produce secondary metabolites from this explant. The leaf of *O. vulgare* regenerated shoots in a small proportion, thus producing direct organogenesis.

Regarding the generation of shoots by growing buds, statistical differences ($P < 0.05$) were found between the treatments evaluated concerning the addition of growth regulators. For both species, the number of shoots and, therefore, the multiplication coefficient benefited when 1.0/0.0 mg L⁻¹ BA/AIB was used; it increased almost twice for *L. graveolens* and triple for *O. vulgare* (Table 3). The length of the shoots of *L. graveolens* increased 22.8 mm with 0.0/0.5 mg L⁻¹ BA/AIB concerning the control, in that same concentration, up to five roots per explant with an average length of 47 mm compared to the control that formed 1.3 roots per explant with approximately 3.5 mm.

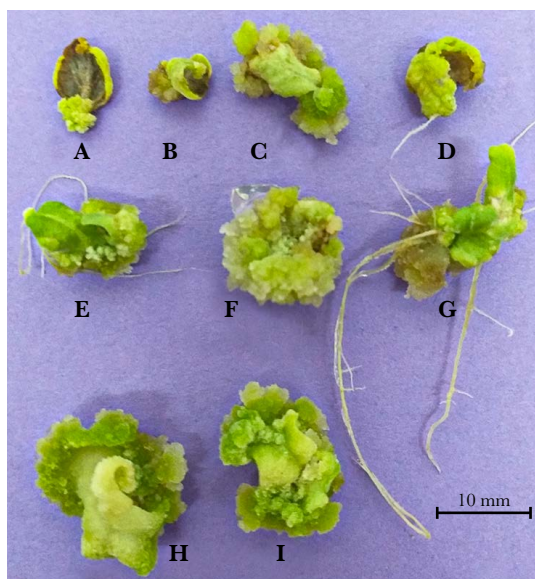


Figure 2. *Lippia graveolens* callus formation from leaf in MS basal medium added with 6-benzyl amino purine/ indole butyric acid in mg L⁻¹. A: Control, B: 0.5/0.0, C: 1.0/0.0, D: 0.0/0.1. E: 0.5/0.1, F: 1.0/0.1, G: 0.0/0.5, H: 0.5/0.5, I: 1.0/0.5.

Table 3. Effect of cytosine/auxin in the multiplication and root formation stage in *Lippia graveolens* and *Oryganum vulgare* from buds.

Treatment BAP/AIB mg L ⁻¹	Number of shoots	Shoot length (mm)	Root number/explant	Root length (mm)	Propagation coefficient
<i>Oryganum vulgare</i>					
0.0/0.0	1.5 ^{BC}	16.6 ^{AB}	0.0 ^A	0.0 ^A	4.4 ^{BC}
0.5/0.0	1.9 ^{ABC}	12.8 ^B	0.0 ^A	0.0 ^A	5.1 ^{ABC}
1.0/0.0	2.5^A	18.2 ^{AB}	0.0 ^A	0.0 ^A	7.3^A
0.0/0.1	1.6 ^{BC}	17.4 ^{AB}	0.0 ^A	0.0 ^A	4.9 ^{ABC}
0.5/0.1	1.6 ^{BC}	12.5 ^B	0.0 ^A	0.0 ^A	3.9 ^{BC}
1.0/0.1	1.8 ^{ABC}	16.2 ^{AB}	0.0 ^A	0.0 ^A	4.8 ^{ABC}
<u>0.0/0.5</u>	1.1 ^C	<u>22.9^A</u>	0.1 ^A	1.0 ^A	3.4 ^C
0.5/0.5	1.4 ^{BC}	18.3 ^{AB}	0.0 ^A	0.0 ^A	3.8 ^C
1.0/0.5	2.0 ^{ABC}	16.3 ^{AB}	0.0 ^A	0.0 ^A	6.5 ^{AB}
<i>Lippia graveolens</i>					
0.0/0.0	1.8 ^{DE}	22.1 ^{BC}	1.3 ^C	3.5 ^C	4.0 ^D
0.5/0.0	3.0 ^{CD}	16.9 ^{CD}	0.2 ^D	0.1 ^C	8.7 ^{BC}
1.0/0.0	4.4^A	14.4 ^{CD}	0.0 ^D	0.0 ^C	13.1^A
0.0/0.1	1.7 ^{DE}	29.4 ^B	2.3 ^B	14.1 ^B	5.1 ^D
0.5/0.1	3.2 ^{BC}	19.2 ^C	0.0 ^D	0.0 ^C	10.1 ^{BC}
1.0/0.1	3.6 ^{AB}	9.0 ^D	0.0 ^D	0.0 ^C	4.3 ^D
<u>0.0/0.5</u>	1.3 ^E	<u>44.9^A</u>	<u>5.1^A</u>	<u>47.0^A</u>	5.9 ^{DC}
0.5/0.5	2.3 ^{CD}	23.0 ^{BC}	0.0 ^D	0.0 ^C	6.0 ^{DC}
1.0/0.5	2.0 ^{DE}	9.5 ^D	0.0 ^D	0.0 ^C	4.0 ^D

Different letters present statistically significant differences $P \leq 0.05$.

The results of this research agree with those reported by García-Pérez *et al.* (2012); via direct organogenesis, they obtained shoots of *O. vulgare* in MS medium enriched with 1 mg L⁻¹ of BA. Castellano-Hernández *et al.* (2013) reported the induction of shoots of *L. graveolens* in MS medium enriched with 2.0 mg L⁻¹ of BA, achieving 2.4±3.8 shoots per explant, due to a combination of 0.1/0.5 mg L⁻¹ of IAA/BA of 6 mm and the induction of roots with 0.1 and 0.5 mg L⁻¹ of IAA with 7.3±4 and 5.3±2.9 roots per explant. The increase in the shoot length (63±17mm) and roots (100.0±47 mm) and up to 15 roots per explant were reported by adding 1.5 mg L⁻¹ of IBA.

The results of this work surpassed those reported for *L. graveolens* and *L. alba* (Bueno, 2014), where DM added with 2 mg L⁻¹ of BA reported three shoots per explant at 28 days in *L. alba* and DM added with 0.5/0.1/3 mg L⁻¹ of BA/AIB/phloridzin, they obtained 2.2 shoots per explant at 22 days. Sanchez-Velázquez *et al.* (2021) reported the micropropagation of *L. graveolens* from axillary buds in MS medium enriched with 2.5 mg L⁻¹ of BA, obtaining shoots with great success.

CONCLUSION

The germination of *L. graveolens* seeds can be achieved in MS medium after 15 days. Microcuttings of both *L. graveolens* and *O. vulgare* were successfully established in MS basal

medium enriched with 1 g L⁻¹ charcoal that showed low oxidation percentages and induced up to 90% the production of shoots in the explants. The mixture of 1.0/0.5 mg L⁻¹ BA/AIB induces callus formation for both species; when this medium is in contact with leaves as an explant, its formation is potentiated, achieving diameters up to 15 mm. In order to achieve the induction of shoots and roots, buds should be established in MS medium enriched with 0.5 mg L⁻¹ IBA for both species; this mixture increased the multiplication coefficients.

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