Article

Development of an efficient method for oregano micropropagation

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Abstract

Lippia graveolens, or Mexican oregano, is a plant of economic importance and the collection of foliage is mainly carried out from natural populations. Micropropagation proposes an alternative to produce material, with high phytosanitary quality and propagation in less time of individuals selected for their characteristics of commercial value. The objective of this work was to establish a protocol for the *in vitro propagation* of *Lippia graveolens.* The research was conducted in 2018-2019. The establishment was achieved with nodal segments in basal medium (MS), at this stage 57.32% of axenic explants were recovered. For the propagation stage four concentrations (0, 1, 2 and 3 mg L^{-1}) of benzylaminopurine (6-BAP) were added to the MS medium, the number of shoots and height of the plan were evaluated, the best results in multiplication were achieved with 3 mg L^{-1} of 6-BAP, obtaining 40.35 shoots per explant. The length of the shoot was longer in treatments without growth regulators. For rooting, it was tested with three concentrations (0, 0.1, 0.3 and 0.5 mg L^{-1}) of auxins (indole acetic acid and indole butyric acid) and the rooting percentage and root length were evaluated, obtaining the same result with and without auxin regulators. In the acclimatization phase, photoperiod (16 h and 24 h) was evaluated with and without mycorrhizas (*Glomus intraradicens*). The best treatment was obtained with 24 h light and the addition of mycorrhizas with 86.11% survival.

Keywords: *Glomus intraradicens*, biotization, photoperiod.

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Introduction

Non-timber forest resources (RFNM) represent an ecological, economic and cultural importance of our country's indigenous and rural communities. The collection of these resources can have negative effects associated with the level of abundance or loss of biodiversity. Therefore, it is necessary to find new sustainable management strategies that allow to minimize the impact. It has been observed that the specialized production of RFNM in an agroforestry system with greater ecological commitment, promotes environmental and subsistence results (Kusters *et al*., 2006).

Oregano is a worldwide distribution RFNM, which is mainly represented by two species: *Origanum vulgare* (Lamiaceace), native to Europe and *Lippia graveolens* (Verbenaceae), native to the Americas. The world production of oregano is estimated at around 15 000 t, with Turkey being the main producer followed by Mexico (García-Pérez *et al*., 2012). *L. graveolens* known as Mexican oregano is an aromatic plant native to the Southeastern USA, Mexico, Guatemala, Nicaragua and Honduras (Martínez-Rocha *et al*., 2008) belonging to the family Verbenaceae.

Its use involves the removal of leaves and stems (Ocampo-Velázquez *et al*., 2009; Granados-Sánchez *et al*., 2013). Due to its high content of essential oil, oregano is frequently used as a seasoning in food preparation and as a medicinal plant, it is used to treat headaches, rheumatic pain and as a diuretic (Laferriere *et al*., 1991; Marques *et al*., 2009).

Thymol and carvacrol are two of the active compounds of the essential oil obtained from Mexican oregano. These compounds are of special interest due to their antimicrobial properties against *Vibrio* species, it shows antifungal effect against *F. oxysporum* in tomato seeds and is acaricide against *Rhipicephalus microplus* (Paredes-Aguilar *et al*., 2007; Cueto-Wong *et al*., 2010; Martínez-Velázquez *et al*., 2011) and antioxidant (Dunford and Vázquez, 2005; Ávila-Sosa *et al*., 2010).

In the state of Coahuila, the utilization of *L. graveolens* is carried out in eight municipalities, however, it is in the southeastern region of the state, in the municipalities of Parras de la Fuente, General Cepeda and Ramos Arizpe, where the largest production is obtained, and an average of 700 t of dried leaf is generated annually (Villavicencio-Gutiérrez *et al*., 2010). It is mainly extracted from natural populations and its utilization coincides with flowering and alters the formation of fruits and seeds (Ocampo-Velázquez *et al*., 2009).

Over time the collection of plants can decrease the size and density of the population, as well as the reduction of genetic diversity in the utilization areas, therefore, the need arises to optimize a propagation protocol that allows the mass propagation of high-quality phytosanitary individuals, in addition to presenting desirable chemotypic characteristics, using biotechnological tools.

The first propagation protocol in *L. graveolens* was performed by Castellanos-Hernández *et al*. (2013), who used seed to generate *in vitro* plants, from which they obtained explants for the induction of shoots via direct organogenesis of axillary buds ero when coming from seeds they

have high variability in the concentration of thymol and carvacrol, which has an impact on their yield and quality (Soto *et al*., 2007). The objective of this work was to optimize an *in vitro* propagation protocol of the species *L. graveolens* by growing nodal segments.

Materials and methods

This research was carried out at the Plant Tissue Cultivation Laboratory of the Department of Plant breeding of the Antonio Narro Autonomous Agrarian University (UAAAN), in Saltillo Coahuila.

Plant material

The explants were collected from plants planted from a commercial plantation of two years old, located in the town of El Amparo (La Peña) municipality of Parras de la Fuente, Coahuila.

Stage 1. Establishment of nodal segments of *L. graveolens*

Explants of nodal segments of mother plants were taken in early spring, in March 2018. A disinfection pre-treatment of the segments was carried out to remove external contaminants, for this they were kept for half an hour in a chlorine solution at 10% (v/v), to which four drops of commercial detergent were added, then they were rinsed with running water. At this stage, the asepsis protocol of the explants was performed on the laminar flow hood.

The nodal segment had a height of approximately 2 to 3 cm, a 70% (v/v) ethanol solution was applied to it for one minute, then they were placed in a 20% sodium hypochlorite (Cloralex®) solution for 20 min and subsequently rinsed three times with sterile distilled water and planted in test tubes with 20 ml of culture medium Murashige and Skoog (1962) regulator-free and supplemented with 100 mg L^{-1} of myo-inositol, 1 mg L^{-1} of thiamine HCl, 1 mg L^{-1} of pyridoxine-HCl, 50 mg L^{-1} of cysteine, 30 g L^{-1} of sucrose, 4 g L^{-1} of phytagel (Sigma Aldrich) adjusting to a pH of 5.7 and sterilized in autoclave for 20 min at 121 °C.

An explant per tube was placed and approximately 250 explants of 2 cm were established. The growing conditions were: temperature of 25 ±1 °C, with 16 h light and 8 h of darkness at 2 500 lux. After 30 days the following variables were assessed: percentage of contaminated explants, percentage of dead explants, and percentage of healthy explants.

Stage II. Multiplication of *L. graveolens*

For this stage nodal and apical segments with four leaves were used, obtained from the previous stage, which were placed in glass bottles 6 cm in diameter and 8 cm high, with 20 ml of MS medium added with four concentrations of 6-BAP (0, 0.1, 2 and 3 mg L^{-1}) supplemented with 100 mg L^{-1} of myo-inositol, 1 mg L^{-1} of thiamine HCl, 1 mg L^{-1} of pyridoxine-HCl, 30 g L^{-1} of sucrose and 4 g L^{-1} of phytagel (Sigma Aldrich) adjusted to a pH of 5.7 and sterilized in autoclave for 20 min at 121 °C.

Four explants were placed per bottle and a total of eight treatments were obtained, each with 5 repetitions. 30 days after incubation in the growth chamber, the parameters were determined: number of shoots/explants and length of the shoot. The explants were continuously subcultured in the same combinations for five subcultures every 30 days. At the time of the subculture, the number of shoots/explants and the length of the shoot were evaluated in each of the treatments.

The shoots obtained at the multiplication stage were subcultured in MS medium added with 0.01 mg L^{-1} naphthalen acetic acid (ANA), 100 mg L^{-1} of myo-inositol, 1 mg L^{-1} of thiamine HCl, 1 mg L^{-1} of pyridoxine-HCl, 30 g L^{-1} of sucrose, 0.025 g L^{-1} of cysteine and 4 g L^{-1} of phytagel (Sigma Aldrich) adjusted to a pH of 5.7 and sterilized in autoclave for 20 min at 121 °C.

Stage III. Rooting

Shoots of 2.5 cm were taken and placed in glass bottles 6 cm in diameter and 8 cm high, with 20 mL of MS medium added with three concentrations of indole acetic acid (AIA), indole butyric acid (AIB) and a witness. Treatments were: IA0.1= 0.1 mg L^{-1} of AIA, IA0.3= 0.3 mg L^{-1} of AIA, IA0.5= 0.5 mg L^{-1} of AIA, IB0.1= 0.1 mg L^{-1} of AIB, IB0.3= 0.3 mg L^{-1} of AIB, IB0.5= 0.5 mg L^{-1} of AIB and AT= witness.

Four explants were placed per bottle and a total of six treatments and one absolute witness were obtained, each with five repetitions and they were placed in the incubation room under the conditions mentioned in the establishment. After 30 days the root length was evaluated.

Stage IV. Acclimation

For this stage, seedlings 3-3.5 cm high were selected, set in a completely random design with five treatments: (T1) witness (with irrigation), (T2) photoperiod of 16 h (2 500 lux), (T3) photoperiod of 16 h (2 500 lux) plus mycorrhizas; (T4) photoperiod of 24 h (2 500 lux) and (T5) photoperiod of 24 h (2 500 lux) plus mycorrhizas.

For the hardening of seedlings, the bottles were opened one week before being placed in a greenhouse, then the seedlings were removed from the culture medium, washed with distilled water and mycorrhizas (*Glomus intraradicens* 46 spores g^{-1}) were applied to the T3 and T5 treatments. Each treatment consisted of 36 seedlings placed in trays with peat-moss:perlite substrate (1:1), the plant material was placed in the greenhouse, with humidifier equipment (Hydrofogger) applying 1.5 L h^{-1} every 4 h and at 30 days the percentage of survival was evaluated.

Statistical analysis

For the propagation stage a completely random design was made with factorial arrangement (2 x 4), where the factors were: a) type of explants (nodal and apical segments); and b) concentrations of 6-BAP (0, 1, 2 and 3 mg L^{-1}). At the rooting stage, a variance analysis (Anova) with completely random design was performed and the means were compared by the Tukey test ($p< 0.05$). The analyses were performed using the statistical computing language R (Core Team 2017). For the acclimatization stage, a survival percentage was assessed.

Results

Stage I. Establishment of nodal segments of *L. graveolens*

The concentration and application time of the disinfectant significantly influenced the results of this experiment (Table 1). The best results show 57.32% survival of the explants when applying the disinfection method (time and concentration). 100% contamination and dead explants were observed in the witness. Contamination in sodium hypochlorite treatment was 32.52%, while dead explants accounted for 10.16%.

Table 1. Effect of sodium hypochlorite on disinfection of nodal explants in *L. graveolens.*

NaClO (%)	Time (min)	(%) contaminated explants	(%) of dead explants	(%) of healthy explants
0%	20	100 b	0	0
20%	20	32.52 a	10.16	57.32

Mean with equal letters do not differ statistically (One-Way Anova, Tukey, $p< 0.05$).

Stage II. Multiplication of *L. graveolens*

The shoots obtained from the two adventitious buds were used as a source of explants in the multiplication process. The dose of 6-BAP used in the culture medium, significant differences ($p< 0.05$) were observed in the multiplication and length of the shoot in both types of explants (nodal and apical segment) *of L. graveolens* (Table 2).

Table 2. Effect of different concentrations of 6-BAP on the multiplication of *L. graveolens* shoots on two types of explants. B0= Witness; B1= BAP 1 mg L^{-1}; B2= BAP 2 mg L^{-1}; B3= BAP 3 mg L^{-1}.

BAP (mg L^{-1})	Apical segment		Nodal segment	
	Number of shoots per explant	Length of the shoot (cm)	Number of shoots per explant	Length of the shoot (cm)
B0= 0	4.9 ±1.92 b	2.2724 ±0.34 a	3.1 ±1.5 b	1.5387 ±0.39 a
B1= 1	26.65 ±7.74 a	1.1103 ±0.07 c	29 ±7.9 a	1.1058 ±0.06 b
B2= 2	24.65 ±6.08 a	1.4971 ±0.08 b	31.5 ±15.82 a	0.7965 ±0.05 c
B3= 3	22.65 ±10.33 a	1.0796 ±0.07 c	40.35 ±17.49 a	0.7083 ±0.03 d

Mean with equal letters do not differ statistically (One-Way Anova, Tuckey, $p< 0.05$).

The highest number of shoots per explant was 40.35 in the nodal segment at a concentration of 3 mg L^{-1}, while the witness had the lowest number of shoots in both the nodal and apical segments with 4.9 and 3.1 respectively (Figure 1). Significant differences ($p< 0.05$) were observed for the shoot length variable in the concentrations of 6-BAP and in the two types of explants.

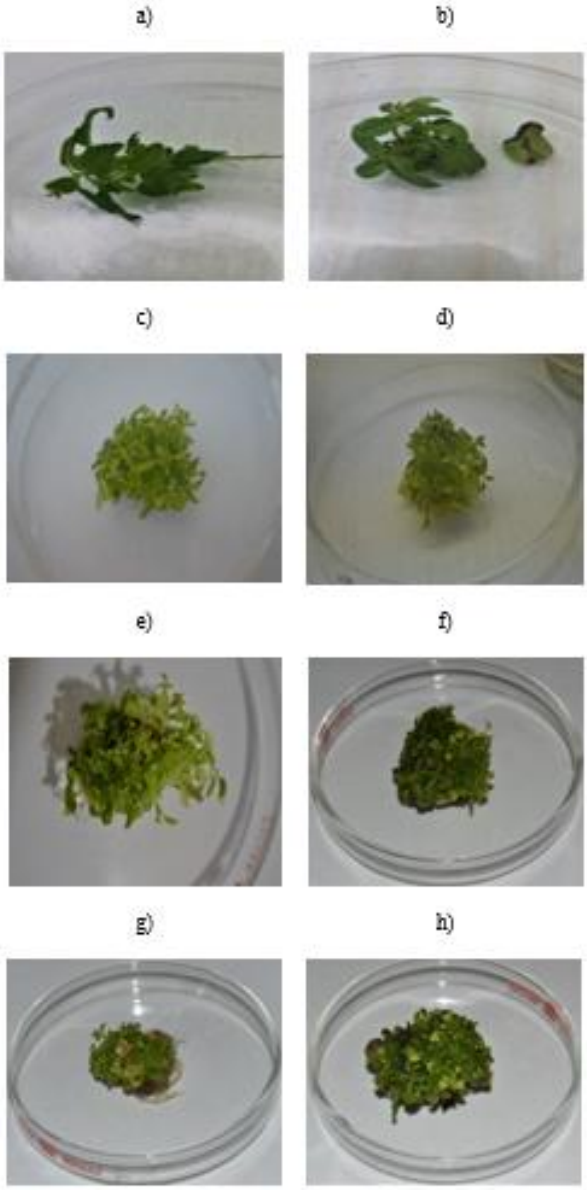

Figure 1. Results obtained by applying different concentrations of 6-BAP in the multiplication of *L. graveolens* in two types of explants (nodal and apical segments). B0 (witness): apical segment (a); nodal segment (b). B1 (1 mg L^{-1}): apical segment (c); nodal segment (d). B2 (2 mg L^{-1}): apical segment (e); nodal segment (f). B3 (3 mg L^{-1}): apical segment (g); and nodal segment (h).

The results show the influence by explant type and concentration of 6-BAP. The witness of the apical segment presented the largest elongation with 2.27 cm. In nodal segments the decrease in shoot length was shown gradually as the concentration of 6-BAP increased, in apical segments this pattern is not explained as the concentration of 2 mg L^{-1} of 6-BAP generates a length greater than the other two concentrations of 6-BAP (Figure 2).

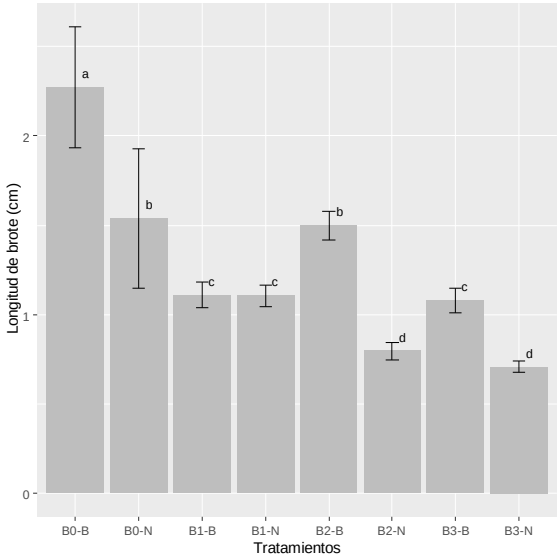

Figure 2. Effect of different concentrations of 6-BAP on the length of *L.* graveolens shoots on two types of explants. B0-B= witness apical segment, B0-N= witness nodal segment; B1-B= BAP 1 mg L^{-1} apical segment; B1-No= BAP 1 mg L^{-1} nodal segment; B2-B= BAP 2 mg L^{-1} apical segment; B2-N= BAP 2 mg L^{-1} nodal segment; B3-B= BAP 3 mg L^{-1} apical segment; B3-N= BAP 3 mg L^{-1} nodal segment.

Stage III. Rooting

In all treatments 100% rooting was obtained, and no significant differences were present ($p < 0.05$) in the root size between treatments (Figure 3).

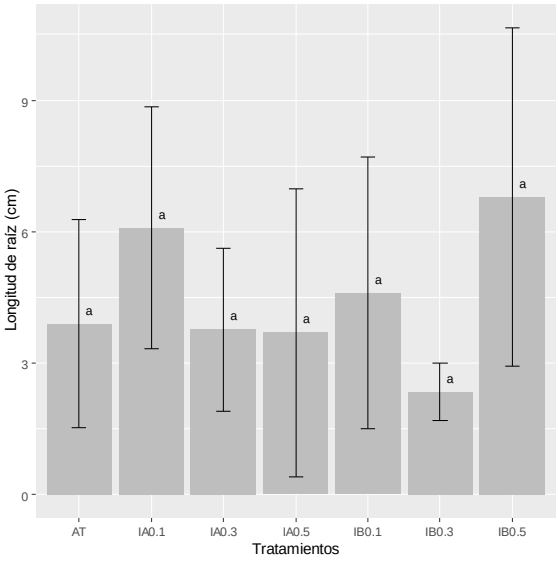

Figure 3. Effect of different concentrations of AIA and AIB on the root length of *L. graveolens* shoots. AT= witness; IA0.1= AIA 0.1 mg L^{-1}; IA0.3= AIA 0.3 mg L^{-1}; IA0.5= AIA 0.5 mg L^{-1}; IB0.1= AIB 0.1 mg L^{-1}; IB0.3= AIBA 0.3 mg L^{-1}; IB0.5= AIB 0.5 mg L^{-1}. Means with equal letters do not differ statistically, Tukey ($p < 0.05$).

Stage IV. Acclimation

Acclimatization results show that the increase in photoperiod increases seedling survival and this result is improved when mycorrhizas are applied in combination with photoperiod, the best treatment (T5) achieved a survival percentage of 86.11% compared to the witness who had only 33.33% (Figure 4).

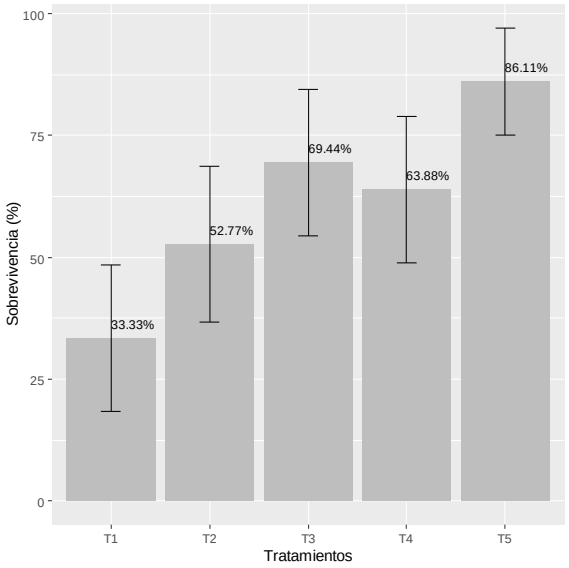

Figure 4. Percentage of survival of *L. graveolens* in different treatments with and without mycorrhizas and two photoperiod times. T1= witness; T2= 16-hour photoperiod; T3= photoperiod of 16 h plus mycorrhizas; T4= 24-hour photoperiod; T5= photoperiod of 24 h plus mycorrhizas.

Discussions

At the stage of establishment of *the in vitro* culture an adequate aseptic technique is required; however, other factors to consider are the source of the explant, its phytosanitary state and the type of explant. The disinfection method used in this experiment during *in vitro* establishment has been used in other species of the genus *Lippia* with different results.

In this study the plant material was obtained from a commercial plantation and the percentage of contaminated explants was 32.52%, similar results were observed in *L. gracilis* where 36.5 5% of contaminated explants were obtained, also from material from the field, where the process of disinfection of ethanol at 70% for one minute was performed, and then disinfection with calcium hypochlorite at 3% was carried out (Marinho *et al*., 2011).

However, in the species *L. grata* the pollution did not exceed 3% using ethyl alcohol at 70% for 1 minute and sodium hypochlorite at 2% with 50 µL of de Tween 20 for 20 minutes with plants coming from greenhouse and with a previous phytosanitary treatment (Palhares-Neto *et al*., 2018). As mentioned above, the source of the explante and the phytosanitary quality of the mother plant are decisive in determining good results.

On the other hand, the type of explant used may present the risks mentioned by Cassells (2011), who points out that apical meristem is the appropriate explant because the leaves, stems and nodal explants have a high risk of contamination since microrganisms can lodge in intercellular and intracellular spaces, in the cuticle of the leaves and in cavities in the stems.

In this study the number of healthy explants obtained allowed the multiplication stage to be established using cytokinin 6-BAP. This type of growth regulator stimulates protein synthesis and participates in cell cycle control and applied to the culture medium for shoot induction overcomes apical dominance and releases the lateral buds from latency (Van Stadent *et al*., 2007). Cytokinins during this stage may be alone or combined with a lower concentration of auxins to generate a better morphogenetic response (Okubo *et al*., 1991; Sjahril *et al*., 2016).

In the case of establishing without auxins, it is considered that there is endogenous supply of auxins that balances hormonal needs to initiate organogenesis (Hu *et al*., 2017). As in the case of citrus explants *Citrus sinensis* and *Citrus limon*, where the presence of auxins causes inhibition of the formation of adventitious shoots making it necessary to remove them (Hu *et al*., 2017).

In marigold it is mentioned that the source of explant is a way to take advantage of the ability to synthesize auxins of apical explants, as they used three types of nodal explants (apical, middle and basal) and it was in the apical explant where more leaves and adventitious shoot size were obtained, but fewer shoots, also rooting was higher in the apical explant and decreased in the middle and basal regions (Pimentel-Victorio *et al*., 2012).

In this research the presence of endogenous auxins of the explants (knots and nodal segments) in combination with three concentrations of 6-BAP generated a hormonal balance that stimulated the induction of axillary shoots in the multiplication stage. The type of explant did not significantly influence in relation to the number of shoots obtained, nor did the concentrations of 6-BAP significantly affect the two types of explants.

However, a difference with the witness was established, where the fewest shoots occurred in both explants. However, the number of shoots obtained with the hormone 6-BAP was higher than the results reported in another research of this species. Muñoz-Miranda *et al*. (2019) reports a maximum of 6.4 shoots with BA at a concentration of 0.5 mg L^{-1}, while Castellanos-Hernández *et al*. (2013) mentions 2.8 shoots per explant with BA at that same concentration.

The 6-BAP growth regulator has also generated a different response at the multiplication stage in each of the species of the genus *Lippia*, where it has been used. In *L. junelliana*, 13.5 shoots per explant were obtained by adding 0.04 µM AIB plus 4.4 µM of BAP (Juliani *et al*., 1999), on the other hand, in *L. alba* the number of shoots obtained was 6.4 shoots per explant with 5 mg L^{-1} of BAP (Gupta *et al*., 2001), *in L. javanica* 2.42 shoots per explant were observed with 0.25 mg L^{-1} of BAP (Ara *et al*., 2010), equally in *L. rotundifolia* 2.6 shoots per explant with 0.33 µM of BAP (Resende *et al*., 2015) and in *L. integrifolia* an average of 16 shoots per explant was obtained with 2.2 µM BAP (Ianicelli *et al*., 2016), as for *L. filifolia*, Pereira-Peixoto *et al*. (2006) obtained 27.03 shoots per explant at a concentration of 4.5 µM of BAP plus 54 nM of ANA.

This difference between apical and nodal segments may be due to endogenous auxins produced by meristem as in the results reported in marigold (Pimentel-Victorio *et al*., 2012). In the results of nodal segments, a decrease in the length of shoot of 45% was observed between the witness and the higher concentration of cytokinin. The effect was directly proportional to the increase in the concentration of 6-BAP.

This effect of the regulator was also reported by Juliani *et al.* (1999) in *L. junelliana*, where they indicate that the concentration of this regulator suppressed the elongation of shoots. On the other hand, Gupta *et al*. (2001) mention that, in the micropropagation of *L. alba*, the concentration of benzylaminopurine affected the growth of the shoots produced. Similarly, Pereira-Peixoto *et al*. (2006) in *L. filifolia* report that the number and size of the shoots was adjusted to a quadratic function in response to the increase of this regulator.

This could be the cause of the decrease in length shoot when the 6-BAP concentration is increased, as they would not only limit water absorption but nutrient absorption. Similarly, Resende *et al.* (2015) report a decrease in shoot length in *L. rotundifolia* when the BAP concentration was higher. The results obtained at the rooting stage showed no significant differences in root length between the witness and AIA and AIB concentrations, 100% rooting was also observed in all treatments, this result had already been previously reported in other species of the genus *Lippia*, as in the case of *L. alba*, where 100% root formation was obtained in the witness (Gupta *et al*., 2001).

In *L. Junelliana* the best rooting percentage was obtained in a hormone-free environment that also produced a long and vigorous root system (Juliani *et al*., 1999). In addition, in *L. rotundifolia* the witness did not exceed the best concentration of ANA (0.44 µM) in root formation and shoot size, since it was similar to those obtained in the other three concentrations used (Resende *et al*., 2015) Similarly, in *L. filifolia* the witness obtained results similar to three concentrations of auxin ANA but did not exceed the best concentration of 0.11 µM ANA in the number of roots and that of 0.22 µM in the root length (Pereira-Peixoto *et al*., 2006).

The results obtained at the acclimatization stage show a synergistic effect between photoperiod and biotization in the acclimatization of *L. graveolens.* Prolonged photoperiod has been observed to modify the morphology and physiology of plants by generating responses that favor plant development. Adams and Langton (2005) mention that, when applying a prolonged photoperiod, an increase in dry weight is observed, and possibly an increase in photosynthesis, it is also accompanied by a larger foliar area with greater number of cells and cell size.

On the other hand, photoperiod favors an increase in stoma and stomatic density. Zacchini *et al*. (1997) mention that in *in vitro* cultivation of fruit tree shoots the density of stomata was higher in leaves exposed to continuous light, on the other hand, when the same number of light/darkness hours were applied at different intervals (4/2, 16/8), modification in density and stomatic index was observed, the most favorable result was when short periods of light/darkness were applied.

Also, in strawberry seedlings subjected to photoperiods of 16 and 24 h light, the growth was greater than in sunlight, although all treatments provided the same integral daily light (Tsuruyama and Shibuya, 2018). Meanwhile, Fortini *et al*. (2020) also observed greater influence on biomass

growth and content, with high photosynthetic rates, affecting primary and secondary metabolism with differences in the production of soluble sugars, glycogen, amino acids and bioactive compounds.

Biotization has also increased plant survival when applied at the acclimatization stage. Mirjani *et al*. (2018) observed at the acclimatization stage of *Satureja khuzistanica* when applying *Glomus fasciculatum*, it facilitated the uptake of different elements, contributing to a rapid recovery of plants during this stage, in addition an increase of 17.4% was observed in the survival of plants.

Similarly, Bidabadi and Masoumian (2017) applied *Glomus intraradicens* and evaluated its effect on micropropagated *Stevia rebaudiana* plants, under salinity conditions, they observed a decrease in damage by reducing oxidative stress and increasing photosynthetic efficiency. On the other hand, in banana the application of mycorrhizas decreased the mortality of micropropagated seedlings and increased the quality of these (Ortas *et al*., 2017); therefore, it can be inferred that photoperiod and the addition of mycorrhizas during acclimatization improve the survival percentages.

Conclusions

An *in vitro* propagation protocol for *L. graveolens* was established, which will allow the conservation of the species in the long term, in addition to the establishment of homogeneous specialized plantations. The multiplication of *L. graveolens* is influenced by the type of explant, its acclimatization is dependent on light that can be successfully achieved with the application of mycorrhizas to improve and reduce stress at this stage.

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