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ORIGINAL ARTICLE

Enhancing drought tolerance in *Lippia graveolens* through ethyl methanesulfonate (EMS)-induced mutagenesis

Araceli Rodriguez-Sahagun*, Osvaldo A. Castellanos-Hernández, Gustavo J. Acevedo-Hernández, Jesus M. Hernández-Zaragoza, Rayn C. Aarland

Centro Universitario de la Ciénega, Universidad de Guadalajara, Av Universidad 1115, Ocotlan, Jalisco, Mexico

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*Corresponding address:
aracelicrs@gmail.com

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Abstract

Mexican oregano (*Lippia graveolens*), belonging to the Verbenaceae family, is an aromatic and perennial herb that produces an essential oil rich in the monoterpenes thymol and carvacrol, widely utilized in various industries. Endemic to Mexico, it predominantly thrives in arid and semi-arid regions, typically displaying notable drought tolerance. However, previous studies reveal that irrigation frequency significantly influences biomass production, prompting the need for further improvement in drought tolerance in this species, especially when considering future climate change scenarios. This study employed chemical mutagenesis with ethyl methanesulfonate (EMS) to create new genetic variants through induced mutations. Seeds of *L. graveolens* underwent EMS treatment at varying concentrations (0.1 and 0.2%) and exposure times (1, 3 and 6 hours), and then aseptically germinated on MS medium. Nodal segments from resulting seedlings were used as explants for multiple shoot proliferation using 50 g $\cdot$ l^{-1} of polyethylene glycol (PEG) as a selective agent for drought tolerance, where non-mutagenized plants displayed severely inhibited development and necrosis. Twenty-five putative mutants tolerant to osmotic stress were recovered, and some of them showed evident morphological alterations and significant changes in the content of phenols and flavonoids, compounds associated with responses to stress. These results highlight the effectiveness of chemical mutagenesis as a strategy for genetically enhancing drought tolerance in Mexican oregano.

Keywords: drought stress, *in vitro* culture, Mexican oregano, phytochemistry, polyethylene glycol

Introduction

Lippia graveolens, commonly known as Mexican oregano, is an aromatic perennial shrub that belongs to the Verbenaceae family. It is considered to be a non-timber forest species, with organoleptic and medicinal properties that make it highly attractive for the pharmaceutical, cosmetic and food industries, being one of the most commercially relevant species within this family. The plant is endemic to Mexico and it is mainly located in arid and semi-arid regions in the central part of the country. It has oval-shaped serrated leaves with pointed tips, a hairy woody stem and small white flowers. The plant is a rich source of bioactive compounds with several properties (e.g., antioxidant, antimicrobial, and anti-inflammatory). Its commercial value stems largely from its essential oil, characterized by a high content of the phenolic terpenes thymol and carvacrol (Muñoz-Miranda *et al.* 2019; Bautista-Hernández *et al.* 2021; Bautista-Hernández *et al.* 2023). One of the most common applications is the use of its essential oil as a conservative for packaged food and as a broad-spectrum antimicrobial due to its remarkable inhibitory effect on the growth of bacteria (Hernández-Hernández 2019; Reyes-Jurado *et al.* 2020) and fungi such as *Candida albicans* (Herrera-Rodriguez *et al.* 2019).

Mexican oregano is adapted to grow under low water availability, which makes it a suitable alternative for agricultural production in arid areas of Mexico. Although oregano plants do not require frequent watering in the early stages of growth, it has been shown that in latter stages there is a linear relationship between the amount of water applied during irrigation and the overall plant biomass production (Llamas-Torres *et al.* 2022). This behavior suggests that water availability significantly influences the harvest yields of oregano. Therefore, projections of future drought affecting the distribution area of this species, as per climate change scenarios, are anticipated to have a considerable adverse impact on oregano production (Balting *et al.* 2021). Climate change directly impacts the agricultural sector by increasing various types of abiotic stress, such as drought and salinity, leading to reductions in crop yields, which can exceed 50% worldwide. The closure of stomata, as a mechanism to protect against water loss, limits the CO_2 concentration, inhibiting the productivity of the photosynthetic process. Lowering of CO_2 levels also promotes the formation of reactive oxygen species (ROS), which, in turn, cause the oxidation of important molecules such as proteins and lipids. Besides its direct effect on plant physiology, abiotic stress also severely affects agricultural productivity by causing a reduction in genetic diversity and increasing the risk of germplasm loss (Muscolo *et al.* 2014; Kopecká *et al.* 2023).

Various biotechnology approaches have been employed to enhance abiotic stress tolerance in plants. One of the most frequently used methods, mutation breeding, relies on applying physical or chemical mutagens to plant material to artificially increase the number of mutations and expand the genetic variability of plant species of agronomic interest. This approach has contributed to the creation of plant varieties with enhanced phenotypic characteristics, including environmental stress tolerance (Channaoui *et al.* 2019b; Mullins *et al.* 2021). In the case of plants containing bioactive compounds, mutagenesis allows for the generation of varieties with a higher content of these metabolites (Shah *et al.* 2020). Ethyl Methanesulphonate (EMS) stands out as one of the chemical mutagens commonly used in mutation breeding, typically inducing random point mutations in the plant genome. The dosage and duration of EMS application are crucial factors influencing mutagenesis efficiency (Channaoui *et al.* 2019a; Olaolorun *et al.* 2019). Noteworthily, EMS mutagenesis has also been effective for the development of novel stress-resistant microbial agents that exhibit plant-beneficial attributes, offering potential solutions for agricultural challenges posed by climate change (Wongwanich *et al.* 2017).

After mutagenesis, to select phenotypes of interest in the laboratory, efforts are made to recreate the conditions to which the plants will be exposed. The goal is to identify those individuals that exhibit better development or fewer impairments in such an environment. In order to mimic drought stress under *in vitro* conditions, a variety of compounds called osmolytes have been used as selective agents when added to the culture medium. Two of the most commonly used osmolytes are mannitol and polyethylene glycol (PEG), which act by lowering water potential, making it more difficult for plants to absorb water from the medium. Using this procedure, drought-tolerant mutants of crops such as alfalfa, sugarcane, rapeseed and radish have been successfully obtained (Khalil *et al.* 2018; Tiryaki *et al.* 2022; Chen *et al.* 2023; Gonzalez *et al.* 2024).

Previous attempts have been made to apply EMS-induced mutagenesis in order to increase genetic diversity in Mexican oregano. In that context, nodal segments were used as plant material and the effect of the mutagen on the *in vitro* regeneration rate was evaluated. The genetic variability of the plants obtained was assessed using molecular markers (Muñoz-Miranda *et al.* 2019). The main goal of the present study was to obtain mutant plants of *Lippia graveolens* through the exposure of seeds to EMS and the selection of lines that showed improved performance under drought stress induced by either mannitol or PEG.

Materials and Methods

Plant material

Seeds were obtained from *L. graveolens* plants collected from wild populations in the municipality of Colotlán, Jalisco, at the following geographic coordinates (via GPS): 22º01'43.12" N 103º15'01.9" W and 22º01'44.2" N 103º14'41.0" W. Seeds from two plants were sampled at each location and pooled to obtain a single bulk used for all experiments. The collection site is located at 1,720 meters above sea level.

In vitro germination

For disinfection, seeds placed in Eppendorf tubes were first soaked in 70% ethanol for 1 min and then rinsed three times with sterile distilled water (1 min each). Subsequently, washed seeds were immersed for 3 min in 30% commercial bleach (1.68% available sodium hypochlorite) and rinsed three times with sterile distilled water for 1 min. Half-strength MS medium (Murashige and Skoog 1962) was used for the germination of disinfected seeds. This medium was supplemented with 1% sucrose and 0.25% Phytagel™, and the pH was adjusted to 5.8 before autoclaving at 121°C for 15 min. Seeds placed on MS medium were incubated at 24°C

with a photoperiod of 16/8 h light/dark under white fluorescent lamps with an intensity of 32 µmol · m^{-2} · s^{-1} (Muñoz-Miranda *et al.* 2019).

Formulation of culture media for selection of mutants

Mannitol and PEG 6000 were added to the culture medium to simulate drought stress. To determine the minimal concentration at which these osmolytes impair plant growth, culture medium was prepared as full-strength MS with 3% sucrose and 0.25% Phytagel™, and the pH was adjusted to 5.8 before autoclaving. This medium was supplemented with either mannitol (50, 100 and 150 mM) or PEG (10, 25 and 50 g · l^{-1}). Nodal segments obtained from *in vitro* germinated seedlings were placed on each medium. MS medium without any of the osmolytes was used as a control. Each treatment was comprised of three explants per flask with 10 replicates. After 30 days, morphological characteristics of the shoots produced from the axillary buds were evaluated. Features such as color of the tissue, presence of hyperhydric malformations (vitrification), necrosis, and overall survival were recorded.

Chemical mutagenesis

After disinfection, selected seeds were exposed to a sterile aqueous solution of EMS at two concentrations (0.1 and 0.2%) with three exposure times (1, 3 and 6 hours). EMS concentrations and exposure times were established based on those commonly used for seeds and previous experience with the mutagenesis of vegetative tissues in *L. graveolens* (Muñoz-Miranda *et al.* 2019; Chen *et al.* 2023). One-hundred seeds were used for each treatment. A control without EMS was also included. After the treatments, the seeds were rinsed seven times with sterile distilled water (1 min each) to remove excess EMS. Mutagenized seeds were then sown on MS medium prepared as previously described for seed germination.

Selection and micropropagation of putative mutant plants

The micropropagation of putative mutant *L. graveolens* plants was carried out according to the procedure described by Castellanos-Hernández *et al.* (2013). M1 plants derived from EMS-mutagenized seeds were used as the source of explants. Three nodal segments from these plants were placed on selective MS medium containing the appropriate concentration of either mannitol or PEG for selection of mutants as determined previously, and supplemented with 2 mg · l^{-1} of benzyladenine (BA). After 100 days, plants that successfully coped with osmotic stress and did not exhibit severe growth impairment were transferred to MS medium without the addition of the osmolyte for regeneration. Following regeneration, plants showing better performance were selected for a second cycle of micropropagation.

Measurement of morphological traits

Two months into the second cycle of micropropagation, the morphological characteristics of the plants such as the number of activated buds, plant height (only aerial part), and the number of leaves were evaluated.

Phytochemical analysis

Preparation of the crude extract

One plant from each putative mutant genotype was selected for phytochemical analysis. For each plant, three replicates, consisting of two leaves, were prepared. The weight of the leaves was recorded, and methanolic extracts were obtained by placing the leaves in Eppendorf tubes and adding 1.5 ml of methanol. The tubes were left for 24 hr with continuous agitation. The liquid was recovered in a new tube and this crude methanolic extract was used for further determinations.

Determination of total flavonoid content (TFC)

The total flavonoid content was quantitatively determined as described by Pedro *et al.* (2023). A standard solution of quercetin in methanol was used to plot a calibration curve in the concentration range of 10–100 µg · ml^{-1}. The determination of TFC was performed by mixing 500 µl of crude methanolic extract with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. This mixture was incubated for 30 min at room temperature, and then absorbance was measured at 415 nm using a spectrophotometer. Results were reported as mg of quercetin equivalents per 1 g of fresh weight (mg QE · gfw^{-1}).

Determination of total phenolic content (TPC)

The total phenolic content of the extracts was estimated using the Folin-Ciocalteu method as described by Waligóra *et al.* (2023) with some changes. First, the Folin-Ciocalteu reagent was diluted to a ratio of 1 : 10 with distilled water; then, 1 ml of this diluted reagent was mixed with 200 µl of the crude methanolic extract and incubated for 1 min at room temperature.

Subsequently, 0.8 ml of 7.5% sodium carbonate was added, and the mixture was incubated for 1 hr at room temperature. Following the same procedure, a standard solution of gallic acid in methanol was used to construct a calibration curve in the concentration range of 25–200 μg · ml^{-1}. The absorbance was measured at 765 nm using a spectrophotometer and results were expressed as mg of gallic acid equivalents per 1 g of fresh weight (mg GAE · gfw^{-1}).

Statistical analysis

Values were expressed as means ± standard deviation (SD). Data were analyzed using one-way analysis of variance ANOVA and Post-hoc analysis was conducted using Tukey's pairwise comparison. A multiple range test was carried out to determine whether significant differences occurred between individual treatments at a significance level of $p < 0.05$. The software Statgraphics Centurion XVI v.16.103 was used for all analyses.

Results

Selective medium

Lippia graveolens seeds were not directly sown on MS medium containing either mannitol or PEG since germination was severely inhibited by both osmolytes (data not shown). Therefore, seeds were first germinated on MS without these compounds, and then nodal explants from the resulting seedlings were taken for micropropagation on medium supplemented with the osmolytes. The explants exposed to mannitol did not show evident alterations in their development at any of the concentrations used. For this reason, this osmolyte was not subsequently used as a selective agent for the identification of putative mutants tolerant to drought stress. On the other hand, PEG did affect the growth of the plants when added to the culture medium, with this effect being more pronounced at 50 g · l^{-1}. At this concentration, plants showed hyperhydricity (formerly known as vitrification), chlorosis, and finally necrosis by the end of the incubation period (Fig. 1a). Hence, MS medium supplemented with PEG 50 g · l^{-1} was used as a selective medium for the identification of mutant plants showing enhanced tolerance to drought stress.

Chemical mutagenesis

Exposure to EMS affected the germination rate of the seeds at all doses and times tested, although there was a general trend of decreased germination rates with increased dose and exposure time (Table 1). Seedlings that germinated and grew after the mutagenesis treatment were used as a source of explants for micropropagation on selective medium containing PEG. Plants developed from explants, showing no evident growth impairment, were considered to be putative mutants tolerant to drought stress. Representative images of the putative mutants are shown in Fig. 1. A total of 25 of these tolerant plants were obtained (Table 1) and transferred to MS medium without PEG for micropropagation and further morphological and phytochemical analyses. Putative mutant plants were labeled using codes LGM01 to LGM25.

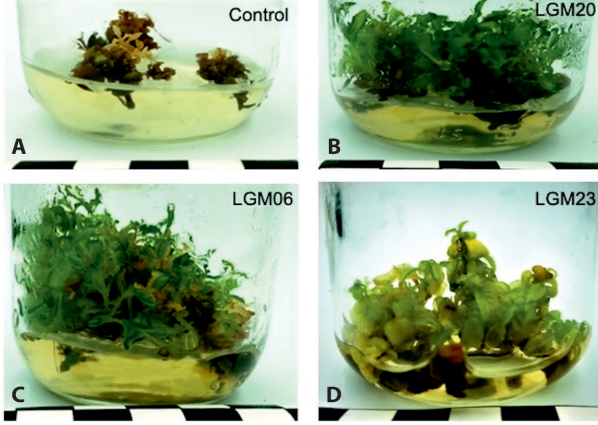

Fig. 1. Selection of putative EMS-induced drought-tolerant *L. graveolens* mutants. Development of nodal segments from A – non-mutagenized oregano plants, and three putative mutants: B – LGM20, C – LGM06 and D – LGM23, after 100 days on MS medium supplemented with PEG 50 g · l^{-1} as a selective agent

Table 1. Effect of EMS treatments on the germination rate of *Lippia graveolens* seeds

	EMS Concentration						
	Control	0.1%			0.2%		
	Exposure time [h]						
	0	1	3	6	1	3	6
Germination rate	65%	26%	11%	4%	18%	6%	5%
Number of putative mutant plants	–	9	4	1	7	3	1

Table 2. Morphological characteristics of selected *Lippia graveolens* mutant plants

	Mutant plants						
	control	LGM06	LGM11	LGM14	LGM20	LGM23	LGM25
Number of leaves	15 ± 1.0 b	14 ± 1.0 b	11 ± 2.0 c	20 ± 3.0 a	9.0 ± 0.5 c	11 ± 1.0 c	20 ± 3.0 a
Number of axillary buds	18 ± 1.0 a	6.0 ± 0.2 b	3.5 ± 1.0 b	20 ± 2.0 a	4.0 ± 1.0 b	4.5 ± 0.5 b	20 ± 2.0 a
Plant height (cm)	13.6 ± 1.0 c	21.3 ± 1.5 a	18.1 ± 1.0 b	4.0 ± 1.5 d	24.3 ± 2.0 a	3.5 ± 0.5 d	4.0 ± 1.5 d

Values are expressed as the mean ± standard deviation (SD). Letters next to the values represent homogeneous groups; within each row, different letters indicate significant difference at $p < 0.05$ using ANOVA and Tukey's HSD test

Morphological characteristics

Putative mutant plants showing optimal development after two cycles of micropropagation on MS medium supplemented with benzyladenine were selected for analysis of their morphological traits. Some of these plants displayed noticeable differences in height, number of leaves, and the proliferation of axillary buds compared to non-mutagenized plants, as shown in Table 2. Even though those characteristics were not measured, in several of the potential mutants obtained, noticeable alterations were observed in the color, size, and shape of the leaves, as well as the abundance of trichomes. Representative images of the putative mutants, alongside the control plants, are shown in Figure 2.

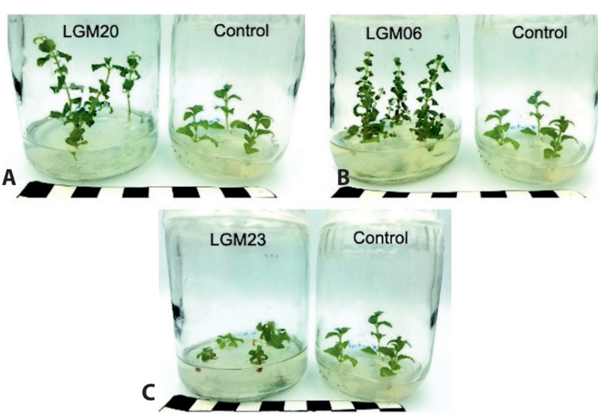

Fig. 2. Morphological variations observed in EMS-induced *Lippia graveolens* mutants during micropropagation. Development of nodal segments from PEG-resistant plants after 1 month on PEG-free medium for micropropagation: A – LGM20, B – LGM06, C – LGM23. In all panels, the flask on the right contains non-mutagenized control plants

Phytochemical analysis

The total flavonoid content (TFC) in the *Lippia graveolens* mutants varied significantly across the different plant samples evaluated (Fig. 3). Mutants LGM14, LGM20, LGM23, and LGM25 exhibited notable increases in TFC compared to the control plants

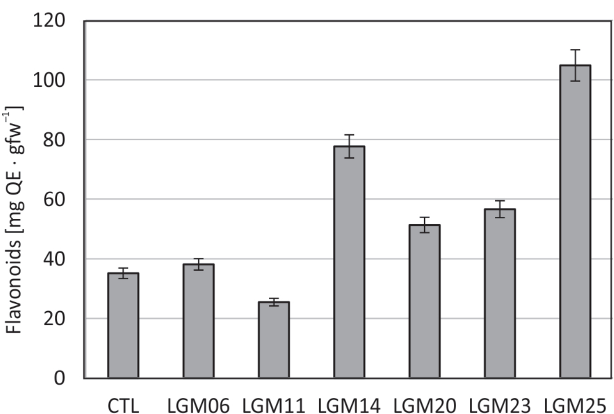

Fig. 3. Quantitative determination of total flavonoids content (TFC) of *L. graveolens* mutants. Means of the TFC for three replicates and standard deviation are shown. Different letters above the bars indicate significant differences ($p < 0.05$). Control (CTL) corresponds to non-mutagenized oregano plants

(CTL). In particular, mutant LGM25 showed the highest flavonoid content, reaching approximately 130 mg QE · gfw^{-1}, which was significantly higher than all other samples ($p < 0.05$). LGM14 and LGM23 also demonstrated a significant increase in TFC relative to the control, with values around 80 and 70 mg QE · gfw^{-1}, respectively. In contrast, plants LGM11 and LGM06 exhibited lower flavonoid levels, with LGM11 showing the lowest concentration, approximately 20 mg QE · gfw^{-1}, with no significant differences compared to the control. These findings suggest that mutagenesis in certain genotypes enhanced flavonoid accumulation, which was potentially linked to an increase in secondary metabolite production as a response to induced genetic changes.

The total phenolic content (TPC), expressed in milligrams of gallic acid equivalents per gram of fresh weight (mg GAE · gfw^{-1}), was evaluated in *Lippia graveolens*, including a non-mutagenized control (CTL) and six mutant lines (LGM06, LGM11, LGM14, LGM20, LGM23, and LGM25) (Fig. 4).

Statistical analysis revealed significant differences ($p < 0.05$) in TPC between the treatments, as indicated by distinct letters above the bars. The mutant line LGM25 exhibited the highest TPC, significantly exceeding all other plants. In contrast, LGM11 displayed

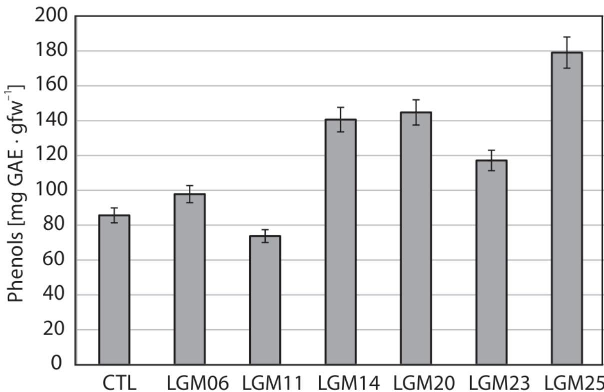

Fig. 4. Quantitative determination of total phenolics content (TPC) of *L. graveolens* mutants. Means of the TPC for three replicates and standard deviations are shown. Different letters above the bars indicate significant differences ($p < 0.05$). Control (CTL) corresponds to non-mutagenized oregano plants

the lowest TPC. These results indicate that mutagenesis can induce substantial variations in the phenolic content of *L. graveolens*, highlighting its potential as a tool for enhancing phenolic compound levels in this species.

Discussion

Ethyl methanesulfonate (EMS) is a potent alkylating agent that induces high-frequency random chemical modifications in nucleotides, particularly guanine, which often results in point mutations by converting GC base pairs into AT pairs. This mutagen has been widely used to generate novel plant varieties, exhibiting both morphological and physiological changes. It has proven effective in improving agronomic traits such as drought tolerance (Khalil *et al.* 2018; Chen *et al.* 2023). While seeds are the most commonly used plant material for chemical mutagenesis, EMS has also been successfully applied to various explants used in *in vitro* vegetative propagation (Mullins *et al.* 2021). Previous work has demonstrated the effectiveness of EMS in promoting genetic diversity in *Lippia graveolens* through mutagenesis of nodal explants from a single *in vitro*-propagated genotype. This approach made it possible to assess genetic variation using molecular markers, confirming the potential of this technique for the genetic improvement of *L. graveolens* (Muñoz-Miranda *et al.* 2019).

In this study, the aim was to generate drought-tolerant mutants using seeds as the starting material for mutagenesis. It is commonly considered that a mutagen dose causing a lethality rate of 50% is appropriate for mutagenesis experiments, as it may result in the highest mutation rates (Chen *et. al.* 2023). Based on the observed reduction in germination rate and the number of putative mutants obtained, 0.1% EMS for 1 hr would be a suitable treatment for mutagenesis of *L.* graveolens seeds. Both polyethylene glycol (PEG) and mannitol were employed to simulate drought conditions. Initial tests revealed that direct germination of *L. graveolens* seeds on media supplemented with these compounds significantly inhibited germination. Therefore, post-mutagenesis seeds were germinated in osmolyte-free MS medium, and potential mutants were selected during micropropagation using nodal explants derived from the seedlings.

During the optimization of osmolyte concentrations, it was observed that mannitol did not significantly impact explant development or shoot regeneration. This rendered it unsuitable as a selective agent. Mannitol's protective role against water stress, as an antioxidant, has been proposed in some plants. It is thought to mitigate reactive oxygen species (ROS) formation under drought stress by scavenging hydroxyl radicals, providing protection against abiotic stresses such as salt and drought (Patel and Williamson 2016). This phenomenon could explain the minimal impact of high mannitol concentrations observed in *L. graveolens*. Conversely, PEG was highly effective in inducing drought-like conditions, particularly at 50 $g \cdot l^{-1}$, which caused the death of wild-type plants. Therefore, PEG was used to screen mutants under selective pressure. The reliability of PEG for mimicking drought has been debated because, despite causing some typical plant responses to drought stress, impairments in nutrient uptake and alterations in carbon metabolism have been observed compared to plants with reduced irrigation (Castañeda and Gonzalez 2021; Gonzalez *et al.* 2024). However, this compound remains useful for *in vitro* screening of EMS-induced drought-tolerant mutants, which maintain this tolerance when subjected to water deficit conditions in greenhouse pot trials (Khalil *et al.* 2018; Tiryaki *et al.* 2022).

Although EMS exposure significantly reduced germination rates, in some cases to less than 10%, the surviving seedlings were sufficient for the selection of potential mutants. The reduced germination observed with EMS can be attributed to chromosomal deletions, mitotic suppression, and other dose-dependent biochemical and physiological effects (Singh *et al.* 2021). Selected plants that survived the micropropagation process displayed noticeable morphological changes, suggestive of genetic alterations. The total flavonoid and phenol content in these mutants was assessed, as these compounds play crucial roles in defense against abiotic and biotic stresses. Under drought conditions, plants produce ROS, leading to protein degradation, lipid peroxidation, and cellular damage. The phenylpropanoid pathway, which synthesizes phenolic and flavonoid compounds, helps mitigate ROS accumulation,

thus protecting the plant (Chowdhary *et al.* 2022; Tiryaki *et al.* 2022).

Interestingly, there was no direct correlation between drought tolerance in the mutants and increased flavonoid or phenolic content, as some mutants exhibited lower levels of these compounds than non-mutagenized plants. However, the positive correlation observed between flavonoid and phenolic content in the mutants suggests that certain mutations may have affected shared regulatory points in the phenylpropanoid pathway. It is possible that mutants with lower levels of phenolic compounds and flavonoids possess other mechanisms to cope with the oxidative stress caused by water deficiency, such as the accumulation of other metabolites with antioxidant activity, including terpenes, glutathione, ascorbate, or the increased activity of enzymatic antioxidants like superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), among others (Mishra *et al.* 2023; Haghpanah *et al.* 2024).

Further analysis of gene expression in mutant plants, particularly genes encoding the enzymes or transcription factors involved in the biosynthesis and regulation of these compounds, would provide valuable insights into the mechanisms of drought tolerance in this species. Unfortunately, the *L. graveolens* genome has not been fully sequenced, and current information on gene sequences is limited. Ongoing work to sequence the transcriptome of *L. graveolens* will provide key data on genes involved in drought responses, making it possible to evaluate their expression in mutant plants. Additionally, it will be crucial to assess the field performance of these mutants by subjecting them to drought conditions under more realistic environmental scenarios.

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