

# An Investigation of the Expression of 1-8-Cineole Synthase and Linalool Synthase Genes of *Salvia mirzayanii* Medicinal Plant by Foliar Spraying of Different Levels of Nano Cerium Oxide

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## Abstract

An experiment was conducted at the research greenhouse of Islamic Azad University, Arak branch to examine the morphological and metabolic alterations, as well as the expression of specific genes involved in the production of secondary metabolites in *Salvia mirzayanii* under conditions of drought, nano cerium oxide, and re-irrigation (Re-watering). The study was executed in a factorial arrangement utilizing a randomized block design with three replications. The study's findings indicate that under conditions of drought stress, there was an increase in proline levels, as well as in the activity of the antioxidant defense system, specifically the antioxidant activity of peroxidase, catalase, and superoxide dismutase enzymes. Additionally, there was an increase in the expression of genes 1-8 cineole synthase and linalool synthase. Conversely, the study observed a decrease in the number of pigments related to photosynthesis, such as chlorophyll a and b, as well as a decrease in the percentage of essential oil and growth indicators. The study utilized gas chromatography/mass spectrometry to analyze and identify the essential oils of *Salvia mirzayanii* plants. The results indicated that the highest concentration of essential oil compounds was observed during various treatments, including decane (19.53), spatuleol (35.86), dodecane (18.4), butyric acid (19.8), octane (17.55%), trimethyl (43.3%), linalool (17.8), and hexyl isovalerate (21.1%).

**Keywords:** Gene expression, Drought stress, Photosynthetic pigments, Real-time PCR

## 1. Introduction

The employment of herbal drugs is progressively gaining traction as a result of the recognition of the adverse effects associated with chemical drugs. Based on data from the World Health Organization, it has been found that a significant proportion of individuals residing in developing countries, particularly those in impoverished regions, rely on herbal drugs to fulfill their primary healthcare and medical requirements (Sedighi Khoidek et al., 2022). The global trend towards the utilization of herbal drugs or medicinal plants has led to an increased

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focus on the identification and therapeutic application of botanicals, as well as a renewed interest in natural remedies. Hossein Pourazad et al. (2022) assert that Iran has the potential to make a substantial contribution to economic growth and entrepreneurship in this sector, given its diverse climate and abundance of talent. *Salvia mirzayanii* (Rech. f. & Esfand) is classified as a biennial or perennial herbaceous plant within the Lamiaceae family, belonging to the *Salvia* genus. The plant in question is indigenous to Iran and is found in various regions of the country. Its medicinal properties and diverse applications have garnered the attention of traditional medicine practitioners and local healers for a considerable duration.

*Salvia* holds significant medicinal value as a type of mint and exhibits noteworthy therapeutic characteristics. People all over the world are aware that different *Salvia* species have potent therapeutic properties (Rahmanian & Khorrami, 2020) that can affect how the human body functions physiologically, particularly the heart (Rastega et al., 2016), liver, and kidney (Samii et al., 2020). Studies indicate that the botanical extract in question possesses anti-anxiety (Kennedy et al., 2006), anti-inflammatory (Abdul Maleki et al., 2015), as well as antifungal and antimicrobial attributes (Mohammadi, 2018). Furthermore, *Salvia* has been found to possess antioxidant properties (Irvani et al., 2020) and to have the ability to lower blood sugar levels (Rastega et al., 2016). According to Murray et al. (2020), *Salvia* extract is presently utilized in the treatment of certain liver and kidney ailments.

Drought stress is a significant limiting factor for plant growth globally and is widely regarded as the most prevalent environmental stressor, affecting approximately 52% of the world's land. The impact of drought stress on agricultural yields has been documented, resulting in a reduction in crop production. According to Hosseinpour et al. (2020), the impact of drought stress has resulted in a reduction in production in approximately 25% of the world's lands, thereby affecting the distribution of plants in relation to water availability.

Conversely, the stationary characteristic of plants has rendered them persistently vulnerable to diverse unfavorable growth and environmental circumstances such as drought and high salinity. Thus, employing strategies that mitigate the harmful effects of this stress may prove beneficial. Consequently, humans must utilize specific materials that prevent their accumulation in the soil, plants, animals, and the human body, and facilitate their natural decomposition in the environment. Furthermore, these materials are not solely imperative for enhancing the plant's resilience against unfavorable biotic and abiotic factors, but also for augmenting the plant yield and refining its characteristics. The utilization of contemporary technologies, such as nanotechnology, has been highlighted as a viable approach by scholars. This method has gained significant traction in diverse fields, including plant sciences and agriculture (Ghahremani et al., 2021).

In recent years, the unique properties and numerous medicinal applications of metal nanoparticles have garnered significant attention from researchers (Rozi et al., 2016). The utilization of nanoparticles in plant sciences is a fundamental application, albeit with limited research and knowledge on the favorable and unfavorable impacts of nanoparticles on vascular plants. The efficacy of certain nanoparticles, such as cerium, on plant growth has been demonstrated through research (García-Caparrós et al., 2019).

Cerium oxide nanoparticles (CeO<sub>2</sub>NP) are a prevalent type of engineered nanoparticle utilized in various industrial applications and are frequently employed in the agricultural sector (Lima et al., 2013).

According to Parian et al. (2019), cerium oxide nanoparticles (CeO<sub>2</sub>) refer to the oxidized variant of the rare element cerium, wherein a cerium atom is encompassed by oxygen networks. The nanoparticle in question exhibits antioxidant properties similar to those of superoxide dismutase enzymes and catalase and is capable of inhibiting nitric oxide and hydroxyl radicals. Additionally, the oxidant properties of the nanoparticle are dependent on the environmental pH (Popov et al., 2017). The application of cerium oxide nanoparticles at low concentrations has been found to promote plant growth, whereas, at high concentrations, it has been observed to impede plant growth. The optimal concentration for enhancing plant growth is contingent upon

various factors, including the specific plant species, the inherent properties of cerium oxide nanoparticles, and the prevailing growth conditions. According to reports, the application of cerium dioxide nanoparticles at low concentrations has been found to enhance biomass production which suggests that such nanoparticles may have potential as a nano fertilizer, albeit at specific concentrations.

Parian et al. (2019) conducted a study to investigate the impact of various concentrations of cerium oxide nanoparticles (0, 250, 500, and 1000 mg/liter) on the morpho-physiological and phytochemical characteristics of red Salvia. The experiment involved foliar spraying and soil feeding during two growth phases, namely vegetative and reproductive. The study findings indicate that augmenting the concentration of cerium oxide nanoparticles through foliar spraying and soil feeding leads to an elevation in the levels of total chlorophyll, carotenoids, phenolic, and flavonoid compounds, as well as the percentage of antioxidant activity. The reproductive phase was found to yield the highest amount of total chlorophyll, while the application of 250 mg/liter of cerium nanooxide through soil feeding and foliar spraying methods resulted in the highest carotenoid levels. The application of foliar spraying at a concentration of 500 nanometers resulted in the highest levels of phenolic and flavonoid compounds, as well as the greatest percentage of antioxidant activity. Meanwhile, the highest levels of rosmarinic acid and tension were observed during soil feeding at a concentration of 500 nanometers in the reproductive phase. The findings indicate that the application of 250 ml of nano during the reproductive and vegetative phases via foliar spraying resulted in the greatest fresh and dry weight of shoots and roots.

The study conducted by Jahani et al. (2019) investigated the impact of varying concentrations of cerium oxide nanoparticles ( $\text{CeO}_2$  NPs) on the biochemical and physiological parameters of *Calendula officinalis* L., a medicinal plant. The concentrations tested were 50, 100, 200, 400, 800, 1600, and 3200 mg/liter. In The findings suggest that *C. officinalis* exhibits a favorable response to low concentrations of  $\text{CeO}_2$  nanoparticles, whereas higher concentrations elicit oxidative stress and toxic reactions. The augmentation of nano  $\text{CeO}_2$  concentration resulted in a proportional rise in the levels of osmotic regulating agents, namely proline, glycine betaine, and soluble sugars, as well as the activity of antioxidant enzymes such as ascorbate peroxidase, catalase, superoxide dismutase, and polyphenol oxidase. Concurrently, the high concentrations of the substance resulted in a decrease in both growth parameters and chlorophyll content, whereas low concentrations (50 and 100 mg/liter) led to an increase in these factors.

Bagheri et al. (2019) conducted a study titled "Investigation of the effect of silicon on some morphological and physiological indicators and the expression of betaine aldehyde dehydrogenase and proline 5-carboxylate synthetase genes in *Echium amoenum* medicinal plant under drought stress" using a factorial design in a completely randomized manner with three replications. The aim of the study was to examine the impact of silicon treatment on various morphological, physiological, and molecular characteristics of *Echium amoenum* plants under drought stress conditions. The findings of the study indicate that drought has a significant impact on growth parameters, chlorophyll pigments, and carotenoids in comparison to the control group. However, the application of silicon to plants mitigates the adverse effects of drought stress and enhances growth parameters and photosynthetic pigment content. The study revealed that under drought stress conditions, there was a significant elevation in the levels of anthocyanin, soluble and reduced carbohydrates, glycine betaine, and proline. Additionally, the expression of betaine aldehyde dehydrogenase (BADH) and proline 5-carboxylate synthetase (P5CS) genes was found to be upregulated. The study demonstrated that the concurrent exposure to drought and silicon resulted in a significant reduction in the expression of BADH and P5CS genes, as well as the corresponding products of these genes, namely proline and glycine betaine. This outcome provides evidence for the efficacy of silicon in mitigating osmotic stress.

Bayat and Nasser Moghadam (2019) conducted a study to examine the impact of drought stress on the growth parameters, proline content, and antioxidant system activity of three cultivars of *Salvia nemorosa*

L, namely Violet Queen, Rose Queen, and Isfahan medicinal plant, over a period of ten days. The findings indicate that the examined traits were significantly impacted by drought stress. Specifically, under drought stress conditions, the number of leaves, leaf area, dry weight of stem and root, total dry weight, and concentration of photosynthetic pigments, including chlorophyll a, b, and total, were observed to decrease in all three cultivars compared to the control conditions. The results of this study reveal that the levels of phenolic and flavonoid compounds, proline content, soluble sugars, and antioxidant activity of catalase and peroxidase enzymes increased in all three cultivars under drought stress.

Since Iran is situated in one of the arid and semi-arid regions globally, it appears that sustainable crop production can be achieved through two main approaches. Firstly, through the optimal utilization of water resources and the enhancement of plant drought resistance mechanisms. Secondly, by addressing the water scarcity crisis and implementing effective water resource management strategies to reduce water consumption.

The objective of the present study was to determine the optimal concentration of nano cerium oxide for enhancing the vegetative traits, as well as the yield and quality of essential oil in *Salvia mirzayanii* under favorable and arid environmental conditions.

## 2. Materials and Methods

The objective of this study was to examine the expression of cineole synthase 1-8 and linalool synthase genes in Iranian sage, a medicinal plant, under varying drought stress treatments, recovery periods, and foliar spraying with differing levels of Nano cerium oxide in 2014-2015. The study was executed as a factorial design utilizing a randomized complete block approach, with two factors and three replications. The experimental design involves the consideration of five pots for each treatment within each repetition. Thus, a total of 300 pots were utilized, with 100 pots being allocated for each repetition. The seeds necessary for the study were procured from the Forestry and Pasture Research Institute of the nation (Fig. 1).



(b)



(b)

Fig. 1 a) Germination of seeds, and b) Transplanting seedlings to pots

### 2.1 Treatments Procedure

The pots were irrigated following their agricultural potential and on a daily cadence until the imposition of stress. During the period spanning from the second to the sixth week after planting, irrigation was carried out under the designated irrigation protocols. The irrigation quantity was ascertained utilizing the pressure cell technique and the calculation of weight-based moisture percentage. The FC value of the soil was determined and various levels of FC were created to induce drought stress. The pots were continuously weighed during

each watering cycle and the amount of water needed to reach the desired treatment level was calculated. The period spanning from the second week to the sixth week after planting was identified as the dry period, followed by a two-week recovery period (from the sixth week to the eighth week) during which all pots were watered. The cerium oxide nanoparticle utilized in the study was procured from Pishgaman Nano Material Iranian Company under the US Nano brand, with particle sizes ranging from 10 to 30 nm and presented in the form of a milky powder. The particles exhibited a spherical morphology, with a surface density of 0.8 to 1.1 g/cm<sup>3</sup> and a purity of 99.97%. The application of Nano cerium oxide treatments was initiated at the 6-leaf stage and repeated once every 15 days through solution spraying. Harvesting was conducted after the onset of flowering during the growth period.

To facilitate the design of primers for the gene (1-8, cineol and linalool) and the control gene 18srRNA (GAPDH) required for this study (Table 1), the sequences documented in the World Bank of Biotechnology were utilized. The genetic data of these specific genes were extracted and subsequently subjected to sequence alignment using Bioedit software. The identification of the safeguarded regions of these genes was conducted and subsequently employed for primer design utilizing the Oligo7 software. The primers that were designed underwent specificity testing using the primer-BLAST tool available on the NCBI website.

**Table 1** Specifications of Primers

Primer name	Primer sequence	Product size (open pair)	Junction temperature (°C)
1-8, Cineole F	ATCCAAACTGGACGACGAAC	106	60
1-8, Cineole R	GCCCTCATCAAGTGCTTCTC		
Linalool F	GAGAAGGACTTGCATGCTACTG	186	60
Linalool R	GACATCTGCACGGATACCT		
GAPDH F	ATGGCATTCCGTGTTCTAC	112	61
GAPDH R	CCTTCAACTTGCCCTCTGAC		

The modified triazole and alcohol precipitation method with DNase I enzyme was employed for the extraction of RNA from total plant tissue.

To determine the concentration of the extracted RNA samples, a volume of 1 microliter was introduced into the Nanodrop device and analyzed using the 260/280 and 230/260 indicators. The concentration of the samples was expressed in units of ng/μl. For gene expression studies, samples that exhibit a concentration exceeding 100 ng/μl and a ratio of 260/280 greater than 1.9 were chosen.

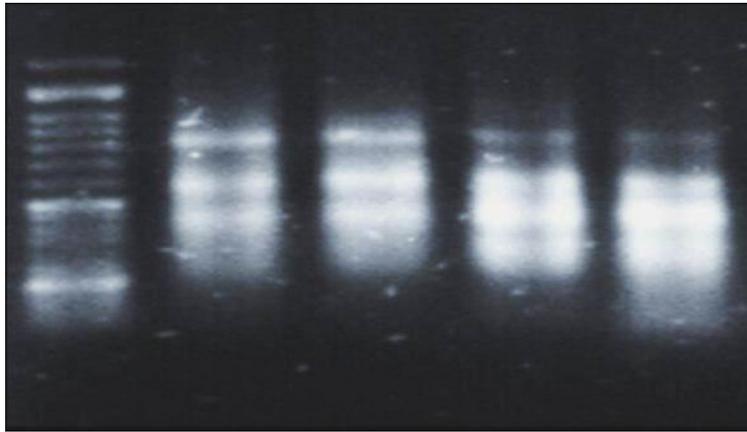
The Real-Time PCR technique was employed for gene expression analysis, utilizing the SYBR green fluorescence dye methodology.

To facilitate the infiltration of cerium Nano oxide into the plant organs, scanning electron microscope (SEM) images were generated from the terminal leaves of the plant following foliar spraying treatments. The SEM model employed for this purpose was the TESCAN S9000.

The  $2^{-\Delta\Delta CT}$  method was employed to evaluate the expression ratio of the target gene relative to the internal control gene across all treatments. Subsequently, statistical analysis of all trait data was conducted using SAS software, and the means were compared using Duncan's multiple range test method. The statistical analysis was conducted at a significance level of 0.05, and the results were presented using various visual aids such as tables, graphs, and charts in Microsoft Word and Excel applications.

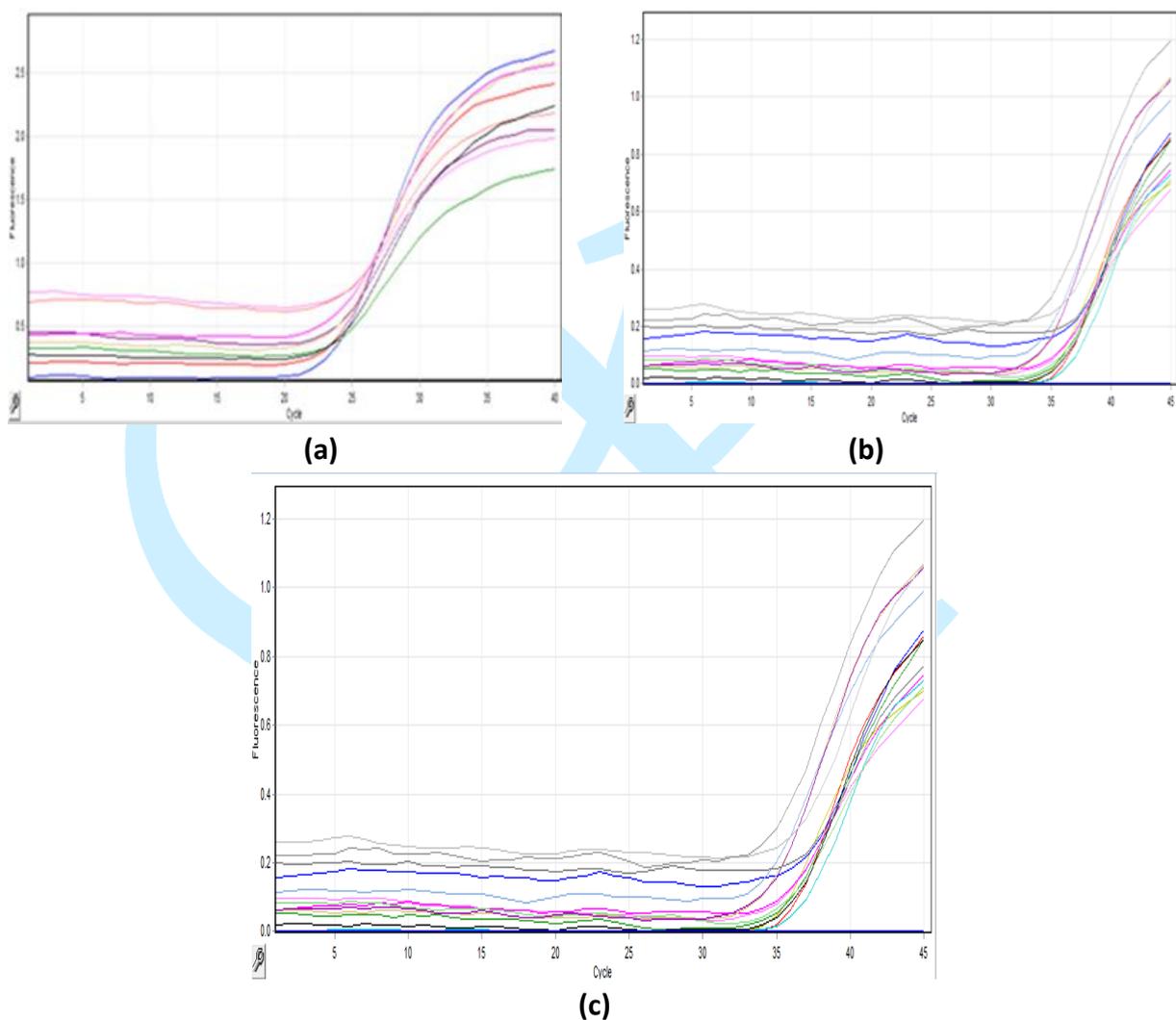
### 3. Results

Fig. 2 displays a selection of extracted samples. The identification of 18s and 28s bands may serve as evidence supporting the validity of the extraction process.



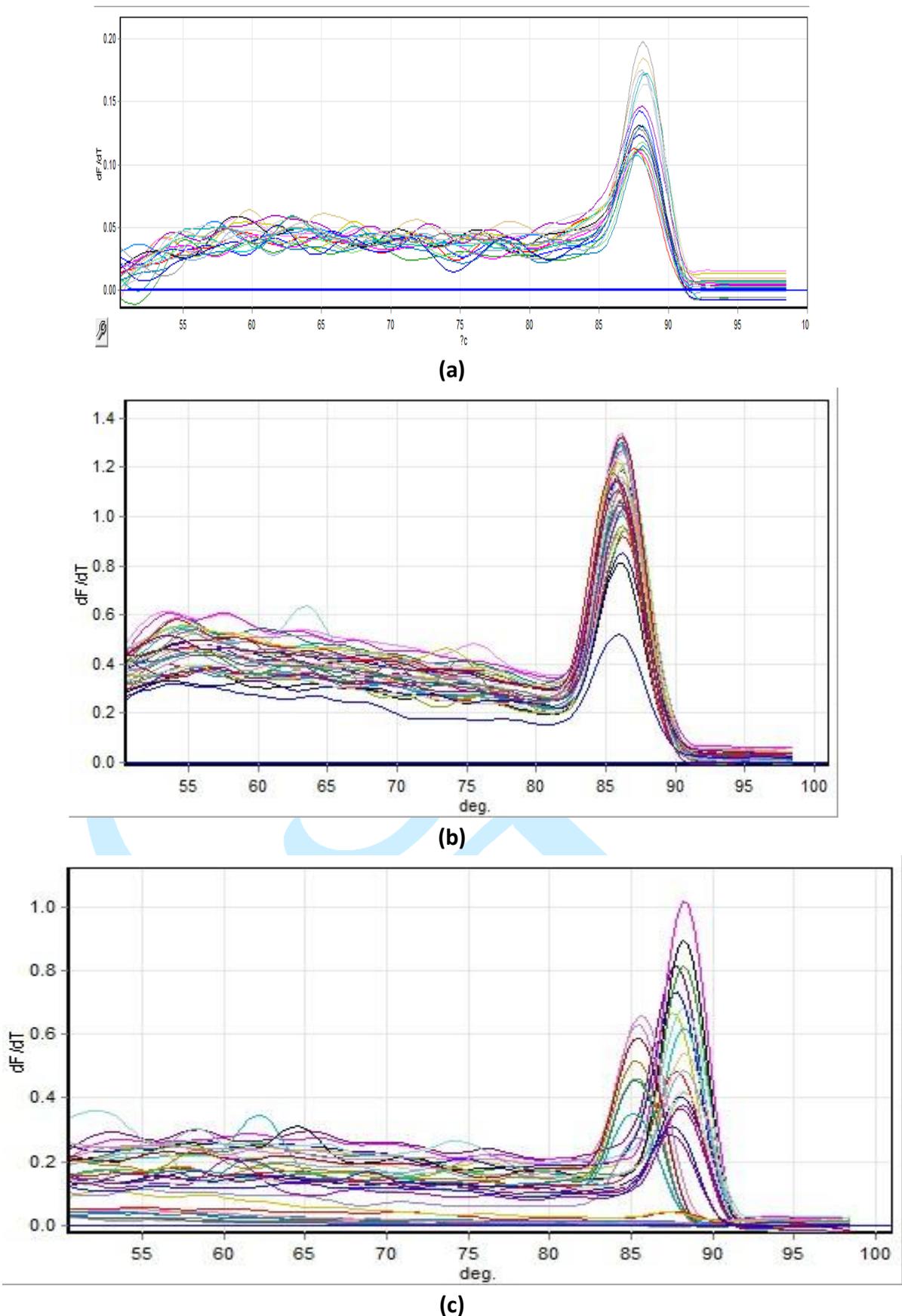
**Fig. 2** RNA extraction results from the total samples

The results of gene proliferation curve are given in Fig. 3.



**Fig. 3.** a) Standard gene amplification curve (GAPDH), b) Cineol synthase 8-1 gene amplification curve, c) Linalool synthase gene amplification curve. (Cycle number on the horizontal axis and light intensity on the vertical axis)

Based on the analysis of the melting curve for each of the three primers, it can be concluded that the primers exhibited specificity and successfully amplified a singular sample derived from cDNA. The lack of a supplementary peak that is smaller in size than the peak of the products is indicative of the non-existence of primer dimer. This suggests that the primers are not interacting with one another and that the reaction is sufficiently efficient for the purpose of quantifying gene expression (Fig. 4).

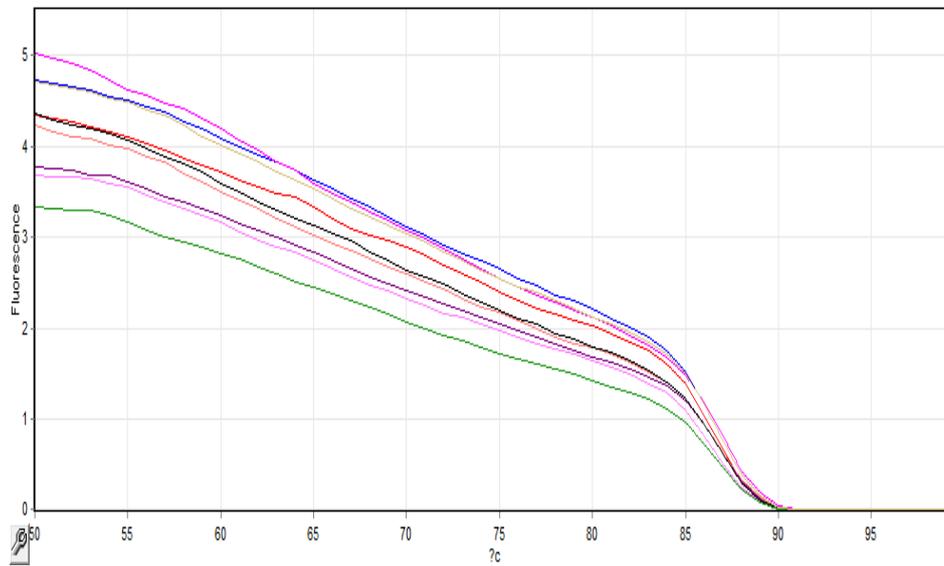


**Fig. 4** a) Melting curve: GAPDH gene, b) 1-8 gene, Cineole, c) Linalool gene. (The current peak's location suggests a temperature of 89 degrees Celsius)

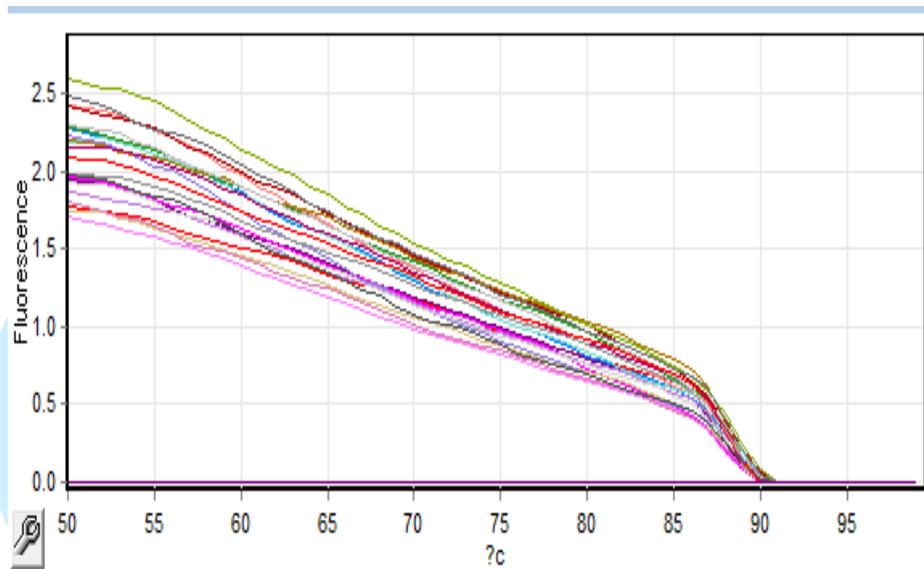
### 3.1 Crossing Line Fluorescence (Threshold)

Typically, thresholds for specific fluorescence are established between cycle 3 and 15 based on the background fluorescence intensity. This cut off is commonly referred to as the Crossing line (Fig. 5).

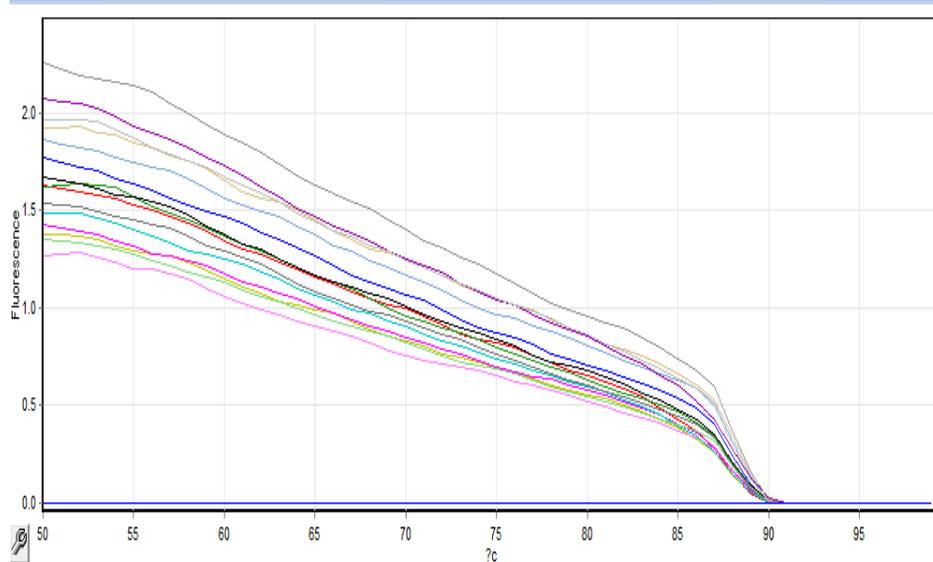
Crossing Point= (CP) or (Cycle threshold) = (Ct)



(a)



(b)



(c)

**Fig. 5** Fluorescence changes as a function of temperature for a) Linalool gene. b) Gene 1-8, Cineole. c) GAPDH gene (horizontal axis temperature and vertical axis fluorescence rate)

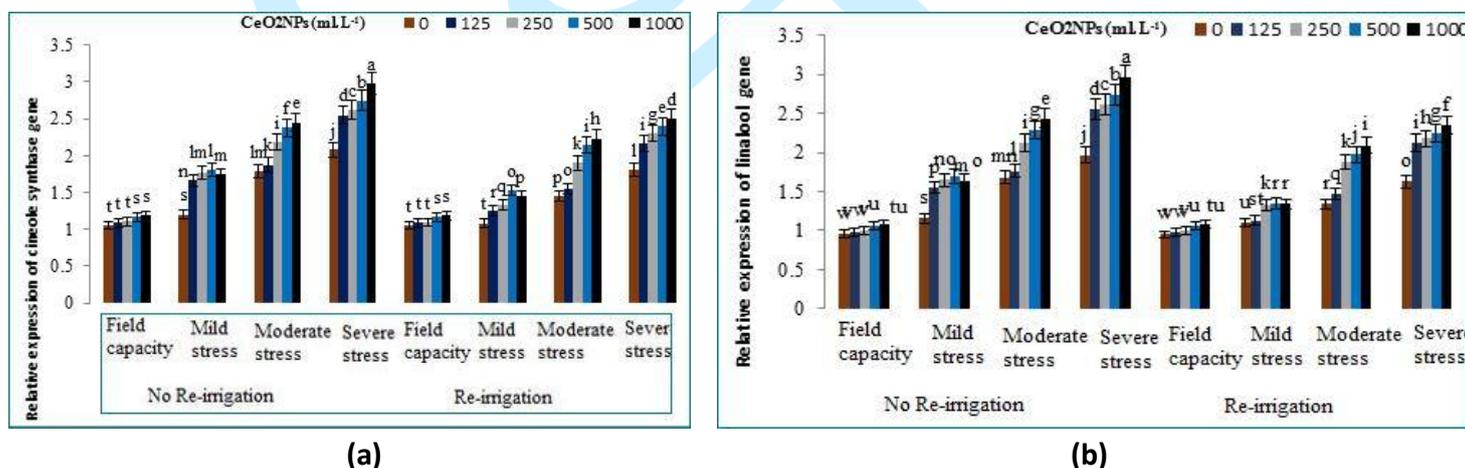
The variance analysis table revealed that the relative expression levels of the 8-1 cineol synthase and linalool genes were significantly impacted by the treatments of drought stress, cerium Nano oxide, re-irrigation, as well as their double and triple interactions in the experiment. The results were deemed statistically significant at a significance level of 1% as indicated in Table 2.

**Table 2** The results of variance analysis of drought and Nano stress and re-irrigation on the relative expression level of 1-8 cineol synthase and linanol genes

Sources of Variations	Degrees of freedom	Relative expression of the cineole 1-8 gene	Relative expression of linalool gene
Block	2	0.0008 <sup>ns</sup>	**0.0026
Drought stress	3	**9.73	**9.98
Cerium Nano oxide	4	**1.09	**1.05
Re-irrigation (Re-watering)	1	**1.67	**2.1
Dryness × Nano	12	**0.11	**0.13
Drought × Re-irrigation	3	**0.19	**0.27
Nano × Re-irrigation	4	**0.009	**0.014
Drought × Nano × Re-irrigation	12	**0.0092	**0.01
Error	78	0.001	0.0006
Coefficient of variation		1.82	1.49

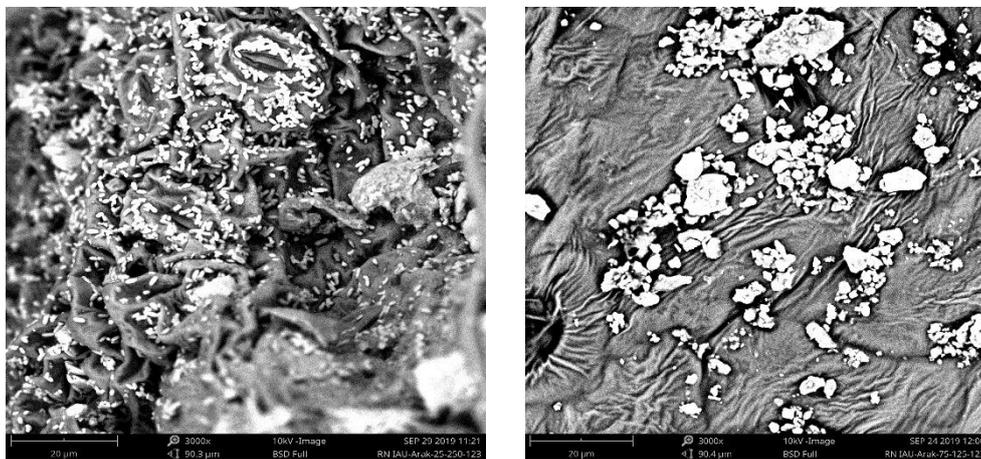
ns, \* and \*\*: no significant difference, significant difference at 5% and 1% level, respectively

Fig. 6 illustrates the mean comparison of distinct Nano levels concerning the quantity of 8-1 cineole synthase and linalool genes. The findings of this study indicate that the act of plant re-irrigation following each instance of stress resulted in their recovery, thereby leading to a reduction in the expression of the aforementioned genes.



**Fig. 6** The results of interaction between drought stress and cerium nanooxide on the relative expression level of: a) cineol synthase gene, b) linalool synthase gene

To facilitate the infiltration of cerium nano oxide into plant organs, scanning electron microscopy (SEM) images were generated and analyzed from the terminal leaves of the plant across varying concentrations. The penetration of Nano cerium into leaf tissue occurs at varying concentrations. At high concentrations, the nanoparticles tend to aggregate upon contact with plant cells, resulting in accumulation surrounding the stomatal cells (Fig. 7).



**Fig. 7** SEM images: Penetration and accumulation of nanoparticles in leaf tissues in the form of white spots

#### 4. Discussion

The study's findings indicate that under drought stress conditions, the expression levels of 1-8, cineol synthase (CS), and linalool synthase (Lis) genes increased in comparison to the control level. Moreover, the severity of drought stress was found to be positively correlated with the level of gene expression. Notably, the highest level of gene expression was observed during severe drought stress. The observed upregulation of CS and Lis genes by 45% and 51%, respectively, aligns with the conclusions drawn by Alimirzai and Chaji (2021) in their study on medicinal plants. Specifically, their research indicated that drought stress can stimulate the expression of genes involved in the production of secondary metabolites in medicinal plants.

The study conducted a comparative analysis of CS gene expression under varying degrees of drought stress, ranging from severe to moderate and mild, as well as after re-irrigation following each stress level, in comparison to a control group. The results indicated a significant increase in CS gene expression across all drought stress levels following the application of stress. Nevertheless, the process of re-irrigation partially mitigated the deleterious impact of drought stress and facilitated the recuperation of the plants. Conversely, intense drought stress resulted in a 45% upsurge in the expression of this gene relative to the control condition. The significance of the CS gene in the sage plant lies in its role as a crucial monoterpene synthase gene. The CS gene encodes the CS enzyme, which facilitates the synthesis of cineole synthase 1-8, the end product, from the precursor geranyl diphosphate (Shaw et al., 2015).

The investigation of the level of LIS gene expression in response to drought stress treatments and subsequent re-irrigation in comparison to the control group revealed a significant increase in the expression of the LIS gene at all levels of drought stress. Nonetheless, the act of re-irrigation after each stress level exhibited a partial ameliorative impact on the deleterious consequences of drought stress, thereby facilitating plant recuperation. Nevertheless, the gene expression was found to be elevated by 51% in response to intense drought stress as compared to the control condition. The findings of the previous research indicate that the expression of the linalool synthase gene (LIS) in various medicinal plant species is positively correlated with adverse growth conditions. Specifically, it has been observed that the expression of LIS tends to increase under such conditions. The expression of this particular element may potentially contribute to the plant's defense mechanism against various stressors such as pathogenic agents and insect infestations. The study conducted by Kabiri et al. (2018) demonstrated that the level of LIS expression exhibited a significant increase of 4.86 times the control condition after 12 hours at a temperature of 10°C.

The impact of cerium oxide nanoparticles on the expression of CS and LIS genes was investigated. The results indicated that the use of cerium oxide nanoparticles alone led to an upregulation of the aforementioned genes. Specifically, as the concentration of cerium oxide nanoparticles increased, the expression levels of CS and LIS genes exhibited a corresponding increase relative to the control level. The data revealed an increase of approximately 12% and 12% in ascending order. Furthermore, the concomitant

administration of Nano cerium under conditions of drought stress, followed by re-irrigation after each stress level, resulted in an augmentation of the expression of both genes. The foliar spraying treatment with ml concentration resulted in the highest level of expression of CS and LIS genes, with mean values of 2.974 and 2.958, respectively. The experimental treatment involved the application of 1000 ml.L-1 of cerium oxide under conditions of severe drought stress.

In recent years, there has been a significant focus on the utilization of nanoparticles as a stimulus, with numerous studies conducted to investigate their impact on the expression of diverse plant genes. An instance of citing relevant research findings is exemplified by the reference to the outcomes of the investigation conducted by Jiang et al. (2018). The researchers posited that the utilization of nanoparticles leads to an elevation in the expression levels of the genes involved in the biosynthesis pathway of vinblastine and vincristine, sanguinarin, and rosmarinic acid, as compared to the control level. The present findings are in line with the outcomes of this investigation, which indicate that the administration of cerium oxide nanoparticles leads to an increase in the expression of CS and LIS genes.

According to reports, the utilization of nanoparticles in unfavorable growth conditions has a beneficial impact on the induction of gene expression in plants. Mousavi Nik et al. (2019) conducted a study to examine the impact of silver nanoparticles and iron chelate nanoparticles on the expression of TpGAS and TpPTS genes in the presence of parthenolide, an effective substance found in *Tanacetum parthenium* L. medicinal plant, during drought stress. The findings indicated that the application of nanoparticles at each stress level increased gene expression. The aforementioned genes were observed to be actively expressed. The combination of water stress treatment with green and chemical synthesis Nano silver resulted in the greatest expression of TpGAS and TpPTS genes, leading to increased parthenolide production. The findings of the previously mentioned research are consistent with those of the present study, which indicate that the utilization of cerium oxide nanoparticles enhances the expression of CS and LIS genes under conditions of stress and re-irrigation. The statistical significance or insignificance of interactions implies that the utilization or non-utilization of stimulants or any other substance is contingent upon the degree of the second factor (i.e., irrigation regime: full irrigation or drought stress). To clarify, the utilization of cerium oxide nanoparticles in the presence of water stress conditions leads to an upregulation of the aforementioned genes in comparison to their expression levels in the absence of said nanoparticles.

Thus, the analysis of the literature suggests that the impact of nanoparticles on the stimulation of gene expression could be directly linked to the activation of plant defense responses and also could be indirectly by activating a cascade of secondary signaling signals in plants. The activation of gene expression and subsequent biosynthesis of secondary metabolites can exert a significant influence on these physiological reactions.

## 5. Conclusion

The findings of this study reveal that the utilization of Cerium Oxide Nanoparticles may prove to be beneficial in mitigating the deleterious consequences of drought stress in the *Salvia mirzayanii* plant. This is achieved by enhancing the antioxidant defense mechanism through the augmentation of superoxide dismutase, peroxidase, and catalase enzymes, as well as the amplification of osmotic regulators such as proline, and the expression of certain genes involved in the defense pathway. Consequently, the cellular water balance and structure are upheld, leading to an improvement and subsequent increase in growth indicators during periods of drought stress and subsequent re-irrigation.

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## Declaration of Conflict

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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