# The effect of biosynthesized silver nanoparticles on FAE1 and FAD2 gene expression in *Camelina sativa*

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## Abstract

Camelina is an oilseed and re-emerging plant that requires a lot of research on its oil production process. This study was conducted to investigate the effect of biosynthesized silver nanoparticles on the expression level of FAE1 and FAD2 in Soheil cultivar of Camelina oilseed plant based on a completely randomized design with four replications in 2018-2019. The aqueous extract of Camelina leaf and silver nitrate salt was used to prepare nanoparticles. Experimental treatments included 0.5, 1, 2, and 3 mg /L silver nanoparticles. After preparing foliar samples for all treatments, RNA extraction, cDNA synthesis, and temperature gradient determination, the Real Time PCR reaction was used to study gene expression patterns. The data were then analyzed using GenEX and SAS software. Results showed that the effects of silver nanoparticles on FAE1 and FAD2 gene expression were significant ($p<0.05$), showing an increase with increasing silver nanoparticle concentration. The highest enhancement was observed at 3 mg/L silver nanoparticles.

**Keywords**: biosynthesize, Camelina, gene expression, nanoparticles, Real Time PCR.

**Mirmoeini, T. L. Pishkar, D. Kahrizi, G. barzin and N. Karimi.2021.** 'The effect of biosynthesized silver nanoparticles on FAE1 and FAD2 gene expression in *Camelina sativa'. Iranian Journal of Plant Physiology,* 11(5), 3911-3918.

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## Introduction

Camelina (*Camelina sativa*) is a flowering plant endemic to the Mediterranean regions in Europe and Asia. The plant belongs to Brassicaceae family and has been considered as a low input plant with greater resistance to spring cold than other Brassicaceae plants. It is also highly resistant to common pests in oilseeds such as pollen beetles. The potential of high yield production in Camelina has been demonstrated and its feasibility has been reported as an appropriate alternative to the rotation with fine grain cereals (Ghamkhar et al., 2010 and Ghamarnian, 2020). Also, Camelina is recognized as a valuable and usable crop in human and animal nutrition and industry sectors (Nelson et al., 2011).

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*Received: January, 2021*
*Accepted: June, 2021*

Nanotechnology is one of the new technologies that has entered the field of agriculture. Nanoparticles are atomic or molecular complexes with a minimum dimension of 1-100 nm that have different physicochemical properties (Monica and Cremonini, 2009; Ansari and Kahrizi, 2018). The uptake of carbon and metal nanoparticles by plants is a new field of research. Adsorption, transport, and accumulation of nanoparticles vary depending on plant species, type, size, chemical composition, structure, and strength of the nanoparticles ( Rico et al., 2011).

Increasing agricultural production on an economic scale without polluting the environment is a global challenge. The agricultural and health sectors have seen great strides in recent years. Many developing countries face the threat of food insecurity and healthcare (Husen and Siddiqi, 2014). With the advent of nanotechnology, many applications of nanoparticles in plant cultivation environments are undeniable and they have had a major impact on the knowledge-based economy, industry, and society. The use of nanoparticles as one of the key aspects of nanotechnology has made significant progress over the past few years (Biswas and Wu, 2005). Silver is classified as a high-risk material and its toxic effects have been reported on many organisms (Fouda, 2020). This toxicity is inhibited in the form of silver nanoparticles (Boxall et al., 2007), which are one of the most popular and widely used nanotechnology products. It is estimated that around 320 tons of nanoparticles are used worldwide annually (Nowack et al., 2011). Among the different types of nanoparticles, silver nanoparticles have wide applications in the fields of nanotechnology and medicine (Jegadeeswaran, et al., 2012).

Silver nanoparticles fabricated based on chemical methods have great threat due to their use of hazardous and toxic chemicals and their environmental damage. Therefore, the production of silver nanoparticles using the principles of green chemistry has recently gained prominence in research (Ahmed et al., 2016 and , Sharma et al., 2009).

One of the recently used non-living elicitors is nanoparticles. These particles are the most important nanotechnology tools that have many applications in the biological sciences. When comparing these particles with natural biomaterials, the usual 10-µm diameter of the cell and its components are predominantly in the sub-micron range (Salata, 2004). Because of their small size, these nanoparticles rapidly penetrate the cell, leading to increased protein levels and stimulation of gene expression in plant cells (Yang et al., 2008). For example, a 4-fold increase in DBOX gene expression 48 h after stimulation with silver nanoparticles, a decrease 72 h after treatment, and no significant effect on expression 168 h after treatment was reported (Khodayari , 2015).

Elicitors can regulate the level of secondary metabolites by affecting the expression of genes involved in the synthesis of primary metabolites that are involved in vacuolar transport. In other words, the transport and storage of primary metabolites play an important role in the production of valuable compounds (Vasconsuelo and Boland, 2007). However, transcriptional activation of genes involved in eliciting immune responses has also been reported (Park et al., 2003). It should be noted that these stimuli also affect the plant in natural conditions and produce a specific metabolite (Schearer, 1984).

Camelina is one of the valuable oilseed crops that is known as low input and adaptable plant. Due to the good oil properties of this plant, it has attracted researchers' attention (Ghamarnian, 2020; Obour et al., 2015; Waraich , 2013).

One of the problems with Camelina oil is that it has erucic acid, which in some varieties is above the permitted level (5% by Canadian standard and 3% by European standard) (Campbell, 2018; Campbell et al., 2013). On the other hand, it has been shown that FAE1 and FAD2 genes are involved in erucic acid biosynthesis (Peng, 2010; Soltani, 2019). This study aimed to investigate the effect of silver nanoparticles on the expression of FAE1 and FAD2 genes in Camelina.

## Materials and Methods

To study the effects of silver nanoparticles (0.5, 1, 2, and 3 g /``L) on the expression of FAE1 and FAD2

genes in Camelina, an experiment was conducted in a completely randomized design with four replications at Razi University during 2018-2019. The plant material included Camelina cultivar Soheil, obtained from Bisetoon Shafa Co., Iran.

To prepare the aqueous extract of Camelina leaves, the leaves were first washed twice with distilled water in the laboratory at 23 ℃. Thirty (30) grams of fresh Camelina leaf were weighed and cut into small pieces. The pieces were poured into an Erlenmeyer flask and distilled in distilled water to a volume of 100 ml and then boiled for 5 minutes, and the extract was filtered after cooling by filter paper. Then, 20 mM silver nitrate was added in a beaker containing 800 ml of the extract. The reaction mixture was stirred at laboratory temperature for one hour before the color of the extract changed from pale yellow to dark brown indicating the production of silver nanoparticles. Then UV-visible spectrophotometer (Cray 50) was used to measure the spectra.

We planted the seeds in 7 kg pots. Within each pot, a combination of clay, sand, leaf soil, and fully rotten manure was applied in equal proportions. To disinfect the seeds and prevent possible fungal contamination, the seeds were immersed in 1% sodium hypochlorite solution for two minutes, then rinsed with ordinary and distilled water, respectively. When the Camelina seedlings reached the 6-8 foliar stage, they were treated with different concentrations of silver nanoparticles for 20 days.

## *Study of gene expression*

To study gene expression, first the treated leaf samples were powdered with liquid nitrogen and then were sent to the Zagros Bioidea Company located at the Razi University to study the effect of nanoparticles on gene expression.

### *First-strand cDNA synthesis*

Synthesis of the first-strand cDNA was performed using a 2-steps RT-PCR kit from Sinaclone according to the following steps: First, the Strand Master Mix st1 including 1 µl oligo dT as primer, 1 µl dNTPs, 3 µl RNA, and 5µl Nuclease-free Water (in the final volume of 10µl) were added (Table 1). The tubes were incubated for 5 min at 65 ℃ in the Ben Murray (hot water bath) apparatus and then kept on ice for 2 min. The samples were then shaken gently.

Table 1
Materials used for the first-strand cDNA synthesis

| Material | Amount (µl) |
|---|---|
| Oligo dT | 1 |
| dNTPs | 1 |
| RNA | 3 |
| Nuclease- free water | 5 |
| The final volume of the reaction | 10 |

Table 2
The cDNA Synthesis Mix

| Material | Amount (µl) |
|---|---|
| 10x Buffer M-Mul V | 2 µl |
| M-Mul V Reverse Transcriptase | 1 µl or 100 unit |
| Nuclease- free water | 7 µl |
| The final volume of the reaction | 10 µl |

The cDNA Synthesis Mix was then added, including 10X Buffer M-Mul V (2 µl), M-Mul V Reverse Transcriptase to 1 µl (unit 100), and 7 µl Nuclease-free Water. Then 10 µl of the above materials were added to the tubes and placed in a Ben Murray at 42 ℃ for 60 minutes and finally placed in a Ben Murray at 85 ℃ for 5 minutes before they were kept on ice for 2 minutes (Table 2). The tubes were then centrifuged at 10,000 rpm for 2 min at room temperature and transferred to a freezer set at -80 ℃. The quality of the samples was evaluated by electrophoresis on 1.2% agarose gel (W / V).

### *Real-Time PCR Steps*

Real-time PCR was performed to determine quantitatively the transcript levels of the selected genes.

### *Primer design*

Designing primers is the most important step in examining the quantitative expression of genes. After selecting the desired genes, a pair of primers for each gene was designed using primer 3 software and the website address for Real-Time PCR reaction as below:

http://biotools.nubic.northwestern.edu/OligoCalc.html (Table 3).

Table 4
Chemicals and quantities used in Real Time PCR reaction

| Chemicals | The amount used | Final concentration |
|---|---|---|
| cDNA | 100 ng | |
| qPCR GreenMaster with lowRox | 10 µl | 1 X |
| Primer F | 0.8 µl | 0.5 µM |
| Primer R | 0.8 µl | 0.5 µM |
| RNase free H2O | Up to 20 µl | |

Absence of any similar pattern in the genome (single expression band), the formation of a single band, temperature proportionality of two primers (Forward and Reverse) with a maximum difference of 2 ℃, lack of dimer primers, lack of heteroduplex formation, GC content between 40-60%, having a good melting temperature (Tm above 50 ℃), a suitable length (20 to 27 bp), and a product length between 200 and 100 bp were the most important characteristics of the designed primers. After designing the primers, their specifications were sent for the manufacture of the primers.

In this research, the FAD2-3 gene with 2755 bp length and accession number HQ008322 and the FAE1 gene with 4125 bp length and accession number KJ461881 were used. The 18S rRNA gene was also used as an internal control (Table 3).

### *Qualitative evaluation of cDNA and its preparation*

To evaluate the quality of the synthesized cDNAs, a 260 nm reading using NanoDrop (Thermo, USA) was used. In this method, 1 µl of each sample was used for reading. After reading, all samples were diluted with DNase and RNase-free water to a concentration of 500 ng/µl and were used for the next step of the experiment.

### *Real-time PCR analysis*

The Real-Time PCR reaction for quantitative evaluation was performed using qPCR GreenMaster with lowRox kit purchased from Takapoo Zist Co. Gene expression analysis was performed using the Real-Time PCR method based on SYBR green fluorescence dye in two device replications and three biological replicates. In this method, the reaction was performed from a 48 well plate for a Real-Time PCR (ABI StepOn, USA) with a final reaction volume of 20 µl containing the materials listed in Table 3-5. The applied temperature program included enzyme activation at 95 ℃ for 10 minutes, 40-cycle at 95 ℃ for 15 seconds, annealing (at 53 and 60 ℃) for 30 seconds, and extension (at 72 ℃) for 1 minute. Finally, the melting point test was performed in a cycle consisting of 95 ℃ for 15 seconds, 60 ℃ for 1 minute and 0.3 ℃ rise to 95 ℃ to plot the PCR product melting curve.

## Statistical Analysis

The data were collected based on a completely randomized design and the means were compared using the LSD test at 1% level using SAS 9.1 software.

To analyze Real-Time PCR data, the relative expression of each gene was calculated based on the relative standard curve method using the $2^{-\Delta\Delta C_T}$ formula (Livak and Schmittgen, 2001) calculated as follows:

Amount of Target Gene= $2^{-(C_T \text{ Gene}-C_T \text{ Ref}) \text{ Stress} -(\text{mean } C_T \text{ gene} - \text{mean } C_T \text{ Ref}) \text{ Normal}}$

where $C_T$ is Cycle Threshold, Ref is reference gene or internal control, and base 2 denotes the amount of cDNA in each PCR cycle doubled.

## Results

### *RNA extraction*

The RNA on 1% agarose gel indicates the high accuracy and quality of the extracted RNAs (Fig. I).

### *cDNA synthesis*

The pattern of I8S gene amplification using cDNA has is shown in Fig. II.

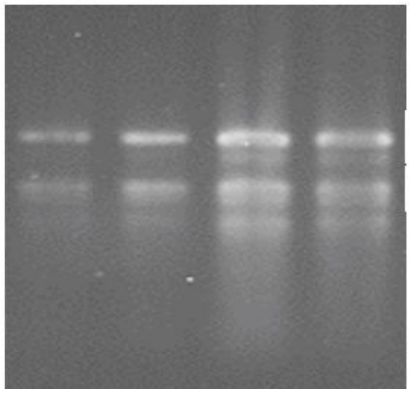


Fig. I. Determination of RNA quality on agarose gel

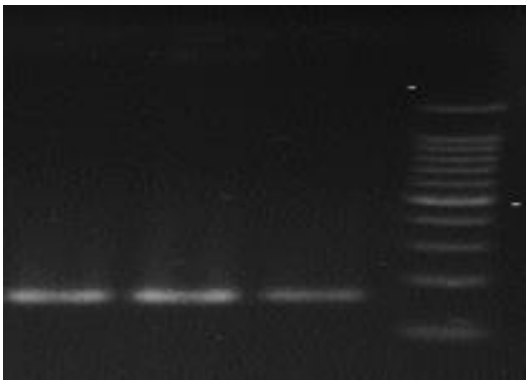

Fig. II. The pattern of I8S gene amplification using cDNA. Lanes: 1, 2, and 3: cDNA samples. 4: DNA size marker (100 bp).

Table 5
Analysis of variance of the effect of biosynthesized silver nanoparticles on the expression of FAE1 and FAD2 genes in Camelina sativa

| Source of variations | Degree of freedom | *FAE1* | *FAD2* |
|---|---|---|---|
| Silver nanoparticles | 3 | $7.82^{**}$ | $0.53^{**}$ |
| Error | 7 | 0.05 | 0.05 |
| CV% | | 2.8 | 4.37 |

** ($p<0.001$)

Table 6
Mean comparison of the effect of biosynthesized silver nanoparticles on the expression of FAE1 and FAD2 genes in Camelina sativa

| Silver nanoparticles ($mg.L^{-1}$) | *FAE1* | *FAD2* |
|---|---|---|
| 0.5 | $6.45^{c}$ | $3.31^{c}$ |
| 1.0 | $7.28^{b}$ | $4.76^{b}$ |
| 2.0 | $7.5^{b}$ | $5.08^{ab}$ |
| 3.0 | $10.72^{a}$ | $5.38^{a}$ |

Based on Duncan's test, the means with similar letters in each column are not significantly different ($p<0.01$).

***Investigation of 18S rRNA gene expression at different concentrations of silver nanoparticles***

The measured expression levels of each gene were recorded and analyzed in real-time by the device. The $C_T$ value for the functional genes was very different at different times.

***Evaluation of FAE1 gene expression at different concentrations of silver nanoparticles***

Analysis of variance (Table 5) showed that silver nanoparticles had a significant effect on the FAE1 gene ($p<0.01$). It was also found that the use of silver nanoparticles increased the expression of this gene and the highest gene expression was obtained in 3 mg / L silver nanoparticle treatment (Table 6).

***Evaluation of FAD2 gene expression at different concentrations of silver nanoparticles***

According to the results of the analysis of variance, the effect of silver nanoparticles on the FAD2 gene was significant ($p<0.01$) (Table 5). Results showed that silver nanoparticles increased expression of this gene and maximum gene expression was obtained in 3 mg/L silver nanoparticles (Table 6).

## Discussion

The small size of the nano-elicitors particles relative to the cell size makes their penetration into the cell easier, forcing the cell to increase gene expression in plant cells in the shortest time (Tran and Le, 2013). A study on cumin showed that FNS I gene expression at 25 and 50 µM concentrations of silver elicitor was significantly increased compared to the control plant (Yousefi et al., 2015). Expression of genes in root suspensions of *Papaver somniferum* L. treated with silver nano-elicitor showed that this elicitor significantly affected the expression of tydc7, DBOX, DIOX2 and bbe1 genes (Khodayari et al., 2015). It seems that nano-stimuli can be used to increase the expression of genes that affect the biosynthetic pathway ( Childs et al., 2016).

Application of nano-elicitors should be done with more cautious in gene up-regulation because they will decrease the expression of desired genes over time and with their continued presence (Babaei, 2019). This is because nanoparticles become more effective and the toxicity of silver nanoparticles increases by reducing the size of the nanoparticles (Gorth, 2011).

The presence of two 18S and 28S bands without fragmentation agarose gel indicates the good quality of the extracted RNA. PCR was performed using the synthesized cDNA as the template for the genes under investigation. Results showed that the amplified products were highly consistent with the expected band size. Also, the presence of a specific amplified single band for each gene indicates the appropriate quality of the synthesized cDNA and specialty of primers, which has a crucial role in the study of gene expression by Real Time PCR. Fig. (II) shows the single bands of the 18S rRNA gene on the agarose gel.

CT value for the 18S housekeeping gene showed that expression of this gene had the same CT value at different time points, indicating that housekeeping gene expression was not affected by environmental conditions. Also, the CT values of the FAE1 gene were obtained for samples, indicating differences in the expression of functional genes at concentrations of silver nanoparticles.

FAE1 or Fatty Acid Elongase 1 is a key gene in the erucic acid biosynthesis in Camelina (Hutcheon et al., 2010). Therefore, it is important to investigate the effect of different substances and conditions on the expression changes of this gene.

Different CT values of FAE1 gene were obtained for different samples, indicating differences in the expression of functional genes at different concentrations of silver nanoparticles.

FAD2 or Fatty Acid Desaturase 2 gene is involved in fatty acid biosynthesis. This gene converts oleic acid to linoleic acid so that, the process of un-saturating and removing hydrogen from the carbon chain causes it to convert a double bond into two double bonds ( Cahoon et al., 2001; Li et al., 2008).

## Conclusion

RNA extraction and cDNA synthesis were performed in high quality. 18S rRNA gene is known to be a suitable housekeeping gene because it had the same expression. The effect of silver nanoparticles on the expression of FAE1 and FAD2 genes was significant ($p<0.05$) and increased as the concentration of silver nanoparticles increased. The highest enhancement was observed on 3 mg/L silver nanoparticles. This suggests that nanoparticles can be used to increase the expression of genes that affect the biosynthetic pathway.

## Acknowledgments

Thanks to Biston Shafa Co. for providing the researchers with Soheil cultivar of Camelina sativa.

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