

1-1-2015

Effects of cobalt nanoparticles on artemisinin production and gene expression in *Artemisia annua*

BITA GHASEMI

RAMIN HOSSEINI

FATEMEH DEHGHAN NAYERI

Follow this and additional works at: <https://dctubitak.researchcommons.org/botany>



Part of the [Botany Commons](#)

Recommended Citation

GHASEMI, BITA; HOSSEINI, RAMIN; and NAYERI, FATEMEH DEHGHAN (2015) "Effects of cobalt nanoparticles on artemisinin production and gene expression in *Artemisia annua*," *Turkish Journal of Botany*. Vol. 39: No. 5, Article 3. <https://doi.org/10.3906/bot-1410-9>
Available at: <https://dctubitak.researchcommons.org/botany/vol39/iss5/3>

This Article is brought to you for free and open access by TÜBİTAK Academic Journals. It has been accepted for inclusion in Turkish Journal of Botany by an authorized editor of TÜBİTAK Academic Journals.

Effects of cobalt nanoparticles on artemisinin production and gene expression in *Artemisia annua*

Bitra GHASEMI, Ramin HOSSEINI, Fatemeh DEHGHAN NAYERI*

Department of Agricultural Biotechnology, Faculty of Engineering and Technology, Imam Khomeini International University, Qazvin, Iran

Received: 10.10.2014 • Accepted/Published Online: 10.03.2015 • Printed: 30.09.2015

Abstract: The low artemisinin content in *Artemisia* has caused this compound to be expensive among medicines. Several attempts have been made to increase its production by altering the expression of different genes. However, no approach has been cost-effective. In this study, the expression levels of *SQS* and *DBR2* genes were quantified by qRT-PCR, and artemisinin content was measured by HPLC in *Artemisia annua* cell suspension culture under nano cobalt particles elicitation. For this purpose, nano cobalt particles were used in 0.25, 2.5, and 5 mg L⁻¹ concentrations and samples were collected after 8, 24, 48, and 72 h. The highest artemisinin content was observed 24 h after 5 mg L⁻¹ nano cobalt treatment. In this case, artemisinin production was 2.25-fold (113.35 mg g⁻¹ dw) higher than that of the control. Simultaneously, the expression levels of *SQS* and *DBR2* genes decreased. It appears that the decrease in the expression of *SQS* and *DBR2* genes caused the artemisinin content to increase by high concentrations of the nano cobalt particles.

Key words: Artemisinin, nano elicitor, cobalt, gene expression, HPLC, QRT-PCR

1. Introduction

Secondary metabolites, which are a unique source of medicines with protective properties, are generally produced by plants exposed to different elicitors and/or signal molecules (Zhao et al., 2005). Artemisinin, as one of the secondary metabolites produced by *Artemisia annua*, is a sesquiterpene lactone used for removing the causes of malaria (*Plasmodium falciparum* and *P. vivax*) (Snow et al., 2005) and for treating different types of cancer such as leukemia, breast cancer, colon cancer, and small cell lung carcinomas (Lei et al., 2011). Artemisinin content in wild *A. annua* plants is very low. Therefore, it causes an increase in the price of medicines made by this product, especially for people in developing countries where malaria is widely spread. The chemical synthesis of artemisinin is expensive and not economical. In spite of the discovery of artemisinin and its valuable medicinal properties, scientists have not yet achieved a commercial method to increase artemisinin content (Ferreira et al., 1995). Cell suspension culture is a powerful tool for producing such compounds that are difficult to extract or synthesize chemically. Investigation of genes involved in a biosynthetic pathway can be helpful to recognize the key genes in order to manipulate the pathway (Zhao et al., 2005). Application of elicitors in cell suspension culture is

one of the most important strategies to increase metabolite content. Elicitation in a proper concentration and at a suitable time can shorten the time needed to achieve the highest level of metabolites (Mulabagal and Tsay, 2004). Modulation of microelements in culture media is also an appropriate strategy to increase the metabolite content in the cell suspension culture (Jimenez-Aparicio and Gutierrez-Lopez, 1999). For example, divalent metal ions, such as iron, zinc, copper, cobalt, and nickel, have been reported to influence secondary metabolites production (Trejo-Tapia et al., 2001). Although these metal ions are micronutrients that play an important role in the activities of proteins involved in maintaining the growth of organisms, at high concentrations they are harmful to living organisms (Viehweger, 2014). Metal ions such as nickel, cobalt, zinc, and manganese are necessary for regulating enzyme activity despite being highly toxic at high concentrations (Ovečka and Takáč, 2014).

The properties of substances will change while in nano size, and, more importantly, the number of atoms will increase on the surface. The important properties and extraordinary features of nanoparticles are mostly attributed to their surface characteristics. By increasing the number of atoms on a surface, the total free energy will also increase, which can alter certain characteristics

* Correspondence: nayeri@eng.ikiu.ac.ir

of the materials. Nanoparticles have the potential to be used as novel effective elicitors in plant biotechnology for the elicitation of secondary metabolites' production (Fakruddin et al., 2012). The study of Zhang et al. (2013) highlighted the potential of nanosilver particles as a novel effective elicitor in plant biotechnology for the production of plant secondary metabolites. Using Ag-SiO₂ core-shell nanoparticles (AgNPs), they increased artemisinin content in the hairy root culture of *Artemisia annua* (Zhang et al., 2013). Recent studies have reported the suitability of lipid nanoparticles for parenteral delivery and the enhancement of the antimalarial activity of artemisinin derivative artemether (Aditya et al., 2010).

New information about the metabolites' production pathway is necessary for discovering the function of the elicitor. The first step in the artemisinin pathway is the production of farnesyl diphosphate (FDP), which can be produced in cytosol or plastids. Sesquiterpene

and sterol production pathways of FDP are controlled coordinately. Sesquiterpene cyclase (SQC) is required for sesquiterpene production, whereas in the other pathway (sterol production) squalene synthase (SQS) is used. By increasing sterol production, artemisinin production will decrease, and vice versa (Lange et al., 2000). The converse regulation of these 2 genes, SQS and SQC, has been demonstrated by using miconazole to inhibit SQS in *A. annua* (Weathers et al., 2004). Amorpha 4,11-diene, the first precursor of artemisinin production, is synthesized by amorpha diene synthase (ADS). FDP is circularized by the ADS gene. The amorpha 4,11-diene is converted to artemisinin alcohol and artemisinin aldehyde sequentially, and to dihydro artemisinic aldehyde eventually (Figure 1) (Teoh et al., 2009). The rest of the artemisinin pathway is not fully known. Artemisinic acid faces 2 pathways: one produces arteannuin B (AB) and the other produces artemisinin. In the AB production pathway, artemisinic

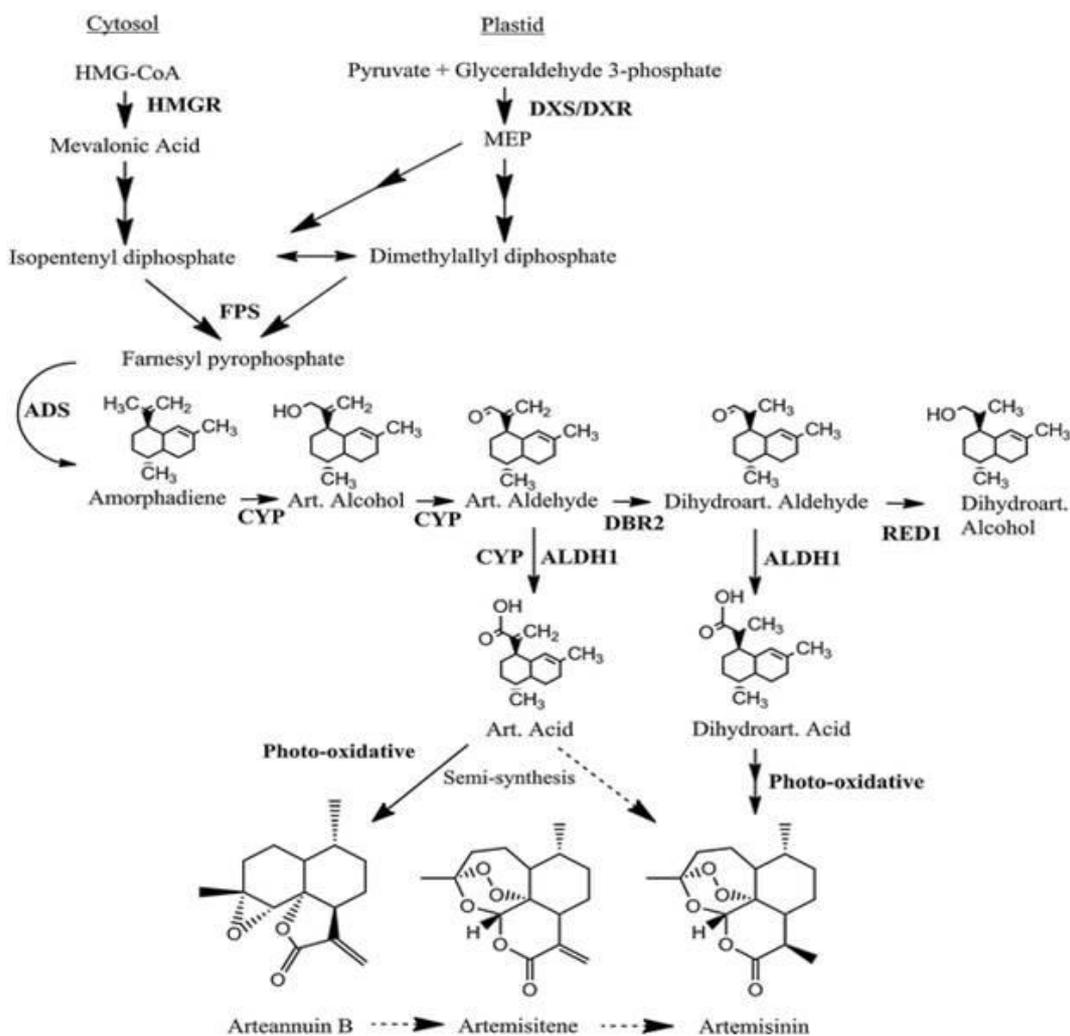


Figure 1. Artemisinin and its byproducts' production pathway (Nguyen et al., 2011).

aldehyde is oxidized and converted to artemisinic acid (AA) by CYP or aldehyde dehydrogenase (Aldh1), and is eventually converted to AB (Teoh et al., 2009). Dhingra and Lakshmi Narasu (2001) reported that AB can be converted to artemisitene (AT) and then to artemisinin. However, Brown and Sy (2007) demonstrated that AA cannot be converted to artemisinin. Based on the work of Nguyen et al. (2011), there is no specific enzyme to convert AB to artemisinin via AT. Therefore, it seems that AB is the only product of seco-cadinanes (containing AB and artemisinin) in *A. annua* (Nguyen et al., 2011). In the other branch of the artemisinin pathway, artemisinic aldehyde is converted to dihydro artemisinic aldehyde by *DBR2* (double-bond reductase) and cytochrome. Artemisinic aldehyde is also converted to dihydro artemisinic acid, which is eventually converted to artemisinin by several nonenzymatic pathways and possibly with photooxidation (Wallaart et al., 2001). It has been reported that dihydro artemisinic aldehydereductase (Red1) converts the dihydro artemisinic aldehyde to dihydro artemisinic alcohol that can conquer artemisinin production, in order to reduce artemisinin production (Rydén et al., 2010). Briefly, the artemisinin pathway enters detour routes by expression of the *SQS* gene, and *DBR2* expression determines whether artemisinin is produced. A survey of expression of genes (*SQS* and *DBR2*) related to artemisinin biosynthesis can be helpful for further increasing artemisinin content. To our knowledge, there are no reports regarding the influence of nano cobalt on the expression level of involved genes and artemisinin content in *Artemisia*, which is the goal of the present study.

2. Experimental design

The seeds of *Artemisia annua* were donated by the Forest, Rangeland, and Watershed organizations of Iran. Seeds were surface-sterilized with 96% ethanol for 3 s and 0.1% (w/v) HgCl_2 for 5 min, followed by 3 rinses with autoclaved distilled water. After surface sterilization, seeds were cultured on solid MS medium containing 30 g L^{-1} sucrose and 8 g L^{-1} agar, and were incubated in a growth chamber at 25 ± 2 °C with a 16-h photoperiod. Explants were detached from 2- to 3-week-old plantlets and cultured on the MS basal medium supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} agar, and different levels of plant growth regulators. Leaf discs were used as explants for callus induction. Friable white calli were obtained when a combination of 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} BAP, and 30 g L^{-1} sucrose was used. Petri dishes containing the explants were incubated in the dark in a growth chamber at 25 ± 2 °C. After 3 subcultures, friable white calli were used for the establishment of homogeneous suspension cultures. Maximum growth rate of suspension culture was achieved in the MS liquid medium supplemented with 0.5 mg L^{-1}

NAA, 0.5 mg L^{-1} BAP, and 30 g L^{-1} of glucose. One gram of friable white callus was excised and carefully transferred into 100-mL narrow-necked Erlenmeyer flasks containing 30 mL of liquid MS medium supplemented with 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} BAP, and 30 g L^{-1} glucose. The pH of the medium was adjusted to 5.8 before autoclaving at 120 °C for 20 min. The flasks were rotated at 120 rpm on a gyratory shaker and incubated at 25 ± 2 °C in the dark. For growth analysis, samples were taken at 48-h intervals from day 1 to day 30 of subculturing. Cultures were maintained routinely by transferring 10 mL into 250-mL narrow-necked Erlenmeyer flasks containing 100 mL of fresh MS medium every 2 weeks.

2.1. Nano cobalt characterization

Citrate-stabilized zero valence cobalt nanoparticles were purchased from Nano Zino Company (Iran). The diameter of nano cobalt was 10 nm and purity was 99.9%. The pH was adjusted to 5.7 and nanoparticles were autoclaved before being added to the *Artemisia* cell cultures.

2.2. Elicitor treatment

A homogeneous cell suspension culture was obtained after several subcultures. Cells were treated with various concentrations of nano cobalt particles (0.25, 2.5, and 5 mg L^{-1}) for 8, 24, 48, and 72 h. The experiment was repeated 3 times. Based on the growth curve of the cells, the best time to apply elicitors was determined. For this purpose, the contents of the flasks were aseptically passed through preweighed Whatman No. 1 filter papers to collect the cells and measure fresh weight. The collected cells were dried for 24 h at 37 °C and their dry weight was recorded every 2 days for a month. Based on the growth curve, day 14 after subculturing was chosen for the application of elicitor (nano cobalt), since cells were at the early stationary phase. Samples were collected at 0, 8, 24, 48, and 72 h after elicitor treatment.

2.3. RNA isolation and first-strand cDNA synthesis

For gene expression analysis, total RNA was isolated from nano cobalt-treated cells using a TRIzol kit (Sigma, Germany) according to the manufacturer's instructions. After treating the extracted RNA with RNase-free DNaseI (Fermentas, Germany), the purity and concentration of RNA were evaluated by the NanoDrop apparatus (Thermo NanoDrop 1000, USA) and its integrity was checked on a 0.8% (w/v) agarose gel before and after DNaseI treatment. RNA (1 μg) was reverse-transcribed with Aid M-Mul V kit (Vivantis, Malaysia) in a reaction volume of 20 μL to generate the first-strand cDNA. The qRT-PCR primers for 2 artemisinin biosynthetic genes, *SQS* and *DBR2*, and *Actin* as internal reference, were designed by Primer3 software (www.embnet.sk/cgi-bin/primer3) based on the *Artemisia* genes available in the NCBI databank (<http://www.ncbi.nlm.nih.gov/>) and listed in the Table. Quantitative RT-PCR was performed in a Bio-Rad iQ5 (USA) sequence detection

Table. Primer design for qRT-PCR reaction.

Gene name	Primer name	Primer sequence F/R [5'-3']	Primer T _m [°C]	% GC	PCR product size icon length [bp]
SQS	AF302464	F-TTTGAAAGCAGTATTGAAACAC	51.3	31.8	192
		R- CAGACAGCATCACGAAGC	52.8	55.6	
DBR2	EU704257	F- CATCAACAAGCAAGCCCATTTC	56.5	45.5	125
		R- GCGATAGTCTTCAACCACCTC	55.7	52.4	
ACT	U36376	F- AGTGCTCCTGGTTAGTTGTC	54.1	50	166
		R- CTTGTTGCCTCGTAATCTTCG	54.7	47.6	

system adopting 96-well plates. Reactions in a final volume of 20 μ L contained 5 μ L of template (cDNA), 2 μ L of the 10 pmol/ μ L primer mix (forward and reverse), and 10 μ L of SYBR green master mix (SYBR Biopars, GUASNR, Iran). The standard thermal profile was used for all reactions as follows: 94 °C for 30 s followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 30 s and generation of dissociation curve. The CT values were determined in 24 treated samples (3 nano cobalt concentrations, 4 time courses, and 2 genes). PCR efficiency was evaluated by Linreg PCR 11.0 software with statistical calculations. The relative fold changes in gene expression were calculated based on the $2^{-\Delta\Delta CT}$ comparative method (Pfaffl, 2001). The relative expression was normalized against *Actin* gene and calculated using the untreated samples as a calibrator. The statistical analysis of ANOVA was performed using MSTATC software, and the comparison of relative gene expression was measured with Duncan's test. Pearson correlation coefficient between the expression profiles of the 2 genes was calculated as well.

2.4. Artemisinin quantification

Artemisinin concentration was analyzed by HPLC (Knauer, Germany) with a K-1001 pump c-8 column, UV-K-2501 detector, and EuroChrom 2000 software. For HPLC analysis, the mobile phase was prepared by combining acetonitrile/acetic acid (30/70), and flow rate was maintained at 0.2 mL/min at a 10-min run time. The standard curve was drawn using standard artemisinin with 98% purity (Sigma, Germany). Each sample was run 3 times and SPSS was used for statistical analysis. The extraction process was carried out according to Caretto et al. (2011).

3. Results and discussion

3.1. Artemisinin production

The *Artemisia* cells were maintained by subculturing every 2 weeks. Cell density was determined daily from day 0 of subculture to day 30. The growth of *Artemisia* cell cultures followed standard growth kinetics, in which the number of cells increased exponentially due to the high rate of

cell division. It was followed by a slow rate of cell increase during the stationary phase. The cell number peaked on day 14 and then declined. To carry out the experiments, the elicitor was added to the cell cultures 14 days after the subculture, when the cells were at the early stationary phase (Figure 2). The production of artemisinin by *A. annua* cells was investigated for 34 days. Results showed that *A. annua* cells were able to naturally produce at least 0.3 mg g^{-1} dw artemisinin on the first day, to a maximum of 31.06 mg g^{-1} dw on day 16 (Figure 3). Considering the HPLC results, different concentrations of nano cobalt increased artemisinin content up to 24 h after elicitation compared to the untreated controls, but had no subsequent effect on artemisinin content. However, after the addition of 0.25 mg L^{-1} nano cobalt, 8 h after treatment a slight increase was observed in artemisinin content, followed by a decrease. Artemisinin concentration showed increases with 0.25 and 2.5 mg L^{-1} nano cobalt addition 24 h after nanoparticle treatment. The content of artemisinin reached its highest level with the addition of 5 mg L^{-1} nano cobalt after 24 h and decreased thereafter (Figure 4). Briefly, cells showed increased artemisinin contents only during the first 24 h of treatment with nano cobalt particles, reaching 120 mg

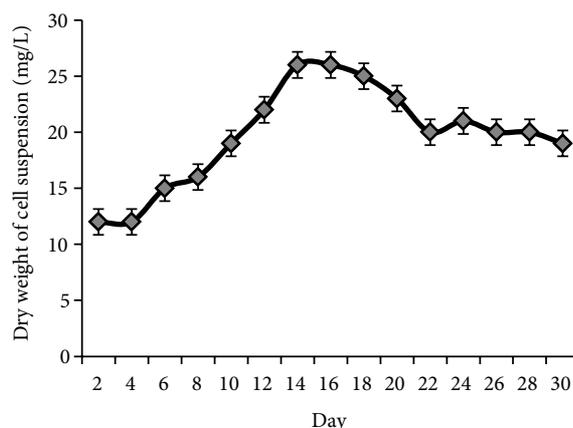


Figure 2. The cell growth curve in suspension culture of *A. annua* containing 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} BAP, and glucose (30 g L^{-1}).

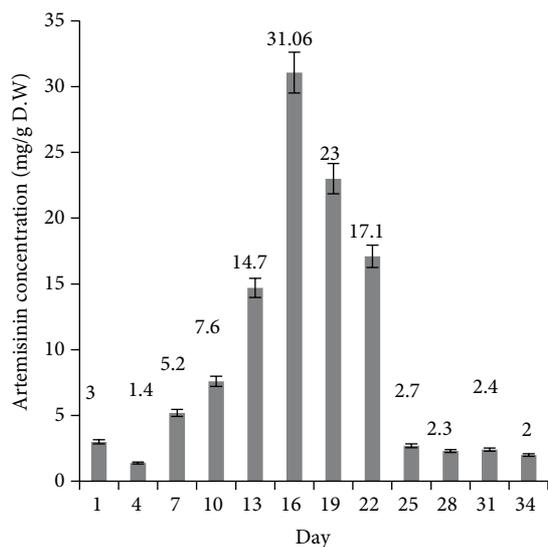


Figure 3. Intracellular artemisinin production level in the suspension culture of untreated *A. annua* in the MS medium containing 0.5 mg L⁻¹ NAA, 0.5 mg L⁻¹ BAP, and glucose (30 g L⁻¹).

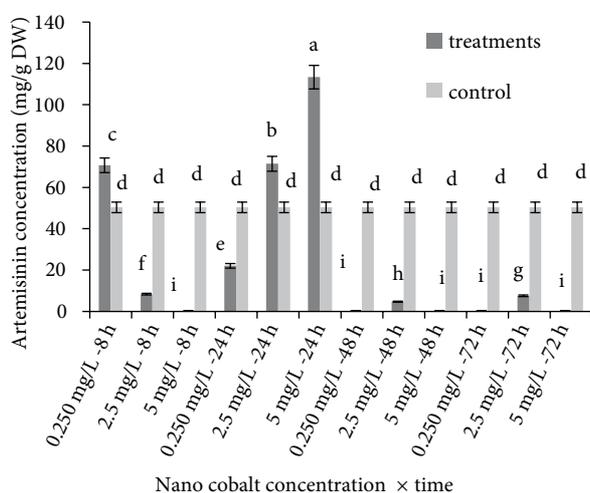


Figure 4. Artemisinin content in treated and untreated (control) samples at different times and concentrations of nano cobalt. Values are mean \pm SD.

g⁻¹ compared to the control, and then the artemisinin declined to a lower level than that of the control (Figure 4).

3.2. Expression of 2 main genes in the artemisinin biosynthesis pathway

3.2.1. The expression pattern of the SQS gene

Elicitors induce genes involved in the biosynthetic pathway of secondary metabolites through their specific motifs in the promoter region. Salicylic acid and methyl jasmonate cause an increase in artemisinin production in *A. annua*

through 2 TGACG motifs in the *DBR2* gene promoter (Pu et al., 2009). In the *DBR2* gene promoter there is a TGA box with a TGACGT/AA sequence for responding to auxin, a GARE element with a TAACAAA/G sequence for responding to gibberellins, and a Wun motif with a TCATTTTCGAA sequence for responding to wounding. Based on the studies of *Artemisia*, artemisinin content increases significantly 2 h after wounding, since the expression of genes involved in its biosynthesis increases remarkably. Moreover, there are two G boxes in the *DBR2* gene promoter with CACGTT and CACATG sequences that have a role in response to light, UV radiation, and abscisic acid. Several W boxes refer to promoter elements with the sequences of TGAC and TGACGTT found in the promoter of the *DBR2* gene, which are binding sites for the WRKY transcription factors. These transcription factors play a role in secondary metabolites' biosynthesis and response to environmental stresses. Furthermore, there are several MRE boxes in the promoter of the *DBR2* gene required for the MYB transcription factor that is involved in light- and drought-dependent responses in plants (Sarvestani et al., 2013). To determine the possible effect of cobalt nano particles on the transcription of *SQS* and *DBR2* genes, 2 main genes in artemisinin biosynthesis and qRT-PCR technique were used. Cells treated with various concentrations of nano cobalt showed changes in the transcription levels of these genes compared to the control. In different time courses, the *SQS* gene showed the highest and lowest expression levels at 8 and 72 h after treatment, respectively (Figure 5). At 8 h, the expression level of *SQS* increased approximately 2.5-fold compared to the control, although it decreased gradually until 72 h after the nano cobalt treatment. Without considering the posttreatment time, the expression level of *SQS* increased 2-fold in 0.25 mg L⁻¹ concentration of nano cobalt and then downregulated slowly (Figure 6).

In the interaction between nano cobalt concentrations and time, *SQS* expression showed 2 distinct peaks: 0.25

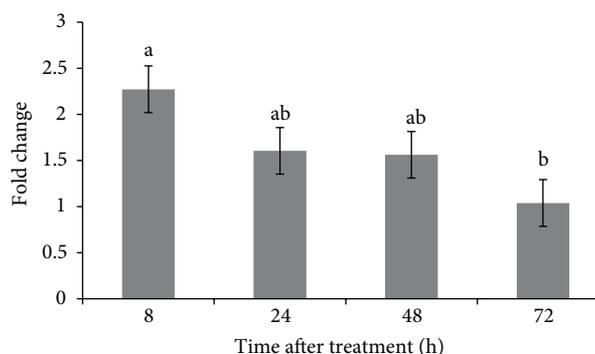


Figure 5. Changes in *SQS* gene expression at different times after treatment by nano cobalt. Data represent the means of $2^{-\Delta\Delta CT} \pm$ SD. Letters represent statistical difference at $P \leq 0.05$.

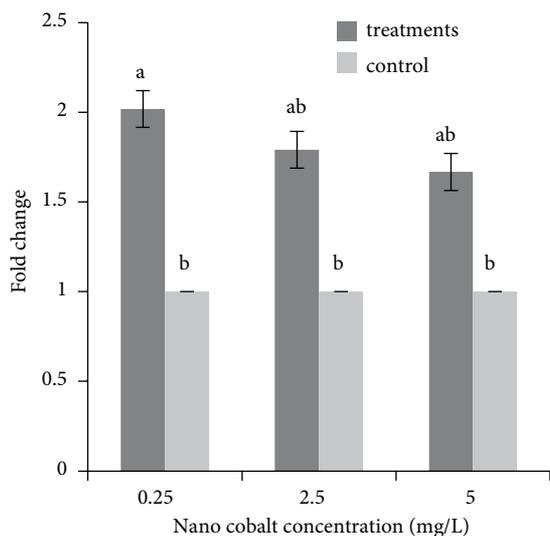


Figure 6. Changes in SQS gene expression at different concentrations of nano cobalt. Data represent the means of $2^{-\Delta\Delta CT} \pm SD$. Letters represent statistical difference at $P \leq 0.05$.

mg L⁻¹ at 72 h and 2.5 mg L⁻¹ at 8 h after nano cobalt application. The highest expression level of SQS (5.2-fold) was observed in 5 mg L⁻¹ nano cobalt after 48 h (Figure 7). After aligning the expression profile of the SQS gene with the artemisinin content, it was observed that overall, and concomitantly with the increase in SQS gene expression, the artemisinin content decreased (Figures 5 and 7).

3.2.2. The expression pattern of the DBR2 gene

Transcript levels of the DBR2 gene were upregulated in 0.25, 2.5, and 5.0 mg L⁻¹ nano cobalt concentrations 4.5-, 3.5-, and 3-fold, respectively, compared to the control.

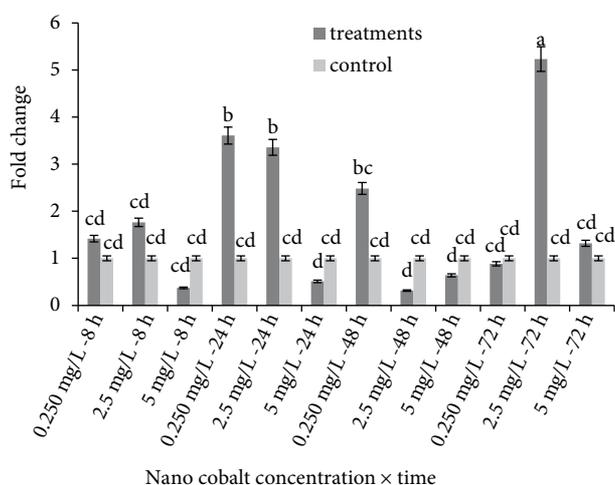


Figure 7. Changes in SQS expression at the interaction of time and concentration of nano cobalt treatments. Data represent the means of $2^{-\Delta\Delta CT} \pm SD$. Letters represent statistical difference at $P \leq 0.05$.

The maximum increase in DBR2 gene expression (4.5-fold) was observed with 0.25 mg L⁻¹ nano cobalt and it continuously declined after that (Figure 8). In different time courses and regardless of nano cobalt concentration, the highest expression level of DBR2 gene was observed 8 h after nano cobalt treatment (~4-fold) and it declined within 72 h. However, this decrease was still 2-fold higher than the control (Figure 9).

In the interaction between nano cobalt concentration and time, the DBR2 gene showed upregulated expressions in 2.5 mg L⁻¹ at 8 h, 0.25 mg L⁻¹ at 24 h, and 5.0 mg L⁻¹ at 72 h after treatment (Figure 10). In other treatments, the DBR2 gene indicated similar expression profiles. The maximum expression of DBR2 gene was detected in 0.25 mg L⁻¹ nano cobalt at 72 h after treatment (~8-fold). In total, there was a positive correlation between the expression levels of SQS and DBR2 genes and a negative correlation between the expression of both genes and artemisinin content. At 72 h, both genes showed a 1.5- to 2-fold downregulation (Figures 5 and 9).

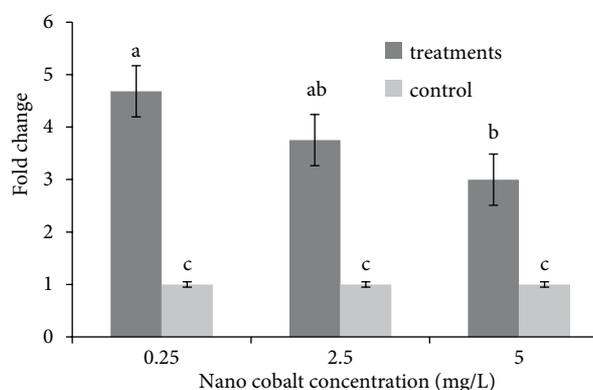


Figure 8. Changes in DBR2 gene expression at different concentrations of nano cobalt. Data represent the means of $2^{-\Delta\Delta CT} \pm SD$. Letters represent statistical difference at $P \leq 0.05$.

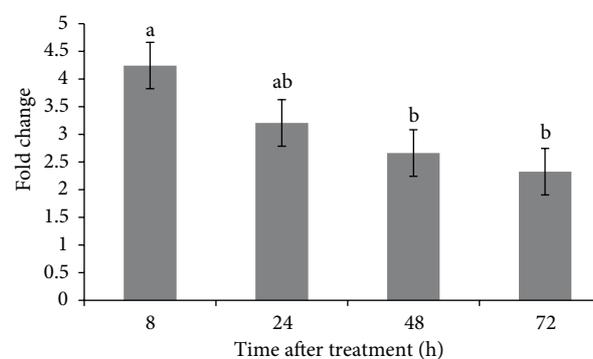


Figure 9. Changes in DBR2 gene expression at different times after treatment by nano cobalt. Data represent the means of $2^{-\Delta\Delta CT} \pm SD$. Letters represent statistical difference at $P \leq 0.05$.

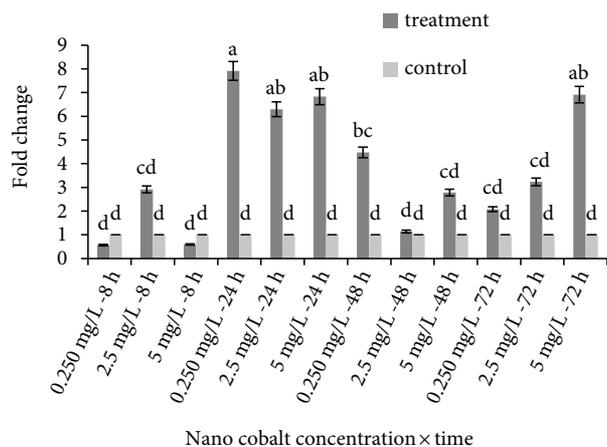


Figure 10. Changes in *DBR2* expression at the interaction of time and concentration of nano cobalt treatments. Data represent the means of $2^{-\Delta\Delta CT} \pm SD$. Letters represent statistical difference at $P \leq 0.05$.

3.2.3. Changes in artemisinin content

Among different nano cobalt treatments, there was an increase in artemisinin content at 8 h and 24 h after the elicitor application, although no considerable changes were observed at 72 h. *Artemisia* cells were in their logarithmic phase between days 14 and 16 of culture and entered the static and death phases afterwards (Figure 2). Since elicitor was applied on day 14, and considering that the increase in artemisinin content occurred 24 h after elicitor treatment (day 15), it appears that the best time for elicitor application for the stimulation of artemisinin production is before the static phase. It was also observed that without elicitor application (Figure 3), and upon stimulation by nano cobalt (Figure 4), artemisinin production began to decline from day 15 onwards. Therefore, it seems that while cells were entering the death phase, the produced artemisinin (naturally produced or elicited) was probably consumed for the activity of the cells or secreted into the culture medium. Thus, it may be easier to isolate and purify artemisinin before cells enter the death phase.

Our data indicated a negative correlation between the expression of the *SQS* and *DBR2* genes with artemisinin production. Nguyen et al. (2011) reported that the *SQS* gene causes FDP to enter a competitive pathway and eventually be converted to sterol that causes artemisinin content to decrease. Olofsson et al. (2011) also reported that squalene synthase (but not other sesquiterpene synthases) appears to be a significant competitor of FDP in artemisinin-producing tissues, and different sesquiterpene synthases compete for the FDP that is a precursor of artemisinin. Moreover, FDP is used for the synthesis of squalene, which is the precursor of sterols and triterpenes (detour pathway of artemisinin synthesis). Therefore, the

activity of various sesquiterpene synthases may influence the yield of artemisinin in the plant. In addition, squalene synthesis probably affects the yield of artemisinin, since the potential utilization of FDP by *SQS* is most likely higher than that of *ADS* (Olofsson et al., 2011).

Transgenic approaches also provide an effective way to manipulate a metabolic pathway. In this regard, overexpression of key enzyme genes in the artemisinin biosynthetic pathway or blockage of the competitive pathways genes led to increased artemisinin content in transgenic *Artemisia* (Tang et al., 2014).

Zhang et al. (2009) reported a 3.14-fold increase in artemisinin content in transgenic *A. annua* in comparison to the content in untransformed control plants by suppressing the expression of *SQS* using the RNAi technique. Therefore, inhibition of *SQS* expression by the application of various methods can be a useful alternative to the increase of artemisinin content. Based on Nguyen et al. (2011), one way for artemisinin regulation is a competing pathway. Our results are in agreement with Towler and Weathers (2007), who mentioned that increased yield of artemisinin can be achieved by inhibiting *SQS* gene expression by miconazole. The highest amount of artemisinin was observed with 5 mg L^{-1} nano cobalt 24 h after treatment, and, interestingly, *SQS* expression level was low under these conditions. The biosynthetic pathway of artemisinin after artemisinic aldehyde production is not well known. Dihydro artemisinin aldehyde (*DBR2* gene product) can be converted to dihydro artemisinin alcohol by the *Red1* gene, or to dihydro artemisinic acid and then to artemisinin by *ALDH* and some photooxidative steps, respectively (Dhingra and Lakshmi Narasu, 2001). The *DBR2* transcript is important because differences in *DBR2* expression can determine whether artemisinin or arteunin B are produced (Nguyen et al., 2013). Based on the negative correlation between the expression of the *DBR2* gene and artemisinin content observed in our research, it is assumed that dihydro artemisinin aldehyde (*DBR2* gene product) is probably converted to dihydro artemisinic alcohol, rather than being converted to dihydro artemisinic acid and subsequently artemisinin, and eventually does not lead to artemisinin production. It seems that *DBR2* also has a negative correlation with *ALDH*, because a decline in artemisinin content (increase in *DBR2* expression) means that decreases have occurred in the conversion of dihydro artemisinin aldehyde to dihydro artemisinic acid (eventually to artemisinin) and artemisinin aldehyde to artemisinic acid (eventually to artemisinin). These 2 reactions (finally leading to artemisinin production) are catalyzed by aldehyde dehydrogenase (*ALDH*). The interaction between the elicitor concentration and time was significantly higher in *SQS* and *DBR2* gene expressions. In contrast to this observation, HPLC results

showed significant increases in artemisinin content with the addition of 2.5 and 5 mg L⁻¹ nano cobalt 24 h after treatment. This may be due to the activation of other genes in the artemisinin biosynthetic pathway by the addition of nano cobalt particles. The mechanism of nano cobalt influence on enhancing artemisinin content will be better understood with an investigation of the expression of all genes involved in artemisinin production. Liu et al. (2011) concluded that there are several limiting points in artemisinin biosynthetic pathway. According to Zhang et al. (2004), it is possible to modulate the biosynthetic pathway of artemisinin. However, in order to increase the production of a metabolite, enhancing the expression of one gene is not sufficient. Divalent cations like Co²⁺ and Cu²⁺ have been noticed for their positive effect on the production of secondary metabolites (Trejo-Tapia et al., 2001). Metal ions such as cobalt, nickel, iron, silver, and vanadium all stimulate the production of a wide range of secondary metabolites in plants (Mithöfer et al., 2004; Zhao et al., 2005). In a study conducted by Baldi and Dixit (2008), a slight increase was observed in the artemisinin content of *Artemisia* cell suspension upon the addition of yeast extract. This increase was attributed to the presence of metal ions Co²⁺ and Zn²⁺. The mechanism of nanoparticles that function as elicitors is still undefined, and more research should be carried out. Studies have reported that the mechanism by which metal ions stimulate secondary metabolite induction is not known (Zhao et al., 2005). However, the production of ROS via heavy metal ions has been shown by Kandlbinder et al. (2004), Mithöfer et al. (2004), and Zhao et al. (2005).

Using 900 mg L⁻¹ AgNPs, artemisinin content in *A. annua* hairy root culture increased from 1.67 mg g⁻¹ dw to 2.86 mg g⁻¹ dw after 3 days. In addition, this elicitor stimulated artemisinin production in 20-day-old hairy

root cultures up to 13.3 mg L⁻¹, a 3.9-fold increase over the control. AgNP treatment induced oxidative stress (H₂O₂ production) resulting in lipid peroxidation (increased malonyldialdehyde accumulation), enhanced activities of catalase, and increased artemisinin content in hairy roots (Zhang et al., 2013).

The stimulating effect of cobalt ions has been proven in some plants. For example, betanin increased up to 60% by enhancing the concentration of Co²⁺ from 1 μM to 5 μM in *Beta vulgaris* suspension culture. Nonetheless, higher concentrations of cobalt did not have a positive effect (Trejo-Tapia et al., 2001). Pitta-Alvarez et al. (2000) reported an increase in scopolamine content with the addition of cobalt chloride (CoCl₂) in the hairy root culture of *Brugmansia candida*. Paclitaxel was increased more than 3-fold by adding 20 μM CoCl₂ (Zhang et al., 2007). There is no report regarding the influence of cobalt nano particles on the production of artemisinin, and this study is the first. We found that cobalt nanoparticles can be an effective elicitor to stimulate artemisinin.

In the present study, it was shown that artemisinin raised to 113.35 mg g⁻¹ dw in the *Artemisia* suspension culture by stimulation with 5 mg L⁻¹ nano cobalt. In this case, artemisinin production was 2.25-fold higher than that of the control. It can be concluded that for artemisinin production, *A. annua* suspension culture is an alternative compared to the whole plant. In addition, by the optimization of concentration and time, nano cobalt can be used as a suitable and economical elicitor for increasing artemisinin content.

Acknowledgments

This work was conducted at the Agricultural Biotechnology Department of Imam Khomeini International University. We thank all staff for their collaboration.

References

- Aditya N, Patankar S, Madhusudhan B, Murthy R, Souto E (2010). Arthemeter-loaded lipid nanoparticles produced by modified thin-film hydration: pharmacokinetics, toxicological and in vivo anti-malarial activity. *Eur J Pharm Sci* 40: 448–455.
- Baldi A, Dixit V (2008). Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. *Bioresource Technol* 99: 4609–4614.
- Brown GD, Sy LK (2007). In vivo transformations of artemisinic acid in *Artemisia annua* plants. *Tetrahedron* 63: 9548–9566.
- Caretto S, Quarta A, Durante M, Nisi R, De Paolis A, Blando F, Mita G (2011). Methyl jasmonate and miconazole differently affect artemisinin production and gene expression in *Artemisia annua* suspension cultures. *Plant Biol* 13: 51–58.
- Dhingra V, Lakshmi Narasu M (2001). Purification and characterization of an enzyme involved in biochemical transformation of arteannuin B to artemisinin from *Artemisia annua*. *Biochem Bioph Res Commun* 281: 558–561.
- Fakruddin MD, Hossain Z, Afroz H (2012). Prospects and applications of nanobiotechnology: a medical perspective. *J Nanobiotechnology* 10: 1–8.
- Ferreira JFS, Simon JE, Janick J (1995). Developmental studies of *Artemisia annua*: flowering and artemisinin production under greenhouse and field conditions. *Planta Med* 61: 167–170.
- Jimenez-Aparicio A, Gutierrez-Lopez G (1999). Production of food related colorants by culture of plant cells. *Adv Exp Med Biol* 464: 195–210.

- Kandlbinder A, Finkemeier I, Wormuth D, Hanitzsch M, Dietz KJ (2004). The antioxidant status of photosynthesizing leaves under nutrient deficiency: redox regulation, gene expression and antioxidant activity in *Arabidopsis thaliana*. *Physiol Plantarum* 120: 63–73.
- Lange BM, Rujan T, Martin W, Croteau R (2000). Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. *P Natl Acad Sci USA* 97: 13172–13177.
- Lei C, Ma D, Pu G, Qiu X, Du Z, Wang H, Li G, Ye H, Liu B (2011). Foliar application of chitosan activates artemisinin biosynthesis in *Artemisia annua* L. *Ind Crop Prod* 33: 176–182.
- Liu B, Wang H, Du Z, Li G, Ye H (2011). Metabolic engineering of artemisinin biosynthesis in *Artemisia annua* L. *Plant Cell Rep* 30: 689–694.
- Mithöfer A, Schulze B, Boland W (2004). Biotic and heavy metal stress response in plants: evidence for common signals. *Febs Lett* 566: 1–5.
- Mulabagal V, Tsay HS (2004). Plant cell cultures—an alternative and efficient source for the production of biologically important secondary metabolites. *Int J Appl Sci Eng* 2: 29–48.
- Nguyen KT, Arsenault PR, Weathers PJ (2011). Trichomes + roots + ROS = artemisinin: regulating artemisinin biosynthesis in *Artemisia annua* L. *In Vitro Cell Dev Biol Plant* 47: 329–338.
- Nguyen KT, Towler MJ, Weathers PJ (2013). The effect of roots and media constituents on trichomes and artemisinin production in *Artemisia annua* L. *Plant Cell Rep* 32: 207–218.
- Olofsson L, Engström A, Lundgren A, Brodelius PE (2011). Relative expression of genes of terpene metabolism in different tissues of *Artemisia annua* L. *BMC Plant Biol* 11: 45–56.
- Ovečka M, Takáč T (2014). Managing heavy metal toxicity stress in plants: biological and biotechnological tools. *Biotechnol Adv* 32: 73–86.
- Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: 45–51.
- Pitta-Alvarez SI, Spollansky TC, Giulietti AM (2000). The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of *Brugmansia candida*. *Enzyme Microb Tech* 26: 252–258.
- Pu GB, Ma DM, Chen JL, Ma LQ, Wang H, Li GF, Ye HC, Liu BY (2009). Salicylic acid activates artemisinin biosynthesis in *Artemisia annua* L. *Plant Cell Rep* 28: 1127–1135.
- Rydén AM, Ruyter-Spira C, Quax WJ, Osada H, Muranaka T, Kayser O, Bouwmeester H (2010). The molecular cloning of dihydroartemisinic aldehyde reductase and its implication in artemisinin biosynthesis in *Artemisia annua*. *Planta Med* 76: 1778–1783.
- Sarvestani R, Peyghambari S, Abbasi A (2013). Isolation and characterization of DBR2 gene promoter from Iranian *Artemisia annua*. *J Agric Sci Technol* 16: 191–202.
- Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI (2005). The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* 434: 214–217.
- Tang K, Shen Q, Yan T, Fu X (2014). Transgenic approach to increase artemisinin content in *Artemisia annua* L. *Plant Cell Rep* 33: 605–615.
- Teoh KH, Devin RP, Darwin WR, Patrick SC (2009). Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in *Artemisia annua*. *Botany* 87: 635–642.
- Towler MJ, Weathers PJ (2007). Evidence of artemisinin production from IPP stemming from both the mevalonate and the nonmevalonate pathways. *Plant Cell Rep* 26: 2129–2136.
- Trejo-Tapia G, Jimenez-Aparicio A, Rodriguez-Monroy M, De Jesús-Sánchez A, Gutierrez-Lopez G (2001). Influence of cobalt and other microelements on the production of betalains and the growth of suspension cultures of *Beta vulgaris*. *Plant Cell Tiss Org* 67: 19–23.
- Viehweger K (2014). How plants cope with heavy metals. *Bot Stud* 55: 1–12.
- Wallaart TE, Bouwmeester HJ, Hille J, Poppinga L, Majiers NCA (2001). Amorpha-4, 11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. *Planta* 212: 460–465.
- Weathers P, De Jesús-Gonzalez L, Kim Y, Souret F, Towler M (2004). Alteration of biomass and artemisinin production in *Artemisia annua* hairy roots by media sterilization method and sugars. *Plant Cell Rep* 23: 414–418.
- Zhang B, Zheng LP, Yi Li W, Wen Wang J (2013). Stimulation of artemisinin production in *Artemisia annua* hairy roots by Ag-SiO₂ core-shell nanoparticles. *Curr Nanosci* 9: 363–370.
- Zhang CH, Fevereiro PS, He G, Chen Z (2007). Enhanced paclitaxel productivity and release capacity of *Taxus chinensis* cell suspension cultures adapted to chitosan. *Plant Sci* 172: 158–163.
- Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, Oksman-Caldentey KM, Xu T, Pi Y, Wang Z, Zhang H (2004). Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. *P Natl Acad Sci USA* 101: 6786–6791.
- Zhang L, Jing F, Li F, Li M, Wang Y, Wang G, Sun X, Tang K (2009). Development of transgenic *Artemisia annua* (Chinese wormwood) plants with an enhanced content of artemisinin, an effective anti-malarial drug, by hairpin-RNA-mediated gene silencing. *Biotechnol Appl Bioc* 52: 199–207.
- Zhao J, Davis LC, Verpoorte R (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol Adv* 23: 283–333.