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Original Article

TiO_2 nanoparticles and salinity stress in relation to artemisinin production and ADS and DBR2 expression in *Artemisia absinthium* L.

Nanopartículas de TiO_2 e estresse de salinidade em relação à produção de artemisinina e expressão de ADS e DBR2 em *Artemisia absinthium* L.

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Abstract

Artemisia absinthium L. is an important herb that is widely cultivated in different parts of the world for its medicinal properties. The present study evaluated the effects of four concentrations of nanoparticles treatment (0, 10, 20 and 30 mg L^{-1}) and NaCl salinity stress (0, 50, 100 and 150 mM NaCl) and their interactions with respect to the expression of two key genes, i.e. DBR2 and ADS, in the biosynthesis pathway of artemisinin in *A. absinthium.* Total RNA was extracted and a relative gene expression analysis was carried out using Real-Time PCR. The amount of artemisinin was also determined by HPLC. All the experiments were performed as factorial in a completely randomized design in three replications. The results revealed that salinity stress and nanoparticles treatment and their interaction affected the expressions of these genes significantly. The highest levels of ADS gene expression were observed in the 30 mg L^{-1} nanoparticles–treated plants in the presence of 150 mM salinity stress and the lowest levels in the 10 mg L^{-1} nanoparticles–treated plants under 50 mM salinity stress. The maximum DBR2 gene expression was recorded in the 10 mg L^{-1} nanoparticles–treated plants in the absence of salinity stress and the minimum expression in the 100 mM salinity-stressed plants in the absence of nanoparticles treatment. Moreover, the smallest amounts of artemisinin were observed in the 150 mM salinity-stressed plants in the absence of nanoparticles and the highest amounts in the 30 mg L^{-1} nanoparticles–treated plants. The maximum amounts of artemisinin and ADS gene expression were reported from the plants in the same nanoparticles treatment and salinity stress conditions. In this regard, the amount of artemisinin was decreased by half in the plants containing the highest DBR2 gene expression. Meanwhile, no significant correlation was observed between these gene expressions and the artemisinin amount in the other nanoparticles–treated plants under different levels of salinity stress. The biosynthetic pathway of secondary metabolites appears to be very complex and dose not directly dependent on these gene expressions.

Keywords: artemisinin, gene expression, salinity stress, titanium dioxide nanoparticles, wormwood.

Resumo

Artemisia absinthium L. é uma erva importante que é amplamente cultivada em diferentes partes do mundo por suas propriedades medicinais. O presente estudo avaliou os efeitos de quatro concentrações de tratamento com nanopartículas (0, 10, 20 e 30 mg L-1) e estresse de salinidade com NaCl (0, 50, 100 e 150 mM NaCl) e suas interações com relação à expressão de dois genes-chave, isto é, DBR2 e ADS, na via de biossíntese da artemisinina em A. absinthium. O RNA total foi extraído, e uma análise de expressão gênica relativa foi realizada usando PCR em tempo real. A quantidade de artemisinina também foi determinada por HPLC. Todos os experimentos foram realizados como fatorial, em delineamento inteiramente casualizado, em três repetições. Os resultados revelaram que o estresse por salinidade e o tratamento com nanopartículas e sua interação afetaram significativamente as expressões desses genes. Os níveis mais altos de expressão do gene ADS foram observados nas plantas tratadas com nanopartículas de 30 mg L-1 na presença de estresse de salinidade de 150 mM, e os níveis mais baixos, nas plantas tratadas com nanopartículas de 10 mg L-1 com estresse de salinidade de 50 mM. A expressão máxima do gene DBR2 foi registrada nas plantas tratadas com nanopartículas de 10 mg L-1 na ausência de estresse de salinidade, e a expressão mínima, nas plantas estressadas com salinidade de 100 mM na ausência de tratamento com nanopartículas. Além disso, as menores quantidades de artemisinina foram observadas nas plantas com estresse de salinidade de 150 mM na ausência de nanopartículas, e as maiores quantidades, nas plantas tratadas com nanopartículas de 30 mg L-1. As quantidades máximas de expressão de genes de artemisinina e ADS foram

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relatadas a partir das plantas no mesmo tratamento com nanopartículas e condições de estresse de salinidade. A esse respeito, a quantidade de artemisinina diminuiu pela metade nas plantas que contêm a expressão gênica DBR2 mais alta. Enquanto isso, nenhuma correlação significativa foi observada entre essas expressões gênicas e a quantidade de artemisinina nas outras plantas tratadas com nanopartículas sob diferentes níveis de estresse de salinidade. A via biossintética dos metabólitos secundários parece ser muito complexa e não depende diretamente dessas expressões gênicas.

Palavras-chave: artemisinina, expressão gênica, estresse por salinidade, nanopartículas de dióxido de titânio, absinto.

1. Introduction

Industrial applications of nanoparticles have rapidly increased due to the new physical and chemical properties of manufactured nanoparticles (Demir et al., 2014). Among the different nanoparticles, titanium dioxide (TiO_2) nanoparticles have greater applications in different industries due to their high chemical resistance, non-toxicity, high shelf life, affordability and low costs (Bavykin et al., 2006). The application of TiO_2 nanoparticles has also increased considerably in recent years due to their several biological properties (Qi et al., 2013). According to some studies, these nanoparticles are a useful ingredient that can stimulate plant growth and increase plant production (Feizi et al., 2012). TiO_2 nanoparticles can increase the fresh and dry weights of plants by increasing their light absorption efficiency, enzymes' function and nitrate absorption and accelerating the conversion of inorganic materials to organic ones. TiO_2 nanoparticles enhance plants' immune system and control plant diseases, thereby increasing plant yields (Mingyu et al., 2007; Nair et al., 2010).

There are approximately 50 species of *Artemisia* over the world, with *Artemisia absinthium* L. being a popular species noted in almost all western herbal medicine books. This plant is native to the temperate Eurasia and North Africa and is used in pharmaceutical industries (Hashimi et al., 2019). This medicinal herb produces a secondary metabolite known as artemisinin, which has an isoprenoid structure and is accumulated in the secretory (glandular) trichomes of the aerial organs (Durante et al., 2011).

Artemisinin has several biological activities, including antitumor, neurotoxic, liver protective, antimalarial, anti-fever, anti-depressant, analgesic, anti-ulcer, nerve protective, antioxidant and antibacterial properties effects (Shafi et al., 2012; Khan et al., 2016; Mubashir et al., 2016; Basiri et al., 2017).

This compound is produced through the condensation and oxidation of three isopentenyl diphosphate (IPP) precursor molecules (Bouwmeester et al., 1999; Covello et al. 2007). According to previous studies, the condensation of these molecules catalyzed by FDP synthase (FPS) first synthesis farnesyl disphosphate (FDP); then, a sesquiterpene cyclase, namely Amorphadiene Synthase (ADS), catalyzes the production of amorpha–4,11–diene(Bouwmeester et al., 1999; Kim et al., 2006). A cytochrome P450, CYP71AV1 (CYP), catalyzes the next three reactions: oxidation of amorpha–4, 11–diene to artemisinic aldehyde and also to Artemisinic Acid [AA] (Teoh et al., 2006), which is then converted by a Double–Bond Reductase (DBR2) to dihydroartemisinic aldehyde, the presumed precursor to Dihydroartemisinic Acid [DHAA] (Zhang et al., 2008). Brown and Sy (2004) argued that the conversion of DHAA to artemisinin has been proven to occur *in vitro* as a photo–oxidative reaction, though this process may not necessarily occur *in vivo*.

Evaluating the means of setting these pathways and identifying the steps affecting the rate of artemisinin production are the first and most important steps for increasing its production. Although several evaluations were available about ADS and DBR2 expression and artemisinin concentrations in different *Artemisia* species under different stress conditions (Arsenault et al. 2010; Kim et al., 2006), no references were found on the effect of TiO_2 nanoparticles and salinity stress on the noted factors in *A. absinthium*.

The present study, evaluated the expression of two key genes, including ADS and DBR2, in salinity-stressed and titanium nanoparticle-treated plants. The results can help better understand the potential role of these genes in the rate of artemisinin production. The most effective concentrations of nanoparticle treatment were determined in stimulating plant growth and secondary metabolites production under salinity stress.

2. Materials and Methods

2.1. Cultivation and treatment

Seeds of *A. absinthium* were obtained from PAKAN BAZR Company (Isfahan, Iran) and were verified according to the descriptions provided by the references. The seeds were disinfected by 2% for 5 min and were washed twice with sterile water. After preparation, the seeds were placed between wet sterile filter papers and transferred to pots containing cocopeat and perlite. The plants were grown under greenhouse conditions on a 12 h light–dark cycle (25±2 °C light period, 20±2 °C night). After four weeks of plantation, only pure water was added for the irrigation of the young seedlings. Then, five plants were kept in each pot to create the appropriate density. After six weeks of plantation, one-fourth of Hoagland solution (Merck Company), followed by one-half of Hoagland solution, was used for feeding and irrigation.

The experiments were carried out with four concentrations of NaCl salinity (0, 50, 100, 150 mM) and TiO_2 nanoparticles (0, 10, 20, 30 mg L^{-1}) in a factorial arrangement in a completely randomized design with five replications. The NaCl salinity treatments were prepared by the addition of sodium chloride (NaCl) solutions (Merck Company) to pots containing 4-leafed plants. In order to prevent osmotic shock, the application of salinities was performed gradually in four steps. The TiO_2 nanoparticles were prepared by Nanotechnology Pishgaman Company (Iran). We determined the morphology and size of

the nanoparticles by TEM (Figure 1). Physicochemical traits of used nanoparticles have been summarized in Table 1.

The nanoparticles were applied at four concentrations on a weekly basis after the induction of NaCl salinity by spraying the plant leaves. Each concentration was prepared in five replications. In order to prevent damage to the leaves, spraying was carried out at sunset. The plants were harvested 72 h after the fifth replication of each nanoparticles concentration.

2.2. Sampling and RNA extraction

Several fresh leaves were harvested randomly from each pot and immediately frozen at -70 °C. In the next step, the complete RNA was extracted using the modified lithium chloride protocol (Peng et al. 2014). The quality of the extracted RNA was investigated by electrophoresis on a 1% agarose gel (Figure 2A).

2.3. Real-Time PCR (RT-qPCR) reactions

The synthesis of cDNA was performed using VIVANTIS kit according to the manufacturer's instructions (Cat. No.: cDSK01–050; Figure 2B). For the primer design, target sequences were obtained by referring to genomic databases with access numbers KJ609176.1 and AB926434.1 for the ADS and DBR2 genes, respectively (Supplementary Material). Specific primers were designed for these genes in the next step using Gene Runner software (Table 2). The reference gene primers were also designed based on the RNA gene sequence with access number AJ297261.1. A classic RT-QPCR was performed to optimize and pre-evaluate the synthesized cDNA using the primers. The RT-qPCR products were examined by agarose gel electrophoresis. Relative and comparative RT-qPCRs were performed according to the method proposed by Livak and Schmittgen (2001). Data are compared based on Fold Changes (FC) for gene expression. The FC is the expression ratio: if the fold change is more than 1, it means that the gene is upregulated; if it is less than 1, it means that the gene is downregulated (Livak and Schmittgen 2001).

2.4. HPLC

The plant samples were dried in the shade for one week after harvesting. To purify the extract, the dried leaves (1 g) were powdered and floated in chloroform at a sample: solvent ratio of 1g: 20 CC (w/v) for 24 h at 25°C. The mixtures were then homogenized at 25°C for 1h using a homogenizer (IKA, Germany). The extracts were then filtered using the filter paper and concentrated at 55°C using a rotary evaporator (Wiggens STRIKE 300, Germany) and freeze-dried for 16 h. collected extracts were stored at 4°C for next experiments. The dried extracts were dissolved in 5 mL of HPLC mobile phase solution (acetonitrile: 0.1%, acetic acid 30:70 V/V). Then, 2 mL of this solution was transferred to microtubes and centrifuged at 5000 rpm for 3 min at room temperature. Next, 20 µL of the extract was subjected to HPLC (WATERS apparatus). The HPLC column dimensions were 5 x 250 mm (5 µm in particle size). The flow rate was 1 ml min^{-1} and the UV detector's (WATERS 2487) wavelength was 216 nm. To draw the standard calibration curve, three concentrations (0.025, 0.05 and

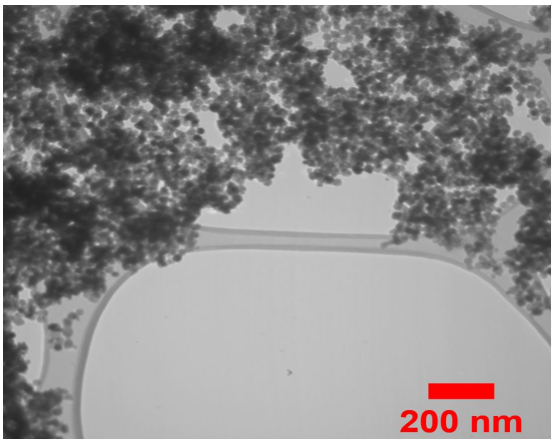

Figure 1. TEM micrograph of the used Titanium dioxide nanoparticles.

Table 1. Physico-chemical characteristics of Titanium dioxide nanoparticles.

Characteristics	Average Particle Size (APS)	Specific Surface Area (SSA)	Purity	Color	Crystal nature		Bulk Density	pH
					anatase	rutile		
TiO_2 NPs	20 nm	10-45 m^2g^{-1}	≤99%	white	80%	20	0.46 gml^{-1}	5.5-6.0

pH: Power of hydrogen.

Table 2. The list of primers applied in this study in real time RT–PCR analysis.

Gene	Direction	Sequence	Product length	Accession number (NCBI)
ADS	Forward	AGGTTTGCTTGAGTTGTACG	100bp	KJ609176.1
ADS	Reverse	CATAATGCTAAGACGAGATCG		
DBR2	Forward	CTTCATGTAACTCAACCACG	129bp	AB926434.1
DBR2	Reverse	GCTGCATATAAAAGTTCCAAC		
RRSS	Forward	CGATGAAGAACGTAGCAAAATG	111bp	AJ297261.1
RRSS	Reverse	AGACGTGCCCTCGGCCAAAAG		

NCBI: National Center for Biotechnology Information.

0.1 mL min^{-1}) of artemisinin (98% Sigma-Aldrich 361593) were injected into the HPLC in three replications. The three curves achieved for each concentration were calculated by the integral of the area under the curves. Based on the concentrations of the standard samples analyzed by the HPLC and the area under each curve, a linear correlation was drawn between the concentration of artemisinin and the area under the curve and its equation. Finally, the concentration of artemisinin was calculated for the different treatments by estimating the under curve area for each extract (Lapkin et al., 2009; Woerdenbag et al., 1991).

2.5. Statistical analyses

All the experiments were run in triplicates. In order to compare the number of the target gene copies and the reference gene, a comparative method was used according to the following formula: $\Delta C_T = (C_{T\ target} - C_{T\ reference})$ and $\Delta\Delta C_T = (\Delta C_T$ test sample $- \Delta C_T$ calibrator sample); (Livak and Schmittgen, 2001). The mean and standard errors were calculated for each parameter. Duncan's Multiple Range test (DMRT) was performed to determine if significant variations existed among the treatments for each measured parameter. Data analyses were performed in SPSS software v. 23.

3. Results

3.1. Molecular studies

TiO_2 nanoparticles treatment affected ADS and DBR2 gene expression significantly (Figures 3, 4). Salinity stress and the interaction of salinity stress and nanoparticles treatment were also reported to have significant effects on ADS gene expression. Meanwhile, no significant variations

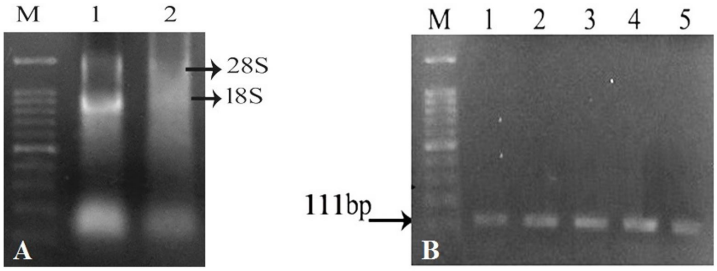

Figure 2. (A) Gel electrophoresis of RNA extracted from the leaf; (B) Gel electrophoresis of Standard RT–PCR product of 16s rRNA. M: 100bp DNA size marker. Numbered wells are samples. Gel agarose 1%.

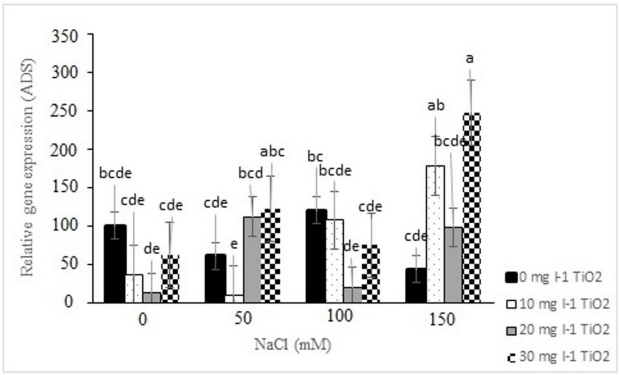

Figure 3. The effect of salinity and titanium dioxide nanoparticles on ADS gene expression in wormwood (non–identical letters indicate significant difference based on Duncan test $P \leq 0.05$).

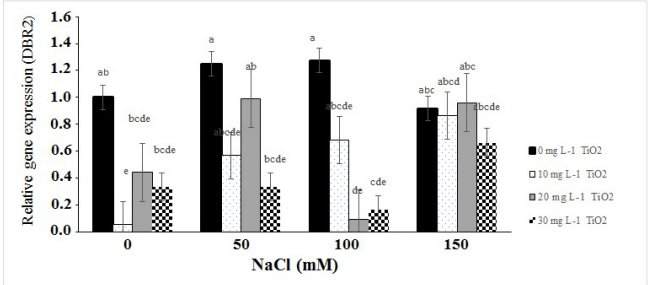

Figure 4. The effect of salinity and titanium dioxide nanoparticles on DBR2 gene expression in wormwood (the non–identical letters indicate significant difference based on Duncan test $P \leq 0.05$).

were observed for DBR2 gene expression in the salinity-stressed plants in the absence/presence of nanoparticles treatment (Table 3).

In the absence of salinity stress, the expression of both genes decreased with an increase in nanoparticles concentration. Under these conditions, the maximum expression was registered for these genes in the control plants; however, the minimum ADS gene expression and the minimum DBR2 gene expression was observed in the 20 and 10 mg L^{-1} nanoparticles--treated plants, respectively. Under 50 mM salinity stress, ADS gene expression was enhanced with an increase in nanoparticles concentration, but the reverse was recorded for DBR2 gene expression, as the highest DBR2 gene expression was found in the 50 mM salinity-stressed plants in the absence of nanoparticles treatment.

In the 100 mM salinity-stressed plants, the expression of both genes decreased with an increase in nanoparticles concentration. Nonetheless, the minimum expression of both genes was observed in the 20 mg L^{-1} nanoparticles-treated plants. Under 150 mM of salinity stress, ADS gene expression increased with an increase in nanoparticles concentration, and the maximum and minimum amounts of gene expression were recorded in 30 and 0 mg L^{-1} nanoparticles–treated plants, respectively. The exact reverse conditions occurred for DBR2 gene expression, as its minimum amount was observed in the 30 mg L^{-1} nanoparticles–treated plants (Table 4).

Table 3. Analysis of variance test of relative genes expression and artemisinin concentration in the samples under salinity stress and titanium dioxide nanoparticles treatment.

Source Variation	Degrees of freedom	Mean Square		
		FC/ADS	FC/DBR2	Artemisinin concentration (mg g^{-1} DW)
Salinity	3	1.720**	0.417 ns	0.0003**
TiO_2 Nanoparticles	3	0.914*	1.195**	0.001**
Salinity*TiO_2 Nanoparticles	9	1.095**	0.243 ns	0.001**
Error	48	0.314	0.170	0.000002
Coefficient of variation		0.546	0.287	0.991

–Data are compared based on Fold Changes (FC) for gene expression. **Significantly at a level of 1% error (p>0.01); *Significantly at a level of 5% error (p>0.05). Ns: Non–significant, DW: Dry weight, FC: Fold Changes.

Table 4. Mean ± standard error of relative genes expression and artemisinin concentration in the nanoparticles–treated and salinity–stressed plant samples of *A. absinthium* (DW: Dry weight, FC: Fold Changes, Non–similar letters represent a significant difference based on the Duncan test $P \leq 0.05$).

Salinity (mM)	Nanoparticles (TiO_2) (mg L^{-1})	FC/ADS	FC/DBR2	Artemisinin concentration (mg g^{-1} DW)
0	0	$1.00^{bcde} \pm 0.00$	$1.00^{ab} \pm 0.00$	$0.0442^{e} \pm 0.0007$
	10	$0.36^{cde} \pm 0.07$	$0.05^{e} \pm 0.05$	$0.0206^{j} \pm 0.0008$
	20	$0.13^{de} \pm 0.02$	$0.44^{bcde} \pm 0.08$	$0.0273^{i} \pm 0.0007$
	30	$0.62^{cde} \pm 0.25$	$0.34^{bcde} \pm 0.27$	$0.0178^{k} \pm 0.0007$
50	0	$0.61^{cde} \pm 0.27$	$1.25^{a} \pm 0.94$	$0.0375^{gh} \pm 0.0038$
	10	$0.10^{e} \pm 0.04$	$0.57^{abcde} \pm 0.49$	$0.0386^{g} \pm 0.0009$
	20	$1.12^{bcd} \pm 0.68$	$0.99^{ab} \pm 0.87$	$0.0358^{h} \pm 0.0009$
	30	$1.22^{abc} \pm 0.26$	$0.34^{bcde} \pm 0.28$	$0.0478^{d} \pm 0.0003$
100	0	$1.20^{bc} \pm 0.17$	$1.27^{a} \pm 0.38$	$0.0283^{i} \pm 0.0025$
	10	$1.07^{bcde} \pm 0.19$	$0.68^{abcde} \pm 0.11$	$0.0410^{f} \pm 0.0009$
	20	$0.20^{de} \pm 0.05$	$0.10^{de} \pm 0.12$	$0.0470^{d} \pm 0.0009$
	30	$0.75^{cde} \pm 0.61$	$0.17^{cde} \pm 0.15$	$0.0370^{gh} \pm 0.0005$
150	0	$0.44^{cde} \pm 0.16$	$0.92^{abc} \pm 0.48$	$0.0144^{l} \pm 0.0000$
	10	$1.78^{ab} \pm 0.68$	$0.86^{abcd} \pm 0.23$	$0.0503^{c} \pm 0.0004$
	20	$0.98^{bcde} \pm 0.37$	$0.96^{abc} \pm 0.10$	$0.0608^{b} \pm 0.0007$
	30	$2.47^{a} \pm 0.13$	$0.66^{abcde} \pm 0.45$	$0.0646^{a} \pm 0.0003$

3.2. Phytochemical studies

There were significant differences (P>0.01) in artemisinin concentrations in the salinity-stressed plants, nanoparticles-treated plants and the nanoparticles-treated plants under salinity stress (Table 3).

The artemisinin concentration differed highly among the nanoparticles-treated plants under salinity stress in comparison with the control plants. The highest concentration was observed in the 150 mM salinity-stressed plants treated with 30 mg L^{-1} nanoparticles, and the lowest concentration was observed in the 30 mg L^{-1} nanoparticles–treated plants in the absence of salinity stress (Figure 5).

In addition, the artemisinin concentration varied for each level of salinity stress. In the absence of salinity stress, the concentration of artemisinin decreased with an increase in nanoparticles concentration. Under these conditions, the highest concentration was recorded in the control and the lowest in the 30 mg L^{-1} nanoparticles-treated plants.

Under 50 mM of salinity stress, the artemisinin concentration increased with an increase in nanoparticles concentration. In these plants, the highest artemisinin concentration was observed in the 30 mg L^{-1} nanoparticles–treated plants and the lowest in the 20 mg L^{-1} nanoparticles treated plants.

In the 100 mM salinity–stressed plants, the artemisinin concentration increased at TiO_2 nanoparticle concentrations up to 20 mg L^{-1}. Meanwhile, the compound diminished at the highest TiO_2 nanoparticle concentration. Therefore, the highest and lowest concentrations of artemisinin were observed in the 20 mg L^{-1} nanoparticles–treated and the control plants, respectively.

In the salinity-stressed plants, the artemisinin concentration increased with an increase in nanoparticles concentration. The highest artemisinin concentration was recorded in the 30 mg L^{-1} nanoparticles-treated plants, while its lowest concentrations were recorded in the control plants (Table 4).

4. Discussion

This study investigated the effects of various concentrations of TiO_2 nanoparticle treatments and different levels of salinity stress and their interactions on the expression of two key genes in the biosynthesis pathway of artemisinin in *A. absinthium*. The present research is the first case study on this plant, as previous studies had mainly focused on the effect of different nanoparticles treatments or salinity stresses on artemisinin concentration and its mediated genes' expression. Artemisinin, a secondary metabolite found in *Artemisia* species, is a sesquiterpenoid lactone (Lei et al., 2011). Artemisinin production in *A. absinthium* tends to be very low (about 0.1% dry weight) and its chemical synthesis is also very difficult and costly. Therefore, increasing the content of artemisinin in plants helps reduce prices and increase the supply (Kumar et al., 2011).

The present findings revealed that artemisinin biosynthesis decreased significantly in the nanoparticles–treated plants compared to the control plants. The lowest concentration was recorded in the plants treated with the highest concentrations of TiO_2 nanoparticles. Reverse findings were reported by Zhang et al. (2013), who found that nanoparticles treatment-induced oxidative stress results in lipid peroxidation and increases malonyldialdehyde accumulation and thus increases the activities of catalase and enhances artemisinin content in the hairy roots of *A. annua*.

The application of salinity stress (in the absence of nanoparticles) also created similar results, as with an increase in salinity stress, the amount of artemisinin decreased. Exogenous factors have strong effect on production of different plant metabolites (Talebi et al., 2019, 2020). For example, Aftab et al. (2010) reported a synergistic relationship between endogenous H_2O_2 and artemisinin contents, as both contents increased under low levels of salinity (50 and 100 mM) and decreased thereafter.

There were some different results in literature. For example, Prasad et al. (1998) reported that artemisinin content was not influenced by salinity stress, but Irfan Qureshi et al. (2005) and Qian et al. (2007) suggested the artemisinin content increases under salinity stress. These findings were in agreement with those reported by Kumar et al. (2011), who suggested that artemisinin production is not only influenced by genotype, but also by environmental factors such as radiation, salinity and cold stress. Similar results were also reported for other secondary metabolites. For example, TiO_2 nanoparticles

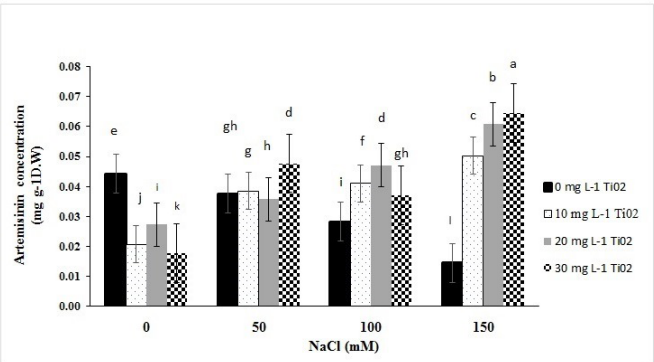

Figure 5. The effect of salinity stress and titanium dioxide nanoparticles on the amount of artemisinin in wormwood (the non–identical letters indicate significant difference based on Duncan test P≤ 0.05).

treatment changed alvine production in *Aloe vera* cell culture suspension (Shukla et al., 2016).

The minimum and maximum amounts of artemisinin synthesis were found in the highest levels of salinity stress in the absence of nanoparticles and in the 30 mg L^{-1} nanoparticles-treated plants, respectively. Yang et al. (2006) suggested that TiO_2 nanoparticles treatment can increase the activities of some enzymes such as glutamine synthetase, nitrate reductase, glutamic-pyruvic transaminase and glutamate dehydrogenase in some plants during the growing stage. Furthermore, these nanoparticles could also promote the plants' absorption of nitrate and accelerate transformation inorganic nitrogen into organic ones.

In another study, Dastmalchi et al. (2007) concluded that mild dehydration increases the secondary metabolites in medicinal plants, but as stress increases, the amount of active compounds decreases greatly.

The application of the highest level of salinity stress decreases the synthesis of artemisinin, and under this condition, the treatment of plants with the highest level of nanoparticles ameliorates the salinity side-effects and increases the amount of artemisinin. It seems that, under such conditions, the accumulation of phytohormones such as Abscisic acid and Salicylic acid, which are involved in plant defense responses, plays an important role in the accumulation of artemisinin. Salicylic acid also regulates the transcriptional process of genes in the artemisinin biosynthesis pathway and ultimately leads to an increase in artemisinin concentration in the plants (Kumar et al., 2011).

The highest and lowest levels of DBR2 gene expression were recorded in the 100 mM salinity-stressed plants in the absence of nanoparticles treatment and in the 10 mg L^{-1} plants without any salinity stress, respectively. The application of salinity stress up to 100 mM thus appears to increase the gene expression. Some similar results were reported for different gene expressions under salinity stress. For example, the level of *bch* and *pds* gene expression in saffron was increased by drought stress treatment (Moshtaghi et al., 2010). Besides, dehydration stress also increased the expression of the RAS gene in fungi (Kamalizadeh et al., 2013).

In contrast, nanoparticles treatment had a negative effect on the expression of the DBR2 gene. In this study, an increase in nanoparticles concentration was associated with a reduction in the expression of the DBR2 gene, except with the application of 20 mg L^{-1} nanoparticles treatment, which increases the gene expression.

The application of nanoparticle treatments and salinity stress decreased ADS gene expression, except for 100 mM salinity stress. However, for all the levels of salinity stress, the application of nanoparticle treatment increased the expression of ADS gene. The present findings were in agreement with the results of previous studies. For instance, Amini et al. (2017) reported that, under stress conditions, chickpea TiO_2 nanoparticles-treated plants showed a significant increase in the expression of some genes such as Receptor-Like Kinases (RLK), ethylene (ERF) transcription factor and VSR receptors. The salinity-stressed plants were more susceptible to cold stress than those treated with TiO_2 nanoparticles. These findings offer a new application for TiO_2 nanoparticles and may result in the stable performance of this plant under stress conditions.

The maximum concentration of artemisinin was reported in the 30 mg L^{-1} nanoparticles–treated plants under 150 mM of salinity stress, which contained the highest level of ADS gene expression. Nonetheless, no significant correlation was observed between the expression of this gene and artemisinin concentration in other nanoparticles–treated plants under different levels of salinity stress.

The amount of artemisinin decreased by half in the plants containing the highest expression of DBR2 gene (100 mM salinity-stressed plants in the absence of nanoparticles treatment). In addition, no significant correlation was found between artemisinin concentration and the expressions of the DBR2 gene. According to Arsenault et al. (2010), artemisinin can inhibit its own biosynthesis; therefore, the concentration of artemisinin decreased significantly in the highest transcript levels of the DBR2 gene.

The present findings were in agreement with previous studies on some gene expressions and metabolite concentration in other species of *Artemisia*. For example, *ADS* and *CYP* gene expression reveal two main steps toward the artemisinic metabolite measurement. Although there are no direct significant correlations between the transcript levels of these two genes and metabolite abundance, the data suggests that the interplay between transcript abundance and product formation is more complicated than previously hypothesized (Arsenault et al., 2010).

According to Arsenault et al. (2010), artemisinin production from the substrates is possible in two ways: (1) the DBR2 pathway that ultimately leads to the production of artemisinic acid through several oxidative processes; (2) the CYP and Aldh1 pathway being more appropriate for cells to reduce the metabolic burden or reduce the toxicity of the intermediate compounds for the producing cells.

The DBR2 is a reducing enzyme and catalyzes the reduction of aldehyde artemisinin to hydro–artemisinic acid as a precursor for artemisinin. In the present study, the expression of the DBR2 gene did not change significantly, even in cases of decreased expression. This change has been observed in other similar studies (Pu et al., 2013). The CYP enzyme is a sesquiterpene cytochrome terpene oxygenase that belongs to the cytochrome P450 family and plays an important role in the biosynthesis of artemisinin (Shen et al., 2018). The DBR2 and CYP enzymes compete for intermediacy in the artemisinin biosynthesis pathway, and if the DBR2 enzyme is reduced, the CYP enzyme wins the competition and artemisinin biosynthesis proceeds in another direction.

Artemisinin has a variety of biosynthetic pathways and can be produced by a different route. The DBR2 enzyme and the CYP enzyme compete for artemisinic aldehyde, which is an intermediate in the biosynthesis pathway of artemisinin, and if DBR2 gene degradation is reduced, enzyme production in the plant decreases and a greater amount of the enzyme CYP becomes available. This event also stimulates an increased *CYP* gene expression and ultimately reduces the expression of the DBR2 gene.

The application of TiO_2 nanoparticles in salinity-stressed plants can increase the transcript level of artemisinin

biosynthesis pathway genes. Other factors may also be involved in the enhancement of artemisinin production. For example, Andersen et al. (2004) suggested that increased levels of ROS enzyme activity or other mediated enzymes can increase artemisinin production.

5. Conclusion

The present findings revealed that the application of nanoparticles treatment and salinity stress, individually, decreased artemisinin production; however, for all the levels of salinity stress, the enhancement of nanoparticle concentrations increased artemisinin production significantly. Furthermore, nanoparticles treatment decreased both genes' expression significantly. Meanwhile, the reverse patterns were recorded for both genes' expression under salinity stress, and there was an increase in transcript levels of DBR2 and a reduction in those of the ADS genes, in most cases. Although the highest ADS gene transcript level and artemisinin production were observed in the plants under the same conditions, no feedback was seen between them. In addition, a reverse pattern was found for the DBR2 gene transcript level and artemisinin production.

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Supplementary Material

Supplementary material accompanies this paper.
Supplementary 1. Melting curve and amplification diagrams related to the ADS and DBR2 as target genes and the 16s rRNA gene as reference gene.
This material is available as part of the online article from http://www.scielo.br/BJB