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Iron and zinc oxide nanoparticles: An efficient elicitor to enhance trigonelline alkaloid production in hairy roots of fenugreek

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum* L.) is a rich source of important medicinal metabolites. This plant belongs to the *Fabaceae* family that grows anywhere around the world. It is extensively used in medicine and as a food additive. Several secondary metabolites are detected in fenugreek, especially a valuable alkaloid called trigonelline. Enhancing the production of secondary metabolites is possible through the application of modern biotechnological techniques such as culturing hairy roots. In the present study, different strains of *Agrobacterium rhizogenes* (ATCC11325, ATCC15834, A_4, A_7, A_{13}, and K599) were used for hairy root induction from fenugreek cotyledon explants. Then, the effect of various levels (x, 2x, and 4x concentrations of B5 medium) of iron oxide and zinc oxide nanoparticles were evaluated on growth, total phenolic, flavonoids, and trigonelline content of hairy roots. The growth curve analysis revealed an increase in the fresh and dry weight of treated hairy roots, as compared with the control. The highest levels of fresh (1.49 g) and dry weight (1.23 g) were obtained in hairy roots which were induced by the ATCC11325 strain and were treated with Zn (2x). The results indicated that using Zn nanoparticles on hairy roots can lead to higher levels of trigonelline, compared with the control group (*i.e.* transformed hairy roots without NPs).

1. Introduction

Fenugreek (*Trigonella foenum graecum* L.) is an important medicinal crop of the *Fabaceae* family. It is widely grown throughout the world and is known for having anti-cancer, anti-diabetic, anti-microbial and hypocholesterolemic properties (Mehrafarin et al., 2010). Fenugreek seeds contain active components that are responsible for the biochemical properties of this plant species. The seeds contain polysaccharides, galactomannan, distinctive saponins (such as diosgenin, yamogenin) and alkaloids (choline and trigonelline) (Aasim et al., 2010). Also, the seeds are a rich source of iron and phosphorus (Billaud and Adrian, 2001). A wide array of secondary metabolites in plants can be produced by the function of substances which act as precursors. The culturing of hairy roots *in vitro* by *Agrobacteriumrhizogenes* and thus genetic transformation are new ways to improve plant metabolite production (Debjani and Bratati, 2011). Hairy roots are produced when *A. rhizogenes* is transferred from the transfer DNA (T-DNA) region (left and right borders DNA) to the genome of the host plant. The T-DNA carries the root oncogenic loci (rol) genes and opine biosynthesis genes. Based on opine type, the strains of *A. rhizogenes* have been divided into the following groups: agropine, cucumopine, mikimopine, and mannopine groups (Shahla Amani et al., 2020). The opine type is defined by the opine synthesis genes of T-DNA (Petit et al., 1983) (Table 1). The genes are specifically expressed where the plant tissue is wounded (Tzfira et al., 2004). Hairy roots are characterized by fast growth and genetic stability. They are capable of growing without plant growth regulators, while secondary metabolites can be synthesized from them in amounts higher than the mother plant (Dehghan et al., 2012).

Plants are a rich source of various bioactive secondary metabolites, which play a significant role in the survival of plants in their environments. *In vitro* plant cell and organ culture is considered for the production of secondary metabolites. The content of secondary compounds in cell and organ cultures was significantly enhanced by optimizing the composition of the culture medium, incorporation of precursors and elicitors and providing appropriate culture conditions. Nanoparticles (NP_s) added to the plant *in vitro* culture medium may act as a nutrient source and an elicitor (Heydari et al., 2020). Recent advances in nanotechnology have provided a new platform for its application in various sciences, especially agriculture and biology (Khodakovskaya et al., 2013). It has been documented that NP_S can alter physiological

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Table 1
Opine biosynthesis genes of pRi-plasmids.

Plasmid type	Opine biosynthesis genes
Agropine	TL: acs (agrocinopine synthase), TR: mas1, mas2 (mannopine synthases), ags (agropine synthase)
Mannopine	mas1, mas2 (mannopine synthases)
Mikimopine	mis (mikimopine synthase)
Cucumopine	cus (cucumopine synthase)

Table 2
A. rhizogenes strains for hairy root induction in *T. foenum graecum* L.

S. No	Bacterial strains	Opine type
1	A. *rhizogenes* ATCC15834	Agropine
2	A. *rhizogenes* ATCC11325	Nopaline
3	A. *rhizogenes* K599	Cucumopine
4	A. *rhizogenes* A_7	Agropine
5	A. *rhizogenes* A_4	Agropine
6	A. *rhizogenes* A_{13}	Mikimopine

processes such as photosynthesis, primary and secondary pathways, and nutrient uptake in plants. The main mechanism through NP_s altered secondary metabolism pathway has been investigated. They provoke reactive oxygen spicies (ROS) production followed by activation of both enzymatic and non-enzymatic antioxidant defense machinery to scavenge excess ROS (Marslin et al., 2017; Jordan et al., 2018). Phenolic compounds are the main antioxidant of plants, and their production increased to scavenging ROS generated through stresse conditions (Vosoughi et al., 2018). As an established science, nanotechnology has enabled researchers to produce valuable substances by triggering a variety of defense responses. By attaching to the cell wall, nanoparticles can disturb the permeability of the cell wall and thus alter cellular respiration. NP_s are capable of penetrating cells and can manipulate proteins and compounds that contain sulfur or phosphorus in the DNA. Nonetheless, when conditions of overdose occur, it is not fully clear how nanotoxicity acts out in plants, although oxidative stress is regarded as a possible way of toxicity when NP_s are absorbed by cells. There are a variety of reports on how secondary metabolites can be produced when nanoparticles are used as treatments. For instance, in fenugreek hairy root cultures, the production of trigonelline was induced significantly by 100 μM of methyl jasmonate and 150 mg L^{-1} of chitosan (Qaderi et al., 2016). The application of Co-60 gamma irradiated chitosan (IC) and phosphorus fertilizer on *T. foenum graecum* seed culture by P_{40}+IC 40 mg L^{-1} led to an enhanced trigonelline content (Ahmad Dar et al., 2015). Different plant species grow and develop variedly when exposed to nanoparticles and they reportedly have both constructive and unwanted effects. For example, iron oxide nanoparticles can have a positive effect on the production of phenolic compounds in zarringiah (*Dracocephalum kotschyi* Boiss) hairy roots when treated with 75 mg L^{-1} after 24 h (Nourozi et al., 2019a,b). In another study on *H. reticulatus*, scopolamine and hyoscyamine peaked in production (five-fold) when 900 and 450 mg L^{-1} $FeNP_s$ were applied as treatments, compared with the control group (Moharrami et al., 2017).

In this research, one aim is to determine how different concentrations of zinc and iron oxide nanoparticles act as elicitors and promote the growth of hairy roots. The hairy roots were grown in exposure to nanoparticles. Flavonoids and total phenol contents in the hairy roots were examined. It was hypothesized that the production of secondary metabolites would be enhanced in hairy roots of fenugreek when culturing them *in vitro* and, specifically, in response to the inclusion of nanoparticles.

2. Materials and methods

2.1. Bacterial strains, plant materials and conditions of culture in vitro

Seeds (collected from Isfahan province, Iran) of fenugreek were immersed in ethanol (96 %, 1 min) and then in sodium hypochlorite solutions (2.5 %, 10 min) as disinfectant treatments. They were finally washed for 15 min with distilled sterilized water. After disinfecting the seeds, they were cultured on MS medium (Murashige and Skoog, 1962) at 25 ± 2 °C, with 16 h of light and 8 h of darkness in a growth chamber. One week after germination, cotyledons that had grown *in vitro* were sampled as explants so as to induce the production of hairy roots. A total number of six *A. rhizogenes* strains were used for genetic transformation (Table 2). All strains were provided by the microbial unit of the National Institute of Genetic Engineering and Biotechnology (Tehran, Iran). A colony of each bacterial strain was grown overnight in 10 mL LB liquid medium containing 50 mg ml^{-1} rifampicin at 28 °C. The medium was shaken constantly at 120 rpm in an incubator for 24 h.

2.2. Establishment of hairy root cultures

The explants (cotyledons) were used at three ages (*i.e.* 7, 14, and 21 days). Transformation by injection method was performed as described by Estrada-Navarrete et al. (2006). The explants were wounded and inoculated by direct injection using a sterile syringe for hairy root induction. After infection, the explants were co-cultivated on hormone-freeB5 solid medium at 25 °C in the dark for 72 h. Three days later, the cotyledons were washed with cefotaxime and were placed on B5 medium containing cefotaxime (500 mg L^{-1}). They were subcultured every 2 weeks until the production of hairy roots was induced.

2.3. Polymerase chain reaction

A molecular analysis of the transformed roots was confirmed by PCR using *rol*B gene-specific primers (Table 3). The total genomic DNA from the transformed and free *rol*B untransformed roots was extracted using the CTAB method. Amplification was performed on a final volume of 12 μl (1.75 μl of each primer), 1 μl of DNA, 6 μl of master mix (Sinna Gen, Iran), and 3.25 μl of distilled H_2O). PCR was performed under the following conditions for *rol*B: 94 °C for 5 min (initial denaturation), 35 cycles of amplification (94 °C for 5 min, 55 °C for 45 s, and 72 °C for 1 min) and a final extension at 72 °C for7 min. Finally, the products were separated by 0.8 % agarose gels (w v $^{-1}$). The amplified PCR products were treated with electrophoresis and exposed to UV light.

2.4. Iron and zinc nanoparticles for hairy root growth

Hairy roots were grown in various concentrations of $FeNP_s$ and $ZnNP_s$ (x, 2x, and 4x concentrations of B5 base medium) with particle size <50 nm (Merck), along with $ZnSo_4$ and $FeSo_4$ (control). They were

Table 3
The sequences of *rol*B primers and PCR condition.

Gene	Primer Sequences	Denaturation Temp. (°C)	Annealing Temp. (°C)	Extension Temp. (°C)	Number of cycle	Amplified Segment (bp)
*rol*B	Forward:5′-ATGGATCCCAAATTGCTATTCCCCACGA-3′ Reverse:5′-TAGGCTTCTTTCATTCGGTTTACTGCAGC-3′	94	55	72	35	760

maintained on a shaker at 110 rpm at 25 °C in the dark. The hairy roots were measured in terms of fresh weight every 7 days for a month. After using NP_s on hairy roots (4 weeks), they were harvested and evaluated in terms of dry weight and total phenolic flavonoid contents.

2.5. Analysis of the total flavonoid content (TFC) and total phenol content (TPC)

The reagent Folin-Ciocalteu was used for evaluating the content of total phenol (TPC). Initially, the sample (0.1 mL) was diluted in Folin-Ciocalteu reagent (0.1 mL, 50 %) (1:1) and water (2.8 mL) for 5 min. Then, sodium carbonate (2 mL) was added and the volume was adjusted to 3 mL with distilled water. After being shaken thoroughly, the solution was allowed to remain in the dark for 30 min. Thereafter, the absorbance measures of the samples were taken with nanodrap at 760 nm. TPC was calculated as mg gallic acid equivalents per g dry root weight. Also, gallic acid was used for making the standard solution (20–100 mg L^{-1}) (Meda et al., 2005).

The content of total flavonoids was evaluated by applying the aluminum chloride colorimetric ($AlCl_3$) assay on hairy roots of *T. foenum*, based on a standard curve of quercetin. An amount of 0.5 mL dried root extract was mixed with methanol (1.5 mL), potassium acetate 1 M (0.1 mL), aluminum chloride (0.1 mL, 10 %) and distilled water (2.5 mL). Subsequently, incubation of the mixture occurred at 25 °C for 30 min. The reaction mixtures were analyzed in terms of absorbance (415 nm) using nanodrap. The data of TFC in samples were expressed as QE (mg of quercetin equivalent/g of the dry weight of roots) (Chang et al., 2002).

2.6. HPLC analysis in hairy roots of T. foenum

The content of trigonelline was determined using HPLC according to the method by Nourozi et al. On a rotary shaker, 5 mL of methanol (80 %) was mixed with dried hairy roots (100 mg) for 24 h. The rotary shaker operated at 100 rpm and the mixture had a temperature of 26 °C. After conducting a centrifuge (10,000 rpm) on the mixture (15 min, 4 °C), the trigonelline in the supernatant was analyzed by HPLC (High-Performance Liquid Chromatography). Fifty µl of the supernatant was inserted into the HPLC. A Knauer K -2501 UV detector was set to operate at 330 nm. The mobile phase consisted of solvents, *i.e.* acetonitrile (30 %) and water (70 %). The mobile phase contained H_3PO_4 (0.1 %). The Knauer K -2501 UV detector operated for 10 min at 1.75 mL min^{-1}. The reference standard of trigonelline matched the guidelines of Sigma-Aldrich (Germany). Using a standard curve, the concentration of trigonelline in each sample was reported as mg g^{-1} DW.

2.7. Statistical analysis

A completely randomized design (CRD) was used and data analyses were carried out accordingly. There were two factors and four replicates per treatment. Three replications were used for the analysis of secondary metabolites. The significance of difference between mean values was obtained using DMRT (Duncan's multiple range test) at a 5 % level with SPSS (Version 16.0).

3. Results and discussion

3.1. Hairy root induction

Six different strains of *A. rhizogenes* (ATCC11325, A_{13}, K599, A_7, A_4, and ATCC15834) were evaluated on the hairy root induction of *T. foenum graecum* L. at various explant ages (7, 14, and 21 days) (Fig. 2). Statistical analysis using (ANOVA) showed significant differences among the results when different strains and explant ages were used, along with the interactions between the two factors ($P < 0.01$). All strains were able to induce hairy root production in 7-day-old explants.

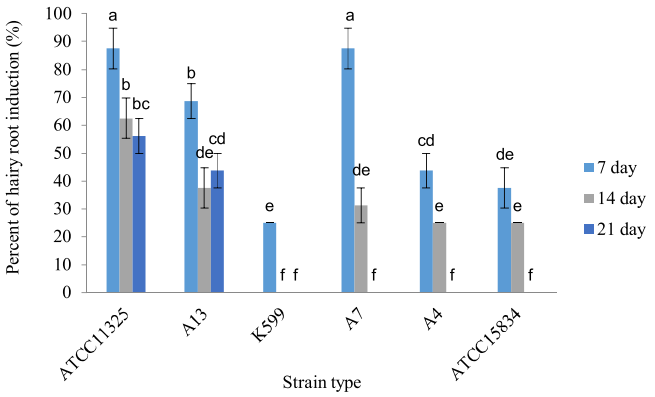

Fig. 1. The percentage of hairy root induction according to explant age and strain type.

The ATCC11325 and A_{13} strain successfully induced hairy root production in all three ages of the explants. The maximum frequency (87.5 %) of hairy root production occurred in response to A_7 strains and ATCC11325 in 7-day-old explants (Fig. 1). Previous research indicated that various factors such as *Agrobacterium* strains (Chabaud et al., 2003; Crane et al., 2006), temperature (Baron et al., 2001), co-culture time (Aggarwal et al., 2012) and type of explants (Geier and Sangwan, 1996) could influence the transfer of T-DNA segments from bacteria to plant genomes for hairy root production (De Buck et al., 1998).

3.2. Molecular verification in determining that transgenic roots contain the rolB gene

Fig. 3 shows that hairy roots successfully acquired the *rol*B gene through the Ri plasmid of *A. rhizogenes* by its T-DNA region. The *rol*B gene is essential for the production and growth of hairy roots (Samadi et al., 2012). The amplicon size (760 bp) was obtained in transformed hairy roots by six strains of bacteria. To probe further, a single colony of A_4 strains of *A. rhizogenes* was applied as a positive control, while normal roots of seedlings served as the negative control. PCR analysis is a usual method for identifying and verifying whether the Ri plasmid has entered the plant genome (Palazon et al., 2003).

3.3. Nanoparticles and biomass accumulation in hairy roots

Among the *A. rhizogenes* strains, four strains were selected based on the percentage of hairy roots that they induced. Each of the four strains (ATCC11325, A_{13}, A_7, and A_4) were used for further study. Hairy roots were not induced (*i.e.* not produced) in control explants. Therefore, the growth of seedling roots *in vitro* were used as the control. According to the results, the concentration of NP_s significantly affected the hairy roots and their fresh weight (Fig. 4).

The analysis of variance showed significant differences among strains, explant age and the interactions between these two factors. Root biomass (*i.e.* fresh weight) increased through the growth period. Hairy roots had a maximum amount of fresh weight when the ATCC11325 strain was used along with Zn (2x) NP_s after 3 and 4 weeks. The A_4 strain (2x concentration) ($ZnNP_s$) resulted in higher amounts of fresh weight after 4 weeks. Higher amounts of fresh weight (0.46 g) were caused by the *A. rhizogenes* A_7 strain and by zinc (2x) nanoparticles in four-week-old hairy roots. In the A_{13} strain, the maximum fresh weight (0.62 g) was obtained by applying zinc (2x) nanoparticles. Nonetheless, there was no significant difference in this regard between three-week-old and four-week-old hairy roots.

The results showed that in using either of the NP_s, the fresh weight increased in comparison with the control explants. Also, increasing the zinc oxide concentration by more than two-fold actually reduced the fresh weight of hairy roots when either of the 4 strains of *A. rhizogenes*

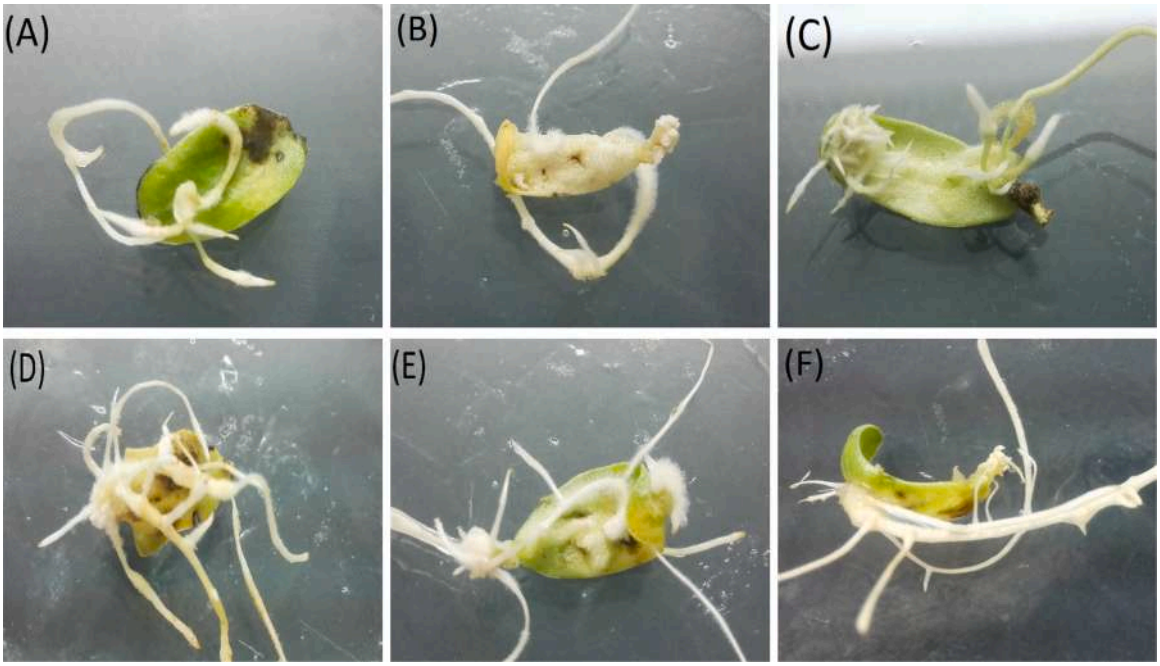

Fig. 2. Hairy roots of fenugreek induced by different bacterial strains (A) Hairy root induction in transformed explants by K599 strain, (B) by ATCC15834 strain, (C) by A_4 strain, (D) by ATCC11325 strain, (E) by A_7 strain.
(F) by A_{13} strain.

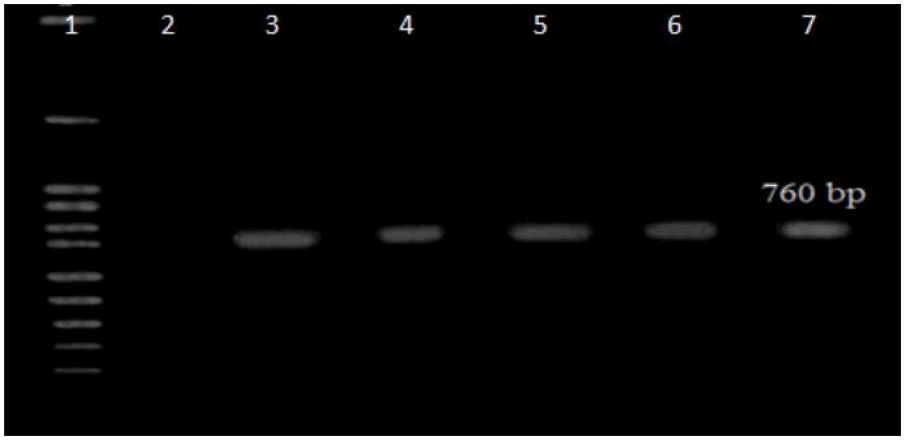

Fig. 3. PCR analysis for verification of *rol*B gene (760 bp) in fenugreek transgenic and normal roots. 1: Molecular size markers (100 bp ladder), 2: negative control (Non- transformed roots), Lanes 3- 6: transgenic roots of fenugreek, 7: positive control (A_4 strain).

Fig. 4. Hairy roots growth on liquid medium, (A) Hairy roots growth 1week after culture, (B) 2 week after culture.
(C) 4 week after culture.

Table 4
Compare means of the effects of strain and duration of nanoparticles treatment on the fresh weight of hairy roots.

Strain	Treatment duration	control	Fe (x)	Fe (2x)	Fe (4x)	Zn (x)	Zn (2x)	Zn(4x)
ATCC 11325	Week1	0.0126^{g}	0.0231^{fg}	$0.0256f^{g}$	0.0273^{fg}	0.0932^{e-g}	0.1424^{c-f}	0.1213^{d-g}
	Week2	0.0185^{fg}	0.0511^{fg}	0.0818^{fg}	0.0831^{fg}	0.2965^{b-e}	0.5786^{b}	0.3906^{b}
	Week3	0.0226^{fg}	0.0777^{fg}	0.0838^{fg}	0.0732^{fg}	0.3208^{b-d}	1.1598^{ab}	0.3290^{bc}
	Week4	0.0272^{fg}	0.0911^{fg}	0.0921^{fg}	0.0617^{fg}	0.3637^{bc}	1.4975^{a}	0.3138^{b-d}
A_4	Week1	0.0126^{g}	0.0677^{e-h}	0.0712^{d-h}	0.0378^{f-h}	0.0493^{e-h}	0.0532^{e-h}	0.0720^{d-h}
	Week2	0.0201^{gh}	0.0696^{e-h}	0.0774^{d-h}	0.0618^{e-h}	0.1118^{c-g}	0.2435^{bc}	0.1267^{c-f}
	Week3	0.0237^{gh}	0.0710^{e-h}	0.0838^{d-h}	0.0596^{e-h}	0.2324^{b-d}	0.3436^{b}	0.2274^{bc}
	Week4	0.0281^{f-h}	0.079^{d-h}	0.0850^{d-h}	0.0512^{e-h}	0.2927^{bc}	0.6991^{a}	0.1663^{b-e}
A_7	Week1	0.0127^{i}	0.0242^{g-i}	0.0280^{g-i}	0.0235^{g-i}	0.0635^{f-i}	0.0779^{e-g}	0.0749^{e-h}
	Week2	0.0174^{hi}	0.0443^{f-i}	0.0508^{f-i}	0.0685^{f-i}	0.1186^{d-f}	0.3068^{ab}	0.1627^{c-e}
	Week3	0.0212^{g-i}	0.0602^{f-i}	0.0645^{f-i}	0.0504^{f-i}	0.2681^{bc}	0.3426^{ab}	0.2610^{bc}
	Week4	0.0263^{g-i}	0.0774^{e-h}	0.0860^{e-g}	0.0432^{f-i}	0.3095^{ab}	0.4689^{a}	0.1745^{cd}
A_{13}	Week1	0.0129^{g}	0.0217^{g}	0.0547^{d-g}	0.0470^{d-g}	0.0511^{d-g}	0.0687^{c-g}	0.0593^{d-g}
	Week2	0.0188^{g}	0.0446^{d-g}	0.0777^{c-g}	0.0516^{d-g}	0.1554^{c-e}	0.1641^{c-f}	0.1045^{c-g}
	Week3	0.0235^{fg}	0.0556^{d-g}	0.0807^{c-g}	0.0542^{d-g}	0.1834^{cd}	0.4023^{ab}	0.0901^{c-g}
	Week4	0.0280^{fg}	0.0649^{d-g}	0.0845^{c-g}	0.0499^{d-g}	0.2116^{bc}	0.6238^{a}	0.0446^{e-g}

Numbers followed by the same letter are not significantly different ($P < 0.05$).

Table 5
Effect of nanoparticles on the dry weight of *T. foenum– graecum* L.

Nanoparticle	Dry weight ATCC11325	Dry weight A_4	Dry weight A_7	Dry weight A_{13}
control	0.0150^{h}	0.0150^{h}	0.0149^{h}	0.0151^{h}
Fe (x)	0.0591^{e-h}	0.0530^{e-h}	0.0519^{e-h}	0.0431^{e-h}
Fe (2x)	0.0684^{e-h}	0.0624^{e-h}	0.0635^{e-h}	0.0606^{e-h}
Fe (4x)	0.0366^{e-h}	0.0315^{f-h}	0.0290^{gh}	0.0303^{f-h}
Zn (x)	0.2744^{cd}	0.2168^{c-e}	0.2417^{cd}	0.1659^{c-f}
Zn (2x)	1.2377^{a}	0.5553^{b}	0.3299^{b-d}	0.4420^{bc}
Zn (4x)	0.2267^{cd}	0.1276^{d-g}	0.1321^{d-g}	0.0265^{gh}

Numbers followed by the same letter are not significantly different ($P < 0.05$).

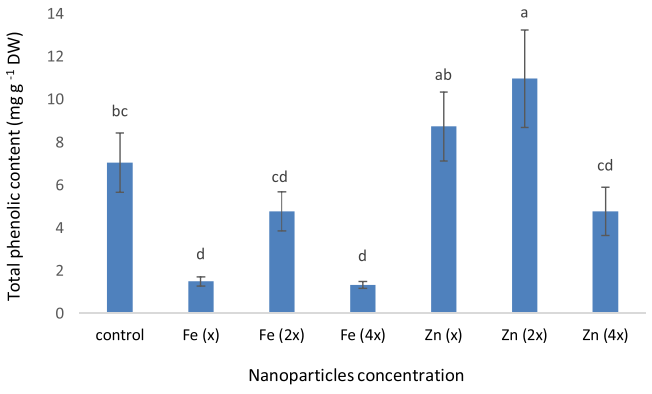

Fig. 5. Effects of different concentrations of nanoparticles on phenolic contents.

were used (Table 4). Plant responses to stimuli are caused by various features of nanoparticles when they are used *in vitro*. These features include surface area, crystal structure, specificity, growth stages, physiochemical traits, particle size and concentrations (Kim et al., 2004; Shin et al., 2015). Plants can rapidly absorb nano-compounds and use the nutrients in them. Therefore, plants can grow more prolifically if nanomaterials are used in appropriate ways (Askary et al., 2017). Following the use of elicitors and nanoparticles, the roots were recorded for their dry weight after remaining for 72 h at room temperature. The results of statistical analysis showed that dry mass was significantly influenced by the type of strain and NP_s and also by the interaction between these factors. The maximum dry weight (1.237 mg g^{-1}) occurred as a result of 2x $ZnNP_s$ by the ATCC11325 strain. Meanwhile, the lowest level of dry weight was observed in the control samples (Table 5).

3.4. Estimation of total phenolic and flavonoid contents

The simultaneous use of nanoparticles and bacterial strains did not cause significant differences compared with their individual use ($P < 0.05$). Nanoparticles were substantially effective when applied individually. There were no significant differences between total phenolic compounds in samples treated with (2x) $ZnNP_s$ and (x) ZnNPs (10.98 and 8.75 mg g^{-1} DW, respectively) (Fig. 5).

As shown in Table 3, the greatest level of flavonoids (53.33 mg g^{-1} DW) were obtained as a result of $ZnNP_s$ (2x) by the ATCC11325 strain (Table 6). Ultimately, the ATCC11325 strain and $ZnNP_s$ (2x) were considered as the best treatments for producing more hairy roots and extracts that could be analyzed by HPLC analysis. It is known that bioactive compounds can be synthesized more when abiotic or biotic elicitors are used in the growth medium, while the defense system becomes more active in response to the treatments (Nourozi et al., 2019a, b). The production of secondary metabolites usually improves in plants when nanoparticles are applied, as a result of which the amounts of ROS increase.

Nanotoxicity and its exact mechanisms of action in plants are not clearly recognized as yet. Nonetheless, it is suggested that plants become prone to NP_s-induced toxicity because of oxidative stress as caused by ROS (Wang et al., 2011). Free radicals are usually produced as a consequence of oxidative stress. Hydroxyl radicals, superoxide anions and hydrogen peroxide are a few examples of free radicals which damage cells, the cellular membrane, nucleic acids and proteins (Ghorbanpour et al., 2015). Phytoalexins such as phenolic compounds

Table 6
Effects of different concentrations of nanoparticles on flavonoid content.

Strain	control	Fe (x)	Fe (2x)	Fe (4x)	Zn (x)	Zn (2x)	Zn (4x)
ATCC11325	20^{de}	11.66^{e-g}	28.33^{a-d}	23.33^{c-e}	28.33^{a-d}	53.33^{a}	33.33^{a-d}
A_4	22.33^{d-g}	15^{d-g}	20^{de}	18.33^{de}	23.33^{c-e}	45^{a-c}	25^{b-e}
A_7	25.66^{d-g}	8.33^{f-h}	18.33^{de}	13.33^{d-g}	28.33^{a-d}	50^{ab}	26.67^{b-e}
A_{13}	20^{de}	1.66^{h}	15^{d-g}	8.32^{fh}	25^{b-e}	51^{ab}	28.33^{a-d}

Numbers followed by the same letter are not significantly different ($P < 0.05$).

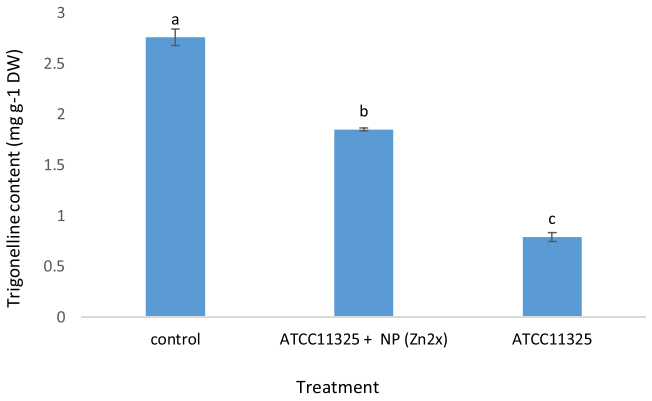

Fig. 6. A comparison of trigonelline content in seedling roots of the control group, hairy roots transformed by the ATCC11325 strain and hairy roots transformed by Zn (2x) and the ATCC11325 strain.

can accumulate in damaged tissues and cells when free radicals exert adverse effects (Wen Wang and Yong Wu, 2010). Phenolic compounds accumulate in roots and assist cellular adaptation to stress when ROS are produced (Janas et al., 2009). In fact, ROS are detoxified partly by phenolic compounds which serve as electron donors in organelle structures and thus enable mechanisms of detoxification (Mittler, 2017). The processes that are induced by the action of elicitors are largely influenced by the concentration of elicitors being used. Hypersensitive reactions usually occur in response to a high dose of an elicitor, sometimes culminating in cell death, whereas the production of hairy roots is induced by an optimum level of elicitor concentration (Kim et al., 2004).

3.5. High performance liquid chromatography (HPLC) analysis

Measuring the trigonelline revealed substantial differences between hairy roots transformed by NP_s and hairy roots not transformed by NP_s. The zinc nanoparticle increased the trigonelline content in transformed hairy root (1.85 mg g^{-1} DW) under treatments with Zn (2x) and

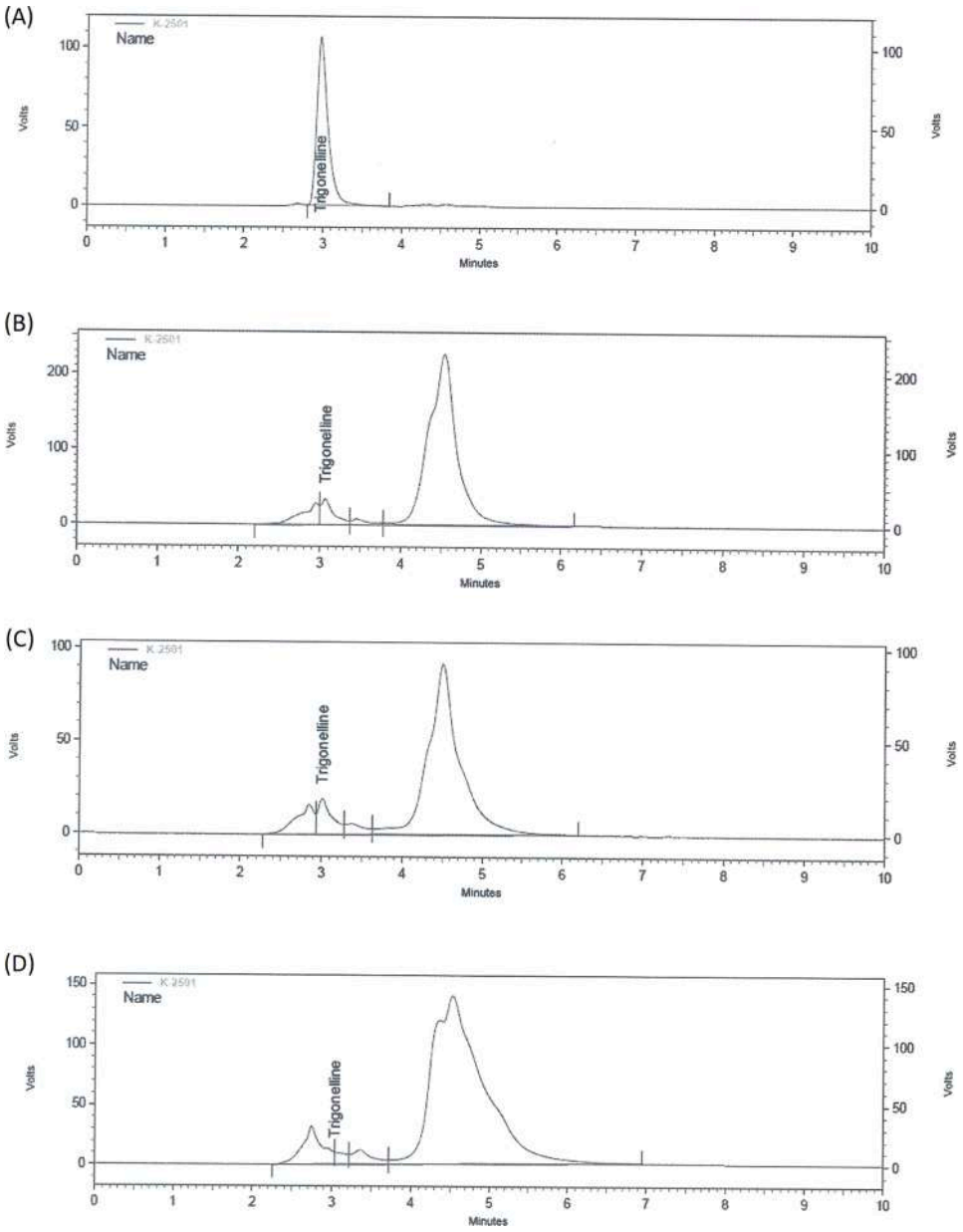

Fig. 7. HPLC chromatograms. Standard trigonelline (A), trigonelline in seedling roots (control) (B), hairy roots transformed by Zn (2x) (C) and hairy roots transformed by the ATCC11325 strain (D).

ATCC11325 strain (Figs. 6 and 7). In previous studies, zinc and iron nano oxides at low concentrations were observed to enhance hypericin and hyperforin production in cell suspension cultures of *H. perforatum* (Sharafi et al., 2013). The application of zinc oxide and copper oxide in licorice)*Glycyrrhiza glabra*(resulted in a decrease in total phenols, glycyrrhizin, flavonoids, proline and anthocyanin when compared with the control (Oloumi et al., 2015). Fenugreek seeds are rich in trigonelline, an alkaloid, which has key medicinal properties (Zandi et al., 2015).

4. Conclusion

Secondary metabolites can be potentially produced from hairy root cultures, as induced by the *A. rhizogenes* strain in this study. There is a variety of *Agrobacterium rhizogenes* strains, each of which has a unique ability to transform plant cells and to induce the production of secondary metabolites. *A. rhizogenes* strains A_7 and ATCC11325 proved to be the strongest elicitors of hairy root induction in *T. foenum*. When compared with transformed hairy roots and seedling roots, hairy roots that had been transformed by NP_s had higher fresh weight. Hairy roots have a tendency to absorb ZnO. High ZnO concentrations, however, can adversely affect hairy root biomass. The trigonelline content in hairy roots that were treated with Zn (2x) and the ATCC11325 strain was twice as much as hairy roots that were transformed without Zn. Accordingly, ZnO can substantially increase secondary metabolites in plants, while nanoparticles and their effects on metabolites in plants need further research before their mechanisms of action can be clearly understood in relation to metabolite production.

CRediT authorship contribution statement

Neda Tariverdizadeh: Conceptualization, Methodology, Writing - original draft. **Mehdi Mohebodini:** Data curation, Supervision, Writing - review & editing. **Esmaeil Chamani:** Visualization, Investigation. **Asghar Ebadi:** Software, Validation.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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