Optimization of hairy root induction in chicory (*Cichorium intybus* L.) and effects of nanoparticles on secondary metabolites accumulation

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Abstract

Hairy root culture is an effective method to produce secondary metabolites. In fact, hairy roots are genetically and biologically stable and also they are able to produce metabolites without hormones**.** Chicory (*Cichorium intybus* L.) is one of the most important medicinal plants with valuable medicinal compounds. In this research, hairy roots were induced by different *Agrobacterium rhizogenes* strains using leaf and petiole explants. In the second experiment, the effect of Iron and zinc oxide nanoparticles was studied on hairy roots biomass accumulation and secondary metabolites production. The results of experiments showed that the highest hairy root induction (60 percent), were observed in the A4 strain and leaf explants. Also the iron oxide nanoparticles. and ATCC15834 strain were superior for highest fresh (4.53 g) and dry weight (0.4 g) productivity, growth index (24.41) and total phenolic content (4.65 mg g^{-1} DW) in hairy roots. Overall, the strain and explant types had considerable effects on the hairy roots induction and iron oxide nanoparticles and ATCC15834 strain were more effective in roots growth and secondary metabolites production.

Key words: *Agrobacterium rhizogenes*, Biomass, Iron and zinc oxide nanoparticles, *Rol*B gene.

INTRODUCTION

Cichorium intybus L. as a member of the *Asteraceae* family, is a biennial plant and is one of the important medicinal plants. It is useful for hepatic disease treatment and promotes appetite and digestion (Saeed *et al*., 2017). All parts of this plant are used medicinally because of the presence of fructose polymer inulin that can be used as a sweetener and prebiotic (Kirchert and Morlock, 2018), chicoric acid inhibited HIV-1 integrase (Liu *et al*., 2017), sesquiterpene lactones, coumarins, flavonoids, phenols and vitamins (Velayutham *et al*., 2006). Over 100 individual and important compounds have been identified from this medicinal plant and the most of them present in roots (Suresh *et al*., 2005). Chicory root extracts have an anti-bacterial and hepatoprotective effect (Rehman *et al*., 2014; Saggu *et al*., 2014). The fresh root is bitter, with a milky Juice. One of the most important secondary metabolites of this plant is phenolic compounds (Innocenti *et al*., 2005; Jaiswal *et al*., 2011; Das *et al*., 2016). Phenolic acids are considered to be liable for antioxidant and antiradical defense mechanisms of chicory and some other plants (Bogucka-Kocka *et al*., 2018).

Hairy root is a plant disease induced by *A. rhizogenes*. The transfer DNA region harbored by the root-inducing (Ri) plasmid of *A. rhizogenes* is integrated into the genome of the host plant, inducing hairy root. Hairy root-derived plants are usually real transgenic (Hudzieczek *et al*., 2018). The inherent genetic stability of hairy roots is reflected in their stable productivity and thereby represents an attractive alternative for *in vitro* production of bioactive compounds, novel compounds that are not synthesized by plants and recombinant proteins

(Ricigliano *et al*., 2014). The result of successful expression of the genes from the T-DNA region in the wound site of plant tissue leads to the hairy roots induction (Singh *et al*., 2014). In hairy roots culture, production of secondary metabolites is more stable and extensive than other plant cell cultures. The cell suspension cultures are genetically and biologically unstable and often produce secondary metabolites at low levels (Hu and Du, 2006). Hairy roots culture is able to grow rapidly without exogenous plant growth regulators (Kim *et al*., 2007). Hairy root production creates a rapid and simple means to integrate and express foreign genes in plant cells, which are capable of synthesizing specific secondary metabolites (Ono and Tain, 2011).

Nanoparticles are the wide class of materials that include particulate substances, which have one dimension less than 100 nm at least (Laurent *et al*., 2010). Depending on the overall shape these materials can be 0D, 1D, 2D or 3D (Tiwari *et al*., 2012). The importance of these materials was realized when researchers found that size can influence the physiochemical properties of a substance e.g. the optical properties. The properties of nanoparticles differ remarkably from small molecules and their chemistry and synthesis necessitate that they are considered more like complex mixtures than small molecules. The ability of the molecules to attach to the surface of nanoparticles and exchange with other molecules already placed there indicates that careful consideration of the chemistry of nanoparticles and how it relates to their fate in surface waters and sediments is a key to predicting their final fate (Christian *et al*., 2008). Prior studies have described the enhancement of hairy roots growth and secondary metabolite production by different nanoparticles. Elicitation of atropine production in hairy root cultures of *Datura metel* by silver nanoparticles was reported (Shakeran *et al*., 2015). The glycyrrhizin yield in hairy root cultures of *Glycyrrhiza glabra* induced with CuO and ZnO nanoparticles was increased comparison to the control samples (Oloumi *et al*., 2015). Nanomaterials can promote some metabolism and reveal physiological answers but the underlying mechanisms are unknown (Hatami and Ghorbanpour 2014). To the best of our knowledge, no previous study has surveyed the influence of iron and zinc oxide nanoparticles as abiotic elicitor on the enhancement of hairy roots growth and secondary metabolites productivity in *C. intibus*. The aim of this study was the optimization evaluation of the hairy roots induction, growth, and production of secondary metabolites by iron and zinc oxide nanoparticles of *C. intybus*.

MATERIALS AND METHODS

Plant material

Seeds (collected from Isfahan province, Iran) of *C. intybus* were used as starting plant material for *in vitro* germinated and subsequently, seedlings were used for tissue culture experiments. Seeds were washed with distilled water and then immersed in 70% ethanol for 1 min followed by 50 ml l^{-1} sodium hypochlorite for 20 min and finally washed three times with distilled sterilized water. The MS basal medium supplemented with 30 g l^{-1} sucrose and 5.5 g l^{-1} agar was autoclaved at 1.06 kg cm^{-2} pressure and 121 ˚C for 20 min. The surface-sterilized seeds were inoculated on the medium and cultures were incubated at 252± ˚C under fluorescent light for a cycle of 16 h light and 8 h dark per day. After 5 weeks, growing plantlets were used for hairy root induction.

Induction and culture of hairy roots

Transformed roots of *C. intybus* were induced from the leaf segments of 35-day-old *in vitro* plantlets by infection with *A. rhizogenes* A4, ATCC11325 and ATCC15834 strains for 15 min. The explants were randomly wounded using a sterile needle and inoculated with *A. rhizogenes* suspension grown in LB medium for 24 h at 282± ˚C on a rotary shaker at 100 rpm. The inoculated explants were placed in MS medium then incubated at (252±) ˚C under the dark condition for 3 days. In the next step, the explants were transferred into the MS solid medium supplemented with 500 mg l^{-1} cefotaxime. The explants were incubated at (252±) ˚C under a 16-h photoperiod condition for four weeks. At the end of incubation time, percent of hairy root induction, root number, and root length were investigated. In order to obtain the root lines, single roots were picked off and placed on new media with cefotaxime. After four weeks biomass accumulation rates were measured in different root lines**.**

PCR analysis of transgenic roots

Total genomic DNA of *C. intybus* hairy root was isolated via the CTAB method (Doyle and Doyle, 1987) and subjected to PCR analysis. Amplification was performed on a final volume of 12 µl (1.75 µl of each primer (50 ng μl^{-1}), 1 µl of DNA (25 ng/µl), 6 µl of master mix (Sinna Gen, Iran), and 1.5 µl of distilled H_2O). Non-transformed adventitious roots' DNA and $pRiA_4$ plasmid DNA were used as negative and positive controls, respectively. The primer pair

specific to the *rol*B fragment sequences included 5-ATGGATCCCAAATTGCTATTCCCCACGA-3 and 5-TAGGCTTCTTTCATTCGGTTTACTGCAGC-3 (Banihashemi *et al*., 2015). The *rol*B genes were amplified by thermal cycler (Qantarus) as follows: denaturation at 94 ˚C for 5 min followed by 35 cycles of a 1 min-denaturation at 94 ˚C, annealing at 55 ˚C for 45 s and then extended by 1 min at 72 ˚C and then 7 min at 72 ˚C. Finally, the products were separated by 0.8% agarose gels (w v^{-1}).

Hairy root culture establishment

First, the root tips of different lines of hairy roots were harvested (almost 2 cm) and cultured in 15 ml of ½ MS liquid medium per flask and placed on a rotary shaker operating at 90 rpm for 30 days at 25 ˚C in dark. Fresh weight of hairy roots was recorded after 6 weeks. Once washed by sterile water, the roots were dried on filter papers and their fresh weight was measured.

Effect of iron and zinc oxide nanoparticles on hairy root growth

First, the root tips of the best line of hairy roots were harvested (almost 2 cm) and cultured in 20 ml of MS liquid medium supplemented with 50 $mg.l^{-1}$ iron and zinc oxide nanoparticles individual or combination. Then approximately, 200 mg of fresh roots of the best line was cultured per flask and placed on a rotary shaker operating at 100 rpm for 30 days at 25 ˚C in dark. Fresh weight of hairy roots was recorded after 6 weeks. Once washed by sterile water, the roots were dried on filter papers and their fresh weight was measured, then the hairy roots were dried for 48 h in a hot air oven at 70 ˚C for dry weight estimation also the growth index of roots and total phenolic content and flavonoids were determined.

Measurement of total phenolic and flavonoid content

The content of phenol was determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965). For this purpose, 1 ml of 95% ethanol was added to 10 mg of dried hairy roots of powdered clones and control; the solution was then subjected to extraction for 48-72 hours. Upon centrifugation at 6000 rpm for 10 min, 0.5 ml of the sample extract solution was mixed with 0.5 ml of 95% ethanol. Then 250 µL of diluted folin reagent (1:10) and 500 µL of 5% Na_2CO_3 were added to the extract and the mixture was shaken thoroughly. Subsequently, the solution was diluted to 3 ml with distilled water and mixed well. After incubation at 23 ˚C in dark for 1 hour, absorbance spectra of the samples were recorded using a spectrophotometer (Jenway 6305) run at 725 nm. Gallic acid standard curve was used to investigate the number of samples. Flavonoids were measured using the Krizek *et al*. (1998) method. For this purpose, 2 g of dried root were weighed and mixed with 3 ml of the mixture of ethanol with hydrochloric acid (ratio 99:1) inside the masonry. It was then passed through a filter paper and placed in a warm water bath at 80 ˚C for 10 minutes. After cooling the samples, the absorbance was read at 270, 300 and 330 nm by spectrophotometer. The extinction coefficients formula was used to determine the amount of flavonoids.

Statistical analysis

The experiments were laid on a completely randomized design (CRD) with three replications and five explants cultured in each Petri dish. Observations were recorded on the frequency (percentage of explants responding for hairy root initiation) and the number and length of roots, root branching and fresh and dry weight and growth index of roots, also secondary metabolites such as phenolics and flavonoids were analyzed. The data collected were subjected to analysis of variance test. The means were compared using Duncan's multiple range tests at a 5% level of significance. The results were analyzed statistically using R open access software.

RESULTS AND DISCUSSION

Induction and culture of hairy roots

The explants (leaf and petiole) were infected with the *A. rhizogenes* strains and co-cultured for 3 days on solid MS medium in dark condition, and then the explants were cultured on the MS solid medium supplemented with 500 mg l^{-1} cefotaxime. The results of experiments showed that between different explants and strains the highest hairy root induction (60 percent) was exhibited by A4 strain and leaf explants, and also the maximum root number, root length and root branching per explant were observed in the leaf explants infected by A4 and ATCC11325 strains (Table 1, 2 and Figure 1, 2). These results indicated that hairy root induction was influenced by different explants and strains types. In the present study, induction of hairy roots was noticed after 9 days of bacterial infection. Kabirnataj *et al*. (2013) indicated that A4, A13 and ATCC15834 strains of *A. rhizogenes* were able to induce hairy roots in *C. intybus* and among MS, Linsmaier and Skoog (LS) and Gamborg (B5) media, MS medium was considered as the best culture medium for co-cultivation of explants with bacteria. Sharafi *et al*. (2014) suggested an efficient hairy root induction system for *Dracocephalum kotschyi*. They developed

Table 1. Analysis of variance of the effect of explant and strain type on hairy root induction characteristics.

Source of variation	df	Means of square			
		Percentage of hairy root induction	Roots number	Root length	Root branch
Explant	1	1088.88**	92.52**	39.04**	57.13*
Strain	2	355.55*	7.52**	5.99**	65.2*
Explant×Strain	2	355.55*	3.16*	5.02*	43.07*
Error	12	88.88	0.77	0.729	10.6

*, **: significant at 5% and 1% probability level respectively.

Table 2. Mean comparisons of the effects of Explants and strains type on hairy roots characteristics.

Explant	Strain	Number of roots/explant	Length of roots/explant	Root branch
Leaf	A4	8.78^{a}	8.66^{a}	18.82^{a}
	ATCC11325	7.21^{a}	7.75^{a}	15.65^{ab}
	ATCC15834	5.58^{b}	5.34^{b}	7.39^{c}
Petiole	A4	2.58^{cd}	5.28^{bc}	0.16^{bc}
	ATCC11325	3.69^{c}	3.94^{bc}	11.6^{bc}
	ATCC15834	1.7^{d}	3.7^{c}	9.41^{c}

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

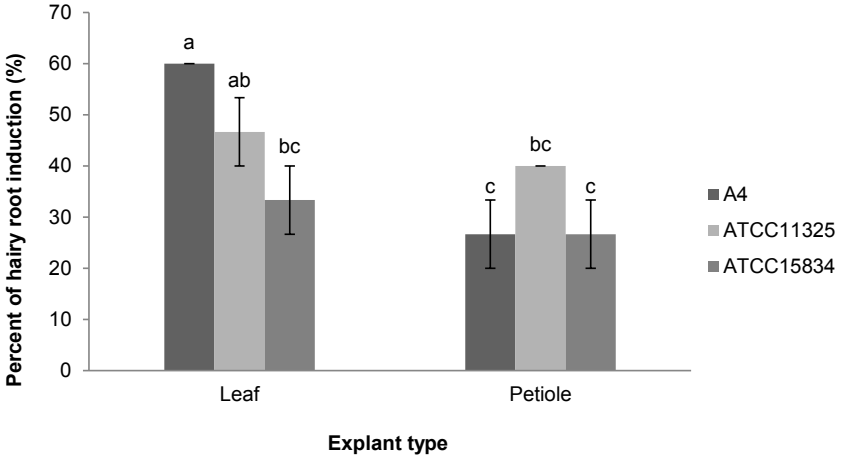

Figure 1. Mean comparisons of the effects of explants and strain type on hairy root induction.

an *A. rhizogenes*-mediated transformation method by modifying the co-cultivation of explants in the MS medium using five bacterial strains, A4, ATCC15834, LBA9402, MSU440, and A13. A drastic increase in transformation frequency was observed when the MS medium lacking NH4NO3 KH2PO4, KNO3 and CaCl2 was used.

PCR analysis of transgenic roots

To probe the existence of the *rol*B gene conveyed from *A. rhizogenes* Ri plasmid, PCR analysis was conducted. PCR reaction with primers for *rol*B genes confirmed the transfer of T-DNA region of Ri plasmid of bacteria into hairy roots genome, while DNA isolated from non-transformed adventitious roots

Hairy root culture establishment

In this research, the obtained hairy root lines showed significant differences in growth rate and biomass production, depending on the strain potential for transformation. The largest biomass production (0.34 g per flask was exhibited by hairy roots resulted from ATCC15834 strain and the third line (Table 3 and Figure 4). Various strategies have been demonstrated for the

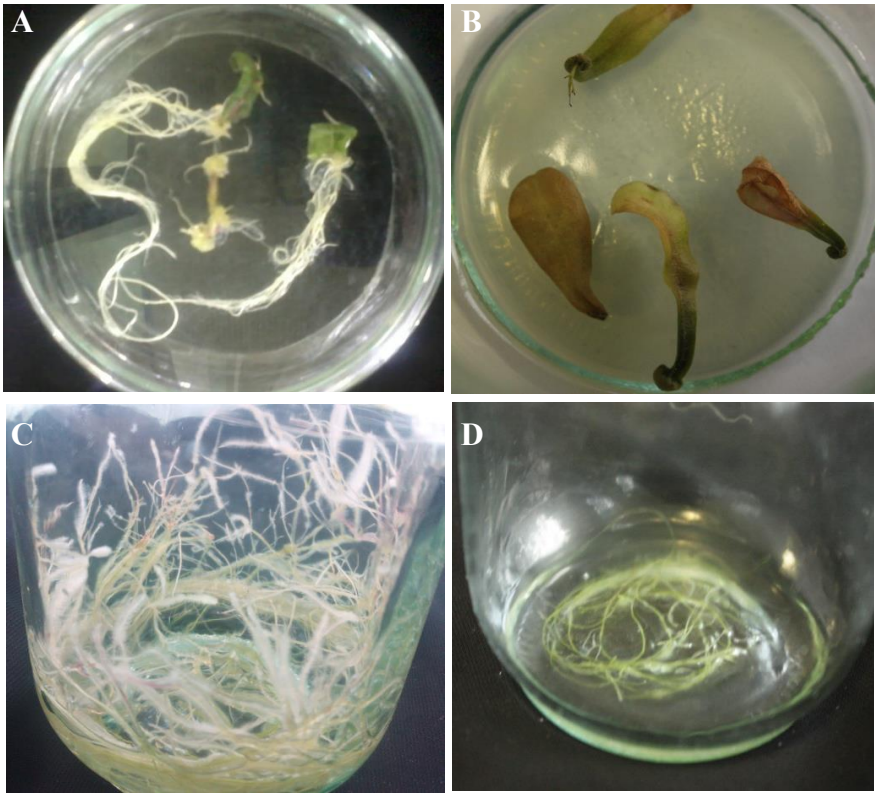

Figure 2. Hairy roots induction by A. rhizogenes in chicory, **A:** Hairy root induction in transformed explants, **B:** Adventitious root induction in non-transformed explants, **C:** Hairy root growth in liquid medium, **D:** Adventitious root growth in liquid medium.

synthesis and production of pharmaceutically important phytochemicals (Nagella *et al*., 2013). These strategies include *Agrobacterium*-mediated gene transfer, *in vitro* cell line establishment, *in vitro* cell suspension cultures, bioreactor cultivation and *in vitro* hairy root cultured (Bourgaud *et al*., 2001). However, among these techniques, *Agrobacterium*-mediated gene transfer has been widely studied as a strategy for producing hairy root lines with high yield for plant compounds (Ali *et al*., 2008). The integration sites of T-DNA within plant genomes are largely random and each transformed cell produces a hairy root line, therefore each line shows a different rate of growth and development (Kim *et al*., 2007). In a research on hairy roots growth in *Nepeta pogonosperma,* five bacterial strains, A4, ATCC15834, LBA9402, MSU440 and A13 and two explant types were used. The maximum growth rate of hairy root lines was obtained from stem explants using MSU440 and ATCC15834 bacterial strains (Valimehr *et al*., 2014). Sharafi *et al*. (2013). Three explant types were used, hypocotyls, leaves and excised shoots of *Papaver bracteatum* for hairy roots induction with strains, A4, ATCC15834, LBA9402, MSU440 and A13., The highest frequency of transformation was achieved using LBA9402 strain in the excised shoots. Also the effect of sucrose concentration and the ratio of NH4: NO3 on

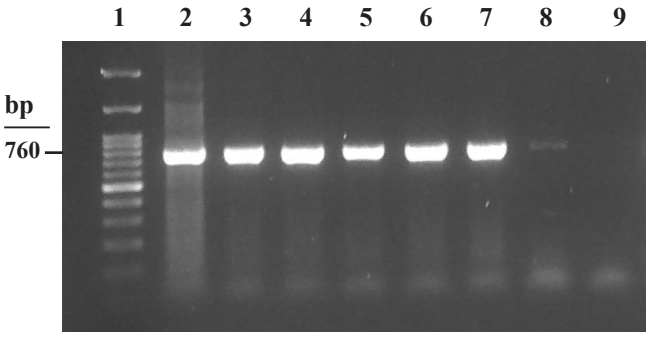

Figure 3. PCR amplified DNA fragments in size (760 bp) using specific primers for the rolB gene of A. *rhizogenes* on chicory hairy root DNA. 1: 100 bp DNA Ladder, 2: A. *rhizogenes* plasmid A4 strain as a positive control, 3- 8: hairy root lines, 9: Adventitious root raised form non-transformed explant as a negative control.

Table 3. Analysis of variance of the effect of strains and hairy root lines on fresh weight of roots.

Source of variation	df	Means of square
Strain	2	0.037**
Line	3	0.035**
Strain×Line	6	0.017**
Error	24	0.001

**: significant at 1% probability level.

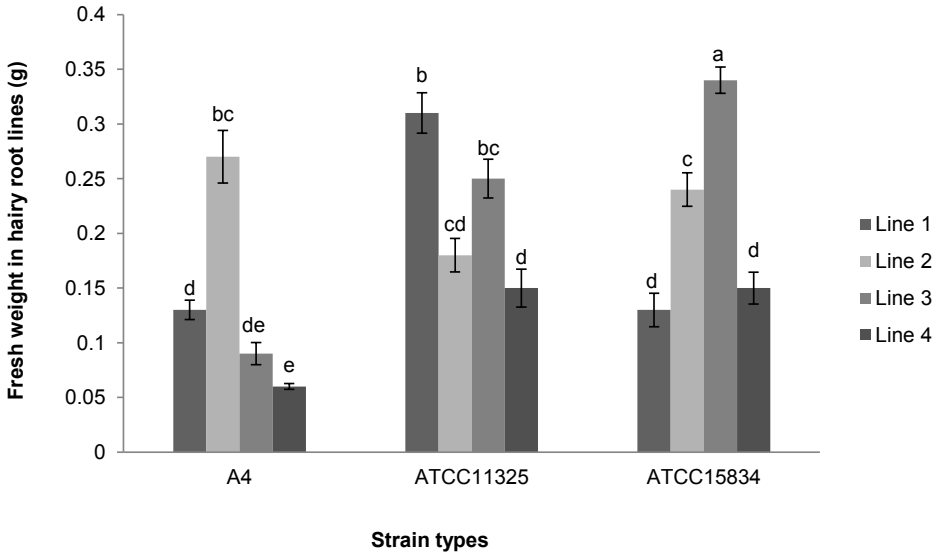

Figure 4. Mean comparisons of the effects of different strains and hairy root lines on hairy roots growth.

hairy root biomass was examined. Maximum biomass was obtained in 30 g l^{-1} sucrose and 20:10 mM ratio of NH4 to NO3 on the MS medium.

Effect of iron and zinc oxide nanoparticles on hairy roots growth

ANOVA showed that the growth of *C. intybus* hairy roots had been significantly affected by iron oxide nanoparticles. The highest fresh and dry weight (4.53, 0.4 g per flask) and growth rate of hairy roots (24.14) were obtained with ATCC15834 strain and iron oxide nanoparticles followed by ATCCC11325 strain and iron oxide nanoparticles. The lowest fresh and dry weight (0.23, 0.025 g per flask) and growth rate of hairy roots were observed in control and iron and zinc oxide nanoparticles combination treatments (0.58) (Table 4, 5 and Figure 5) caused. Moharram *et al*. (2017) elicited the hairy roots derived from *Hyoscyamus reticulatus* L. cotyledon explants by iron oxide nanoparticles at different concentrations (0, 450, 900, 1800, and 3600 mg l^{-1}) and different exposure times (24, 48 and 72 h). The highest hairy root fresh and dry weights were obtained in the medium supplemented with 900 mg l^{-1} iron oxide compared to non-transgenic roots. The highest hyoscyamine and scopolamine production was achieved with 900 and 450 mg l^{-1} iron oxide nanoparticles at 24 and 48 h of exposure time, respectively.

Measurement of Total phenolic and flavonoid content

Transfer of an appropriate combination of *rol* genes from bacteria to the plant is necessary for hairy root induction; on the other hand, transfer of only one of these genes cannot bring about all features of hairy root syndrome (Hanafy *et al*., 2016). The most important feature of phenolic acids is their function in defense mechanisms. Stress conditions, including injury and microbial contamination, increase the biosynthesis of phenolic compounds. Therefore, environmental factors impose significant effects on the content of phenolic acids. Since *Agrobacterium* can be considered as a plant pathogen when used as inoculum for hairy roots induction, the plant seems to adopt a phenol production-based defense mechanism against bacterium (Fu *et al*., 2018). Many secondary metabolites are derivatives of the phenolic compounds and may also be increased by enhancing the phenol production. Results showed significant ($P \leq 0.01$) differences between the phenolic content of dried ethanol extract in hairy roots resulted from different treatments. The total phenolic content was calculated using the Folin-Ciocalteu method. For this purpose, absorbance spectra of the samples were recorded using a spectrophotometer at 725 nm. A standard curve of Gallic acid at various concentrations was used to quantify the samples. The results showed significant differences between the phenolic content of dried ethanol extract from hairy root resulted from different treatments. The total phenolic content was higher (4.65 mg g^{-1} DW) in the hairy roots obtained from the ATCC15834 strain and iron oxide nanoparticles. The results of flavonoid measurement showed significant differences between flavonoid content in hairy root resulted from nanoparticles in comparison with medium control. Flavonoid content in 270, 300 and 330 nm wavelengths was higher (60.35, 70.9 and 77.34 μg g^{-1} DW) in the hairy roots obtained from the leaf explant infected by ATCC11325 strain and iron oxide nanoparticles, (Table 4, 5).

Table 4. Analysis of variance of the effect of strain and nanoparticles type on hairy roots growth.

Source of variation	df	Means of square						
		Fresh weight	Dry weight	Grow index	Phenolic content	Flavonoid 270 nm	Flavonoid 300 nm	Flavonoid 330 nm
Strain	3	6.54**	0.05**	197.23**	6.40**	108.66*	149.96**	178.46*
nanoparticle	2	13.35**	0.1**	410.22**	7.68**	1398.08**	1929.43**	2296.18**
Strain×nanoparticle	6	3.44**	0.02**	106.55**	1.45**	175.13**	241.69**	287.63**
Error	24	0.25	0.003	9.95	0.018	38.62	53.3	63.43

*, **: significant at 5% and 1% probability level.

Table 5. Mean comparisons of the effects of strain and nanoparticles on hairy root characteristics.

Strain	Nanoparticle	Dry weight (g)	Growth index	Phenol (mg g^{-1} DW)	Flavonoid 270 nm (µg g^{-1} DW)	Flavonoid 300 nm (µg g^{-1} DW)	Flavonoid 330 nm (µg g^{-1} DW)
A4	Zn	0.15^{b}	8.85^{b}	4.03^{b}	40.88^{d-f}	48.02^{d-f}	52.39^{d-f}
	Fe	0.14^{bc}	8.33^{bc}	3.56^{c}	56.8^{a-c}	66.72^{a-c}	72.79^{a-c}
	Fe+Zn	0.067^{bcd}	3.2^{bcd}	1.68^{g}	30.48^{fg}	35.80^{fg}	39.6^{fg}
ATCC11325	Zn	0.119^{bcd}	6.45^{bcd}	3.4^{c}	35.23^{e-g}	41.39^{e-g}	45.15^{e-g}
	Fe	0.38^{a}	22.84^{a}	3.87^{b}	60.35^{a}	70.9^{a}	77.34^{a}
	Fe+Zn	0.072^{bcd}	3.41^{bcd}	2^{f}	27.22^{g}	31.97^{g}	34.88^{g}
ATCC15834	Zn	0.112^{bcd}	5.96^{bcd}	2.78^{d}	33.94^{fg}	39.87^{fg}	43.5^{fg}
	Fe	0.4^{a}	24.41^{a}	4.65^{a}	58.92^{ab}	69.22^{ab}	75.52^{ab}
	Fe+Zn	0.87^{bcd}	4.45^{bcd}	2.46^{e}	45.67^{c-e}	53.65^{c-e}	58.53^{c-e}
Control	Zn	0.055^{cd}	2.45^{cd}	1.87^{fg}	48.52^{b-d}	57^{b-d}	62.19^{b-d}
	Fe	0.036^{d}	1.24^{c}	1.37^{h}	51.99^{a-d}	61.08^{a-d}	66.63^{a-d}
	Fe+Zn	0.025^{d}	0.58^{c}	1.21^{b}	45.56^{c-e}	53.53^{c-e}	58.39^{c-e}

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

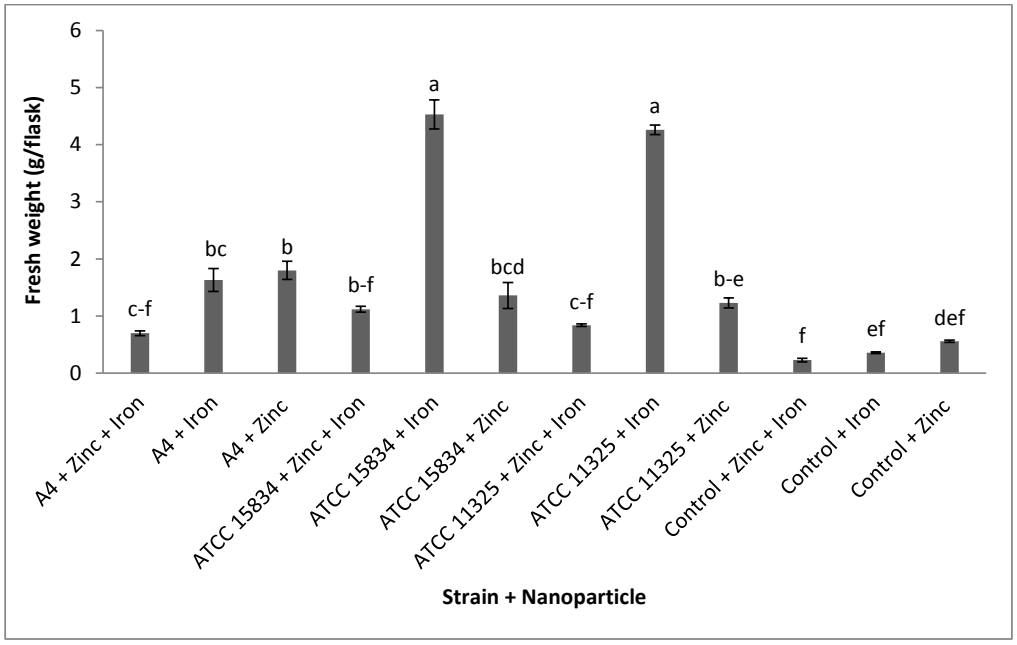

Figure 5. Mean comparisons of the effects of strains and nanoparticles on hairy root growth rate.

CONCLUSION

The present study describes the successful genetic transformation and establishment of a highly productive hairy root culture of *C. intybus*. The establishment of hairy root cultures represents a promising technique for the production of secondary metabolites. The results showed that the use of iron oxide nanoparticles as abiotic elicitor was an effective method for enhancement of growth and secondary metabolites production in hairy root culture of *C. intybus*. The exposure of hairy roots induced by ATCC15834 and ATCC11325 strains to iron oxide nanoparticles, showed the best results, respectively. This study is the first report of the application of nanoparticles in hairy root culture of this plant. It seems that the use of nanoparticles as abiotic elicitors could be an effective strategy to increase the productivity of pharmaceutical compounds in medicinal plants.

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