Industrial Crops & Products 128 (2019) 572–580

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

High-efficiency *Agrobacterium rhizogenes*-mediated genetic transformation in *Cichorium intybus* L. via removing macronutrients

Roghayeh Fathi, Mehdi Mohebodini*, Esmaeil Chamani

Department of Horticultural Sciences, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran

ARTICLE INFO

Keywords:
Co-culture medium
Hairy roots
Macronutrients
Phenolic content
Regeneration

ABSTRACT

Transformed hairy roots technology provides new opportunities for mass production of pharmaceutical metabolites. Hairy roots culture has been considered as an alternative method for producing medicinal biomolecules in plants. Chicory (*Cichorium intybus* L.) is a medicinal herb from *Asteraceae* family. It contains many important metabolites including chicoric acid, inulin, scoline, coumarin, and flavonoids. In this study, for the first time, a reliable gene transfer system via modifying co-cultured medium using different strains of *Agrobacterium rhizogenes* and explants were established for *C. intybus*. Different co-culture media were used for this purpose, including MS basal medium and MS medium without either of KNO_3, NH_4NO_3, KH_2PO_4, $CaCl_2$, and $MgSO_4$. The results showed increases in hairy roots induction percentage, biomass production, and phenolic content of the hairy roots. Maximum hairy root induction percentage obtained by using A_4 strain when a KNO_3-free MS medium was used. High frequency of hairy root induction percentage with ATCC11325 and ATCC15834 strains was achieved when MS medium had KH_2PO_4 removed. In all of the experiments, very low hairy root induction was observed when NH_4NO_3 was removed from co-culture MS medium. Molecular confirmation of transgenic hairy roots was done with PCR using gene-specific primers for *rol*B gene. Total phenols, flavonoids, anthocyanins and chicoric acid contents of hairy root clones and the control (untransformed) roots were also determined. The results indicated significant increases in total metabolites content of hairy root clones induced by A_4, ATCC11325 and ATCC15834 strains with some macronatrient–free co-culture MS media and also directly-regenerated shoots from hairy roots were spontaneous without using any plant growth regulators.

1. Introduction

Transformed hairy roots induced by *A. rhizogenes* strains are used as a tissue culture explants for secondary metabolite production and recombinant proteins (Kim et al., 2002), as the roots are genetically and biologically stable and can produce metabolites within a short time (Georgiev et al., 2012). A part of root-inducing Ri plasmid, namely T-DNA region, from *A. rhizogenes* is integrated into the plant genome. Successful expression of the genes from the T-DNA region in the wound site of plant tissue induces hairy roots (Chilton et al., 1982). In hairy roots culture, production of secondary metabolites follows a more stable and extensive process than other plant cell cultures. Cell suspension cultures are genetically and biologically unstable and often produce secondary metabolites at low levels (Hu and Du, 2006). Hairy roots culture is, however, able to grow rapidly without exogenous plant growth regulators (Kim et al., 2002). Production of recombinant proteins via plants transformation by *Agrobacterium* has been referred to as a new technology in recent years. This technology had been successful in the production of recombinant human proteins in whole leafy plants, such as tomato (Sandhu et al., 2000) *Nicotiana* species (Matoba et al., 2010; Whaley et al., 2011), lettuce (Mohebodini et al., 2015), carrot (Rosales-Mendoza et al., 2007), etc. In terms of safety, economic, and operational aspects, the use of hairy roots for production of recombinant proteins and secondary metabolites has many advantages over other expression systems. Some literature explored the possibility of human or bacterial genes transferring in *C. intybus* by a different strain of *A. rhizogenes* and *A. tumefaciens* to optimization of hairy roots and transgenic plants production. Matvieieva et al. (2015) successfully transferred human interferon *ifn_α2b* gene and telomerase reverse transcriptase *hTert* gene into this plant. They showed that the highest root formation frequency was demonstrated using a pCB161 vector with the *ifn_α2b* target gene and *nptII* selective gene.

As a member of the *Asteraceae* family, *C. intybus* L. is an important biennial medicinal plant. It is useful for hepatic disease treatment and promotes appetite and digestion (Malarz et al., 2002). All parts of this plant have been used in medicinal applications because of the presence

* Corresponding author.
E-mail address: mohebodini@uma.ac.ir (M. Mohebodini).

https://doi.org/10.1016/j.indcrop.2018.11.050
Received 23 October 2017; Received in revised form 25 July 2018; Accepted 19 November 2018

Table 1
Protocol for induction and establishment of hairy roots in *C. intybus* L. under *in vitro* conditions.

Stage	Used explant	Nutrient medium	Cultural conditions
1. *In vitro* seed germination	Seeds	Full strength MS supplemented. with 3% sucrose, 0.7 % agar	25 days at 25 ± 2 °C temperature, 16/8 h photoperiod, 3000 light intensity and 70% humidity
2. Co-cultivation	Cotyledons and Leafs that infected by A4, ATCC11325 and ATCC15834 strains	Full strength MS MS free KNO_3 MS free NH_4NO_3 MS free KH_2PO_4 MS free $CaCl_2$ MS free $MgSO_4$	4 days at 25 ± 2 °C, under dark condition, and 70% humidity
3. Hairy root induction	Cotyledons and Leafs that infected by A4, ATCC11325 and ATCC15834 strains in stage 2	Full strength MS supplemented with 500 mg l^{-1} cefotaxime	4 weeks at 22 ± 2 °C temperature, 16/8 h photoperiod, 3000 light intensity and 70% humidity
4. Hairy roots culture establishment	Hairy roots lines	Half strength MS liquid medium	6 weeks on rotary shaker (90 rpm) at 25 °C in dark condition

of fructose polymer inulin that can serve as sweetener and prebiotic ingredient (Baert and Van Bockstaele, 1992), Chicoric acid, sesquiterpene lactones, coumarins, flavonoids, phenols and vitamins (Velayutham et al., 2006). Chicoric (dicaffeyltartaric) acid, one of the important phenolics of chicory is an important cinnamic acid that use for treating AIDS. The enzyme HIV integrase causes the integration of viral DNA into host DNA. Chicoric acid can inhibit HIV integrase type 1 activity and is known as an anti-HIV agent (Lin et al., 1999). Over 100 individual and important compounds have been identified in this medicinal plant, most of which occur in its roots (Suresh et al., 2005). Chicory root extracts are known to exhibit anti-bacterial and hepatoprotective properties (Bais and Ravishankar, 2001; Nandagopal and RavjithaKumari, 2007). The fresh root is bitter, with a milky Juice. Two of the most important secondary metabolites of this plant are sesquiterpene lactones (Kisiel and Zielinska, 2001) and phenolic compounds (Innocenti et al., 2005; Jaiswal et al., 2011; Rees and Harborne, 1985). Phenolic acid contents of chicory and some other plants are responsible for their antioxidant and antiradical defense mechanisms (Heimler et al., 2009).

There exist six operons on *vir* genes on the left side of the T-DNA region, whose function is essential for the transfer of the T-DNA to the genome of the plant. The *vir*G and *vir*A regions encode a protein that is responsible for activating transcription of other *Vir* genes. Research works have shown that type and concentration of co-culture medium have direct impacts on transformation with *Agrobacterium*. Some macronutrients negatively affect gene transfer such as PO_4, while others (e.g. $MgSO_4$ and Na_2EDTA) affect it positively (Azadi et al., 2010). The activators of the *vir*A region include acidic pH, phenolic compounds (Acetosyringone), and some groups of monosaccharides; these further modify the co-culture medium by, for example, eliminating PO_4 (Palmer et al., 2004). In this research, different types of explants were infected with various *A. rhizogenes* strains. The objective was to obtain information on the effect of modifying MS medium (Murashige and Skoog, 1962) on hairy root induction efficiency and morphology. The hairy roots were cultured in a liquid medium, then investigating their fresh and dry weight and secondary metabolites production. This is the first report on the improved transformation of *C. intybus* L. by modifying MS medium via removing macronutrients from the co-culture medium.

2. Materials and methods

2.1. Plant material

Chicory seeds were surface-washed with running water for 30 min; those were then sterilized with 700 ml l^{-1} ethanol for 90 s and 5% (v v^{-1}) sodium hypochlorite solutions for 20 min then washed four times using sterilized water. The MS basal medium supplemented with 30 g l^{-1} sucrose was adjusted to pH 5.8 and then introduced by 7 g l^{-1} plant agar before being autoclaved for 20 min. The seeds were cultured on the medium under the sterile condition and maintained at 25 ± 2 °C for 16-h in light and 8 h in dark on a daily basis. After 25 days, growing plantlets were used for hairy root induction.

2.2. Preparation of strains and co-culture media

Strains of A_4, ATCC11325 and ATCC15834 were inoculated in Luria-Bertani (Bertani, 1952) medium containing 10 g l^{-1} tryptone, 5 mg l^{-1} yeast extract and 10 mg l^{-1} NaCl at pH 7.2 supplemented with 50 mg l^{-1} rifampicin. A_4, ATCC11325 and ATCC15834 strains are groping types of *A. rhizogenes* that include two separate parts in T-DNA region namely TR-DNA and TL-DNA. TR-DNA region contains two genes, iaaM, and iaaH, responsible for the biosynthesis of auxins and the genes responsible for the synthesis of the opine agropine. TL-DNA carries 18 open reading frames (ORF), four of which are essential for hairy roots formation; ORF10, ORF11, ORF12, and ORF15 are corresponding to the genes *rol*A, *rol*B, *rol*C, and *rol*D, respectively. The *rol*B gene is absolutely essential for the induction of hairy roots. Even when expressed alone, it can induce significant hairy roots production (Nilsson and Olsson, 1997).

Overnight culture was shaken at 28 °C on a shaker incubator (Sahand Azar, 554D) operating at 120 rpm and was used for inoculate the plant at an optical density of 0.6. Effects of macronutrients in co-culture media on hairy root induction were studied. For this purpose, MS basal medium (Medium 1) and modified MS media by removing either of KNO_3 (Medium 2), NH_4NO_3 (Medium 3), KH_2PO_4 (Medium 4), $CaCl_2$ (Medium 5) and $MgSO_4$ (Medium 6) were used.

2.3. Hairy root induction and culture conditions

The explants were wounded by a sterile scalpel and then gently inoculated with *A. rhizogenes* suspension for 30 min. After that, the explants were drained on sterile filter paper and then transferred to co-culture medium. The explants were incubated at 25 ± 2 °C in dark for four days. In the next step, the explants were cultured on MS solid medium supplemented with 500 mg l^{-1} cefotaxime. The explants were incubated at 25 ± 2 °C under 16-h photoperiod condition for four weeks. Several subcultures were prepared on fresh medium with cefotaxime until the bacteria were eliminated. Transformed roots were induced from segments of cotyledons and leaves by infection with *A. rhizogenes* A_4, ATCC11325 and ATCC15834 strains. At the end of incubation time, induction percentage, average number, length, and branching of hairy roots were investigated.

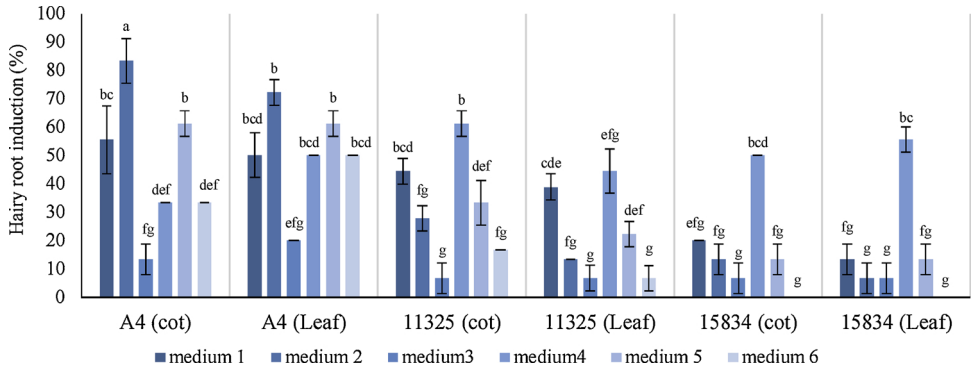

Fig. 1. The effects of strain, explant type and co-cultivation medium on hairy root induction.
Medium 1: MS basal medium, Medium 2: MS free KNO_3, Medium 3: MS free NH_4NO_3, Medium 4: MS free KH_2PO_4, Medium 5: MS free $CaCl_2$ and medium 6: MS free $MgSO_4$ (Medium 6). Vertical lines represent standard deviation.

Table 2
Compare means of the effects of strain and explant type and co-cultivation media on some characteristics of hairy roots.

Strain	Explant	Medium	Root number	Root length (cm)	Root branching
A_4	Cotyledon	MS	5.33 $^{b-d}$	7.23 ^{b}	10.90 ^{b}
		-KNO_3	6.36 ^{b}	**8.63** ^{a}	**13.24** ^{a}
		-NH_4NO_3	1.66 $^{f-i}$	1.86 $^{f-j}$	2.10 $^{g-j}$
		-KH_2PO_4	3.92 $^{b-g}$	4.56 $^{b-g}$	6.78 $^{c-f}$
		-$CaCl_2$	4.43 $^{b-f}$	0.50 ^{ij}	1.00 ^{ij}
		-$MgSO_4$	1.01 $^{g-i}$	1.00 ^{ij}	1.08 ^{ij}
	Leaf	MS	5.10 $^{b-e}$	5.53 $^{b-d}$	8.59 $^{b-d}$
		-KNO_3	3.33 $^{c-h}$	6.23 $^{b-c}$	10.77 ^{b}
		-NH_4NO_3	3.13 $^{c-h}$	2.73 $^{d-j}$	5.43 ^{dh}
		-KH_2PO_4	2.96 $^{d-i}$	0.66 ^{ij}	1.00 ^{ij}
		-$CaCl_2$	3.23 $^{c-h}$	2.03 $^{e-j}$	2.75 $^{f-j}$
		-$MgSO_4$	1.33 ^{gi}	1.00 ^{ij}	1.00 ^{ij}
ATCC11325	Cotyledon	MS	3.4 $^{c-h}$	3.50 $^{c-i}$	5.24 ^{di}
		-KNO_3	2.06 $^{f-i}$	2.16 $^{f-i}$	2.66 $^{f-j}$
		-NH_4NO_3	0.66 ^{hi}	1.06 ^{ij}	1.33 $^{h-j}$
		-KH_2PO_4	3.16 $^{c-h}$	2.46 $^{e-j}$	3.99 $^{e-j}$
		-$CaCl_2$	1.33 $^{g-i}$	1.26 ^{ij}	2.19 $^{g-j}$
		-$MgSO_4$	2.02 $^{f-i}$	1.00 ^{ij}	1.00 ^{ij}
	Leaf	MS	5.16 $^{b-e}$	2.83 $^{d-i}$	4.88 $^{d-i}$
		-KNO_3	5.10 $^{b-e}$	5.00 $^{b-e}$	9.91 $^{b-c}$
		-NH_4NO_3	1.03 $^{g-i}$	1.50 $^{g-j}$	2.00 $^{g-j}$
		-KH_2PO_4	6.00 $^{b-c}$	0.56 ^{ij}	1.00 ^{ij}
		-$CaCl_2$	3.96 $^{b-g}$	4.56 $^{b-f}$	7.91 $^{b-e}$
		-$MgSO_4$	1.41 $^{g-i}$	1.66 $^{f-j}$	1.00 ^{ij}
ATCC15834	Cotyledon	MS	3.00 $^{d-h}$	4.23 $^{c-h}$	4.10 $^{e-j}$
		-KNO_3	2.33 $^{e-i}$	2.33 $^{e-j}$	3.06 $^{f-j}$
		-NH_4NO_3	1.33 $^{g-i}$	1.16 ^{ij}	2.00 $^{g-j}$
		-KH_2PO_4	**8.12** ^{a}	1.40 $^{h-j}$	1.83 $^{g-j}$
		-$CaCl_2$	2.00 $^{f-i}$	2.00 $^{f-j}$	2.00 $^{g-j}$
		-$MgSO_4$	0.00 ^{i}	0.00 ^{j}	0.00 ^{j}
	Leaf	MS	1.66 $^{f-i}$	2.66 $^{e-j}$	2.50 $^{g-j}$
		-KNO_3	1.33 $^{g-i}$	1.66 $^{f-j}$	2.10 $^{g-j}$
		-NH_4NO_3	1.00 $^{g-i}$	1.66 $^{f-j}$	1.50 $^{h-j}$
		-KH_2PO_4	5.73 $^{b-d}$	5.50 $^{b-d}$	5.93 $^{d-g}$
		-$CaCl_2$	2.33 $^{e-i}$	2.50 $^{e-j}$	2.36 $^{g-j}$
		-$MgSO_4$	0.00 ^{i}	0.00 ^{j}	0.00 ^{j}

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

2.4. PCR analysis of transgenic roots

Genomic DNA of *C. intybus* L. hairy root was extracted via the CTAB method (Cai et al., 1997) 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^{-1}), 6 µl of master mix, and 1.5 µl of distilled water. Non-transformed adventitious roots' DNA and $pRiA_4$ plasmid's DNA were used as negative and positive controls, respectively. The primer pair specific to the *rol*B fragment sequences included

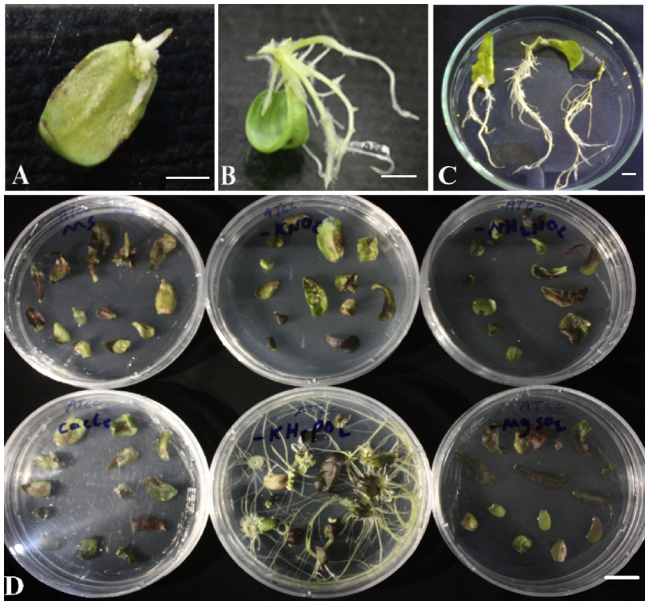

Fig. 2. Hairy roots induction by *A. rhizogenes* in Chicory, A: Hairy root induction one week after infection, B: Increase in hairy root number induced three weeks after infection, C: Hairy root growth five weeks after infection D: Comparison the effect of different co-cultivation on hairy root induction by ATCC15834 strain. Bars in A,B and C: 0.5 cm. Bar in D: 2 cm.

5-ATGGATCCCAAATTGCTATTCCCCACGA-3 and 5-TAGGCTTCTTTCA TTCGGTTTACTGCAGC-3. The protocol followed to amplify the *rol*B gene was 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 by 7 min at 72 °C for 7 min. Finally, the products were separated by 0.8% agarose gels (w v^{-1}).

2.5. Hairy roots culture establishment

At first, the root tips of different lines of hairy roots were harvested (almost 2 cm) and cultured in the 1/2 MS liquid medium. After 30 days, the best line was selected based on the increase in fresh weight, Then approximately, 50 mg of fresh roots of the best line was cultured in 15 ml of 1/2 MS liquid medium per flask and placed on a rotary shaker operating at 90 rpm for 6 weeks 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 (Paradise, 125/36) at 70 °C for dry weight estimation and also the growth index of roots was determined. The protocol of induction and establishment of

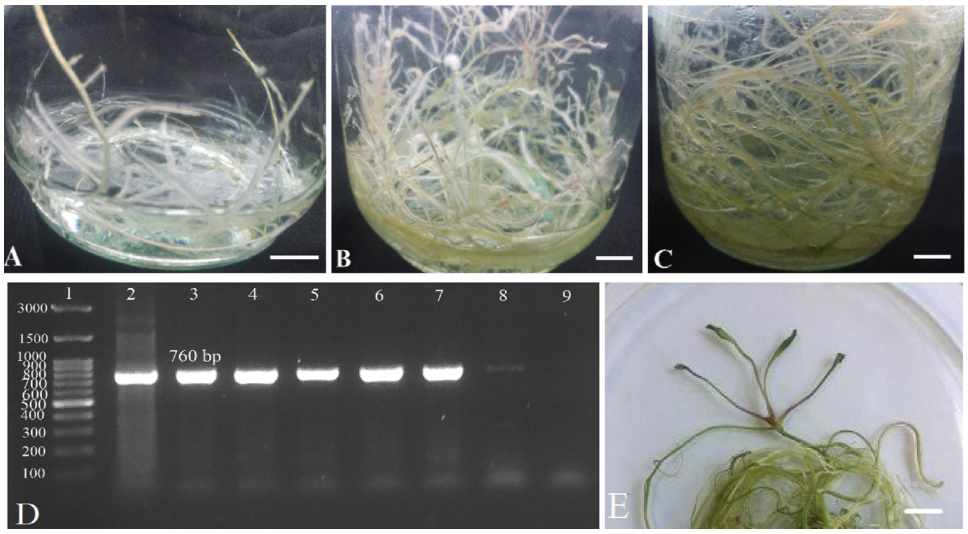

Fig. 3. Hairy roots growth on liquid medium, A: Hairy roots growth 2 weeks after culture, B: 4 weeks after culture, C: 6 weeks after culture. D: PCR amplified DNA fragments (760 bp) using specific primers for *rol*B gene of *A. rhizogenes* on chicory hairy root DNA. 1: 100 bp DNA Ladder, 2: *A. rhizogenes* plasmid A_4 strain as positive control, 3- 8: hairy roots, 9: Adventitious root raised form non-transformed explant as negative control. E: Regenerated plants obtained from hairy roots spontaneously. Bars: 1 cm.

hairy roots in *C. intybus* L. was shown in the Table 1.

2.6. Determination of secondary metabolites content

2.6.1. Total phenolic assay

The content of phenol was determined by the Folin-Ciocalteu method (Ronald and Laima, 2001). 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 centrifuging 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 h, absorbance spectra of the samples were recorded using a spectrophotometer (Jenway 6305) operated at 725 nm. Gallic acid standard curve was used to investigate the number of samples.

2.6.2. Measurement of flavonoid content

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 acid ethanol inside the masonry. It was then passed through a filter paper and placed in a warm water bath at 80 °C for 10 min. After cooling the samples, the absorbance by spectrophotometer was read at 270, 300 and 330 nm in three wavelengths. The extinction coefficients formula was used for determine amount of flavonoids.

2.6.3. Total anthocyanins assay

The total anthocyanin was measured using the Hara et al. (2003) method. The total anthocyanin compounds were extracted with 3 ml of acid ethanol, then place in dark condition for 24-h. In the next step, the extracts were centrifuged at 12,000 rpm. The absorbance by spectrophotometer was read at 550 nm wavelength. The extinction coefficients formula was used for determine amount of anthocyanins.

2.6.4. Determination of chicoric acid in treated roots by HPLC

Extraction and quantification of chicoric acid from the dried hairy root cultures were carried out according to Heimler et al. (2009) with 70% ethanol overnight. Samples were centrifuged for 15 min at 10,000 rpm. The supernatants were filtered and then analyzed the amount of cichoric acid by high performance liquid chromatography (HPLC). Equal amounts (50 µl) of each replication manually were injected into the HPLC system (Knauer, pump K-1001) equipped with a C18 column (250 × 4.6 mm, pore size 5 µm; Teknokroma). The mobile phase was an isocratic 30% (v v^{-1}) acetonitrile: 70% water containing 0.1% (v v^{-1}) phosphoric acid at rate of 1.75 ml/min for 10 min. The UV detector K -2501 (Knauer) was set at 330 nm. Reference standard of cichoric acid was purchased from Sigma- Aldrich (Germany). The calibration curve was made with standard chicoric acid and co-chromatograms of the standards and samples obtained. Chicoric acid were

Table 3
The effects of strain and explant type and co-cultivation media on fresh and dry weight and growth index in hairy roots.

Strain	Explant	Medium	Fresh weight (g)	Dry weight (g)	Growth index
A_4	Cotyledon	MS	1.25 $^{b-e}$	0.106 $^{b-e}$	25.58 $^{b-e}$
		-KNO_3	**1.98** ^{a}	**0.185** ^{a}	**45.41** ^{a}
		-NH_4NO_3	0.63 $^{g-l}$	0.062 $^{d-h}$	14.53 $^{d-h}$
		-KH_2PO_4	1.08 $^{b-g}$	0.103 $^{b-f}$	24.75 $^{b-f}$
		-$CaCl_2$	0.29 $^{k-m}$	0.028 ^{hi}	6.01 ^{hi}
		-$MgSO_4$	0.3 $^{k-m}$	0.031 ^{hi}	6.98 ^{hi}
	Leaf	MS	1.29 $^{b-d}$	0.119 ^{bc}	28.98 ^{bc}
		-KNO_3	**1.57** ^{ab}	0.132 ^{b}	32.08 ^{b}
		-NH_4NO_3	0.22 $^{f-l}$	0.064 $^{d-h}$	15.15 $^{d-h}$
		-KH_2PO_4	0.7 ^{lm}	0.22 ^{hi}	4.5 ^{hi}
		-$CaCl_2$	0.54 $^{h-l}$	0.5 $^{f-i}$	11.66 $^{f-i}$
		-$MgSO_4$	0.34 $^{i-m}$	0.032 ^{hi}	7 ^{hi}
ATCC11325	Cotyledon	MS	0.85 $^{c-j}$	0.094 $^{b-g}$	22.58 $^{b-g}$
		-KNO_3	0.63 $^{g-l}$	0.051 $^{f-i}$	11.83 $^{f-i}$
		-NH_4NO_3	0.67 $^{f-l}$	0.054 $^{e-h}$	12.58 $^{e-i}$
		-KH_2PO_4	0.91 $^{c-h}$	0.074 $^{c-h}$	17.58 $^{c-h}$
		-$CaCl_2$	0.57 $^{g-l}$	0.06 $^{d-h}$	14.08 $^{d-h}$
		-$MgSO_4$	0.35 $^{i-m}$	0.036 ^{hi}	8.16 ^{hi}
	Leaf	MS	1.26 $^{b-e}$	0.098 $^{b-f}$	23.66 $^{b-f}$
		-KNO_3	1.15 $^{b-f}$	0.109 $^{b-d}$	26.41 $^{b-d}$
		-NH_4NO_3	0.23 $^{b-e}$	0.111 $^{b-d}$	26.76 $^{b-d}$
		-KH_2PO_4	1.3 ^{lm}	0.131 ^{b}	31.5 ^{hi}
		-$CaCl_2$	1.1 $^{b-d}$	0.134 ^{b}	32.7 ^{b}
		-$MgSO_4$	0.71 $^{f-l}$	0.073 $^{c-h}$	17.38 $^{c-h}$
ATCC15834	Cotyledon	MS	0.8 $^{d-k}$	0.069 $^{c-h}$	16.33 $^{c-h}$
		-KNO_3	0.39 $^{h-m}$	0.034 ^{hi}	7.63 ^{hi}
		-NH_4NO_3	0.32 $^{j-m}$	0.031 ^{hi}	6.95 ^{hi}
		-KH_2PO_4	1.56 ^{b}	0.128 ^{b}	31.0 ^{b}
		-$CaCl_2$	0.61 $^{g-l}$	0.043 $^{g-i}$	9.91 ^{ghi}
		-$MgSO_4$	0.00 $^{\circ}$	0.00 ^{i}	0.00 ^{i}
	Leaf	MS	0.85 $^{c-i}$	0.062 $^{d-h}$	14.71 $^{d-h}$
		-KNO_3	0.77 $^{e-k}$	0.06 $^{d-h}$	14 $^{d-h}$
		-NH_4NO_3	0.61 $^{g-l}$	0.057 $^{d-h}$	13.35 $^{d-i}$
		-KH_2PO_4	1.33 ^{bc}	0.119 ^{bc}	28.83 ^{bc}
		-$CaCl_2$	0.79 $^{d-k}$	0.069 $^{c-h}$	16.33 $^{c-h}$
		-$MgSO_4$	0.00 $^{\circ}$	0.00 ^{i}	0.00 ^{i}

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

Table 4
Compare means of the effects of strain and explant type and co-cultivation media on phenol, flavonoid and anthocyanin in hairy roots.

Strain	Explant	Medium	Total phenolic content (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)	Anthocyanin (µg g^{-1} DW)
A_4	Cotyledon	MS	3.90 ^{bc}	39.69 $^{e-g}$	41.21 $^{g-l}$	45.26 $^{g-k}$	12.62 ^{fg}
		-KNO_3	**4.46** ^{a}	**59.19** ^{a}	**66.82** ^{ab}	**70.49** ^{ab}	13.55 ^{ef}
		-NH_4NO_3	2.93 $^{d-h}$	29.59 $^{i-l}$	35.60 $^{h-l}$	38.6 $^{k-n}$	12.97 ^{f}
		-KH_2PO_4	2.84 $^{d-i}$	34.21 $^{h-l}$	35.70 $^{h-l}$	37.54 $^{k-n}$	14.94 $^{d-f}$
		-$CaCl_2$	3.09 $^{d-g}$	**58.59** ^{a}	**67.12** ^{a}	**70.18** ^{ab}	**21.88** ^{a}
		-$MgSO_4$	3.24 ^{de}	**57.21** ^{a}	65.49 ^{b}	68.04 $^{b-d}$	18.06 ^{bc}
	Leaf	MS	3.42 ^{cd}	32.95 $^{h-l}$	34.68 $^{i-l}$	38.46 $^{k-n}$	15.05 ^{def}
		-KNO_3	3.27 ^{de}	44.34 $^{b-h}$	44.78 $^{f-h}$	43.45 $^{h-m}$	16.01 $^{c-e}$
		-NH_4NO_3	3.1 $^{d-g}$	26.42 $^{k-l}$	31.72 ^{l}	32.54 ^{n}	14.01 ^{ef}
		-KH_2PO_4	2.62 ^{fg}	47.11 $^{b-g}$	48.56 $^{e-g}$	46.72 $^{g-k}$	17.25 $^{b-d}$
		-$CaCl_2$	4.27 ^{ab}	50.47 $^{b-e}$	67.12 ^{b}	65.83 $^{a-d}$	**19.57** ^{ab}
		-$MgSO_4$	3.14 $^{d-f}$	44.24 $^{b-h}$	48.76 $^{e-g}$	54.61^{e-g}	12.98 ^{f}
ATCC11325	Cotyledon	MS	3.09 $^{d-g}$	40.18 $^{e-g}$	39.48 $^{g-l}$	38.66 $^{k-n}$	7.77 $^{h-l}$
		-KNO_3	**4.45** ^{a}	48.59 $^{b-e}$	65.29 ^{b}	64.27 $^{b-d}$	9.79 ^{hi}
		-NH_4NO_3	2.77 $^{e-i}$	54.14 $^{a-c}$	56.41 $^{c-e}$	58.86 $^{d-f}$	7.87 $^{h-l}$
		-KH_2PO_4	3.2 $^{d-f}$	50.48 $^{a-e}$	59.57 $^{b-d}$	60.19 $^{c-e}$	8.02 $^{h-l}$
		-$CaCl_2$	3.24 ^{de}	33.94 $^{h-l}$	37.74 $^{h-l}$	42.51 $^{i-m}$	10.20 $^{g-h}$
		-$MgSO_4$	3.24 ^{de}	39.88 $^{e-g}$	36.52 $^{h-l}$	38.76 $^{k-n}$	6.26 $^{k-o}$
	Leaf	MS	3.24 $^{d-h}$	41.96 $^{c-i}$	43.86 $^{f-i}$	49.13 $^{g-j}$	9.59 ^{hi}
		-KNO_3	4.09 ^{b}	55.14 ^{b}	**67.84** ^{ab}	68.96 $^{a-c}$	6.96 $^{i-o}$
		-NH_4NO_3	2.77 $^{e-i}$	27.80 $^{i-l}$	32.95 $^{k-l}$	34.58 $^{l-n}$	9.49 ^{hi}
		-KH_2PO_4	2.93 $^{d-h}$	41.76 $^{d-i}$	40.60 $^{g-l}$	41.62 $^{i-n}$	5.25 $^{l-o}$
		-$CaCl_2$	2.85 $^{d-i}$	47.54 $^{b-f}$	48.76 $^{e-g}$	52.33 $^{e-h}$	7.57 $^{h-n}$
		-$MgSO_4$	2.93 $^{d-h}$	38.20 $^{e-k}$	42.95 $^{f-j}$	46.41 $^{g-k}$	9.29 $^{h-j}$
ATCC15834	Cotyledon	MS	2.93 ^{def}	33.74 $^{h-l}$	42.44 $^{g-k}$	42 .13 $^{i-n}$	8.58 $^{h-k}$
		-KNO_3	4.02 ^{b}	55.53 ^{b}	64.27 ^{bc}	65.41 $^{b-d}$	10.20 ^{gh}
		-NH_4NO_3	2.62 ^{f}	28.59 $^{j-l}$	33.05 ^{kl}	37.03 $^{k-n}$	8.38 $^{h-k}$
		-KH_2PO_4	4.04 ^{b}	54.73 ^{b}	65.63 ^{b}	65.70 $^{b-d}$	7.87 $^{h-l}$
		-$CaCl_2$	3.09 $^{d-f}$	56.21 ^{b}	62.13 ^{abc}	63.66 $^{b-d}$	4.34 $^{\circ}$
		-$MgSO_4$	0.00 ^{j}	0.00 ^{m}	0.00 ^{m}	0.00 $^{\circ}$	0.00 ^{p}
	Leaf	MS	2.85 $^{d-i}$	39.19 $^{e-k}$	44.27 $^{f-i}$	41.62 $^{i-n}$	7.27 $^{h-o}$
		-KNO_3	4.02 ^{b}	**58.69** ^{a}	64.65 ^{b}	63.72 ^{c}	7.67 $^{h-m}$
		-NH_4NO_3	2.85 $^{d-i}$	32.55 $^{h-l}$	36.01 $^{h-l}$	33.66 ^{mn}	6.46 $^{j-o}$
		-KH_2PO_4	4.27 ^{b}	53.84 $^{b-d}$	**70.18** ^{a}	**73.55** ^{a}	6.06 $^{k-o}$
		-$CaCl_2$	3.42 ^{cd}	49.49 $^{b-e}$	51.92 $^{d-f}$	50.60 $^{f-i}$	7.93 $^{h-l}$
		-$MgSO_4$	0.00 ^{j}	0.00 ^{m}	0.00 ^{m}	0.00 $^{\circ}$	0.00 ^{p}
Control	Cotyledon	MS	2.92 $^{d-h}$	33.07 $^{h-l}$	33.71 $^{j-l}$	40.38 $^{j-n}$	4.34 $^{\circ}$
		-KNO_3	2.43 ^{hi}	30.82 $^{i-l}$	33.66 $^{j-l}$	43.21 $^{h-m}$	5.28 $^{l-o}$
		-NH_4NO_3	2.47 ^{h}	30.15 $^{i-l}$	32.88 ^{kl}	43.90 $^{h-l}$	4.68 ^{no}
		-KH_2PO_4	2.80 $^{e-i}$	34.08 $^{h-l}$	33.46 $^{j-l}$	39.29 $^{j-n}$	4.82 $^{m-o}$
		-$CaCl_2$	2.29 ^{i}	25.29 ^{l}	36.87 $^{h-l}$	42.14 $^{i-n}$	4.80 $^{m-o}$
		-$MgSO_4$	2.44 ^{hi}	40.10 $^{b-g}$	38.41 $^{h-l}$	37.58 $^{k-n}$	4.41°
	Leaf	MS	2.75 $^{e-i}$	34.57 ^{g}	41.29 $^{g-l}$	42.94 $^{h-m}$	7.36 $^{h-n}$
		-KNO_3	2.37 ^{hi}	30.41 $^{i-l}$	43.69 $^{f-i}$	51.18 $^{f-i}$	5.95 $^{k-o}$
		-NH_4NO_3	2.30 ^{i}	36.56 $^{f-l}$	38.63 $^{h-l}$	43.95 $^{h-l}$	5.18 $^{l-o}$
		-KH_2PO_4	2.49 ^{hi}	38.05 $^{e-l}$	36.88 $^{h-l}$	44.95 $^{h-k}$	6.07 $^{k-o}$
		-$CaCl_2$	2.46 ^{hi}	34.56 $^{g-l}$	35.11 $^{h-l}$	46.74 $^{g-k}$	5.4 $^{l-o}$
		-$MgSO_4$	2.52 $^{g-i}$	36.35 $^{f-l}$	35.50 $^{h-l}$	42.48 $^{j-m}$	6.19 $^{k-o}$

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

quantified as mg g^{-1} DW.

2.7. Statistical analysis

All experiments were performed based on completely randomized design (CRD). The experiment for hairy root induction were laid with four replications and eight explants cultured in each replication. Observations of hairy roots induction percentage, number and length of roots and root branching were recorded. The experiment for best hairy roots line selection and culture in liquid medium were performed with five replications and also secondary metabolites were analyzed in three replications. The data 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.

3. Results and discussion

3.1. Effect of different co-culture media, A. rhizogenes strains, and type of explants

A. rhizogenes strains, namely A_4, ATCC11325, and ATCC15834 were investigated to determine their capability in terms of root transformation and induction of hairy roots. The highest percentage of hairy roots induction (83.33%) was obtained with A_4 strain and cotyledon explants co-cultivated in Medium 2 followed by those cultured in Medium 5 and Medium 1, respectively, whereas Medium 3 produced the lowest percentage of hairy root induction when used with any of the strains and explants (Fig. 1). The ATCC11325 strain showed the maximum percentage of hairy roots induction in Medium 4 (lacking KH_2PO_4). ATCC15834 strain and medium 4 (lacking KH_2PO_4) were able to highest hairy roots induction in both explants in comparison with another medium. The corresponding percentage was low in other media and even zero (0%) in Medium 6.

Cotyledon and leaf explants obtained from 25-day old *C. intybus*

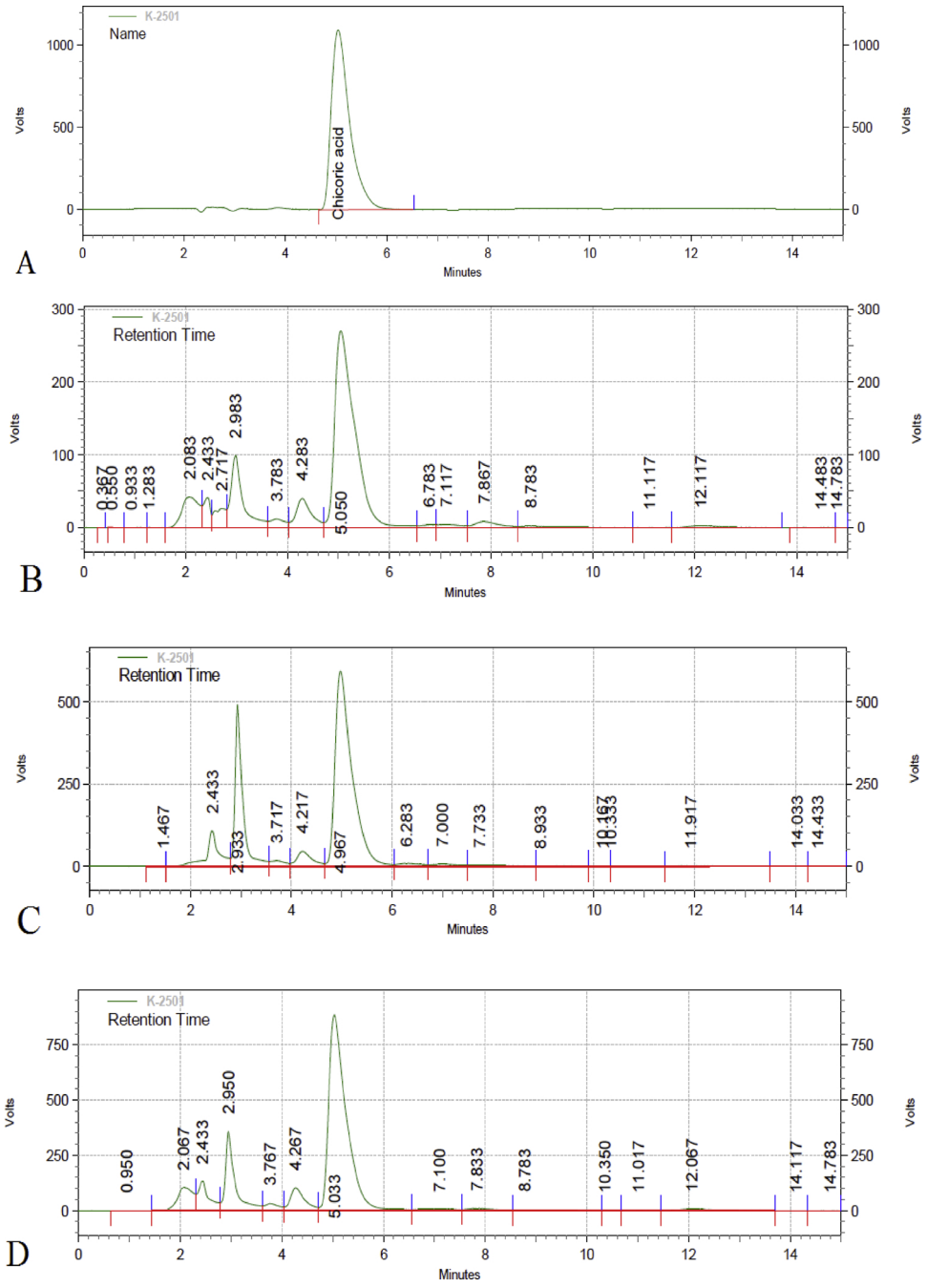

Fig. 4. HPLC chromatograms. Standard chicoric acid (A), chicoric acid in extract of non-transformed root (control) (B), hairy roots resulted from A_4 strain (C) and hairy roots resulted from A_4 in MS medium without KNO3 macronutrient (D).

seedlings were infected by *A. rhizogenes*. Effects of three different strains, two different explants, and six different co-culture MS media on transformation and rate of hairy root induction in chicory were investigated. Accordingly, different percentages of hairy root induction were obtained from different strains and explants. A_4 strain and Medium 2 showed the best result in hairy root induction percentage. Average number, length, and branching of the hairy roots were seen to be influenced by types of strain, explant, and the used compound as co-culture media. Accordingly, the highest roots number (8.12) was observed in cotyledon explants inoculated with ATCC15834 strain and cultured on Medium 4, that is almost 2.7 times more than MS medium while the highest root length and root branching induced from cotyledon explants inoculated with A_4 strain and placed on the medium 2 (Table 2).

Conditions of co-culture medium are very important in the transformation process because T-DNA region transfer from bacteria to the plant occurs in this process so that any change in co-culture medium can affect both quantity and quality of hair root induction. Kabirnotaj et al. (2013) indicated that A_4, A_{13} and ATCC15834 strains of *A. rhizogenes* able to hairy roots induction in *C. intybus* and among MS, Linsmaier and Skoog (1965) (LS) and Gamborg et al. (1968) (B5) media, MS medium was considered as the best culture medium for developing and establishment of hairy root cultures but there are reports confirming effectiveness of removing particular macronutrients, such as NH_4NO_3 (Hoshi et al., 2004), KH_2PO_4 (Valimehr et al., 2014), $CaCl_2$ (Flego et al., 1997; Montoro et al., 2000), and mineral components (Dupre et al., 2000) from co-culture medium for increasing the efficiency of the transformation. In this study, it is suggested that some

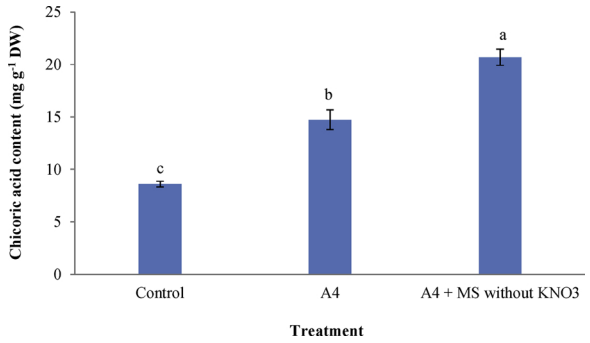

Fig. 5. The comparisons of chicoric acid content in non-transformed (control), transformed hairy roots by A_4 and transformed hairy roots by A_4 in MS medium without KNO_3 macronutrient. Vertical lines represent standard deviation.

macronutrients including KNO_3 and KH_2PO_4 have negative impacts on transformation with *Agrobacterium* depending on the used strain so that their removal increases hairy roots induction percentage by about 4.16, 1.5 and 1.37 folds over those with basal MS medium when ATCC15834, A_4, and ATCC11325 are used as strain, respectively. Removal of KH_2PO_4 from the co-culture medium significantly increased hairy roots induction percentage by ATCC strains (Fig. 2). Removal of PO_4 enhanced both strain performance in binding to the host (Danhorn et al., 2004) and *vir* genes expression (Winans, 1990).

The intra-cellular concentration of *vir*G protein is known to be a rate-limiting factor for *vir* gene induction, and increased expression of *vir*G by phosphate starvation or heat shock inducers can greatly stimulate the expression of the *vir* genes. Sharafi et al. (2013) showed that the efficiency of transformation with *A. rhizogenes* increases by removing macronutrients in co-culture media. They reported that NH_4NO_3, KNO_3, and $CaCl_2$ impose inhibitory effects on the transformation of *Dracocephalum kotschyi*. In hairy roots induction by A_4 strain using Medium 2 (without KNO_3), average root length and root branching enhanced obviously in comparison with those of Medium 1 (MS medium) while root number was highest in medium 1 comparison with the medium 2 in leaf explants.

3.2. Molecular analysis

PCR reaction with primers for *rol*B gene was used to investigate integrated T-DNA into isolated DNA from hairy roots of *C. intybus* L. PCR analysis confirmed the transfer of T-DNA region of Ri plasmid of bacteria into hairy roots genome, while DNA isolated from non-transformed adventitious roots (negative control) did not show any amplification (Fig. 3D).

3.3. Establishment of hairy root liquid cultures

The largest biomass production (1.98 and 1.57 g per flask for fresh weight) was exhibited by transgenic hairy roots resulted from cotyledon and leaf explants infected by A_4 and cultured on Medium 2 (lacking KNO_3); also highest dry weight (0.185) and growth index (45.41) obtained from cotyledon explants infected by A_4 and Medium 2. Fresh and dry weight of the hairy roots were 1.58 and 1.74 times, respectively as large as those resulting from Medium 1 (full-strength MS medium). The lowest fresh weight of hairy roots (0.22 g per flask) was achieved with the leaf explant co-cultured with A_4 and cultured on Medium 3 (lacking NH_4NO_3). In ATCC11325- and ATCC15834-derived hairy roots cultured on Medium 4 (lacking KH_2PO_4), growth and fresh weight were maximal in both of the explants (Table 3). In this research, the obtained hairy root lines showed significant differences in growth rate and biomass production, depending on strain's potential for transformation, removal of macronutrients with inhibitory effects on T-DNA integration, and type of the explant used. Hairy roots growth on liquid medium was shown in Fig. 3A B C.

3.4. Determination of secondary metabolites content

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. Phenolic content was higher (4.46 and 4.45 mg g^{-1} DW) in the hairy roots obtained from the cotyledon explant infected by A_4 and ATCC11325 strains and co-cultured on Medium 2 (without KNO_3), i.e. (Table 4).

Transfer of an appropriate combination of *rol* genes from bacteria to the plant is necessary for hairy roots induction; on the other hand, transfer of only one of these genes cannot bring about all features of hairy root syndrome (Park et al., 2007). 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 acid. 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 (Winans, 1992). Many secondary metabolites are derivatives of the phenolic compounds and may also be increased by enhancing of phenol production. Results showed significant differences ($P \leq 0.01$) between the phenolic content of dried ethanol extract in hairy roots resulted from different treatments.

The results of flavonoid measurement showed significant differences between flavonoid content in hairy root resulted from macronutrients removing from the medium in comparison with medium 1 and control. Flavonoid content in 270 nm wavelengths was higher (59.19 µg g^{-1} DW) in the hairy roots obtained from the cotyledon explant infected by A_4 strain and co-cultured on Medium 2 (without KNO_3) and had no statistically significant different with some treatment. In 300 and 330 nm wavelengths, flavonoid content was higher (70.18 and 73.55 µg g^{-1} DW, respectively) in the hairy roots obtained from the leaf explant infected by ATCC15834 strain and co-cultured on Medium 4 (without KH_2PO_4), i.e. 1.41 and 1.58 and 1.76 times larger than that cultured on Medium 1, respectively and had no statistically significant different with some treatment

Anthocyanin content in 550 nm wavelengths was higher (21.88 µg g^{-1} DW) in the hairy roots obtained from the cotyledon explant infected by A_4 strain and co-cultivated on Medium 5 (without $CaCL_2$), i.e. 1.73 times larger than that cultured on Medium 1. The result of this research shows that the hairy roots obtained from A_4 strain have significantly high anthocyanin content (Table 4). The results of chicoric acid measurement showed significant differences between chicoric acid content in hairy root resulted from macronutrients removing from the medium in comparison with hairy roots and adventitious roots (control). Chicoric acid content was higher (20.7 mg g^{-1} DW) in the hairy roots obtained from the cotyledon explant infected by A_4 strain and co-cultivated on Medium 2 (without KNO_3) (Figs. 4 and 5). Azarmehr et al. (2013) treated the hairy root lines with 0, 1, 5 and 10 mM Zn, as $ZnSo_4.7H_2O$, at three time course levels including 24, 48 and 72 h. Results showed increased chicoric acid in response to higher concentrations of Zinc at higher time courses. Also, an increase in chicoric acid release into the culture media was observed that is important for industrial uses.

3.5. Regeneration of transgenic plants

Regenerated plants obtained from hairy roots are called transgenic plants (Mcknight et al., 1987). Those can be formed from hairy roots

Table 5
Comparison of the amount of flavonoid and anthocyanin in natural plant, regenerated plant from hairy roots and hairy roots.

metabolites	Total phenolic content (mg/g DW)	Flavonoid 270 nm (µg/g DW)	Flavonoid 300 nm (µg/g DW)	Flavonoid 330 nm (µg/g DW)	Anthocyanin (µg/g DW)
Natural plant	2.69 ^{b}	31.28 ^{b}	37.07 ^{b}	37.30 ^{b}	12.42 ^{b}
Regenerated plant from hairy roots	3.88 ^{a}	40.60 ^{a}	45.52 ^{ab}	47.09 ^{ab}	13.85 ^{a}
Hairy roots	4.37 ^{a}	44.99 ^{a}	50.21 ^{a}	55.26 ^{a}	14.46 ^{a}

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

either directly or indirectly after callus induction (He-Ping et al., 2011). According to literature, successful direct regeneration of plants has been reported for *Armoracia lapathifolia* hairy root segments (Noda et al., 1987), *Centaurium erythraea* (Subotic et al., 2003), *Coffea canephora* P ex. Fr. (Kumar et al., 2006), and *Plumbago indica* hairy roots (Gangopadhyay et al., 2010). Also, shoot formation from the callus obtained from hairy roots has been reported in *Alhagi pseudoalhagi* (Wang et al., 2001) and *Malus baccata* (Wu et al., 2012). Results of this research show that direct shoot regeneration from hairy roots was spontaneous on MS supplemented with 500 mg l^{-1} cefotaxime, without using any plant growth regulators in three weeks of culturing (Fig. 3 E). The results of metabolites measurement showed the amount of metabolites in hairy roots was highest and had no significant different with regenerated plant from hairy roots. (Table 5).

4. Conclusion

Hairy roots culture was developed as the innovative path for bulky production of secondary metabolites and phytochemicals which find relevance in the pharmaceutical, food and flavor industries. This technique is of massive significance to develop a large number of roots and secondary metabolites in a short time to a continuous supply of improved value products. This study suggested, for the first time, that particular combinations of macronutrients in MS medium cause significant reductions in transformation efficiency of chicory hairy roots with *A. rhizogenes*, so that removing the macronutrients from MS medium can enhance the transformation efficiency considerably while adding to biomass production, and secondary metabolites content of the resultant hairy roots. High percentages of hairy roots induction were achieved by A_4 (83.33%), ATCC11325 (61.1%) and ATCC15834 (55.55%) strains when KNO_3 and KH_2PO_4 were removed from the co-culture medium, respectively. The results of Establishment of hairy root liquid cultures showed that the hairy roots obtained from cotyledon explants that were infected by A_4 strain and cultured on Medium 2 (lacking KNO_3); had maximum fresh and dry weight and growth index of the hairy roots. Also, high phenolic, flavonoid and anthocyanin compound production was achieved in hairy roots in comparison with the control. Further research is needed to find practical approaches to improve chicory transformation efficiency.

References

Azadi, P., Chin, D.P., Kuroda, K., Khan, R.S., Mii, M., 2010. Macro elements in inoculation and co-culture medium strongly affect the efficiency of *Agrobacterium*-mediated transformation in Lilium. J. Plant Cell Tissue Organ Cult. 101 (2), 201–209.

Azarmehr, B., Karimi, F., Taghizadeh, M., Mousavi Gargari, S.L., 2013. Secondary metabolite contents and antioxidant enzyme activities of *Cichorium intybus* hairy roots in response to zinc. J. Med. Plants By-Prod. 2, 131–138.

Baert, J.R.A., Van Bockstaele, E.J., 1992. Cultivation and breeding of root chicory for inulin production. Ind. Crops Prod. 1, 229–234.

Bais, H.P., Ravishankar, G., 2001. *Cichorium intybus* L. cultivation, processing, utility, value addition and biotechnology, with an emphasis on current status and future prospects. J. Sci. Food Agric. 81, 467–484.

Bertani, G., 1952. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. 62, 293–300.

Cai, D., Kleine, M., Kifle, S., Horloff, H.J., Sandal, N.N., Marcker, K.A., Lankhorst, R.M.K., Salentijn, E.M.J., Lange, W., Stiekema, V., 1997. Positional cloning of a gene for nematode resistance in sugar beet. Science 275, 832–834.

Chilton, M.D., Tepfer, D.A., Petit, A., David, C., Casse-Delbart, F., Tempe, J., 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. Nature 295, 432–434.

Danhorn, T., Hentzer, M., Givskov, M., Parsek, M.R., Fuqua, C., 2004. Phosphorus limitation enhances biofilm formation of the plant pathogen *Agrobacterium tumefaciens* through the PhoR–PhoB regulatory system. J. Bacteriol. 186, 4492–4501.

Dupre, P., Lacoux, Y., Neutelings, G., Mattar-Lavrain, D., Fliniaux, M.A., David, A., Jacquin-Dubreuil, A., 2000. Genetic transformation of *Ginkgo biloba* by Agrobacterium tumefaciens. J. Physiol Plant. 108, 413–419.

Flego, D., Pirhonen, M., Saarilahti, H., Palva, T.K., Palva, E.T., 1997. Control of virulence gene expression by plant calcium in the phytopathogen Erwinia carotovora. J. Mol Microbiol. 25, 831–838.

Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. J. Exp. Cell Res 50 (2), 151–158.

Gangopadhyay, M., Chakraborty, D., Bhattacharyya, S., Bhattacharya, S., 2010.

Regeneration of transformed plants from hairy roots of *Plumbago indica*. J. Plant Cell Tissue Organ Cult. 102, 109–114.
Georgiev, M.I., Agostini, E., Ludwig-Müller, J., Xu, J., 2012. Genetically transformed roots: from plant disease to biotechnological resource. J. Trends Biotechnol. 30, 528–537.
Hara, M., Oki, K., Hoshino, K., Kuboi, T., 2003. Enhancement of anthocyanin biosynthesis by sugar in radish (*Raphanus sativus*) hypocotyl. J. Plant Sci. 164, 259–265.
Heimler, D., Isolani, L., Vignolini, P., Romani, A., 2009. Polyphenol content and anti-radical activity of *Cichorium intybus* L. from biodynamic and conventional farming Food. J Chemistry 114, 765–770.
He-Ping, S., Yong-Yue, L., Tie-Shan, S., Eric, T.P.K., 2011. Induction of hairy roots and plant regeneration from the medicinal plant Pogostemon cablin. J. Plant Cell Tissue Organ Cult. 107, 251–260.
Hoshi, Y., Kondo, M., Mori, S., Adachi, Y., Nakano, M., Kobayashi, H., 2004. Production of transgenic lily plants by *Agrobacterium*-mediated transformation. J. Plant Cell Rep. 22, 359–364.
Hu, Z.B., Du, M., 2006. Hairy root and its application in plant genetic engineering. J. Integr. Plant Biol. 48 (2), 121–127.
Innocenti, M., Gallori, S., Giaccherini, C., Ieri, F., Vincieri, F.F., Mulinacci, N., 2005. Evaluation of the phenolic content in the aerial parts of different varieties of *Cichorium intybus* L. J. Agric. Food Chem. 53, 6497–6502.
Jaiswal, R., Kiprotich, J., Kuhnert, N., 2011. Determination of the hydroxycinnamate profile of 12 members of the Asteraceae family. J. Phytochem. 72, 781–790.
Kabirnotaj, S., Zolalla, J., Nematzadeh, G., SHokri, E., 2013. Optimization of hairy root culture establishment in Chicory plants (*Cichorium intybus*) through inoculation by *Agrobacterium rhizogenes*. J. Agric Biotechnol. 4 (2), 61–75.
Kim, Y., Wyslouzil, B.E., Weathers, P.J., 2002. Secondary metabolism of hairy root cultures in bioreactors. J. In Vitro Cell Dev. Plant 38, 1–10.
Kisiel, W., Zielinska, K., 2001. Guaianolides from *Cichorium intybus* and structure revision of Cichorium sesquiterpene lactones. J. Phytochem. 57, 523–527.
Krizek, D.T., Mirecki, R.M., Britz, S.J., 1998. Inhibitory effects of ambient levels of solar UV-A and UV-B radiation on growth of cv. New Red Fire lettuce. J. Physiol. Plant. 103, 1–7.
Kumar, V., Satyanarayana, K.V., Sarala Itty, S., Indu, E.P., Giridhar, P., Chandrashekar, G.A., Ravishankar, G.A., 2006. Stable transformation and direct regeneration in *Coffea canephora* P ex. Fr. by *Agrobacterium rhizogenes* mediated transformation without hairy root phenotype. J. Plant Cell Rep. 25, 214–222.
Lin, Z., Neamati, N., Zhao, H., Kiryu, Y., Turpin, J.A., Abraham, C., Strebel, K., Kohn, K., Wityrouw, M., Pannecouque, C., Debyser, Z., De Clercq, E., Rice, W.G., Pommier, Y., Burke, R.T., 1999. Cichoric acid analogs as HIV-1 integrase inhibitors. J. Med. Chem. 42, 1401–1414.
Linsmaier, E.M., Skoog, F., 1965. Organic growth factor requirements of tobacco tissue cultures. J. Physiol. Plant. 18, 100–127.
Malarz, J., Stojakowska, A., Kisiel, W., 2002. Sesquiterpene lactones in a hairy root culture of Cichorium intybus. J. Z. Naturforsch. C. 57, 994–997.
Matoba, N., Husk, A.S., Barnett, B.W., Pickel, M.M., Arntzen, C.J., Montefiori, D.C., 2010. HIV-1 neutralization profile and plant-based recombinant expression of actinohivin, an Env glycan-specific lectin devoid of T-cell mitogenic activity. PLOS ONE 5 (6), e1114.
Matvieieva, N., Shakhovsky, A., Kvasko, O., Kuchuk, N., 2015. High-frequency genetic transformation of Cichorium intybus L. using the nptII gene as a selective marker. J. Tsitol Genet. 49 (4), 11–16.
Mcknight, T.D., Lillis, M.T., Simpson, R.B., 1987. Segregation of genes transferred to one plant cell from two separated *Agrobacterium* strains. J. Plant Mol Biol. 8, 439–445.
Mohebodini, M., Jalali-Javaran, M., Alizadeh, H., Mahboudi, F., Yarbakht, M., 2015. Agrobacterium-mediated transformation of lettuce (*Lactuca sativa* L.) to express IgG-binding protein A and human pro-insulin as a fusion protein. J. Hortic Sci Biotechnol. 89 (6), 719–725.
Montoro, P., Teinseree, N., Rattana, W., Kongsawadworakul, P., Michaux- Ferriere, N., 2000. Effect of exogenous calcium on *Agrobacterium tumefaciens*-mediated gene transfer in *Hevea brasiliensis* (rubber tree) friable calli. J. Plant Cell Rep. 19, 851–855.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. J. Physiol. Plant. 15, 473–497.
Nandagopal, S., RavjithaKumari, B.D., 2007. The effectiveness of auxin-induced *in vitro* root culture in chicory. J. Central Eur. Agric. 20, 173–180.
Nilsson, O., Olsson, O., 1997. Getting to the root: the *role* of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. J. Physiol. Plantarum. 100 (3), 463–473.
Noda, T., Tanaka, N., Mano, Y., Nabeshima, S., Ohkawa, H., Matsui, C., 1987. Regeneration of horse radish hairy roots incited by *Agrobacterium rhizogenes* infection. J. Plant Cell Rep. 6, 283–286.
Palmer, A.G., Gao, R., Maresh, J., Erbil, W.K., Lynn, D.G., 2004. Chemical biology of multi-host/pathogen interactions: chemical perception and metabolic complementation. J. Annu. Rev. Phytopathol. 42, 439–464.
Park, K., de Oliveira, R., Brod, F., 2007. Drying operational parameters influence on chicory roots drying and inulin extraction. J. Food Bioprod. Process. 85, 184–192.
Rees, S.B., Harborne, J.B., 1985. The role of sesquiterpene lactones and phenolics in the chemical defense of the chicory plant. J. Phytochem. 24, 2225–2231.
Ronald, S.F., Laima, S.K., 2001. Phenolics and cold tolerance of Brassica napus. J. Plant Agric. 1, 1–5.
Rosales-mendoza, S., Soria-Guerra, R.E., Olivera-Flores, M.J., Lopez-Revilia, R., Arguello-Astorga, G., Jimenezbremont, J.F., Garciadela Cruz, R.F., Loyolarodriguez, J.P., Alpuche-Solis, A.G., 2007. Expression of *Escherichia coli* heat-labile enterotoxin b subunit (LTB) in carrot (*Daucus carota* L.). J. Plant Cell Rep 26, 969–976.
Sandhu, J.S., Krasnyanski, S.F., Domier, L., Korban, S.S., Osadjan, M.D., Buetow, D.E., 2000. Oral immunization of mice with transgenic tomato fruit expressing respiratory syncytial virus- F protein induces a systemic immune response. J. Transgenic Res. 9, 127–135.
Sharafi, A., Hashemi Sohi, H., Azadi, P., Sharafi, A.A., 2013. Hairy root induction and plant regeneration of medicinal plant Dracocephalum kotschyi. J. Physiol. Mol. Biol. Plants. 26, 1–6.
Subotic, A., Budimir, S., Grubisic, D., Momcilovic, I., 2003. Direct regeneration of shoots from hairy root cultures of *Centaurium erythraea* inoculated with *Agrobacterium rhizogenes*. J. Biologia Plantarum. 47 (4), 617–619.
Suresh, B., sherkhane, P.D., Kale, S., Ravishankar, G.A., 2005. Uptake and degradation of DDT by hairy root cultures of *Cichorium intybus* and *Brassica juncea*. J. Chemosphere 61, 1288–1292.
Valimehr, S., Sanjarian, F., Sohi, H.H., Sabouni, F., Sharafi, A., 2014. A reliable and efficient protocol for inducing genetically transformed roots in medicinal plant Nepeta pogonosperma. J. Physiol. Mol Biol Plants. 20, 351–356.
Velayutham, P., Ranjithakumari, B.D., Baskaran, P., 2006. An efficient *in vitro* plant regeneration system for *Cichorium intybus* L.–an important medicinal plant. J. Agric. Technol. 2 (2), 287–298.
Wang, Y.M., Wang, J.B., Luo, D., Jia, J.F., 2001. Regeneration of plants from callus cultures of roots induced by *Agrobacterium rhizogenes* on *Alhagi pseudoalhagi*. J. Cell Res. 11 (4), 279–284.
Whaley, K.J., Hiatt, A., Zeitlin, L., 2011. Emerging antibody products and *Nicotiana* manufacturing. Hum. Vaccin 7, 349–356.
Winans, S.C., 1990. Transcriptional induction of an Agrobacterium regulatory gene at tandem promoters by plant-released phenolic compounds, phosphate starvation, and acidic growth media. J. Bacteriol. 172, 2433–2438.
Winans, S.C., 1992. Two-way chemical signaling in Agrobacterium-plant interactions. Microbiol. Mol. Biol. Rev. 56, 12–31.
Wu, J., Wang, Y., Zhang, L.X., Zhang, X.Z., Kong, J., Lu, J., Han, Z.H., 2012. High-efficiency regeneration of *Agrobacterium rhizogenes*-induced hairy root in apple rootstock *Malus baccata* (L.) Borkh. J. Plant Cell Tissue Organ. Cult. 111, 183–189.