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RESEARCH ARTICLE

Assessment of the efficiency of hairy roots induction using soybean, sugar beet and tobacco explants

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ABSTRACT: *Agrobacterium*-mediated gene transfer method is one of the used methods for genetic transformation in the plant regeneration program. Transformation efficiency can be optimized depending on the strain of bacteria, the genotype of plant and conditions of growth. In this study, the *gfp* gene was transferred into sugar beet, tobacco, and soybean by *Agrobacterium rhizogenes* strain AR15834. The effects of bacterial concentrations, antibiotic concentrations and the types of explants and genotypes on the gene transfer efficiency and transgenic hairy roots production were investigated. The explants were inoculated with the bacteria at the adjusted concentrations and two days after the transformation, the explants were transferred to a solid MS medium containing different concentrations of kanamycin antibiotic. According to the results and the examined factors, the optimal conditions to achieving of the maximum production of transgenic hairy roots included bacterial concentration with OD_{600} = 0.2, cotyledon explant, 50 mg/L kanamycin concentration and Djakel genotype for soybean; bacterial concentration with OD_{600} = 0.2, leaf with petiole explant and SBSI004 genotype for sugar beet, and bacterial concentration with OD600nm = 0.8 and 100 mg/L kanamycin concentration for tobacco.

KEYWORDS: *gfp*, transgenic hairy roots, soybean, sugar beet, tobacco

INTRODUCTION

A. rhizogenes-mediated gene transformation is a method for the production of transgenic hairy roots, and optimizing gene transfer conditions contribute to large scale production. The hairy roots have a high potency as a system for the production of plant metabolites. Some valuable drugs [1], pigments such as polyketides [2] and anticancer drugs such as naphthoquinones [3] have been produced in hairy roots. In addition, the transgenic hairy roots can regenerate the entire plant and maintain its genetic stability throughout the later subcultures [4]. Today, the most important issue is the use of transgenic hairy roots for the production of drugs and environmental uses [5-7].

It has been reported in most of the previous studies that gene transfer by *Agrobacterium* is influenced by factors such as plant genotype, bacterial concentration and inoculation, temperature and plant age [6, 8, 9]. Hairy roots production from different soybean genotypes has been reported [10-12]. Cao et al. [13] showed that *in vitro* production of transgenic hairy roots is faster in younger soybean seedlings, there is no significant difference between bacterial concentrations and the best temperature for hairy roots production is 25-28 °C. Compared to some dicotyledonous plants, the hairy roots of sugar beet are a suitable substrate for biological plant research, even if the hairy roots do not regenerate. These hairy roots are used to produce secondary metabolites to study the interaction of plant cells with root pathogens in *in vitro* conditions [14, 15]. On the other hand, Tobacco plant is a good model for Biotechnology and Plant Physiology due to rapid growth, high compatibility, and generous seed production and cost-effective. Therefore, this plant is used abundantly in genetic transformation and gene expression studies.

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Due to the influence of various factors on the efficiency of gene transfer, the optimum gene transfer conditions in sugar beet, tobacco, and soybean for determine the most effective concentration of *A. rhizogenes*, the best genotype, the best explant and the most effective concentration of antibiotic for gene transfer have been studied in this study.

MATERIALS AND METHODS

Plant materials

To the production of hairy roots, six sugar beet cultivars (SBSI004, SBSI002, Briggita, F-20537, Zarghan, and Jolgeh), two soybean cultivars (Djakel and TMS) and one tobacco cultivar (Turkish) were selected. The seeds were obtained from Seed and Plant Improvement Institute, Karaj, Iran. Surface sterilization of the seeds of sugar beet, soybean and tobacco were done with 70% ethanol for 1 minute and afterward with 5% sodium hypochlorite for 10 minutes, then washed several times with sterilized water. The seeds of sugar beet and soybean germinated in sterilized water containing 1% clotrimazole fungicide for a few days, transferred to the half-strength and complete MS (Murashige and Skoog) medium, and used for genetic transformation by A. rhizogenes after 2 months and 7 days, respectively. Tobacco seeds germinated on MS medium transferred to new MS medium and used for genetic transformation by A. rhizogenes after 1 month.

A. rhizogenes and gene construct

The pK7GWIWG2D (II) vector was transferred into the *A. rhizogenes* strain AR15834 by electroporation method [16]. The vector contains *gfp*, kanamycin resistance and chloramphenicol resistance genes under 35 S promoter and terminator inside T-DNA and streptomycin gene outside T-DNA.

Agrobacterium-mediated plant transformation

A. rhizogenes strain AR15834 was grown in LB medium supplemented with rifampicin (50 mg/L) and kanamycin (50 mg/L) antibiotics and was shaken at 120 rpm at 28 °C for 24 h. Then, the bacterial suspension was centrifuged at 3000 rpm at 4 °C for 20 minutes. The pellet of bacteria was dissolved in the liquid MS medium to obtain different concentrations (OD 600 nm) of 0.2, 0.4, 0.6, 0.8, 1, 1.2, and 1.4 for sugar beet transformation; of 0.4, 0.6, 0.8, and 1 for tobacco transformation and of 0.2, 0.4, 0.6, 0.8, 1, 1.2, and 1.4 for soybean transformation.

The explants of the leaf with petioles and pieces of 1.5 cm of stems from 2-month old sugar beet and leaf explants of one-month-old tobacco were scraped and transferred to the different concentrations of prepared bacterial suspensions and shaken for 5 minutes. Then, the inoculated explants were put on the solid MS medium and after the emergence of bacterial halo around the explants (about 2 to 3 days), washed with sterilized water. The sugar beet explants were transferred to the new MS medium containing meropenem (30 mg/L) and kanamycin (50 mg/L) antibiotics. The tobacco explants were transferred to the new MS medium containing meropenem (30 mg/L) and the different concentrations of kanamycin (0, 50, and 100 mg/L) antibiotics. All explants were placed in darkness at 25°C until the appearance of initial hairy roots.

To soybean transformation, the different concentrations of bacteria suspensions were injected into the subcutaneous axis via insulin syringe and the seedlings were placed in the glasses containing autoclaved sand. After 48 h, the different concentrations of kanamycin (0, 50, 100, 150, and 200 mg/L) and cefotaxime (400 mg/L) antibiotics were added into the glasses. The seedlings were grown under a 16/8 h light/dark photoperiod (1000 lux) at 25°C until the appearance of the hairy roots from the injection sites.

PCR analysis

The DNA extraction from the established hairy roots was carried out using the Dena Zist kit (S-1030-1) according to the manufacturer's instructions. To confirm the presence of *gfp* and *rolB* in hairy roots, PCR was performed using specific primers (Table 1). To confirm the absence of bacterial contamination of the hairy roots, bacterial *virG* specific primers were used. Finally, the PCR products were run on the 1% agarose gel.

Table 1. The sequences of specific primers used in PCR reactions.

Gene	Primer sequence	Tm (°C)
gfp	5'- AAGTGCTGAAGGAACAATC-3'	53.15
	5'-CAAGTGAATGAACAAGGAAC-3'	52.7
rolB	5'-AAGTGCTGAAGGAACAATC-3'	53.15
	5'-CAAGTGAATGAACAAGGAAC-3'	52.7
virG	5'- CCTTGGGCGTCGTCATAC -3'	57.9
	5'- TCGTCCTCGGTCGTTTCC -3'	59.04

RNA extraction and cDNA synthesis

After confirmation of putative transgenic hairy roots, the RNA extraction was done using the RNX-Plus kit of CinnaGen Co. (S-1020-1) according to the manufacturer's instructions. Afterward, DNA removed by DNase enzyme at 37 °C for 30 minute and the synthesis of cDNA was performed using cDNA synthesis kit of Thermo Fisher Scientific Co. (K1622). After the synthesis of cDNA, the PCR was performed using *gfp* specific primers and the PCR products were run on the 1% agarose gel for the confirmation of *gfp* expression.

Statistical analysis

Analysis of variance based on a completely randomized factorial design and comparison of mean traits with Duncan test was performed at P value ≤ 1%. Statistical analysis was performed using SAS 9.0 and Minitab 16 software and the charts were drawn by Excel software.

RESULTS

Formation of hairy roots

The inoculated explants produced hairy roots in the selective medium, after one week for the cultivars of soybean (Fig 1), after two weeks for the cultivars of sugar beet (Fig 2) and after twenty days for the tobacco (Fig 3).

Confirmation of established transgenic hairy roots

To confirm the established transgenic hairy roots, the extracted DNA was used as the template of PCR using specific primers of *rolB*. The presence of 194 bp fragment showed that the evaluated hairy roots were produced from the infection with *A. rhizogenes* (Fig 4a-c). DNA from non-transgenic hairy roots of each plant were used as negative control. Then, putative transgenic hairy roots were selected for further studies.

Confirmation of the absence of *Agrobacterium* contamination

The PCR for the extracted DNA from putative transgenic hairy roots carried out using specific primers of *virG*. The absence of 529 bp fragment confirmed the absence of contamination of the hairy roots with *A. rhizogenes* (Fig 5a-c).

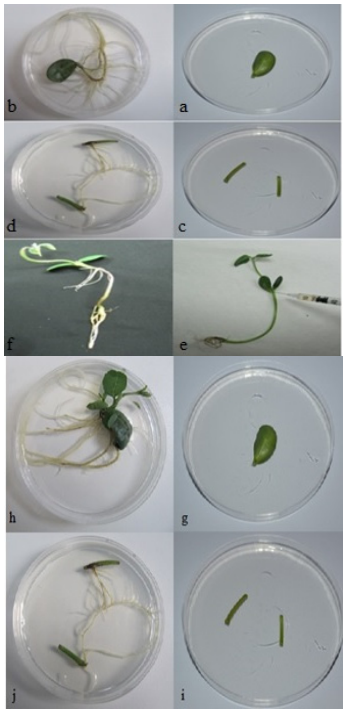

Fig 1. Formation of hairy roots after inoculation of the different explants of soybean with *A. rhizogenes* (a-b: cotyledon, c-d: stem and e-f: whole plant for TMS cultivar and g-h: cotyledon, i-j: stem for Djakel cultivar).

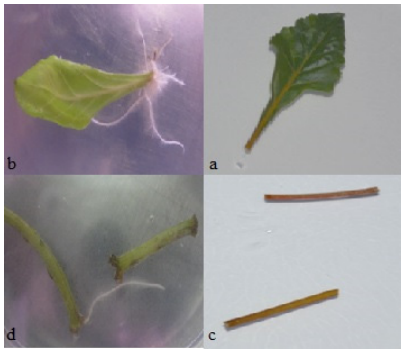

Fig 2. Steps for the formation of hairy roots after the inoculation of different sugar beet explants (a-b: leaves with petiole, c-d: stem) with *A. rhizogenes*.

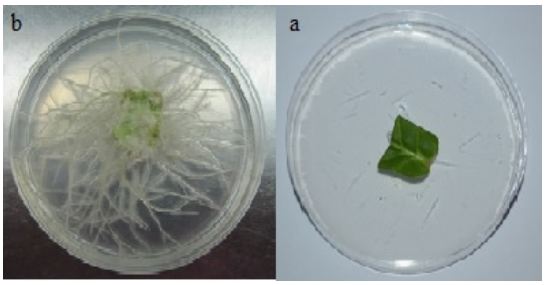

Fig 3. Steps for the formation of hairy roots after the inoculation of different tobacco explants (a-b: the leaves) with *A. rhizogenes*.

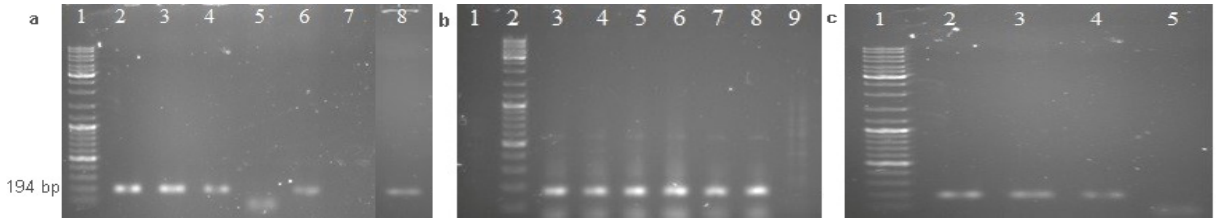

Fig 4. Confirmation of transgenic hairy roots using *rolB* specific primers in **a)** soybean; Lane 1: 100 bp plus DNA ladder, Lane 2-4: DNA from transgenic hairy roots of the whole plant, cotyledon and stem of TMS cultivar, respectively, Lane 5: DNA from non-transgenic root, Lane 6: *A. rhizogenes*, Lane 7: PCR negative control, Lane 8: DNA from transgenic hairy roots of Djakel cultivar. **b)** sugar beet; Lane 1: PCR negative control, Lane 2: 1 kb molecular marker, Lane 3-8: DNA from transgenic hairy roots of SBSI004, Briggita, F-20537, Zarghan, Jolgeh and SBSI002 cultivars, respectively. Lane 9: DNA from non-transgenic root. **c)** tobacco; Lane 1: 1 kb molecular marker, Lane 2-3: DNA from transgenic hairy roots. Lane 4: *A. rhizogenes,* Lane 5: DNA from non-transgenic root.

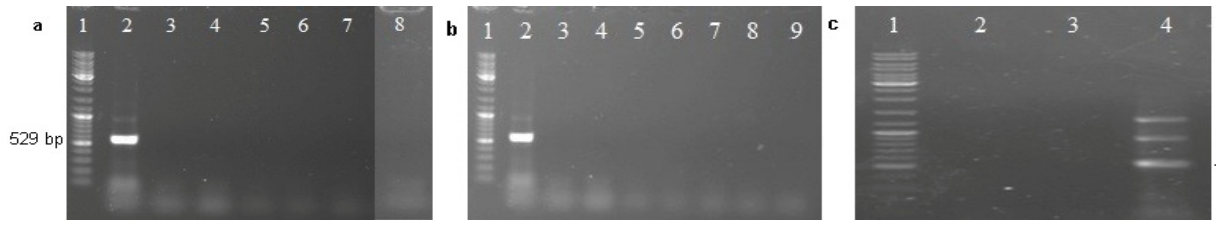

Fig 5. Confirmation of the absence of *Agrobacterium* contamination in **a)** soybean hairy roots; Lane 1: 100 bp plus DNA ladder, Lane 2: *A. rhizodgenes*, Lane 3-5: DNA from transgenic hairy roots of the whole plant, cotyledon, and stem of TMS cultivar, respectively, Lane 6: DNA from non-transgenic roots, Lane 7: PCR negative control, Lane 8: DNA from transgenic hairy roots of Djakel cultivar. **b)** sugar beet hairy roots; Lane 1: 1 kb molecular marker, Lane 2: *A. rhizogenes,* Lane 3-8: DNA from transgenic hairy roots of SBSI004, SBSI002, Zarghan, Jolgeh, F-20537 and Briggita cultivars, respectively, Lane 9: DNA from non-transgenic root. **c)** tobacco hairy roots; Lane 1: 1 kb molecular marker, Lane 2: DNA from transgenic hairy roots, Lane 3: DNA from non-transgenic root, Lane 4: *A. rhizogenes*.

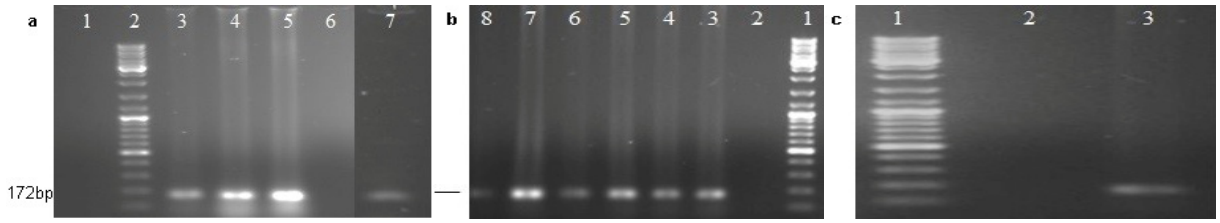

Fig 6. Confirmation of *gfp* gene insertion in transgenic hairy roots of **a)** soybean; Lane 1: PCR negative control, Lane 2: 100 bp plus DNA ladder, Lane 3-5: DNA from transgenic hairy roots of cotyledon, stem and whole plant of TMS cultivar, respectively, Lane 6: DNA from untransformed soybean, Lane 7: DNA from transgenic hairy roots of Djakel cultivar. **b)** sugar beet; Lane 1: 1 kb molecular marker, Lane 2: DNA from non-transgenic roots, Lane 3-8: DNA from transgenic hairy roots of SBSI004, Briggita, F-20537, SBSI002, Zarghan and Jolgeh cultivars, respectively. **c)** tobacco; Lane 1: 1 kb molecular marker, Lane 2: DNA from non-transgenic root. Lane 3: DNA from transgenic hairy roots.

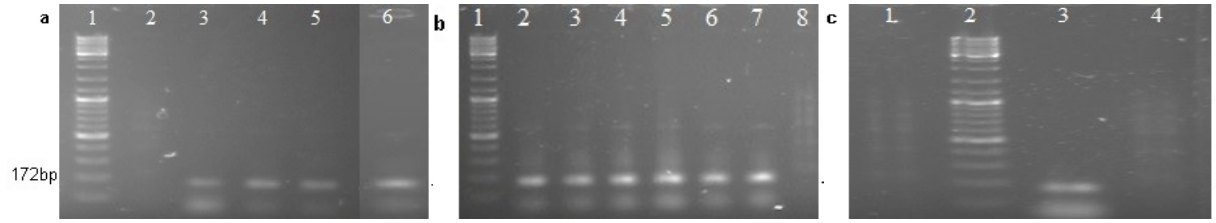

Fig 7. *gfp* expression analysis by semi-qPCR in **a)** soybean; Lane 1: 100 bp plus DNA ladder, Lane 2: cDNA from non-transgenic roots, Lane 3-5: cDNA from transgenic hairy roots of cotyledon, stem and whole plant of TMS cultivar, respectively, Lane 6: cDNA from transgenic hairy roots of Djakel cultivar. **b)** sugar beet; Lane 1: 1 kb molecular marker, Lane 2-7: cDNA from transgenic hairy roots of SBSI004, Briggita, F-20537, Zarghan, Jolgeh and SBSI002 cultivars, respectively, Lane 8: cDNA from non-transgenic root. **c)** tobacco; Lane 1: PCR negative control, Lane 2: 1 kb molecular marker, Lane 3: cDNA from transgenic hairy roots, Lane 4: cDNA from non-transgenic roots.

Table 2. Results of the analysis of variance for the effect of three factors of bacterial concentration, antibiotic concentration, and type of explant on the production efficiency of non-transgenic and transgenic hairy roots in soybean

Source of changes	DF	Mean square	
		Non-transgenic hairy roots	Transgenic hairy roots
Explant	2	67.33**	77.67**
Bacterial concentration	6	1.18ns	0.39ns
Antibiotic concentration	4	9.48**	3.84**
Explant × Bacterial concentration	12	0.94ns	0.49ns
Explant × Antibiotic concentration	8	1.92ns	1.84ns
Bacterial cons. × Antibiotic cons.	24	0.93ns	0.25ns
Explant × Bacterial cons. × Antibiotic cons.	48	0.92ns	0.29ns
Error	945	1.33	0.39

** and ns, significantly at P value ≤ 1% and insignificantly, respectively

Table 3. Results of the analysis of variance for the effect of three factors of genotype, bacterial concentration, and type of explant on the production efficiency of non-transgenic and transgenic hairy roots in soybean

Source of changes	DF	Mean square	
		Non-transgenic hairy roots	Transgenic hairy roots
Genotype	1	21.6**	52.82**
Bacterial concentration	6	0.31ns	0.77ns
Type of explant	1	84.86**	71.42**
Genotype × Bacterial concentration	6	0.27ns	0.07ns
Genotype × Type of explant	1	5.2ns	9.25ns
Bacterial cons. × Type of explant	6	0.29ns	0.41ns
Genotype × Bacterial cons. × Type of explant	6	0.24ns	0.17ns
Error	112	0.78	0.65

** and ns, significantly at P value ≤ 1% and insignificantly, respectively

Screening of putative transgenic hairy roots for *gfp*

In order to the identification of the transgenic hairy roots containing *gfp*, PCR was carried out using genomic DNA. Results showed that a single DNA fragment of 172 bp specific for the *gfp* (Fig 6a-c) was amplified. Genomic DNA of untransformed plants and sterile water were used as negative controls.

To confirm *gfp* expression, semi-qPCR was done using transgenic hairy roots cDNA. Amplification of the 172 bp fragment, confirmed the expression of this gene in transgenic hairy roots (Fig 7a-c).

Transgenic hairy roots of soybean cultivars

Results of the analysis of variance show that there is no significant difference among treatments with different concentrations of bacteria, while there is a significant difference among explants or among the different concentrations of kanamycin antibiotic for TMS cultivar (Table 2).

The results showed that there is a significant difference between the genotypes (TMS and Djakel), and also between the explants of the cultivar Djakel (cotyledon and stem) in terms of producing transgenic and non-transgenic hairy roots (Table 3).

After sliding from each of TMS cultivar hairy roots, the highest and the least number of transgenic hairy roots were produced from cotyledon and whole plant explants respectively, but no significant difference was observed between the stem and whole plant (Fig 8).

According to Table 4, the highest yield of production non-transgenic and transgenic hairy roots in the cotyledon explant for the cultivar TMS was 94.28% and 56.97%, respectively.

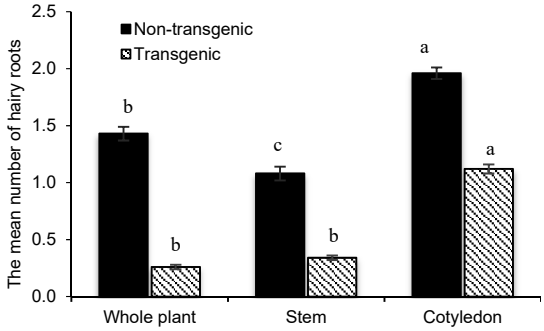

Fig 8. The mean±SD of the non-transgenic and transgenic hairy roots obtained from 1050 soybean explants at P ≤ 1%.

Table 4. The frequency of produced non-transgenic and transgenic hairy roots according to the type of explant in TMS cultivar

Explants	The number of explant with produced hairy root / the total number of explants	The percentage of produced hairy roots	The number of transgenic hairy roots / the total number of roots	The percentage of produced transgenic hairy roots
Cotyledon	330 / 350	94.28	392 / 688	56.97
Stem	194 / 350	55.42	122 / 381	32.02
Whole plant	255 / 350	72.85	93 / 503	18.48

In addition, to determine the best genotype, TMS and Djakel genotypes were used. The results of mean comparison between treatments showed that there is a significant difference between the two genotypes. The mean number of transgenic and non-transgenic hairy roots in Djakel genotype was more than TMS genotype (Fig. 9).

Results of the culture of explants in selective medium containing different concentrations of kanamycin antibiotic after about twenty days showed that kanamycin antibiotic prevented the growth of non-transgenic hairy roots. The highest number of non-transgenic hairy roots was observed in control (without kanamycin). The number of non-transgenic hairy roots decreased with increasing kanamycin concentration (Fig. 10).

Four concentrations (50, 100, 150, and 200 mg/L) were not significantly different in terms of the number of produced transgenic hairy roots. After sliding from each of the transgenic hairy roots, the lowest number was observed in control treatment (without kanamycin) and the highest number in different concentrations of kanamycin antibiotic (Fig 10), but no significant difference was observed at levels above 50 mg/L. Therefore, higher concentrations need not be used to select the transgenic hairy roots in the selective medium and the effective level of kanamycin concentration was 50 mg/L. In Djakel genotype, the frequency of production non-transgenic and transgenic hairy roots is 72.85% and 100%, respectively (Table 5), and this genotype can be used for research on gene transformation.

The mean number of transgenic and non-transgenic hairy roots of cotyledon explants was significantly higher than stem explants (Fig 11, Table 6).

Transgenic hairy roots of sugar beet cultivars

Results of the analysis of variance showed that there is significantly different among genotypes and between the leaves with petiole and the stem explants on production transgenic and non-transgenic hairy roots, while there

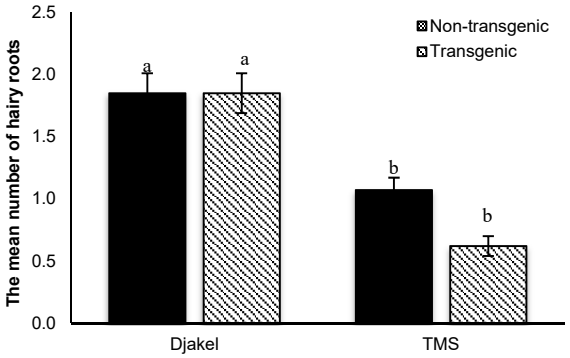

Fig 9. The mean±SD of the non-transgenic and transgenic hairy roots obtained from 140 explants of two genotypes at P value ≤ 1%.

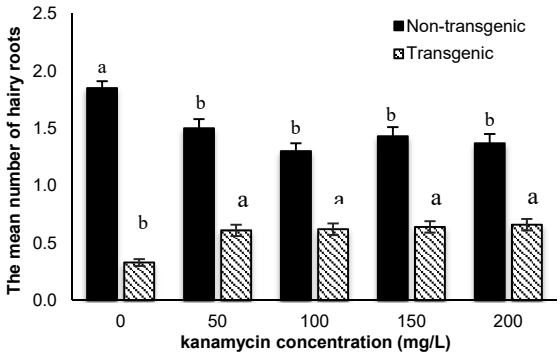

Fig 10. The mean±SD of the non-transgenic and transgenic hairy roots obtained from 1050 soybean explants in the different concentration of kanamycin at P value ≤ 1%.

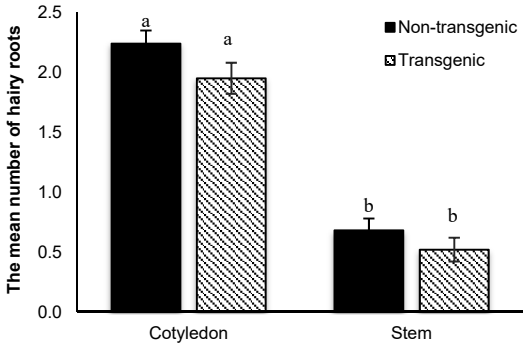

Fig 11. The mean±SD of the non-transgenic and transgenic hairy roots obtained from 140 explants of Djakel cultivar at P value ≤ 1%.

Table 5. The frequency of produced non-transgenic and transgenic hairy roots according to the type of genotype

Genotype	The number of explant with produced hairy root / the total number of explants	The percentage of produced hairy roots	The number of transgenic hairy roots / the total number of roots	The percentage of produced transgenic hairy roots
Djakel	51 / 70	72.85	130 / 130	100
TMS	48 / 70	68.85	44 / 75	58.66

Table 6. The frequency of produced non-transgenic and transgenic hairy roots according to the type of explant in Djakel cultivar

Explant	The number of explant with produced hairy root / the total number of explants	The percentage of produced hairy roots	The number of transgenic hairy roots / the total number of roots	The percentage of produced transgenic hairy roots
Cotyledon	70 / 70	100	137 / 157	87.26
Stem	33 / 70	47.14	37 / 48	77.08

Table 7. Results of the analysis of variance for the effect of three factors of genotype, bacterial concentration, and type of explant on the production efficiency of non-transgenic and transgenic hairy roots in sugar beet

Source of changes	DF	Mean square	
		Non-transgenic hairy roots	Transgenic hairy roots
Genotype	5	477.25^{**}	374.25^{**}
Bacterial concentration	3	0.89^{ns}	0.74^{ns}
Type of explant	1	196.2^{**}	165.004^{**}
Genotype × Bacterial concentration	15	1.84^{ns}	1.45^{ns}
Genotype × Type of explant	5	15.99^{ns}	14.75^{ns}
Bacterial cons. × Type of explant	3	0.95^{ns}	1.24^{ns}
Genotype × Bacterial cons. × Type of explant	15	1.05^{ns}	7.48^{ns}
Error	192	10.22	9.55

** and ^{ns}, significantly at 1% level and insignificantly, respectively

are no significant differences among different concentrations of bacteria (Table 7).

The results of the study on the effect of different genotypes on the production amount of transgenic and non-transgenic hairy roots showed a significant increase in the SBSI004 genotype compared to the genotypes F-20537, Brigitta, SBSI002, Jolgeh and Zarghan (Fig. 12).

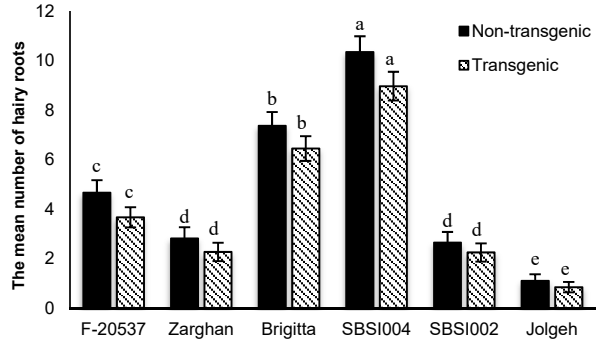

Fig 12. The mean±SD of the non-transgenic hairy roots obtained from 240 explants of six sugar beet genotypes at P value ≤ 1%.

In addition, the percentage maximum and minimum of production transgenic hairy roots were related to SBSI002 and Jolgeh genotypes, respectively (Table 8). According to the results of the mean comparison, the mean number and production frequency of transgenic and non-transgenic hairy roots in the leaves with petiole are higher than the stem (Fig. 13, Table 9).

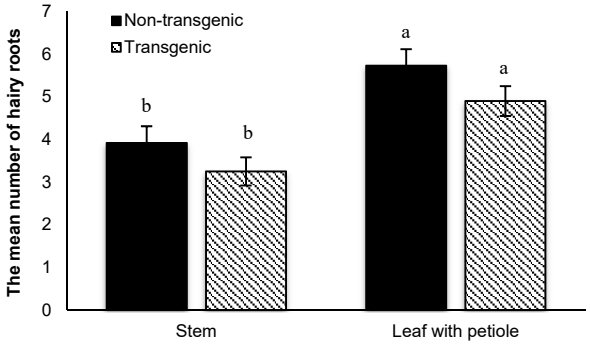

Fig 13. The mean±SD of the non-transgenic hairy roots obtained from 240 explants of the sugar beet at P value ≤ 1%.

Table 8. The frequency of produced non-transgenic and transgenic hairy roots according to the genotype in sugar beet

Genotype	The number of explant with produced hairy root / the total number of explants	The percentage of produced hairy roots	The number of transgenic hairy roots / the total number of roots	The percentage of produced transgenic hairy roots
F-20537	28 / 40	70	147 / 187	78.6
Zarghan	21 / 40	52.5	91 / 113	80.53
Briggita	33 / 40	82.5	258 / 295	87.45
SBSI004	36 / 40	90	359 / 414	86.71
SBSI002	20 / 40	50	90 / 106	90.84
Jolgeh	12 / 40	30	34 / 44	77.27

Table 9. The frequency of produced non-transgenic and transgenic hairy roots according to the type of explant in the sugar beet

Explant	The number of explant with produced hairy root / the total number of explants	The percentage of produced hairy roots	The number of transgenic hairy roots / the total number of roots	The percentage of produced transgenic hairy roots
Leaf with petiole	88 / 120	73.33	589 / 688	85.61
Stem	62 / 120	51.67	390 / 471	82.8

Table 10. Results of the analysis of variance for the effect of two factors of optical density (OD 600nm) and kanamycin concentration on the production efficiency of non-transgenic and transgenic hairy roots in tobacco

Source of changes	DF	Mean square	
		Non-transgenic hairy roots	Transgenic hairy roots
Optical density	3	7.83^{**}	12.49^{**}
Kanamycin concentration	2	174.17^{**}	18.35^{**}
Optical density × Kanamycin cons.	6	0.52^{ns}	0.18^{ns}
Error	108	2.68	1.35

** and ^{ns}, significantly at P value ≤ 1% and insignificantly, respectively

Table 11. The frequency of produced non-transgenic and transgenic hairy roots according to the different concentration of bacteria in the tobacco

Bacterial con.	The number of explant with produced hairy root / the total number of explants	The percentage of produced hairy roots	The number of transgenic hairy roots / the total number of roots	The percentage of produced transgenic hairy roots
0.4	30 / 30	100	100 / 175	57.14
0.6	30 / 30	100	104 / 191	54.45
0.8	30 / 30	100	129 / 221	58.37
1	30 / 30	100	82 / 163	50.3

Transgenic hairy roots of tobacco cultivars

The results of the analysis of variance showed that there is a significant difference between different concentrations of bacteria and between different concentrations of kanamycin (Table 10-11).

The maximum and the minimum number of transgenic and non-transgenic hairy roots was in treated samples with bacterial concentration (OD_{600}) 0.8 and 1, respectively (Fig. 14).

The results of different concentrations of kanamycin in selective culture medium on the production of transgenic and non-transgenic hairy roots were similar to those reported for soybean explants, so that at the concentration of 100mg/L, the max number of transgenic

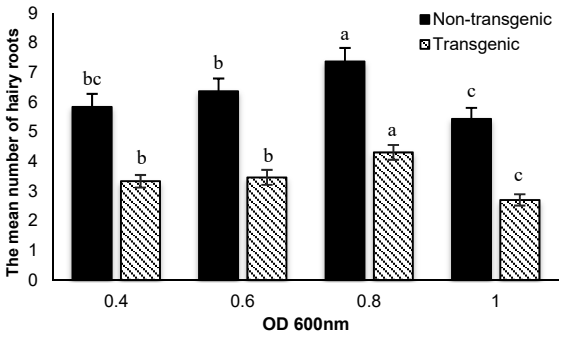

Fig 14. The mean±SD of the non-transgenic hairy roots obtained from 120 explants of the tobacco at P value ≤ 1%.

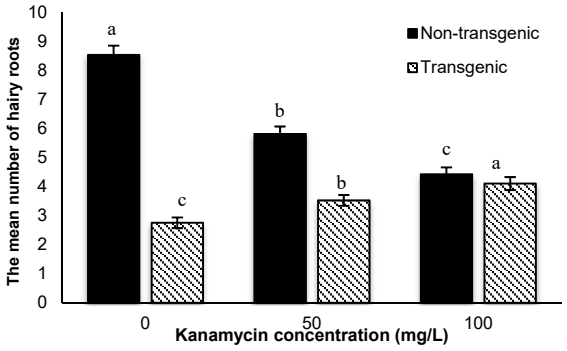

Fig 15. The mean±SD of the non-transgenic hairy roots obtained from 120 tobacco explants in the different concentration of kanamycin at P value ≤ 1%.

hairy roots were created, for this reason, the effective level of kanamycin concentration was determined 100 mg/L (Fig. 15).

DISCUSSION

A. rhizogenes strain AR15834 is a highly pathogenic bacterium and even the low concentrations (OD600 nm) of this bacterium can induce hairy roots in explants. Kereszt et al. [17] and Cao et al. [13] reports that different concentrations of *A. rhizogenes* are not significantly different for the production of hairy roots in soybean plants, which is similar to our results. Weber and Bodanese-Zanettini [18] reported that the percentage of transgenic hairy roots in *in vivo* conditions was higher than that of *in vitro*, but the frequency of *in vivo* gene transfer method was strongly dependent on the genotype of the plant, and in some of the genotypes, *in vitro* gene transfer method has more frequency. Therefore, perhaps, for this reason, the *in vitro* transformation rate is more than *in vivo* in the TMS genotype. Previous studies have shown that there is a significant difference between soybean genotypes in terms of the efficiency of hairy roots production. The results of this experiment are consistent with previous reports [10, 12, 18]. It is noteworthy that in Djakel genotype, the frequency of production non-transgenic and transgenic hairy roots is 72.85% and 100%, respectively, and this genotype can be used for research on gene transformation.

According to the results of the experiment, the frequency of hairy roots production in the cotyledon is higher than the stem, which can be due to different factors such as the plant tissue and the hairy root production system. Cho et al. [19] reported that inoculation of cotyledon explant with *A. rhizogenes* produced the highest number of transgenic hairy roots that is consistent with the results of this study. Dogan et al. [20] showed that there was a significant difference in the production of hairy roots from stem and cotyledon explants in *Lens culinaris*. Cao et al. [13] and Kereszt et al. [17] reported that different concentrations of bacteria don't have the effect on the production of transgenic and non-transgenic hairy roots and these reports are consistent with the results of this experiment. Because the stem tissue is very soft and juicy, it quickly destroyed by bacteria and produces fewer hairy roots. The results of many researchers have shown that the production of hairy roots in sugar beet from the petiole of *in vitro* sterilized seedling [21], leaf [22], cotyledon of nine-day sterilized seedlings [23], hypocotyl of seven-day sterilized seedlings and the leaves with petiole of eight-week greenhouse plants [24, 25] have been successful.

These results can be justified by the fact that the number of *Agrobacterium* cells in the inoculum is considered as a critical factor in gene transfer efficiency and the effect of bacterial concentration varies depending on the strain of bacteria and the type of explants. Optical density 1 causes a high growth rate of bacteria on explants and destroys them in a few days, while low-concentration bacteria also reduce the frequency of T-DNA transmissions [8]. Based on our results, optical density 0.8 is better than other concentrations and is used to inoculate treatments. These results are consistent with the findings of Costa et al. [8].

REFERENCES

[1] Srivastava, S. and A.K. Srivastava, Hairy root culture for mass-production of high-value secondary metabolites. Critical Reviews in Biotechnology, 2007. 27(1): p. 29-43.

[2] KO, K.S., et al., Production of polyketide pigments in hairy root cultures of Cassia plants. Chemical and pharmaceutical bulletin, 1995. 43(2): p. 274-278.

[3] Jeziorek, M., K. Sykłowska-Baranek, and A. Pietrosiuk, Hairy Root Cultures for the Production of Anti-cancer Naphthoquinone Compounds. Current medicinal chemistry, 2018. 25(36): p. 4718-4739.

[4] Bonhomme, V., et al., Tropane alkaloid production by hairy roots of Atropa belladonna obtained after transformation with Agrobacteriumrhizogenes 15834 and Agrobacterium tumefaciens containing rolA, B, C genes only. Journal of biotechnology, 2000. 81(2-3): p. 151-158.

[5] Forde, B.G., Local and long-range signaling pathways regulating plant responses to nitrate. Annual Review of Plant Biology, 2002. 53(1): p. 203-224.

[6] Georgiev, M.I., A.I. Pavlov, and T. Bley, Hairy root type plant in vitro systems as sources of bioactive substances. Applied Microbiology and Biotechnology, 2007. 74(6): p. 1175.

[7] Guillon, S., et al., Harnessing the potential of hairy roots: dawn of a new era. TRENDS in Biotechnology, 2006. 24(9): p. 403-409.

[8] Costa, M., W. Otoni, and G. Moore, An evaluation of factors affecting the efficiency of Agrobacterium-mediated transformation of Citrus paradisi (Macf.) and production of transgenic plants containing carotenoid biosynthetic genes. Plant Cell Reports, 2002. 21(4): p. 365-373.

[9] Guillon, S., et al., Hairy root research: recent scenario and exciting prospects. Current opinion in plant biology, 2006. 9(3): p. 341-346.

[10] Mazarei, M., Z. Ying, and R. Houtz, Functional analysis of the Rubisco large subunit ÂN-methyltransferase promoter from tobacco and its regulation by light in soybean hairy roots. Plant cell reports, 1998. 17(12): p. 907-912.

[11] Owens, L.D. and D.E. Cress, Genotypic variability of soybean response to Agrobacterium strains harboring the Ti or Ri plasmids. Plant Physiology, 1985. 77(1): p. 87-94.

[12] Savka, M., et al., Induction of hairy roots on cultivated soybean genotypes and their use to propagate the soybean cyst nematode. Phytopathology, 1990. 80(5): p. 503-508.

[13] Cao, D., et al., Assessment of conditions affecting Agrobacterium rhizogenes-mediated transformation of soybean. Plant Cell, Tissue and Organ Culture, 2009. 96(1): p. 45-52.

[14] Damgaard, O. and O. Rasmussen, Direct regeneration of transformed shoots inBrassica napus from hypocotyl infections withAgrobacterium rhizogenes. Plant molecular biology, 1991. 17(1): p. 1-8.

[15] Shahin, E., et al., Transformation of cultivated tomato by a binary vector in Agrobacterium rhizogenes: transgenic plants with normal phenotypes harbor binary vector T-DNA, but no Ri-plasmid T-DNA. Theoretical and Applied Genetics, 1986. 72(6): p. 770-777.

[16] Weigel, D. and J. Glazebrook, Transformation of agrobacterium using electroporation. CSH protocols, 2006. 2006(7): p. 1-13.

[17] Kereszt, A., et al., Agrobacterium rhizogenes-mediated transformation of soybean to study root biology. Nature Protocols, 2007. 2(4): p. 948.

[18] Weber, R.L.M. and M.H. Bodanese-Zanettini, Induction of transgenic hairy roots in soybean genotypes by Agrobacterium rhizogenes-mediated transformation. Pesquisa Agropecuária Brasileira, 2011. 46(9): p. 1070-1075.

[19] Cho, H.-J. and J.M. Widholm, Improved shoot regeneration protocol for hairy roots of the legume Astragalus sinicus. Plant Cell, Tissue and Organ Culture, 2002. 69(3): p. 259-269.

[20] Dogan, D., K.M. Khawar, and S. Özcan, Agrobacterium mediated tumor and hairy root formation from different explants of lentils derived from young seedlings. International Journal of Agriculture and Biology, 2005. 7: p. 1019-1025.

[21] Mishutkina, Y.V. and A. Gaponenko, Sugar beet (Beta vulgaris L.) morphogenesis in vitro: effects of phytohormone type and concentration in the culture medium, type of explants, and plant genotype on shoot regeneration frequency. Russian Journal of Genetics, 2006. 42(2): p. 150-157.

[22] Pavlov, A., V. Georgiev, and M. Ilieva, Betalain biosynthesis by red beet (Beta vulgaris L.) hairy root culture. Process Biochemistry, 2005. 40(5): p. 1531-1533.

[23] Pavli, O.I., et al., BNYVV-derived dsRNA confers resistance to rhizomania disease of sugar beet as

evidenced by a novel transgenic hairy root approach. Transgenic research, 2010. 19(5): p. 915-922.

[24] Kifle, S., et al., An improved transformation protocol for studying gene expression in hairy roots of sugar beet (Beta vulgaris L.). Plant cell reports, 1999. 18(6): p. 514-519.

[25] Samuelian, S., et al., Cloning and functional analyses of a gene from sugar beet up-regulated upon cyst nematode infection. Plant molecular biology, 2004. 54(1): p. 147-156.

ارزیابی کارایی القا ریشه‌های موئین با استفاده از ریزنمونه‌های سویا، چغندر قند و تنباکو

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چکیده

روش انتقال ژن به واسطه آگروباکتریوم یکی از گسترده‌ترین روش‌های مورد استفاده برای ترانسفورماسیون ژنتیکی در برنامه اصلاح نباتات است. کارایی ترانسفورماسیون می‌تواند بسته به سویه باکتری، ژنوتیپ گیاه و شرایط رشدی بهینه شود. در این مطالعه، ژن *gfp* به چغندر قند، تنباکو و سویا از طریق سویه AR15834 آگروباکتریوم رایزوژنز منتقل شد. فاکتورهای غلظت باکتری، غلظت آنتی‌بیوتیک و نوع ریزنمونه و ژنوتیپ بر روی کارایی ترانسفورماسیون و تولید ریشه‌های موئین تراریخته مورد بررسی قرار گرفت. ریزنمونه‌ها با غلظت‌های تنظیم شده باکتری تلقیح شدند و دو روز بعد از ترانسفورماسیون، ریزنمونه‌ها به محیط MS جامد حاوی غلظت‌های مختلف آنتی‌بیوتیک کانامایسین منتقل گردیدند. با توجه به نتایج و فاکتورهای مورد آزمایش، شرایط بهینه برای دستیابی به حداکثر تولید ریشه‌های موئین تراریخته شامل غلظت باکتری با OD_{600nm} = ۰/۲، ریزنمونه لپه، غلظت کانامایسین ۵۰ میلی‌گرم در هر لیتر و ژنوتیپ Djakel برای سویا؛ غلظت باکتری با OD_{600nm} = ۰/۲، ریزنمونه برگ با دمبرگ و ژنوتیپ SBSI004 برای چغندر قند؛ و غلظت باکتری با OD_{600nm} = ۰/۸ و غلظت آنتی‌بیوتیک کانامایسین ۱۰۰ میلی‌گرم در هر لیتر برای تنباکو بود.

کلمات کلیدی: *gfp*، ریشه‌های موئین تراریخته، سویا، چغندر قند، تنباکو