Original Article

# Production of Recombinant LSC Protein in *Nicotiana tabacum* Hairy Root: Direct vs. Indirect, a Comparison

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**Abstract**

**Introduction**: The production and purification cost of a recombinant protein in plants is lower than other conventional systems, such as bacteria. Comparing different parts of the plant, the hairy root is known to be a suitable bioreactor to produce recombinant proteins including vaccine candidate immunogens. Among the most important causes of diarrhea, *E. coli* and *Vibrio cholerae* cause the disease by producing a toxin. The B subunit of this toxin is an appropriate candidate for vaccine development because of its non-toxicity and exposure to the immune system.

**Materials and Methods**: To investigate the efficiency of transgenic hairy roots in the production of recombinant protein, two methods were considered: direct (induction of hairy root with recombinant *Agrobacterium rhizogenes*) and indirect (production of transgenic plant with *Agrobacterium tumefaciens* and then polluting it with non-transgenic *A. rhizogenes*). To compare the 2 methods, GUS protein expression was used as a reporter and chimeric protein LSC (consisting of *ltB-stxB-ctxB*) as an antigenic protein. Transformation of tobacco hairy root was confirmed by genomic Polymerase chain reaction (PCR). Gene expression comparison was investigated by semi-quantitative ELISA and enzymatic activity.

**Results**: The results show that the activity of GUS reporter enzyme in the indirect method is seven times more than that of the direct method. Likewise, the expression of LSC recombinant protein in the indirect transformation was 1.5 times more than in direct method.

**Conclusions**: Comparing of these two methods indicated that the hairy roots in the indirect method yield higher protein content than in the direct method.

**Keywords:** Hairy Root, *Agrobacterium Rhizogenes*, Direct and Indirect Transformation

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## Introduction

Plants have a high capability in production of various recombinant proteins, such as vaccines and enzymes. Among their advantages over other systems are affordability, easy transfer of research findings to production stages, capability of producing multipartite proteins, not contamination of plants with human pathogens, and their safety.[1] Moreover, plant cells can be cultured under simple conditions and new products can be easily accumulated in target organelles such as the endoplasmic reticulum, protein storage vacuoles and plastids away from proteases.[2] Due to the presence of mucosal immune system in the body of animals, in the case of the utilization of transgenic plant, it can be expected that these organisms will be immunized against the plant recombinant protein.[1] Among different plant tissues hairy root is considered as an efficient bioreactor because of the advantages such as high protein production ability with the possibility of cultivation in industrial fermenters without the need for light and expensive hormones.[3,4] The production and development of a successful vaccine against human and animal pathogens in hairy roots require selecting correct immunogens and appropriate designing of the synthetic genes for proper expression in the plant system. The bacteria causing intestinal infections and diarrheal disease, which is one of the major causes of death in children under-5 years, are good candidates for studying this system and selection of proper immunogens.[5] The most important bacterial factors causing diarrheal infections are enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), and *Vibrio cholerae* that are major contributors to the development of endemic and epidemic diarrhea around the world.[6,7] The toxin produced by these bacteria is the main factor causing diarrhea. The toxin of enterohemorrhagic *E. coli* (EHEC) is similar to the *Shigella dysentery* toxin named Stx.[8] The ETEC bacteria produce a heat-labile (LT) toxin and the *Vibrio* bacteria produce Cholera enterotoxin (Ctx). These toxins produce diarrhea by causing disorders in the mechanism of ion channels of intestinal cells.[9,10] All these three toxins (LT, Stx, and Ctx) are members of the AB5 toxin family. In this group of toxins, the same pentameric B subunit mediates the toxin binding to the host cell surface receptor.[11] Considering the structure of AB5 toxins, B subunits, as the binding subunit, is the best candidate for vaccine development, which is expected to prevent their operation by blocking the toxin-receptor binding. On the other hand, the B subunit of both LT

and Ctx toxins have an adjuvant activity which can activate the immune system, then their use in a recombinant protein will simultaneously enhance this effect.[12] In previous studies, the binding (B) subunit is known as a strong immunogen that is capable of producing an appropriate blood and mucosal immune response in an animal model.[13-16] There has not been yet any research reported on the production of a chimeric protein including the binding subunits of all three toxins or their binary combination in the hairy root system. In our previous study, a three-partit gene construct containing the binding subunit of ltB-stxB-ctxB (LSC) after bioinformatics studies was synthesized.[17] Here, the expression ability of this chimeric gene in transgenic hairy roots of tobacco plant was investigated by direct and indirect transformation. Expression measurement of GUS enzyme and LSC chimeric protein in the tobacco hairy roots was conducted with the aim of producing an oral vaccine based on hairy roots and comparing two direct and indirect methods in transgenic hairy roots as a new method. The performing stages of this study are presented as the following (Figure 1).

## Materials and Methods
### Preparation of pBI121-*lsc* Gene Construct
The *lsc* gene construct was synthesized according to the previous studies.[17] In order to clone the *lsc* gene construct, the pUC57 vector containing this synthetic gene and the pBI121 vector were digested with XbaI/SacI restriction enzymes. The ligation of the *lsc* to pBI121 was performed and the construct was transformed to 2 strains of *Agrobacterium* by freezing and thawing.[18] The stctxPF primer: 5'CCATACTCTAGAAAAACAATGGCTGATTGT-GCTAAG3' and stctxPR: 5'GAATTCGAGCTCTCA-CAATTCATCCTTCTC3' were used to confirm the transformation of *Agrobacterium*. MCAMF primers: 5'CTATCCTC-CGCAAGACCCTTCCTC3' that are located on CaMV35s



**Figure 1.** A Schematic Diagram of the Direct and Indirect Transformation Methods.

promoter and MPLBR1 primer: 5' GAAGGTGCAAGC-TACCTCTCT3' located on the left border of pBI121 were used to confirm the cloning and sequencing of the cloned genes in pBI121. *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* containing pBI121-*gus* and pBI121-*lsc* gene constructs were used for transformation of tobacco.

### Transgenic Tobacco Plant With Recombinant *Agrobacterium rhizogenes* (Direct Method)
Using standard methods, tobacco seeds were cultured in sterile conditions and explants were prepared from leaves of the tobacco and their transformation was performed.[19] After the emergence of hairy roots, every 7 days, one 3-4 cm slice from end of root from each line was transferred to a new selective medium with a higher concentration of kanamycin (75 and 100 mg/L, respectively). After the final selection, roots were transferred to a liquid MS medium without antibiotic in Erlenmeyer flask at 24° C and 140 rpm for further growth.

### Transformation of Transgenic Tobacco With Non-recombinant *Agrobacterium rhizogenes* (Indirect Method)
In the indirect method, for the formation of hairy roots, first, the transgenic tobacco plant was obtained using *A. tumefaciens* LBA4404 containing the pBI121 + *lsc* gene construct and pBI121-*gus* plasmid.[20] After evaluation of transgenic plants, leaf explants were prepared and hairy root was obtained using *A. rhizogenes* strain ATCC-15834.[19]

### Transgenic Plant and Hairy Roots Confirmation
Genomic DNA was extracted from tissue samples of the leaves and hairy roots of plants.[21] In order to confirm the presence of the recombinant (*lsc*) and reporter (*gus*) gene, PCR reaction with *lsc* gene and construct specific primer of the pBI121 vector was carried out. To ensure the presence of effective genes in the formation of transgenic hairy roots, *rolB*F: 5'GCTCTTGCAGTGCTAGATTTG3' and *rolB*R: 5'GAAGGTGCAAGCTACCTCTCT3' primers, which are specific for the *rolB* gene, were used in PCR reaction.

### Gus Assay
The 20 mg/mL solution of X-Gluc (Fermentas) in DMSO (Merck) was formulated according to Terada et al.[22] The qualitative study of GUS expression was performed in leaf and hairy root samples of transgenic plants and samples were photographed macroscopically. Also, the quantitative assay of *gus* in the cellular extracts of transgenic plants' tissues was measured according to Blazquez.[23] After 10 and 20 minutes, the reaction was stopped by using 1M sodium carbonate solution, and the created color was measured at 455 nm. The activity rate of *gus* in the samples was calculated based on the standard curve drawn for the 4-MUG.

### Evaluation of the Recombinant Proteins Expressed in the Culture Medium of Transgenic Hairy Roots
Quantitative ELISA was used to determine the amount of LSC protein in the total extracted protein. In this regard, a serial dilution of 10 pg to 5 μg of the purified protein LSC and the antibody against this protein was prepared.[17] The endobiotic
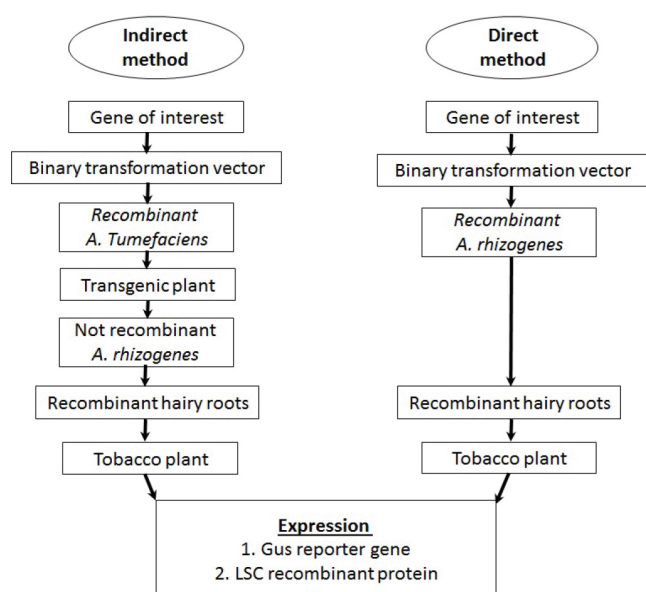
peroxidation activity in root tissues was stopped using 0.3% hydrogen peroxide. A protein solution from not-transformed hairy roots was used as negative control. The protein amount in the hairy root tissues of tobacco was calculated by processing the results obtained from the standard curve which was drawn based on the pure protein LSC.

### Ethical Considerations

This study was approved by the ethics committee of the National Institute of Genetic Engineering and Biotechnology with IR.NIGEB.EC.1394.8.10 code.

### Results

#### Cloning the *lsc* Gene in the pBI121 Plasmid and Transformation to *Agrobacterium*

The pBI121 vector containing *lsc* gene was extracted from *E. coli* DH5α bacteria and evaluated with XbaI and SacI enzymes digestion (Figure 2A). The results of the colony PCR with specific primers of recombinant genes on *A. rhizogenes* and *A. tumefaciens* colonies represents the 650-bp (Figure 2B) and 2200 bp (Figure 2C) bands of the *lsc* and *gus* genes in correct sizes.

#### Tissue Culture and Transgenesis of Tobacco Plant

The tobacco plant was transformed using *A. rhizogenes* containing the constructs (Figure 3). About 7-10 days after transformation of tobacco with recombinant *A. rhizogenes*, the roots began to develop and their growth continued in the

medium containing kanamycin (Figure 3A). The resistant single roots were transferred to solid culture medium with higher concentrations of kanamycin (Figure 3B). Roots were sub-cultured every 10 days by transferring one piece of root to the new medium, and then each line was transferred to the Erlenmeyer for further growth (Figure 3D). The remarkable point in the hairy roots of tobacco was their direct regeneration to seedlings and then the complete plant, as shown in Figure 3C. These seedlings became a complete plant, and also developed reproductive and seed production stages.

Various stages of tobacco transformation by *A. tumefaciens* are represented in Figure 4. In order to develop hairy roots, the leaf of transgenic plants, after validation of transformation, was exposed to non-transgenic *A. rhizogenes*.

#### Genomic PCR of Transgenic Hairy Roots

DNA was extracted from leaves and hairy roots of transgenic plants grown in the selected medium. The result of PCR with primers specific for *lsc* and *gus* genes confirms transgenic plants at the genome level. The amplification of the 636 bp segment with stctPF and stctPR primers confirms the *lsc* gene transfer to the genome of transgenic tobacco plants (Figure 5B). Using the MCAMF (located in the promoter region) and stctPR primers in the PCR reaction for the *lsc* gene, the amplification of 1106 bp segment is observable (Figure 5A). Using the pair primers of MCAMF/MPLBR1 for transgenic samples with *gus* genes resulted in the proliferation of
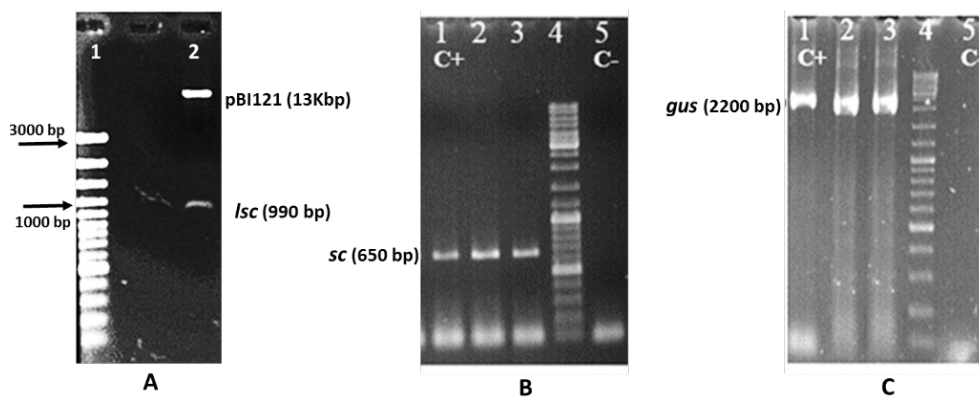


**Figure 2.** (A) Electrophoresis of the enzymatic digestion of pBI121-*lsc* recombinant vectors on Agarose gel 1%, 1) DNA molecular marker, 2) recombinant pBI-*lsc* vector cut with XbaI and SacI enzymes, (B) Electrophoresis of the PCR product of *Agrobacterium rhizogenes* ATCC15834 strain colonies transformed by pBI121 + *lsc*, and (C) pBI121 + gus on the 1% Agarose gel, the molecular size marker was the Mix (Fermentas). C+ : positive control using pUC57 vector containing LSC construct, C- : negative control, the product of PCR without DNA template, 2 and 3) PCR product of two different colonies of *Agrobacterium rhizogenes*.
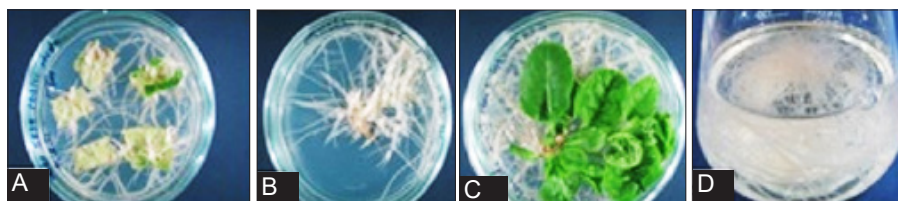


**Figure 3.** Transformation of Tobacco by *Agrobacterium rhizogenes*. (A) formation of hairy roots in solid culture medium, (B) development of a line of hairy roots in the solid medium, (C) formation of seedling from hairy roots, and (D) growing hairy roots in Erlenmeyer flask in a liquid culture medium.
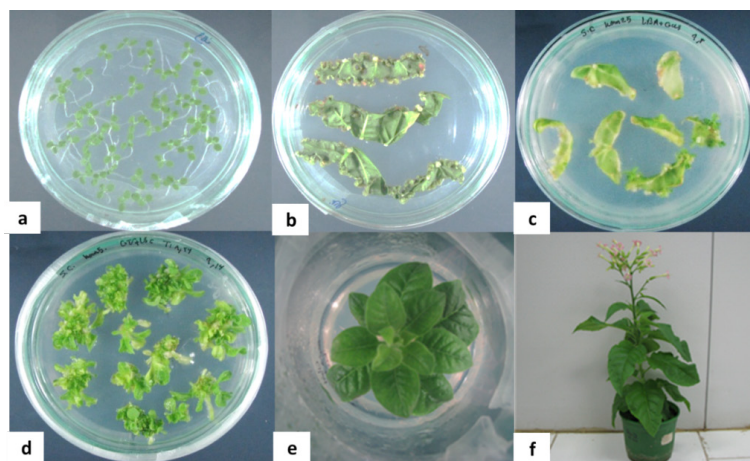
**Figure 4.** Different Stages of Transformation of Tobacco by *Agrobacterium tumefaciens*. (a) Seed culture, (b) Initial calluses on leaf explants, (c) and (d) Callus regeneration, (e) Development of transgenic plants, and (f) Reproductive stage of the transgenic tobacco.
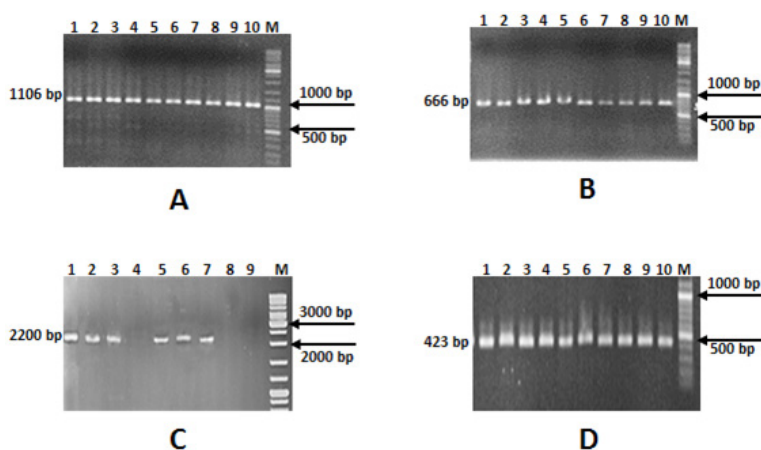


**Figure 5.** Molecular Confirmation of Hairy Roots by PCR Method. The PCR product using the genome of transgenic tobacco root containing lsc gene by MCAMF and stctPR primers (A), and the stctPF and stctPR primers (B), 1 to 5) transgenic hairy root of direct method and 6 to 10) transgenic hairy root of indirect method; PCR product of transgenic tobacco containing *gus* gene by MCAMF and MPLBR1 primers (C); PCR product of transgenic hairy roots using *rolB*f and *rolB*R primers (D), 1 to 5) hairy root of direct method and 6 to 10) transgenic hairy root by indirect method.

2200 bp segment in these samples (Figure 5C). To confirm transferring the *rolB* gene of *A. rhizogenes* involved in rooting, the PCR reaction was performed by *rolB*F and *rolB*R primers and resulted in the observing of the 423 bp band related to the *rolB* gene (Figure 5D).

### Quantitative and Histochemical Evaluation of GUS in Transgenic Samples

The GUS assay was performed on leaf and hairy roots of transgenic plants. In this evaluation, blueness was observed in leaf tissues of leaves and roots of the transgenic plants, but not in the control plant (Figure 6). The qualitative study of the leaves of the transgenic plant that was used in the indirect method represents the expression of GUS protein. In the qualitative comparison of hairy root between the direct transgenic method (Figure 6C) and indirect transgenic method (Figure 6B), it seems that GUS protein was more produced in the root tissue with indirect transgenesis, as the color is observed throughout the hairy root, but in the hairy root of the direct method it was seen as scattered spots on

some points of the root. However, in the hairy root in which the *gus* gene was not transferred, no blue color was observed at all (Figure 6D).

In order to compare the direct and indirect methods in the hairy roots production and the gene expression, the quantitative study of GUS enzyme activity was conducted. The result of this evaluation is presented in a column diagram (Figure 7), which shows hairy root produced in the indirect method had more enzymatic activity. Accordingly, the average GUS activity in the ten lines of examined hairy root for the indirect method after 10 minutes was 326 nmol/min, while for the same number of transgenic hairy root of direct method was 41 nmol/min. A similar trend is observed with a decrease in GUS activity in 20 minutes after starting the enzyme measurement (Figure 7).

### Evaluation of Recombinant Protein Production content in Transgenic Plants

ELISA was used for measuring LSC protein in hairy roots and its culture medium, and the percentage of LSC protein from
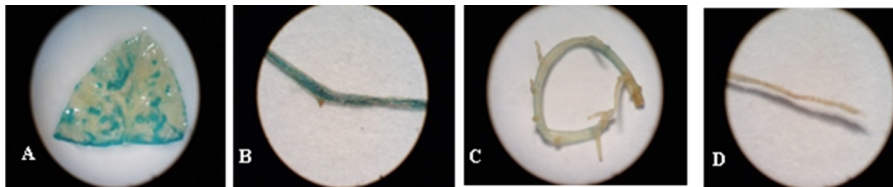
**Figure 6.** GUS Assay in Tissue of Tobacco Plant. (A) The leaves of transgenic plants with *Agrobacterium tumefaciens* containing pBI121 + *gus*, (B) The hairy root obtained from indirect method, (C) The hairy root resulted from direct method, (D) The transgenic sample transformed by *Agrobacterium rhizogenes* containing *lsc* + pBI121 (negative control).
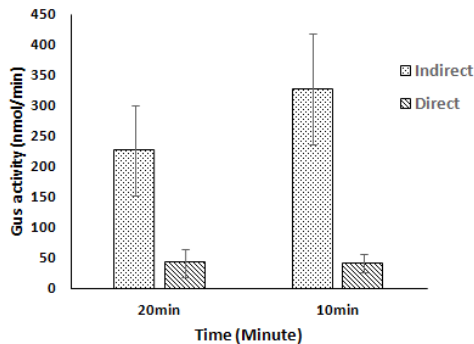


**Figure 7.** Comparison of GUS Enzyme Activity in Hairy Roots of Tobacco Plant Resulted From Direct and Indirect Methods.

total soluble proteins was calculated.[17] The results showed that the average amount of LSC produced in the direct method in the hairy roots was 4/5 µg/g Fw (0.13% of TSP), whereas this value was 5.5 µg/g Fw (0.24% of TSP) in the indirect method. The amount of secreted LSC in the culture medium of hairy roots in the direct method was observed to be 1.1 µg/g Fw 1.0 (0.1% of TSP) and in the indirect method was 1.87 µg/g Fw (0.17% of TSP). The sampling was performed in a 35-day period and the results are shown in Figure 8. The diagram of Figure 8A shows that the intracellular expression of the LSC protein in the indirect method is greater than the direct method. Similar result is observed for the secreted protein in the diagram of Figure 8B.

**Discussion**

Plants, as a biological reactor, are exploited in the mass production of vaccines, antibodies, protein drugs, enzymes,

and biological polymers for medical and industrial purposes.[2] Among different parts of the plant, hairy roots are of great interest because of their advantages such as ability to produce high biomass in a relatively short time, genetic stability, and no need for expensive hormone and culture medium.[4,25] In this study, the simultaneous transferring of T-DNAs (from Ri and pBI121 plasmid) in one step (direct transformation) or two-step (indirect transformation) was investigated. The hairy root was also used to express a vaccine candidate (LSC), a triple gene construct including the binding domain of heat sensitive toxin (LT) of ETEC bacteria, Shiga-like toxin (STX) of EHEC bacteria, and Cholera toxin (CTX) of *Vibrio cholerae*.

The required time for rooting in the direct method was about 8 days and in the indirect method was about 5 days. The reason for this difference may be the presence of kanamycin antibiotics in rooting medium of the direct method.[26] Another observation was that better rooting cases were obtained from larger leaves than the smaller ones. It may be due to the releasing phenolic compounds found in the cut site, and also the secretion of the compounds such as glucose and pH-lowering agents from the vacuoles which, according to the mechanism of gene transfer by *Agrobacterium*,[27] can stimulate cells to receive external DNA more efficiently. Therefore, larger leaves seem to be more susceptible for rooting due to more material accumulation in their vacuoles.

Investigating the growth of hairy roots in the culture medium by gradual increase of the antibiotic concentration or the sudden pressure of the antibiotic indicates that sequential selection on the mediums containing kanamycin 25, 50, 75 and 100 mg/L leads to the production of more roots and provides more varieties, while the sudden pressures with kanamycin 100 severely reduces the density of the roots in each explant.
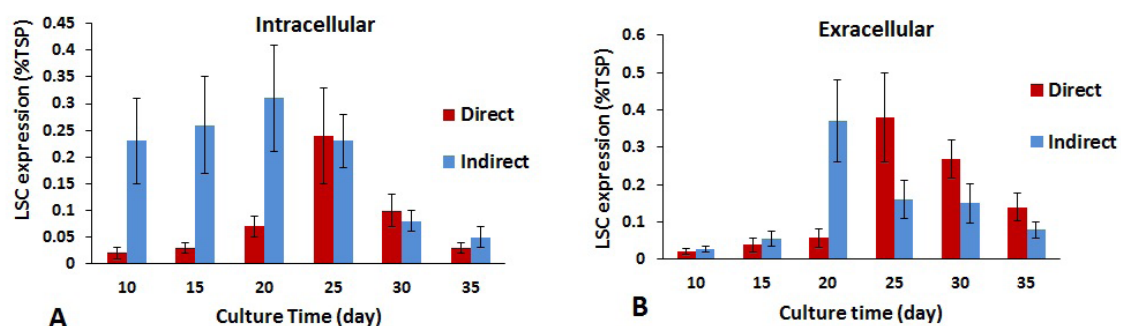


**Figure 8.** Measuring the Amount of LSC Recombinant Protein at Different Times in the Cell Extract (A) and in the Culture Medium (B) of the Hairy Root of Tobacco Plant in 2 Direct and Indirect Methods.

It seems that the sudden pressure of antibiotics destroys the cells rapidly and releases acidic and phenolic substances that lead to the destruction of probably transformed cells, and results in fewer lines of the hairy root. This is while in the gradual increase of the selected factor, lower cell death occurs in the early stages, and a more favorable condition will be provided for the expression of the antibiotic-resistance gene in the transformed cells. In addition, the conducted studies also show the growth decline in high concentrations.[28]

The qualitative evaluation of GUS showed that the reporter gene expression is higher in the indirect method. The quantitative evaluation of GUS indicates that its expression in the indirect method was more than 7 times greater than in the direct method. In the indirect method, the transgenic sample selection was performed in 2 steps, first in the regeneration of transgenic plant and then in the induction of hairy root, so the hairy root developed from a transgenic plant with the adequate expression of antibiotic resistance gene (which is found in the same T-DNA with the gene of interest). While in the direct method, a mosaic of cells can occur among which some cells only have the root-inducing gene (*rolB*) and the other cells receive both genes (*gus* and *rolB* genes), so the resulted hairy roots will be heterogeneous. On the other hand, a root may be selected in which the target gene has not been entered to a proper position, despite the fact that the rooting genes have entered to a good position. This means that there may even be a good root growth, but the target gene expression is weak. These genetically different hairy roots are selected as a transfromant while no difference can be detected in the initial roots. The results of the conducted studies also confirmed the superiority of the indirect method.[29] Another study showed that hairy roots could produce 1 to 7 times more recombinant protein in comparison to the original transgenic plants.[3]

Considering the difference between qualitative and quantitative expression of GUS reporter gene in these two methods, expression evaluation of another recombinant protein (LSC) was considered. Based on our results, the maximum expression of the intracellular recombinant protein in different lines of hairy root in the indirect method is 0.37% TSP which is 1.5 times higher than the direct method (0.23% TSP). Another study has shown that expression in the hairy root is six and seven times more than its production in the leaf and root of the transgenic plant respectively which can be due to the more synthesis of protein in the hairy root.[30]

The study of the secreted protein into the culture medium in the direct and indirect method indicates that the amount of secreted protein into the medium (on days 25 and 20) is the same, however the amount of LSC produced into the hairy root cells in these two methods is significantly different. It seems that since in the design of the gene construct no specific mechanism has been used to secrete the recombinant protein out to the environment, the capacity of the plant to secrete the protein out of the cell is limited and does not depend on the amount of protein in the cell.[31] According to a study, the expression of the recombinant ranalexin protein in transgenic plants was higher in the case of using the secretory signal peptide.[32,33]

The highest level of the recombinant protein expression in the direct method was found on day 25 and in the indirect method on day 20. These findings are in accordance with the previous reports about the expression of the recombinant protein in the hairy roots. After 25 days, the hairy root culture is still alive, but the expression level is significantly reduced, which is probably due to the decrease in the stability of enzymes and the reduction of translation.[33] Also, extracellular factors such as loss of nutrients, significant increase in the proteases in the culture medium, and increase of the harmful substances due to cellular destruction can be referred, as in a ranalexin protein expression study was reported.[32] The secreted recombinant protein into the culture medium can be collected by continuous replacement of the culture medium. In addition, protein stabilizers can be used to stabilize the secreted proteins.[30]

## Conclusions

Comparing these 2 methods indicated that the hairy roots in the indirect method produce higher recombinant protein content than the direct method. Also, it seems that despite a longer tissue culture process, indirect hairy root induction method is a more suitable method for achieving greater recombinant protein expression.

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