Molecular farming: Expression of recombinant Ocriplasmin in genetically transformed root culture of *Nicotiana tabaccum*

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ABSTRACT

Ocriplasmin, also known as microplasmin (JetreaTM), is a novel recombinant drug approved by the FDA. It is a truncated human plasmin used for the treatment of vitreoretinopathies. In the present study, ocriplasmin was expressed in *Nicotiana tabacum* hairy roots as a molecular farming platform. The gene of this recombinant protein was designed, synthesized and sub-cloned from pUC57 to plant expression vector pBI121 at the XbaI and the SacI sites. Then, the *Agrobacterium rhizogenes* strains MSU440, A4 and ATCC15834 were transformed by the obtained plasmid. For plant transformation and hairy root induction, tobacco seeds were cultivated in MS culture medium for 4 weeks at a phytotron. Leaf pieces were infected by the abovementioned *A. rhizogenes* strains. The induction of hairy roots and the co-transformation was confirmed by molecular analysis. The highest hairy root induction percentage, 92% were found in *A. rhizogenes* strain ATCC15834. After confirming the presence of the gene of interest by PCR, total protein was extracted from the obtained transgenic roots and the recombinant protein was purified by the Ni-NTA column due to the presence of His-tag. The obtained proteins were subjected to SDS-PAGE, ELISA and Western blot tests. According to the results, 56 µg mL^{-1} of recombinant protein were found in the purified protein solution based on the Bradford method. The total protein content across three lines of hairy roots was measured at 67.2 µg. The highest total protein concentration and recombinant protein yield were determined to be 61 µg mL^{-1} and 1732 µg mL^{-1}, respectively. Based on our results, we suggest that tobacco transgenic roots may be suitable sources for the production of this human protein.

Keywords: *Nicotiana tabaccum*, Ocriplasmin, Recombinant protein, Molecular farming/pharming, Hairy root.
Article type: Research Article.

INTRODUCTION

Ocriplasmin, also known as microplasmin (JetreaTM), represents a novel recombinant drug approved by the Food and Drug Administration (FDA). It is a truncated human plasmin used for the treatment of vitreomacular traction (VMT), an eye disease that can cause severe visual disturbance (Freund *et al.* 2013; Tibbetts *et al.* 2014; Fahim *et al.* 2014). Abnormal symptomatic VMT is an age-related disorder. This can put traction on the retina and can lead to visual distortions and even macular holes. In severe cases, it can lead to vision loss (Noppen *et al.* 2014). Until the FDA approved ocriplasmin, the only treatment for VMT was a surgical approach such as vitrectomy (Han *et al.* 1988; Vander *et al.* 1992; Sebag 1998; Trese 2000; Bhisitkul 2001; Trese 2002). Plasmin, the enzyme from which ocriplasmin is derived, is a member of the trypsin family and cleaves proteins into the basic amino

acids, lysine and arginine (Beebe 2015). Ocriplasmin has some advantages over plasmin; more especially, ocriplasmin is significantly more stable and also smaller than plasmin, leading to better tissue penetration (Stefanini *et al.* 2014). Ocriplasmin can induce posterior vitreous detachment due to its potential to degrade fibronectin and laminin after intravitreal injection (Gandorfer *et al.* 2004; de Smet *et al.* 2009; Benz *et al.* 2010). It contains two polypeptide chains of 230 and 19 residues joined by two disulfide bonds. Four intrachain disulfide bonds stabilize the residue 230 domain. Initially, it has restricted stability after injection into the vitreous humor, whereas it is stable in its formulation buffer (Aerts *et al.* 2012). The evaluation of the therapeutic efficacy provides after the expression and purification of recombinant ocriplasmin as a precursor in *Pichia pastoris* followed by activation using recombinant staphylokinase or urokinase (Nagai *et al.* 2003). Among various expression systems such as bacteria, yeast, mammalian cells, and insects, plants have become an attractive system for the production of recombinant proteins, drugs, industrial proteins, due to many advantages including safe use, rapid scale-up, long-term storage, and low production cost. Monoclonal antibodies and vaccine antigens have been produced in plants (Thomas *et al.* 2011). In addition, plant cells have the ability to carry out post translational modifications of proteins, which are essential in eukaryotic organisms (Webster & Thomas 2012). Recombinant protein expression platforms in plants include a variety of seed products, leafy products, plant cell cultures, hairy root cultures, and aquatic plants as well as moss and green algae (Wilken & Nikolov 2012). One of the main plant systems for the production of recombinant proteins is tobacco.

The genus *Nicotiana* belongs to the Solanaceae family, which includes 76 species originating in North America, South America, Australia, and Africa (Niknejad 2018). In general, the production of recombinant proteins in plant expression systems is more economical, more and more sustainable, and also the risks of contamination are less and the products are more biologically active similar to the natural form. Plants can perform proper glycosylation, appropriate folding, and disulfide bond formation, which are often necessary for the biological activity of recombinant proteins (Khademi *et al.* 2019; Shams *et al.* 2019). Stably and transiently transformed plants can be used to produce recombinant proteins. The temporary expression of the production of peptides and proteins reduces the negative effects of transgene expression on growth and shortens the time of product production (Hoelscher *et al.* 2022). To date, all systems used for recombinant protein production have limitations, including the inability to produce or secrete functionally complex proteins (bacterial systems), the risk of transmission of viruses and toxic molecules (bacterial systems and mammalian cells), or very high production costs (mammalian cells; Cardon *et al.* 2019). Since the early 2000s, the production of therapeutic proteins using transgenic plants (called molecular farming) offer a number of major advantages over other expression systems, including safety (without the risk of human-threatening viral contamination), lower upstream costs, as well as post translational modifications such as glycosylation (Gutierrez-Valdes *et al.* 2020). Genetically transformed roots so called "hairy roots" are capable of producing complex compounds on a high scale, in this context; the production of recombinant proteins is a promising application of hairy roots. Hairy root cultures allow recombinant proteins production performed in the bioreactor. It can be expressed under controlled conditions (Gutierrez-Valdes *et al.* 2020). *Agrobacterium rhizogenes* is a gram-negative soil bacterium, which lives near plant roots and causes "hairy root syndrome" in the host (infected plant). This syndrome includes excessive growth of non-geotropic branched roots at the site of infection (Gutierrez-Valdes *et al.* 2020). T-DNA contains two independent sequences, i.e. left and right borders. TL-DNA and TR-DNA are usually independently transferred and stably integrated into the host plant genome. However, the presence of TL-DNA alone is critical and sufficient. After determining the sequence of TL-DNA, four (open reading frames) ORFs (*rol A*, *rol B*, *rol C* and *rol D*) that are necessary for hairy root induction were identified and discovered (Gutierrez-Valdes *et al.* 2020). The products of rol genes have specific functions in the formation of hairy roots. Since the production of transgenic crop lines before analyzing the activity of recombinant proteins is expensive and time-consuming (Shams *et al.* 2019), hairy roots and model plants such as tobacco are suitable alternatives for producing these proteins.

Effective production, phenotypic and biochemical stability, rapid production of biomass at commercial level, no need for adding plant hormones, the possibility of producing recombinant protein on a large scale and stability in gene expression for a long time make hairy root culture a suitable tool for rapid evaluation. In addition, hairy roots are not only easy to transform and grow, but are also significantly used to produce secondary metabolites and important therapeutic recombinant proteins (Ron *et al.* 2014; Holaskova *et al.* 2015). In this study, the recombinant ocriplasmin was cloned and expressed in *Nicotiana tabacum* transgenic hairy roots as a molecular farming platform.

MATERIALS AND METHODS

Plant materials

Seeds of *Nicotiana tabacum* cv. Xanthi-nc were obtained from the Iran Tobacco Research Institute, Mazandaran, Iran. The seeds were sterilized by soaking them in 70% (v/v) ethanol for 30 sec, washed with sterile distilled water, then, soaking them in 1.5% hypochlorite sodium for 10 min, and finally rinsing three times using sterile distilled water. The sterilized seeds were grown on solid Murashige and Skoog (MS) medium containing 30 g L^{-1} sucrose and 7 g L^{-1} agar with pH 5.8 (Caizhen *et al.* 2015) for 4 weeks at 25 ± 1 °C with a 16/8 h light/dark photoperiod (Badrhadad *et al.* 2018; Khademi *et al.* 2020).

Construction of the expression vector for ocriplasmin

The coding region of ocriplasmin, containing 749 bp, was obtained from Drug bank database (www.drugbank.ca). Its codon optimization was performed based on the codon-usage preference of the host *N. tobacco* by Genscript database (www.genscript.com). In addition, a 6 × His tag and the ER-retention signal KDEL were fused at the N-terminus, respectively, in-frame with the ocriplasmin gene sequence. The designed gene was synthesized and cloned in the pUC57 cloning vector (Shinegene Co, China). The resulting plasmid was sequenced (Shinegene Co, China) to confirm the existence of ocriplasmin gene in pUC57. The plasmid harboring ocriplasmin gene was transferred to the *E. coli* strain TOP10, using the heat shock method (Russell & Sambrook 2001). Bacterial culture was carried out on LB medium containing ampicillin antibiotic. The designed gene sequence was inserted into the pBI121 expression vector through the XbaI and the SacI sites and a recombinant pBI121-Ocriplasmin expression vector was designed. In this vector, which contained kanamycin-resistance genes, the expression of the ocriplasmin was under the control of the CaMV 35S promoter and the nopaline synthase (NOS) terminator (www.expasy.org; Fig. 1).

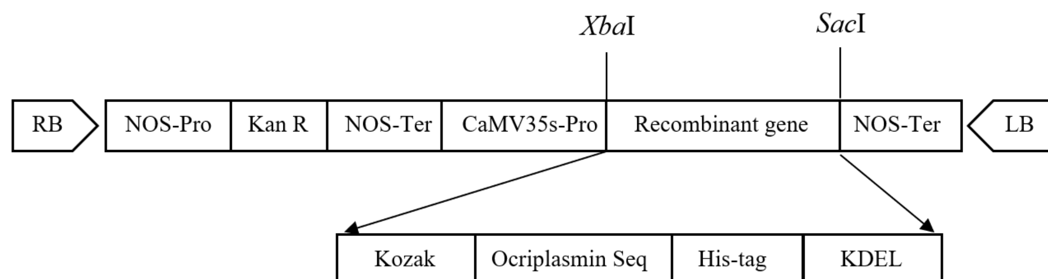

Fig. 1. The gene cassette used in T-DNA of pBI121; CaMV 35S: Cauliflower Mosaic Virus 35S promoter, NOS-ter: nopaline synthase terminator, His-Tag: Histidine tag (His)6, RB: Right border, LB: Left border. (The size of different parts in the figure does not correspond to their actual size).

Also, the culture of *E. coli* Top10 bacteria containing pBI121-GUS+ plasmid was carried out in liquid LB culture medium containing the 50 mg L^{-1} antibiotic kanamycin. In order to confirm the bacterial transformation, colony PCR was performed for TOP10 bacteria containing pUC57-Ocri plasmid. pBI121-GUS+ plasmid extraction and pUC57-Ocri plasmid extraction were performed according to the Exprep Plasmid SV Mini-Kit (GeneAll Co. Korea) protocol, and then pBI121-GUS+ plasmid digestion and pUC57-Ocri plasmid digestion were performed by XbaI and SacI enzymes. The desired fragments of pBI121 and Ocri were purified from gel extraction kit after cutting from 1% agarose gel. Then, the fragment containing Ocri gene was ligated to pBI121 plasmid by T4 ligase enzyme. The pBI121- Ocriplasmin expression vector was diluted 10 times, and 5 µL of it was used to transform *E. coli* strain TOP10 using the freeze-thaw method (Russell & Sambrook 2001). These bacteria were then dispersed on agar solidified Luria-Bertani (LB) supplemented with 50 mg L^{-1} kanamycin and were incubated at 37 °C overnight. Single colonies were selected and cultured in liquid LB medium supplemented with 50 mg L^{-1} kanamycin with shaking at 37 °C, 160 rpm overnight. Bacterial transformation was confirmed using colony PCR assay with a specific primer and also by digestion of the extracted plasmids from the colonies. In order to confirm the transfer of plasmid pBI121 containing ocriplasmin gene to bacteria, colony PCR was performed and plasmid pUC57-Ocri was used as a positive control and the results were observed on 1% agarose gel. From the master plate, 2 positive colonies were selected and cultured in liquid LB culture medium containing the antibiotic

kanamycin. Then the plasmid was extracted from TOP10 bacteria containing pBI121-Ocri plasmid and observed on agarose gel.

Transformation of *Agrobacterium rhizogenes* strains

The cultivation of 3 strains of *Agrobacterium rhizogenes*, including MSU440, A4 and ATCC15834, was carried out on LB solid culture medium containing 50 mg L^{-1} antibiotic rifampicin. All bacteria possess the gene of resistance to rifampicin antibiotic and grow on LB solid culture medium containing rifampicin antibiotic. For plant transformation and inoculation preparation, a single colony of each strain was cultured in liquid LB medium containing rifampicin. The plasmid pBI121-Ocri was extracted from transformed *E. coli* TOP10 using an Exprep Plasmid SV mini-Kit (GeneAll Co.). The recombinant plasmid was used to transform 100 µL of the competent cells of *A. rhizogenes* strains MSU440, A4 and ATCC15834 using the freeze-thaw method (Russell & Sambrook 2001). One mL of liquid LB was added, and incubated at 28 °C in the dark for 2 h. Then, they were dispersed on agar solidified LB containing kanamycin and rifampicin (50 mg L^{-1}) and were incubated at 28 °C in the dark for 48 h. In order to check the contamination from each of the strains, a single colony was cultured in liquid LB culture medium containing 50 mg L^{-1} antibiotic rifampicin and it was shown that they are free of contamination. The transformation of obtained colonies was confirmed using colony PCR assay with a specific primer pair and also by digestion of the extracted plasmid from the bacterial colonies.

Plant transformation and establishment of hairy roots

Leaf explants (3-week-old plants) of 1–2 cm was inoculated for 5 min with an *A. rhizogenes* inoculation culture (OD 600 = 0.5), and then these materials were co-cultured on solid MS medium at 24 ± 1°C in dark condition. After three days, the explants were transferred to fresh MS medium supplemented with 300 mg L^{-1} cefotaxime and 50 mg L^{-1} kanamycin and maintained at 25± 1°C under a 16/8 h light/dark photoperiod for two weeks. The hairy roots that formed at the incision sites of the leaf fragments were subsequently transferred at two-weeks intervals to fresh agar solidified MS containing 400 mg L^{-1} cefotaxime and 100 mg L^{-1} kanamycin at the concentrations noted above and were incubated at 25 ± 1°C (Badrhadad *et al.* 2018; Khademi *et al.* 2020). After several sub-culturing of the obtained hairy root clones, DNA was extracted to perform PCR for molecular confirmation of the hairy roots. Transgenic roots were grown in 250-mL Erlenmeyer flask containing liquid MS medium without antibiotics and were incubated at 28 °C in the dark with mild shaking for one to two months for protein extraction; the medium was refreshed weekly.

DNA extraction

Genomic DNA was extracted using the modified CTAB method (Richards *et al.* 1994). Then, the quantity and concentration of the DNA were measured using a Nanodrop instrument (Thermo Fisher Scientific, USA).

Screening the hairy root lines by polymerase chain reactions (PCR)

Specific primers for *Ocriplasmin*, *rolC*, and *virD* were designed using Oligo7 and Primer 3 software (Table 1). So, the specific primers for the amplification of *rolC* in the putative transgenic hairy root lines were used for PCR reaction. On the other hand, the specific primers for the amplification of *virD* were used to confirm that the *A. rhizogenes* infection had been fully eliminated. PCR were performed in 25 µL volumes comprising 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.1 mM of each forward and reverse primer, 1 unit of Taq DNA polymerase and 25 ng of genomic DNA. The amplifications were performed with a first denaturation at 95 °C for 5 min followed by 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C, and 30 sec at 72 °C, with a final extension at 72 °C for 5 min. The results of the PCR reaction were separated on a 1% agarose gel electrophoresis.

Extraction of total protein from hairy roots

The total proteins of transgenic hairy root clones (5 g) were extracted using phosphate buffer (50 mM, pH 7). The hairy root clones were ground under liquid nitrogen, and the powder was suspended in 1:1 phosphate buffer w/v. Then, the supernatant was prepared by centrifugation at 4000 rpm for 10 min at 4 °C. The amount of extracted total protein was determined using the Bradford method (Bradford 1976).

Purification of recombinant protein

The recombinant protein was purified using a Ni-NTA spin column (Qiagen Co.). First, the Ni-NTA spin column was equilibrated by loading 600 µL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and centrifuging it for 2 min at 1200 rpm. Next, up to 600 µL of concentrated root extract containing 6 × His

tagged ocriplasmin was loaded onto the column and it was centrifuged for 5 min at 300 rpm, then the flow-through was discarded. Furthermore, the column was washed twice with 600 µL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) by centrifugation for 2 min at 1200 rpm. Finally, the protein was eluted twice using 300 µL elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0).

Table1. The sequence of primers for PCR amplification.

Primers	Sequence (5'-3')	Annealing temperature (°C)
Ocri-F	ATTCATGAATTCCCTTCTTTTGATTGTGGAAAGC	60
Ocri-R	ATATATGTCGACATTATTTCTCATAACTCCTTCAATCCA	60
*rol*C-F	TGCTTCGAGTTATGGGTACA	55
*rol*C-R	CTCCTGACATCAAACTGCTC	55
*vir*D-F	ATGTCGCAAGGCAGTAAG	54
*vir*D-R	CAAGGAGTCTTTCAGCATG	54

SDS-PAGE

The total proteins extracted from the transgenic and non-transgenic root clones and the purified ocriplasmin were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel. Following electrophoresis, the gel was stained using Coomassie brilliant blue R-250.

Western blot analysis

To perform a western blot analysis, samples of proteins (10 µg each) were separated on the 12% SDS-PAGE gel and transferred to the PVDF (polyvinylidene fluoride) membrane. Based on the protocol, before adding the anti-His antibody, the PVDF paper was blocked with 5% skim milk in TBS (Tris-buffered saline) for 1 h at room temperature followed by washing with TBS-T (TBS + 0.05% Tween 20). The membrane was incubated at room temperature with 1:2000 dilution of the mouse anti His–HRP conjugated antibody (Santa Cruz Biotech). Finally, the membrane was exposed to DAB (diaminobenzidine) solution and incubated at room temperature until bands appear followed by washing the membrane in distilled water to stop the reaction.

Immunological detection by enzyme-linked immunosorbent assay

The specificity of the recombinant protein, ocriplasmin, produced in genetically transformed root cultures of *N. tabacum*, was assessed using an enzyme-linked immunosorbent assay (ELISA) with an anti-His primary antibody (Abcam, USA). Purified protein (50 µg mL^{-1}) was coated onto a 96-well microplate (100 µL per well) and incubated at 4 °C for 16 h. The plate was then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (v/v), followed by three additional washes with PBS alone. Each well was blocked with 100 µL of PBS-T (PBS + 0.05% Tween 20) and incubated at 37 °C for 1 h, after which the washing steps were repeated as described above. Next, 100 µL of anti-His primary antibody, diluted 1:1000 in PBS-T supplemented with 2% bovine serum albumin (BSA), was added to each well and incubated at 37 °C for 1 h. The plate was washed again under the same conditions. Finally, 100 µL of 1% tetramethylbenzidine (TMB) substrate solution was added to each well and incubated in the dark for 15 min. The enzymatic reaction was terminated by adding 100 µL of 1N sulfuric acid, and the optical density (OD) was measured at 450 nm using a microplate reader (Bio-Rad, Richmond, CA, USA).

RESULTS

Plant materials

Seeds were germinated for 1 weeks successfully, and leaf explants from 4 week-old tobacco seedlings were used for transformation (Fig. 2).

Construction of the expression vector pBI121-Ocriplasmin and transformation of *A. rhizogenes* strains

Digestion of pUC57 containing ocriplasmin gene and pBI121 containing *gus* gene was performed by SacI and XbaI (Fig. 3 a and b). After confirming the presense of ocriplasmin gene in pUC57, the plasmid was transformed to *E. coli* TOP10. After gel recovery of pBI121 *gus*— and gene of interest (ocriplasmin) from digestion process, they were ligated by T4 DNA ligase. The ligation product was transferred to *E. coli* TOP10 and the molecular confirmations were performed by colony PCR and plasmid extraction and digestion by SacI and XbaI. The plasmid pUC57-Ocri was used as a positive control in the colony PCR and the results were observed on a 1% agarose gel. (Fig. 3c and d). The pBI121 harboring ocriplasmin was transferred to three different *A. rhizogenes* strains, i.e., MSU440, A4 and ATCC15834 for use in hairy root induction and the molecular confirmations were performed by colony PCR for each of the three *A. rhizogenes* strains and the expected band was observed (Fig. 3

e). Selected clones from *A. rhizogenes* master plates MSU440, A4 and ATCC15834 were cultured in liquid LB medium containing the antibiotics rifampicin and kanamycin.

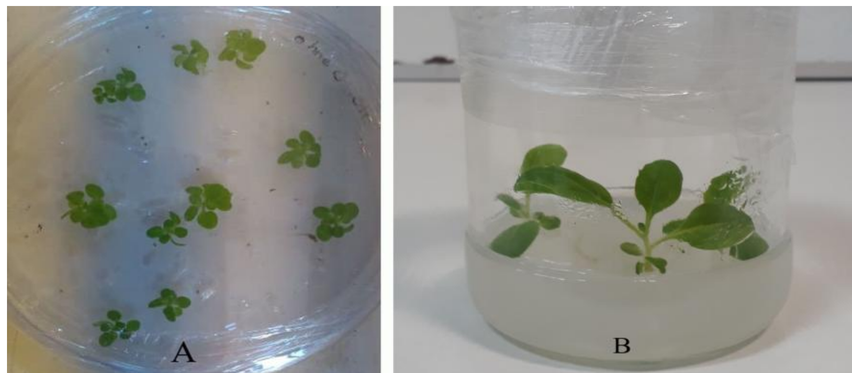

Fig. 2. Seeds were germinated for 1 week (A) 4-week-old tobacco plant used for transformation (B).

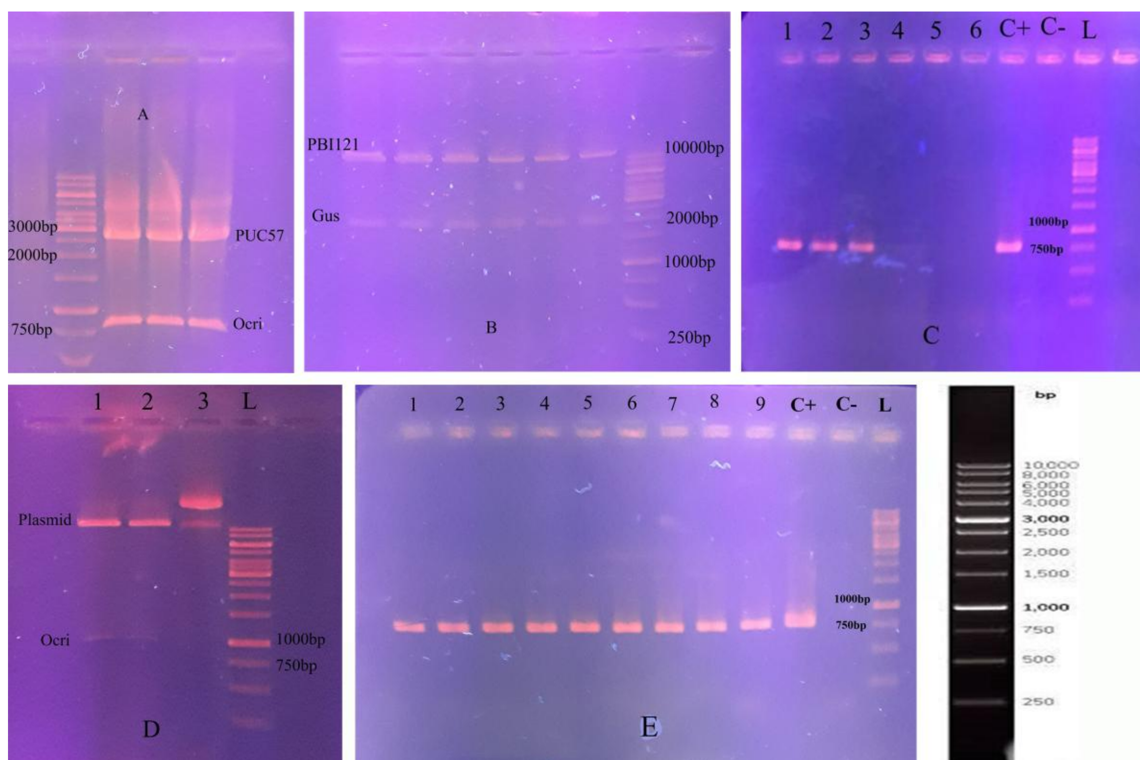

Fig. 3. pUC57-Ocri digestion; ocriplasmin gene and pUC57 plasmid were separated (A). pBI121-*gus*$^{+}$ digestion; *gus* gene and pBI121 vector were separated (B). Colony PCR with ocriplasmin gene-specific primers for *E. coli* TOP10 (C) Confirmatory digestion; Wells 1 and 2 are the confirmed digestion product and well 3 is the undigested plasmid (D). Colony PCR with ocriplasmin gene-specific primers for three different *A. rhizogenes* strains: MSU440, A4 and ATCC15834 (E); DNA Ladder 1 kb Thermo Scientific has been used.

Plant transformation and establishment of hairy roots

Leaf explants were inoculated in the infection medium containing bacteria for 5-10 minutes and then transferred to the co-culture medium for 48 hours. All of the treatments led to hairy root induction. Explants were transferred to fresh media every two weeks to avoid browning (Fig. 4). The percentages of hairy root induction for the three different *A. rhizogenes* strains, A4, MSU440 and ATCC15834 were 73%, 87%, and 92%, respectively.

Molecular confirmation and screening of the hairy root lines by PCR analysis

Genomic DNA was extracted and the quantity and concentration of the DNA were measured using a Nanodrop instrument. The extracted DNA concentration was between 120 and 180 ng μL^{-1} and the optical absorbance at wavelengths of 260 to 280 nm was between 1.7 and 1.9. In order to confirm that hairy roots are not contaminated with *A. rhizogenes*, PCR reaction using specific primers of *Vir*D2 gene (expected size of 438 bp) was performed

(no band observed). To confirm the presence of the *rol* genes in the formation of hairy roots, the specific primers of *rol* C gene were used to amplify the 534 bp fragment, and the transfer of T-DNA in obtained hairy root lines was confirmed (Fig. 5). The presence of the ocriplasmin gene with its specific primers was confirmed by the amplification of the target fragment in transgenic hairy roots (Fig. 5).

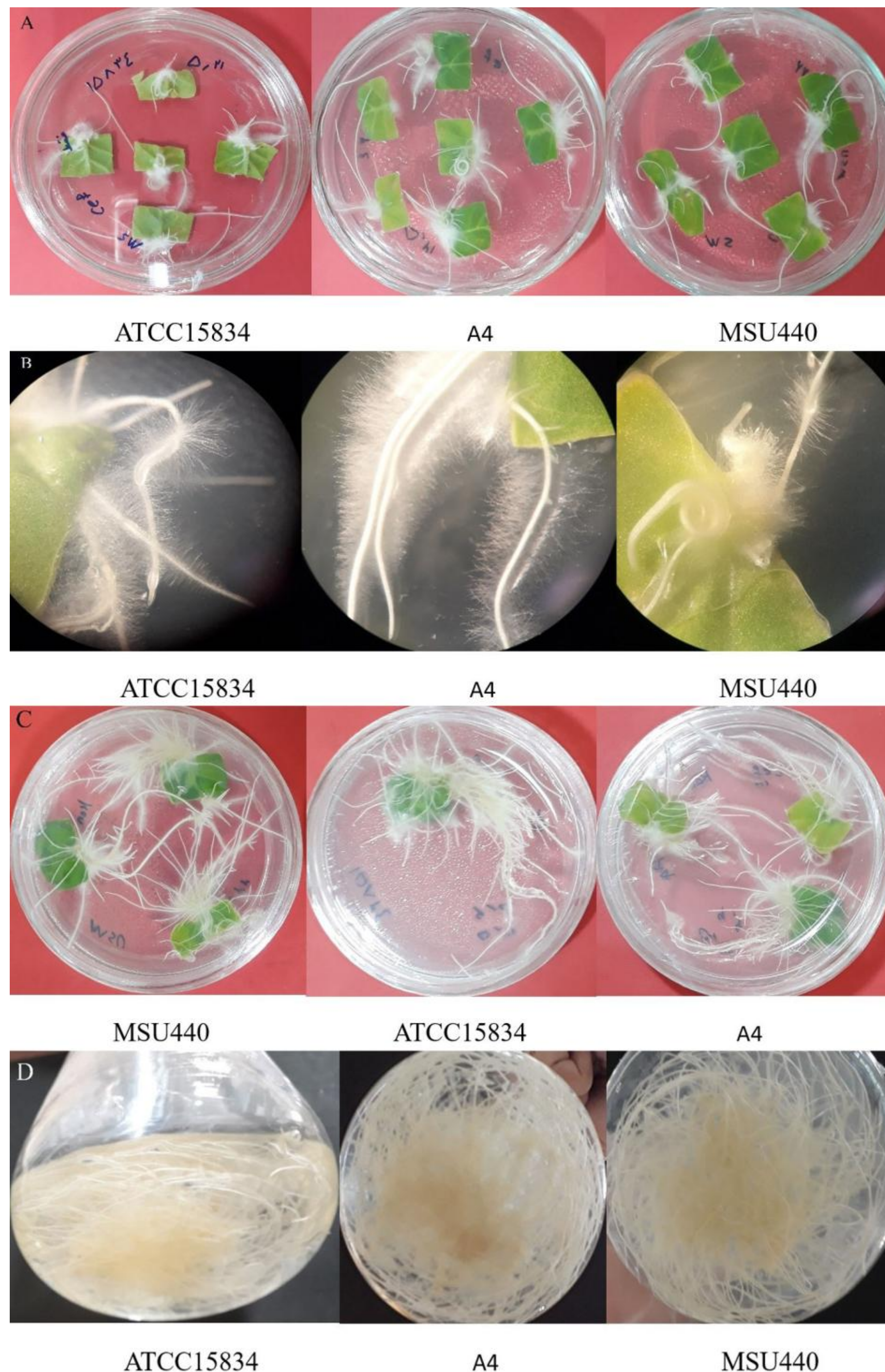

Fig. 4. Induction and growth of *N. tabacum* hairy roots on solid MS medium supplemented with 400 mg L^{-1} cefotaxime based on the results of different *A. rhizogenesis* strains: three weeks after infection (A); High-magnification view of an explant showing the development of hairy roots (B); Induction and propagation of hairy roots after sub-culturing (C). Growth of hairy roots cultivated in a 250-mL Erlenmeyer flask containing MS liquid medium after one month (D).

Protein extraction and purification under native conditions

The protein extraction of transgenic and non-transgenic roots was done by potassium phosphate buffer. The optical absorption of standard values was measured at a wavelength of 595 nm by a spectrophotometer. According to the amount of optical absorption of the standard samples at the wavelength of 595 nm, the standard diagram was

presented (Fig. 6). The concentration of the total protein extract of different lines of hairy roots and transgenic and non-transgenic plants was prepared by spectrophotometric method, and their concentration was estimated by the standard graph using the Bradford method. The average total protein concentrations in the three different *A. rhizogenes* strains, A4, MSU440 and ATCC15834 were 1412, 1407, and 1732 μg mL, respectively.

According to the results, 56 μg mL^{-1} of recombinant protein were found in the purified protein solution based on the Bradford method. The total content of protein in 3 lines of hairy roots was 67.2 μg. The recombinant protein was purified using a Ni-NTA column.

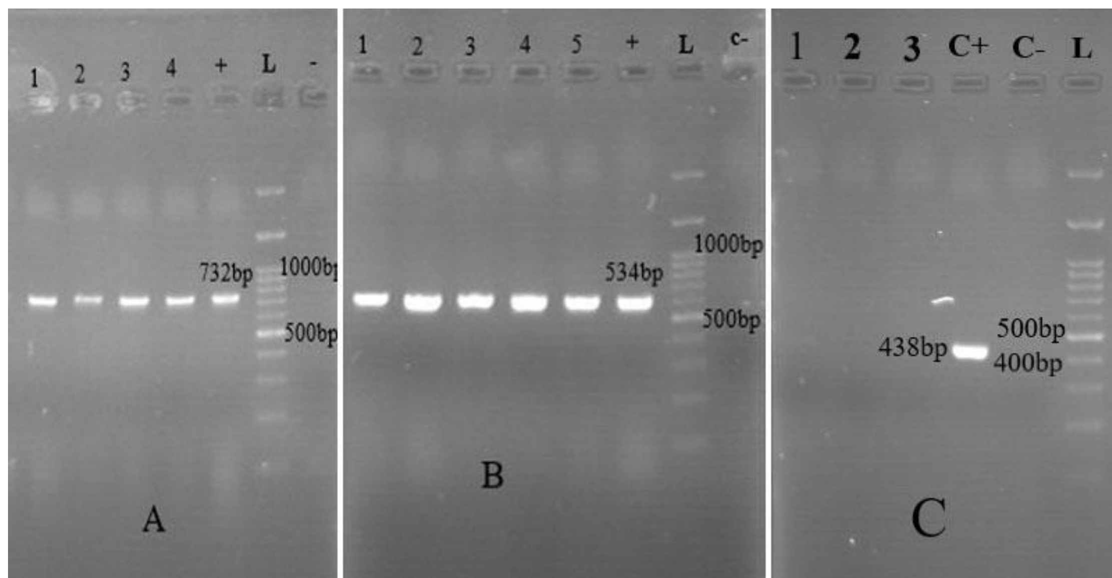

Fig. 5. Screening transgenic hairy roots by PCR: the presence of the ocriplasmin gene with its specific primers was confirmed by the amplification of the target fragment in transgenic hairy roots (A). *rol*C PCR products in different hairy root lines (B); *Vir*D PCR, indicating the absence of bacterial contamination (C); DNA Ladder 100bp Plus Sinaclon has been used.

SDS-PAGE

SDS-PAGE electrophoresis was performed on a 12% acrylamide gel for the total protein extract containing the recombinant protein, followed by Coomassie Brilliant Blue R-250 staining based on Laemmli's method (Laemmli 1970). The recombinant protein with an approximate weight of 27 kilodaltons was observed (Fig. 7), which confirms the presence of recombinant protein in the total protein extract of the hairy root lines.

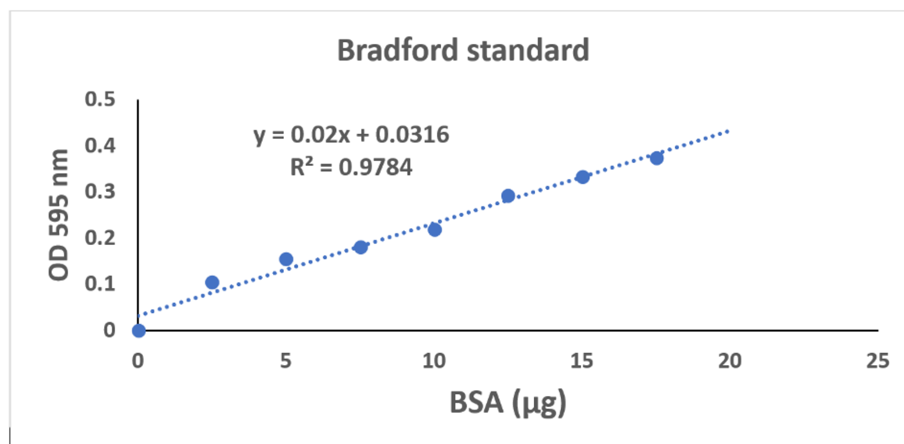

Fig. 6. Bradford Standard Curve, the standard diagram is drawn based on BSA.

Western blot analysis and ELISA assay

Recombinant ocriplasmin protein was purified with the NTA-Ni Sepharose, NTA-Ni affinity column and using equilibrium buffers, washing buffer and separation buffer. Then, SDS-PAGE was performed on 12% acrylamide gel for purified recombinant protein and non-transgenic samples as controls, followed by Coomassie Brilliant Blue R-250 staining based on Laemmli's method (Laemmli 1970). In the electrophoresis, Ocriplasmin recombinant protein with an approximate weight of 27 kilodaltons was observed (Fig. 7), which confirms the

presence of the recombinant protein in the hairy roots. In order to check and ensure the production of recombinant protein containing the signal sequence (His)6 of the hairy root lines that were confirmed in the SDS-PAGE electrophoresis, the ELISA test, analyzed using His-tag antibody, showed that recombinant protein contained (His)6 signal sequence. Also, western blotting test was performed using His-tag antibody. It showed that recombinant protein is produced in the hairy roots (Fig. 8) and the expected bands of approximately 27 kilodaltons for ocriplasmin recombinant protein were observed.

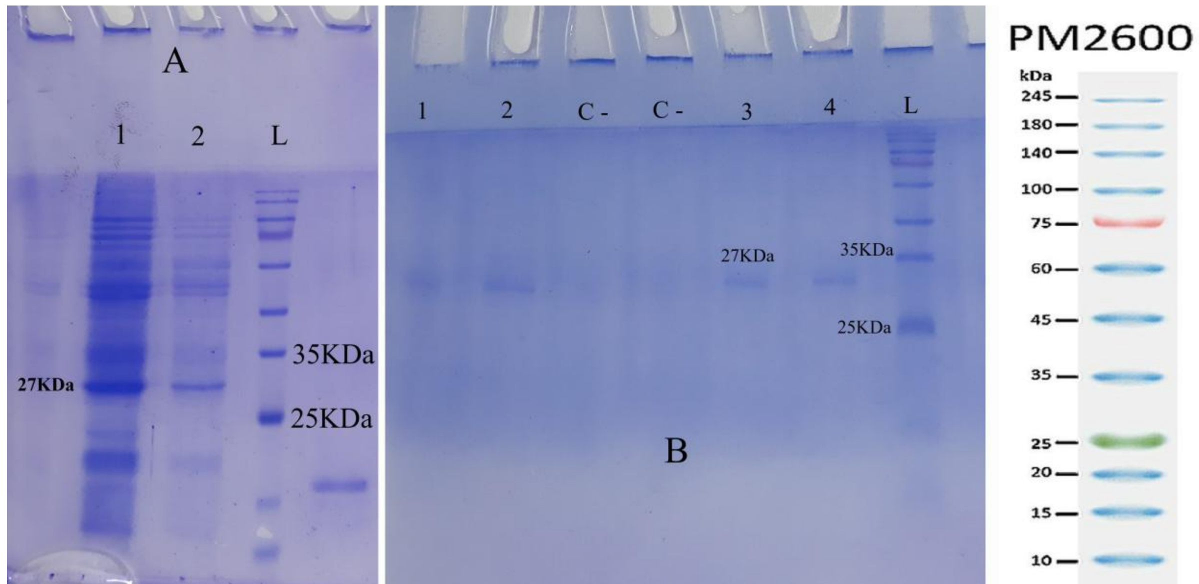

Fig. 7. SDS-PAGE analysis of total extracted proteins (A); The recombinant protein was purified using a Ni-NTA column and analyzed by SDS-PAGE (B); Protein Marker PM2600 Smobio has been used.

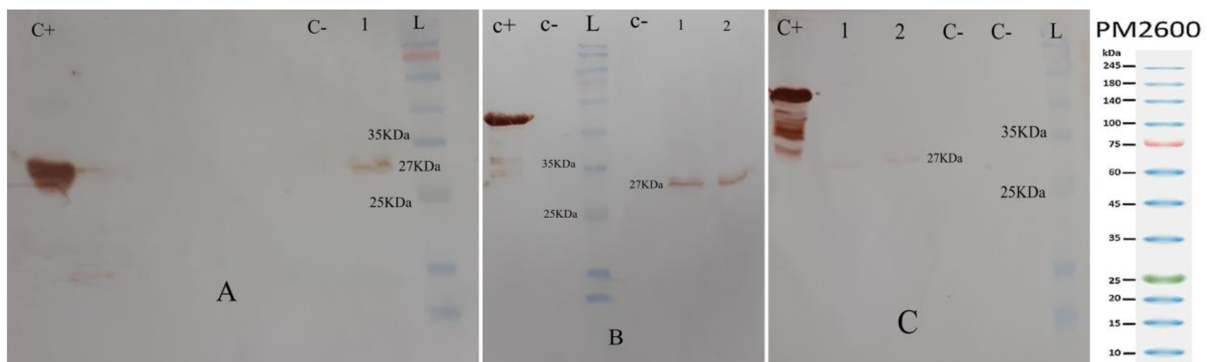

Fig. 8. Western blot analysis using monoclonal antibodies was used to identify recombinant proteins and peptides in transgenic tobacco plants. The presence of the 27-kDa fragment of ocriplasmin was accurately confirmed. A, B and C for three different *A. rhizogenes* strains, MSU440, A4 and ATCC15834 respectively. Protein Marker PM2600 Smobio has been used.

DISCUSSION

A novel technique for producing pharmaceuticals called "molecular farming" enables the industrial mass production of beneficial recombinant proteins in genetically modified organisms (Bharathi *et al.* 2024; Mirmazloum *et al.* 2024). Protein-based pharmaceuticals are rising in significance because of a diversity of factors, including their bioreactivity, precision, safety, and efficacy rate. Heterologous expression methods for the manufacturing of pharmaceutical products have been formerly employed using yeast, bacteria, and animal cells. However, the high expense of mammalian cell system, and production, the chance for product complexity, and contamination, and the hurdles of scaling up to commercial production are the limitations of these traditional expression methods (Moon *et al.* 2020; Kumari *et al.* 2022). Plants have been raised as a hopeful substitution system for the expression of biopharmaceutical products due to their potential benefits, which include low production costs, simplicity in scaling up to commercial manufacturing levels, and a lower threat of mammalian toxin contaminations and virus infections. Since plants are widely utilized as a source of therapeutic chemicals, molecular farming offers a unique way to generate molecular medicines such as recombinant antibodies, enzymes,

growth factors, plasma proteins, and vaccines whose molecular basis for use in therapy is well established (Fischer *et al.* 2020; Siridewa *et al.* 2021; Liu *et al.* 2022; Mirmazloum *et al.* 2024). So far, many studies have been conducted to achieve genetic transformation and stable production of proteins in plants. To achieve these goals, bacterial strains and various plant sources have been used (Bharathi *et al.* 2024). According to the results of this study, it can be said that the use of all three bacterial *A. rhizogenes* strains, MSU440, A4 and ATCC15834, has led to stable transformation and continuous protein production in hairy roots. The highest total protein concentration, was 1732 μg mL^{-1}. Also, the highest recombinant protein production was 61 μg mL^{-1}. The highest hairy root induction percentage (92%) was found in transformation protocol by *A. rhizogenes* strain ATCC15834. On the other hand, it has been determined that leaf explants of tobacco plants, in addition to their acceptable ability to create hairy roots, can be suitable sources for the production of ocriplasmin protein in plants. According to the obtained results, the recombinant ocriplasmin protein produced in this study did not suffer from damage such as hydrolysis and agglomeration during the extraction process using phosphate buffer and purification using NTA-Ni column. This is because the electrophoresis of the isolated protein had only one band with the characteristics of the produced protein. This is while in the process of production and extraction of recombinant proteins, events such as aggregation and enzymatic hydrolysis cause the failure to achieve the desired results (Liu *et al.* 2022). This result was also observed during the collection and detection of recombinant protein in ELISA and Western blot. Another point that can be obtained from the results of electrophoresis, ELISA and Western blot tests is that protein production using this method has led to the production of appropriate amounts of protein. High levels of protein expression in an expression system are considered one of the important factors in the production of recombinant protein. In general, it can be said that the ocriplasmin gene together with the 35S promoter and the NOS terminator effectively causes the expression of the ocriplasmin protein in *Nicotiana tabaccum* hairy roots.

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