

Shikonin and Luteolin Production in Hairy Root Culture of *Perilla frutescens* (L.)

Mahdi Mahmoudi

University of Tehran

Seyed Ahmad Sadat-Noori

Professor of Genetic and Plant Breeding, University of Tehran

Moslem Bahmankar

M.Bahmankar@areeo.ac.ir

Safiabad Agricultural Research and Education and Natural Resources Center, Agricultural Research, Education and Extension Organization (AREEO)

Mohsen Ebrahimi

University of Tehran

Ali Fadavi

University of Tehran

Article

Keywords: *Perilla frutescens* (L.), Transgenic hairy root, Rol genes, Bioreactors, HPLC analysis

Posted Date: August 6th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7080593/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Perilla frutescens (L.) Britton, a medicinal plant from the Lamiaceae family, was investigated for hairy roots induction and development of through two distinct experiments. The first experiment's initial phase examined the effects of explant type (leaf, cotyledon, hypocotyl, and stem internode) and co-cultivation duration (48 and 72 hours) on the efficiency of hairy root induction. The findings showed that the highest transformation efficiency, 66%, was attained by cotyledon explants co-cultivated for 48 hours.

Agrobacterium rhizogenes strains (A4, ATCC 15834), explant age (7, 14, and 21 days old), and two medium types (MS and MS/2) were all optimized in the second phase. The results suggested that cotyledon explants with the ATCC15834 strain in MS/2 medium yielded the highest transformation efficiency, root count per explant, shortest rooting time, and maximum secondary hairy root development. In the second experiment, the impact of light on hairy root development was evaluated both on shaker incubators and FA-Bio bioreactor. Results showed that flask and light treatments produced the least biomass (4.14 ± 0.5 gr), while bioreactor and darkness treatments resulted in the highest biomass (20.96 ± 0.61 gr). PCR analysis confirmed the integration of *rolA*, *rolB*, and *rolC* genes in transgenic hairy roots, validating successful T-DNA integration. According to HPLC result, hairy roots had similar amounts of luteolin and higher concentrations of shikonin than perilla leaves, indicating that hairy root cultures are a good substitute for producing these important compounds. This study highlights the potential of cultured hairy roots as an effective method for producing essential secondary metabolites in *Perilla frutescens*.

Introduction

Perilla frutescens (L.) Britton is a medicinal plant and member of the Lamiaceae family. This fragrant, bushy annual species is grown for commercial purposes in East Asia especially in China and Japan^{1,2}. There are several uses for *Perilla frutescens* (L.), such as using the seeds as food for humans, animals, or birds; using the oil from the seeds as fuel, cooking oil, or frying oil; using the leaves as medicine, food coloring, or as an herb in pots; and distilling the leaves to extract essential oil for flavoring³. *Perilla* is known to have numerous medicinal properties, including anti-allergy, antiseptic, anti-viral, bacterial and fungal infections, anti-HIV-1 activity, anti-spasmodic, anti-tussive and sedative^{4,5}. *Perilla* contains two important secondary metabolites: luteolin and shikonin. Shikonin causes cancer cells to undergo apoptosis, necrosis, and senescence and possesses antibacterial and antioxidant qualities⁶⁻⁸. Luteolin directly inhibits viruses such respiratory syncytial virus, influenza virus, and coronavirus. It provides protective effects on several organ systems by lowering pulmonary inflammation and edema^{9,10}. Because of its anticancer qualities, luteolin has been investigated as an adjuvant in antitumor therapy¹¹. Depending on the plant type, growing some medicinal plants involves a lot of time and traditional farming procedures. Furthermore, a number of variables, such as environmental circumstances and pathogen attack, affect the rate at which secondary metabolites are created. Certain secondary metabolites are not produced by undifferentiated cells; instead, they are made by certain cells or at a particular stage of plant growth. Cell suspension culture has been used mainly for this purpose,

replacing traditional agricultural methods in the production of secondary metabolites. The cultivation of hairy roots has therefore piqued the interest of researchers^{4,12}. Because of their rapid growth and genetic stability, hairy root cultures made by *Agrobacterium rhizogenes* offer a major advantage over cell suspension cultures for the large-scale production of these therapeutic compounds^{13,14}. Optimizing the induction of hairy roots in the target plant is the beginning of exploiting its benefits. Age of the tissue, bacterial strains, co-cultivation time, type of contamination, culture media composition, and various plant species all affect *A. rhizogenes* transformation effectiveness¹⁵. Optimizing the hairy root culture medium's composition is necessary to boost secondary metabolite development and synthesis. Hairy root growth is impacted by the kind and quantity of nutrients present in the culture media^{16,17}. Hairy roots resulting from *Agrobacterium* inoculation have a strong potential for producing secondary metabolites and grow noticeably faster than the surrounding tissues. The insertion of a portion of the bacterial vector genome, called T-DNA, into its host plant genome is the main factor responsible for the rapid growth of these roots. Because this DNA comprises the *rolA*, *rolB*, and *rolC* gene loci as well as genes related to the synthesis of hormones like auxin, it causes root transformation and the appearance of hairy root symptoms at the location of plant contamination with *Agrobacterium*^{18,19}. Bioreactors enable the mass production of hairy root cultures, which is necessary for the commercialization of secondary metabolite synthesis. By offering regulated and suitable conditions for their growth, bioreactors can significantly accelerate the growth of hairy roots^{20,21}. Better oxygen transmission, continuous nutrient supply, continuous administration of nutrients and growth regulators, enhanced medium control (pH, temperature, agitation modulation), more uniform growth, and large-scale hairy root development are only a few advantages of using bioreactors^{21,22}. Various bioreactor types can be adjusted to maximize the production of metabolites and hairy root biomass, frequently leading to better yields than shaking flasks^{13,23}. The temporary immersion systems bioreactor is one of the bioreactors used in hairy root research. It improves nutrient absorption and root growth by regularly immersing the roots in a nutrient solution^{24,25}. The impact of different parameters, including explant age, bacterial strain, co-culture time, and explant type, on the induction and growth of hairy roots has not been thoroughly examined, despite the importance of perilla. Along with the aforementioned, the current study was to investigate the metabolites of luteolin and shikonin in hairy roots as well as to create and enhance hairy roots using temporary immersion systems.

Results and discussion

As far as we are aware, no thorough reports on perilla hairy roots similar to ours have been published. Consequently, there was no all-inclusive technique for perilla hairy root production and proliferation. We were able to create an appropriate technique for growing perilla hairy roots by examining multiple approaches and utilizing a bioreactor.

Molecular analysis of transgenic hairy roots

It is essential to affirm the transgenic nature of roots using the PCR method, even though the specific morphology and developing traits of roots that had been actuated by *Agrobacterium rhizogenes* within the hormone-free medium almost proved the transmission of T-DNA into the genome of host cells and the transformed nature of these roots. In spite of the fact that the *rol B* gene is vital for the advancement of hairy roots, the *rol A* and *rol C* qualities as well as other open reading frames (ORFs) upgrade the creation of hairy roots²⁶. Greater development and appropriate branching of hairy roots are the results of these three genes' combined existence in the transgenic hairy root's genome and their expression^{27,28}. In the present study, PCR testing was able to detect the presence of genes effective in hairy root induction, including *rol A*, *rol B*, and *rol C* (Fig. 1).

To confirm that the hairy root tissue was free of *Agrobacterium*, PCR was used to find the *virD* gene outside the T DNA region, which is often not transposable into the plant cell. The need of amplification of this gene within the PCR consider affirms the transgenic nature of the hairy roots and the nonattendance of bacterial contamination¹⁵. Consequently, the use of complex and expensive analysis methods like southern blotting is not required; nonetheless, it might be required to ascertain the quantity of inserted copies of *rol* genes²⁹. The PCR outcome indicated that the transgenic roots treated with *Agrobacterium rhizogenes* had the expected DNA segments for the *rol A*, *rol B*, and *rol C* genes. This illustrates the effective addition of the T-DNA parcel of the bacteria's plasmid into the hairy roots' DNA. No amplification was detected in DNA samples taken from non-transgenic roots (Fig. 1).

Impact of explant type and co-cultivation duration on transformation efficiency

Since the bacterial strain and explant interaction effect on the days to hairy root induction was significant, the mean interaction effect of the aforementioned factors was examined using the slicing technique (Table 3) (Fig. 2). There were significant differences ($P < 0.01$) in the number of days required for each treatment to induce hairy roots (Table 3). The cotyledon explants that were 48 hours of co-cultivation period had the lowest days to rooting (7.33). The treatment combination of hypocotyl and 24 hours of co-cultivation had the longest number of days to rooting induction, followed by the internodal explant over the same co-cultivation period (Table 3). While the main effects of the bacterial strain and explant were significant in the current investigation, their interaction effect on transformation efficiency was not. Consequently, an evaluation was conducted to compare the mean main effect of the two factors (Table 3). The cotyledon explant that received 48 hours of co-cultivation had the highest transformation efficiency, which varied from 0.125 to 0.66. At 72 hours, the cotyledon explant's transformation efficiency was 0.41 (Table 3). All through the co-cultivation period, the induction of hairy roots with the hypocotyl and stem internode explants had the least success rate. Furthermore, they grew slowly. Other researchers have used leaf explants to produce hairy roots in perilla with *A. rhizogenes*¹⁷. Our study revealed that compared to other explants, cotyledon and leaf explants were more effective at genetic transformation (Fig. 2, Table 3). Various explants exhibit distinct responses to bacterial transformation, leading to variability in the effectiveness of the transformation process. The hypersensitive reaction, a plant defense mechanism, may have been used by the cell walls of the

hypocotyl and internodal explants in response to damage. Through necrosis of tissue, cellular death, and the buildup of antimicrobial chemicals, this mechanism stopped their cells from undergoing more cell transformation^{30,31}. Following injury, the vascular system in the hypocotyl and stem internode explants would have been disturbed, which would have reduced transformation success and increased the induction of day-to roots^{32,33}. T-DNA transport into the cell genomes requires cell-bacterial cooperation, and bacteria use chemotaxis to find the right plant cells.

Researchers assert that the specific type of cell wall receptors is essential for the subsequent entry of bacteria into the plant cell³⁴. During the 24-hour co-culture phase, the majority of the explants in the current study displayed the lowest percentage of hairy root induction (Table 3). The bacterial plasmid's T-DNA portion is thought to be carried into the plant's cores amid the transformation method utilizing *A. rhizogenes*, where it blends with the have plant's genome. Subsequently, a vital calculate in surveying the transformation's efficacy is the term of the co-culture stage^{35,36}.

Table 3
Comparison of the mean interaction of explant type and co-culture duration on perilla hairy root induction

Explant Type	Co-culture	Days to Rooting	Transformation Efficiency
Hypocotyl	24	17 ± 0.85 a (a)	0.125 ± 0 c (c)
	48	14 ± 0.85 cd (c)	0.25 ± 0 bc (a)
	72	15.66 ± 0.49 abc (b)	0.16 ± 0.06 bc (b)
Cotyledon	24	8.33 ± 0.49 gh (b)	0.375 ± 0.106 bc (b)
	48	7.33 ± 0.49 h (c)	0.66 ± 0.061 a (a)
	72	9.66 ± 0.49 fg (a)	0.416 ± 0.061 ab (b)
Leaf	24	12.67 ± 0.49 de (a)	0.208 ± 0.061bc (b)
	48	8.66 ± 0.49 gh (c)	0.416 ± 0.061ab (a)
	72	11.33 ± 0.49 ef (b)	0.25 ± 0.1bc (b)
Stem Internode	24	14.33 ± 0.49 bcd (b)	0.125 ± 0.0 c (b)
	48	9.33 ± 0.49 fgh (c)	0.291 ± 0.12 bc (a)
	72	16.33 ± 0.49 ab (a)	0.208 ± 0.06 bc (ab)

According to the LSD test, numbers that have the same letter in each column do not differ significantly at the 5% level. Comparisons of means by the slicing method are indicated by letters inside parentheses, whereas comparisons of means for overall interaction effects are shown by letters outside parentheses.

Impact of bacterial strain, medium type, and explant age on transformation efficiency

The first experiment's second phase was conducted following the determination of the optimal explant (cotyledon) and co-cultivation length (48 hours). Diverse strains of *A. rhizogenes* were developed in cotyledon explants of distinctive ages (14, 21, and 28-day-old) employing an assortment of mediums in order to decide the leading combinatorial treatment for transformation success. The data indicated significant differences among the various treatments regarding transformation efficiency, average hairy root count, secondary branch count, and rooting duration ($P < 0.01$) (Table 4). The data indicate that, although the factors in the study had a significant main influence on the number of days to rooting, their interaction effect was not significant (Table 4). Figure 3 (a), (b), and (c) illustrates that the treatments employing the 14-day-old cotyledon, ATCC 15834 strain, and MS/2 media exhibited the shortest duration for rooting induction. Transformation is strongly influenced by the explant's age; tissues with younger cells have fewer vacuoles and a greater potential for transformation^{16,37}. Within this examination, root emergence in 14-day-old explants was speedier than other treatments (Fig. 3).

Table 4

Analysis of variance of the studied factors including explant age, bacterial strain, and medium type on day to rooting, transformation efficiency, average count of hairy roots, and secondary branches count.

S.O. V	df	Day to Rooting	Transformation Efficiency	Average Count of Hairy Roots	Secondary Branches Count
Explant age	2	**	**	**	**
Bacterial Strain	1	**	**	**	**
Medium Type	1	**	**	**	**
Explant age * Bacterial Strain	2	ns	**	ns	*
Explant age * Medium Type	2	ns	ns	ns	ns
Bacterial Strain * Medium Type	1	ns	ns	*	ns
Explant age * Bacterial Strain* Medium Type	2	ns	ns	ns	ns
Error	24	-	-	-	-
Total	35	-	-	-	-

ns not significant. *, and **Significant at $p < 0.05$ and $p < 0.01$ respectively.

The only significant double interaction effect on transformation efficiency was the interaction between bacterial strain and explant age; the triple interaction effect of the parameters under investigation was not significant (Table 4). Meanwhile, the transformation efficiency was significantly impacted by the medium type's main effect (Fig. 3 (d)). According to Fig. 4 (a), the transformation efficiency varied from 0.125 for the 28- day-old cotyledon and A4 strain to 0.48 for the 14-day- old cotyledon and ATCC 15384

strain. Important parameters such as the type of explant, the age of the explant, and the used strain of bacterial affect the percentage of transgenesis and transformation^{38,39}. Due to differences in their cell wall structures, physiological conditions, and molecular markers, different plant tissues may have varying capacities for transformation^{40,41}. The *Agrobacterium* strains in the current research confirmed differing talents to switch genes to plant cells, with strain ATCC 15834 showing the highest transformation efficiency. The wild-kind *A. rhizogenes* strain ATCC 15834 contains the agropine-type plasmid pRi 15834. This plasmid has two T-DNA areas, TR-DNA and TL-DN, which input the plant independently after being separated via more or less 24 kilobases of non-transferred DNA. The TL-DNA is about 20 kilobases long and contains at least 18 open reading frames (ORF).

Rol A, *rol B*, *rol C*, and *rol D* are represented via ORFs 10, 11, 12, and 15, respectively^{42,43}. The wild-kind strain of *Agrobacterium* ATCC 15834, of the agropine kind, has 18 repeats of T-DNA switch stimulator sequences (TSS) on its Ri plasmid.

It is anticipated that this strain will exhibit strong transgenic performance due to the function of the TSS region in T-DNA transfer⁴⁴. Among the double interaction effects, only the interaction between medium type and bacterial strain was significant, while the triple interaction of the components examined had no significant impact on the average count of hairy roots (Table 4). The main effect of explant age significantly influenced on the average count of hairy roots (Fig. 3 (e)). Figure 4 (b) shows that the ATCC 15384 strain and MS/2 medium type produced the highest hairy roots (10.33 ± 1.58). Figure 4 (b) shows that the combination of the A4 strain and MS medium type treatment produced the lowest count of hairy roots (3.77 ± 1.39). The triple interaction effect of the parameters under investigation on the count of secondary branches was not significant, and the only significant double interaction effect was that between bacterial strain and explant age (Table 4). As shown in Fig. 4 (c), the count of secondary branches of hairy roots varied during the treatment, ranging from 3.66 (21-day-old cotyledon and A 4 strain) to 6 (14-day-old cotyledon and ATCC 15384 strain) (Fig. 4 (c) and Fig. 5).

Additionally, the main effect of medium type had a significant impact on the count of secondary branches (Table 4) and varied from (3.5 ± 0.98) in MS to (5.5 ± 1.29) in MS/2 (Fig. 3 (f)). Since there were more secondary branches and hairy roots per explant in the $\frac{1}{2}$ MS media than in the MS medium, confirming the impact of ammonium ion concentration on hairy root formation. Numerous studies have demonstrated that a high ammonium concentration in the MS culture medium lowers the pH of the medium, it inhibits and reduces growth^{45,46}. This variation in transformation and morphology can be attributed to a variety of things, such as the variety of added T-DNA parts, the quantity of different replicas of added T-DNA, the location of the T-DNA in the genome of the plant, the plasmids embedded within the bacterial strain, and the variable T-DNA gene expression of the transgenic roots³⁴. Inner auxin has a specific effect on hairy root growth, lush branching, and halting linear increase due to the fact the aux genes of TR-DNA that are gift inside the plasmid can also result in expanded auxin production⁴⁴. In general, the cotyledon explants, ATCC15834, and MS/2 culture media exhibited the highest

transformation efficiency, the highest count of roots per explant, the lowest day to rooting, and the highest secondary of the hairy root.

Comparison of Hairy root Biomass in Bioreactor and Shake Flasks

To maximize biomass and enhance culture situations, the best developed hairy root line selected from the section earlier changed into used on this portion of the experiment. For this purpose, the interaction effect of two factors including light (light or dark) and the growth environment (shake flasks and bioreactor) was investigated. The outcomes cautioned that the biomass of hairy roots turned into extensively impacted by using the aggregate of light conditions and growth environment (Fig. 6). Combining the bioreactor and darkness treatments resulted in the maximum quantity of root biomass (20.96 ± 0.61 gr), whereas the flask and light treatments produced the lowest amount of biomass (4.14 ± 0.5 gr) (Table 5).

Different researchers reported that extra growth and density of hairy roots have been discovered in dark than light conditions⁴⁷. In contrast to darkness, light can encourage root growth by creating sugars and auxins in the plant's aerial portions and also has a favorable impact on biomass; however, this effect can occasionally be reversed if sucrose is present in the culture medium⁴⁸. Under dark conditions, hairy roots produced more biomass and were white. Hairy root cultures in light conditions, on the other hand, had less biomass and started to turn pale green. Given that hairy root performance has improved when bioreactors are used, it can be said that using bioreactors has increased hairy root growth by creating conditions that are suitable and controlled (Fig. 6). Improved oxygen transfer, better culture medium control, uniform hairy root growth and development, and a steady supply of nutrients and growth regulators are some advantages of employing bioreactors to speed up and boost hairy root growth ^{21,22}.

Table 5
Mean comparison of the studied factors including propagation chamber type, and Lighting status on FGI index

Culture condition	Lighting status	FGI (gr)
Bioreactor	Dark	20.96 ± 0.61 a (a)
	Light	14.77 ± 0.7 b (b)
Shaker	Dark	7.16 ± 0.24 c (a)
	Light	4.14 ± 0.5 c (b)

The similar letters in the FGI column are not statistically significant.

According to the LSD test, numbers that same a letter in the FGI column do not differ significantly at the 5% level. Comparisons of means for overall interaction effects are indicated by letters outside parentheses, while comparisons of means by slicing method are indicated by letters inside parentheses.

Shikonin, and Luteolin production in hairy roots of *Perilla frutescens* (L.)

Hairy root culture is a feasible alternative strategy for synthesizing medicinal metabolites in plants since it can produce important secondary metabolites in a shorter time (Jakovljevic et al., 2023). Hairy roots from cotyledon explants inoculated with strain ATCC-15834 and perilla leaves grown in a greenhouse were used in this study to assess the levels of luteolin and shikonin using HPLC. The findings indicated that the shikonin-associated peak was seen in transgenic hairy roots at a wavelength of 269 nm at 5.42 minutes and in leaf at 256 nm at 6.5 minutes (Fig. 7). The study's findings found out that the amount of shikonin in hairy roots was more than twice that of the leaf, with dried hairy root powder having a concentration of 0.54 mg/gr and dry leaf powder having a concentration of 0.21 mg/gr (Fig. 8). The results also showed that the peak related to luteolin was observed in hairy roots at wavelengths 370 nm and minutes 2.08 and in leaf at wavelengths 345 nm and minutes 2.62 (Fig. 7). The findings demonstrated that there was little difference in the amount of luteolin in the hairy root and the leaf, with the hairy root having 21 mg/gr and the leaf having 16 mg/gr (Fig. 8).

The current study's results support the belief held by researchers that if a particular metabolite is produced in a plant's aerial tissues, it can also be produced in hairy roots⁵⁰.

According to this study, the perilla plant's hairy roots may be a good choice for shikonin production because shikonin has been found in them. It is important to note that there has only been one study on shikonin in perillas⁸, and that study only included a few information. Although this is one of the earliest reports of shikonin's existence in the hairy roots of the perilla plant, the study's findings shed additional light on the compound's occurrence in perillas. The current study compared the quantity of shikonin and luteolin in the leaves and hairy roots, in addition to identifying it in the hairy roots. It should be mentioned that several medicinal plants have been found to be able to create secondary metabolites through the cultivation of hairy roots^{50–52}. Secondary metabolite formation in plants has been the subject of several investigations, and the extraction of these molecules from plant tissues under natural conditions has proven difficult due to factors like growing season limitations, environmental stresses, and species extinction⁵³. Consequently, the cultivation of hairy roots in medicinal plants, like perilla, can be regarded as a great substitute for the synthesis of therapeutic secondary metabolites.

Conclusion

This study successfully optimized the induction and growth of hairy roots in *Perilla frutescens*, setting the stage for more efficient production of valuable secondary metabolites. By carefully testing different *Agrobacterium rhizogenes* strains, explant types, and growth conditions, established a reliable protocol for cultivating hairy roots. The present study indicated that the cotyledon explants inoculated with ATCC15834 strain that were cultivated in MS/2 media exhibited the most secondary hairy root development, the maximum transformation efficiency, the shortest rooting time, and the largest root count per explant. The biomass yield was considerably higher using a temporary immersion bioreactor in a dark condition than using a shaker incubator. The current study is one of the few that has examined

luteolin and shikonin in perilla hairy roots. It suggests that luteolin concentrations in hairy roots were comparable to those in normal plants, while shikonin levels were greater. The potential of *P. frutescens* hairy roots as a source of shikonin production is highlighted by this finding. The current study's findings also provide a basis for future research that aims to investigate different approaches for employing hairy root cultures in a bioreactor to increase the yield of shikonin and other important metabolites.

Material and Methods

Plant materials, Surface Sterilization, and In vitro Germination

In this study, the seeds of *Perilla frutescens* var. *crispa* were subjected to a sterilization process to ensure a clean starting point for cultivation. Initially, the seeds were immersed in 70% ethanol for one minute, followed by a ten-minute immersion in 5% sodium hypochlorite⁵⁴. Following three rounds of sterilized distilled water washing, they were cultivated on basic MS medium supplemented with sucrose (3 %W/V) and agar (0.7 %W/V) without the addition of hormones. After that, they were maintained at $24 \pm 2^{\circ}\text{C}$ with a 16/8-hour light/dark photoperiod. The leaf, cotyledon, hypocotyl, and stem internode of the 8-day-old seedlings were collected and utilized as explant in order to induce root growth.

Designing experiments to induce hairy roots and optimization of growth condition

A. rhizogenes transformation and growth were optimized in two different investigations to encourage the induction and growth of hairy roots in *Perilla frutescens* (L.) Britton (Fig. 9). The two conducted experiments are mentioned below.

First experiment: Optimization of hairy root induction

The first effort to improve genetic transformation mediated by *Agrobacterium* was conducted in two stages: In the first phase, two parameters were optimized using *A. rhizogenes* ATCC15834 in *Perilla frutescens* (L.) Britton: (1) explant types (hypocotyl, cotyledon, leaf, stem internode) and co-culture periods (24, 48 and 72 hours). Hypocotyl, cotyledon, leaf, and internodal explants have been used as explants. Small wounds were made to the explant using a sterile scalpel blade. After that, for ten minutes, these slices were co-cultured in bacterial suspension. As controls, some explants were infected with sterile distilled water rather than bacterial suspension (under the same circumstances). After the infection turned into dealt with as described above, the inflamed tissues had been transferred on filter out paper to put off any ultimate germs. Immediately afterwards, the explants were placed on solid culture medium. Co-culture periods included 24, 48, and 72 hours. Taking after three washing steps with sterile water, the explants were exchanged to MS strong culture medium containing 200 mg/L cefotaxime. Petri dishes were set in a growth chamber with a 16-hour photoperiod and a light intensity of 3000 LUX at a temperature of $24 \pm 2^{\circ}\text{C}$ to induce hairy roots. The transformation efficiency was calculated based on the percentage of transgenic explants producing hairy roots. To ensure hairy roots free of bacteria, they were subculture onto a MS medium with cefotaxime antibiotics every two weeks. Within the second stage, in order to decide the foremost compelling method of hairy root induction, three

parameters were optimized: (1) explant age (7, 14, and 21 days-old); (2) *A. rhizogenes* (A4, ATCC 15834); and (3) media (MS or MS/2).

Second experiment: Optimization of the Growing Condition

To optimize biomass production, the culture conditions were improved using the hairy root line that grew the fastest, which was selected from the previous stage. The treatments were as follows: (1) Type of growth system were including shaker incubators and bioreactors; and (2) presence or absence of light. FA-Bio bioreactor, a temporary bioreactor similar to RITA® bioreactor, was fabricated in the Department of Food Science and Technology, the details explained somewhere else⁵⁵. The FA-Bio bioreactor device was programmed for 8 sec min of immersion followed by 4 hr of aeration.

Molecular Characterization of Hairy roots by Polymerase Chain Reaction (PCR)

DNA was extracted from the putative transgenic roots utilizing the CTAB technique to confirm the presence of the Ri T-DNA⁵⁶. The *A. rhizogenes* plasmid was utilized as positive control tests, though typical roots that had not experienced change were utilized as negative control tests.

To recognize the *rol A*, *rol B*, *rol C*, and *Vir D* genes of *A. rhizogenes* within the hairy roots, the consequent primer sets were utilized for PCR. (Table 1 and Table 2). Ethidium bromide staining and electrophoresis on agarose (1.5% w/v) were used to analyze the PCR amplicons.

Table 1
The primer sequence utilized to identify the target genes

Gene	Forward sequence	Reverse sequence
<i>rol A</i>	5'-ACGGTGAGTGTGGTTGTAGG - 3'	5'-GCCACGTGCGTATTAATCCC-3'
<i>rol B</i>	5'-ATGGATCCCAAATTGCTATTCCTTCCACGA-3'	5'-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3'
<i>rol C</i>	5'-CTCCTGACATCAAACGTC-3'	5'-TGCTTCGAGTTATGGGTACA-3'
<i>Vir D</i>	5-TGTGCGCAAGGCAGTAAGCCC - 3	5-GGAGTCTTTCAGCATGGAGCAA - 3

Table 2
lists the PCR reaction parameters used to affirm the *rol A*, *rol B*, *rol C*, and *Vir D* genes in *P. frutescens* hairy root culture

PCR Step	Temperature (°C)				Time	Number of cycles
	<i>rol A</i>	<i>rol B</i>	<i>rol C</i>	<i>Vir D</i>		
Initial denaturation	94	94	94	94	5 min	1
Denaturation	92	94	92	92	1 min	35
Annealing	54	56	54	54	1 min	35
Extension	72	72	72	72	2 min	35
Final extension	72	72	72	72	7 min	35

Detection of Shikonin and Luteolin in hairy roots by High-Performance Liquid Chromatography (HPLC)

Shikonin and luteolin content was measured in derived hairy roots of cotyledon explants induced by ATCC 15834 strain which had produced more biomass and the normal root of the perilla that was grown under greenhouse conditions. 0.1 grams of dried hairy roots were chopped to tiny particles and extracted for this purpose using the method outlined by researchers⁵⁷, and then the products of extracted samples were filtered using a 0.45-µm PTFE syringe filter for analysis by high-performance liquid chromatography system (KNAUER company, HPLC PUMP K-1001) equipped with a UV detector (number: RIFR-WI-PHYT-29). Subsequently, the shikonin and luteolin content was estimated in 100 gr of a sample.

Growth Rate

Using fresh weight measurement, the growth of hairy roots was detected. Sterile paper towels were used to remove excess moisture from the hairy roots. The following equation was used to measure the growth rate of hairy roots:

$$FGI = (\text{Final fresh weight of hairy root biomass} - \text{Initial fresh weight of inoculum}) / \text{Initial fresh weight of inoculum}.$$

Declarations

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Funding

No funding.

Acknowledgements

We appreciate the Agronomy and Plant Breeding Department of Tehran University for their Comprehensive Support in this research.

Author contributions

M.M. Conducting experiments, Data curation, Drafting the manuscript, S.A.S.N: Supervise the execution of experiments and, Guide the project, Editing the manuscript, M.B: Consultant in the project, Statistical analysis and Writing the final version of manuscript, M.E: Guide the project, A.F.: Bioreactor designer, Editing the manuscript, All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

References

1. Zhou, Y. *et al.* Genome-Wide Analysis of Glycerol-3-Phosphate Acyltransferase (GPAT) Family in *Perilla frutescens* and Functional Characterization of PfGPAT9 Crucial for Biosynthesis of Storage Oils Rich in High-Value Lipids. *Int J Mol Sci* **24**, (2023).
2. Ma, S. J. & Lee, J. K. Morphological variation of *Perilla* crop and their weedy types from northern and southern areas of China. *Genetics and Molecular Research* **16**, (2017).
3. Wu, X. *et al.* *Perilla frutescens*: A traditional medicine and food homologous plant. *Chinese Herbal Medicines* vol. 15 Preprint at <https://doi.org/10.1016/j.chmed.2023.03.002> (2023).
4. Zhu, Y. *et al.* Plant hairy roots: Induction, applications, limitations and prospects. *Ind Crops Prod* **219**, 119104 (2024).
5. Wu, X. *et al.* *Perilla frutescens*: A traditional medicine and food homologous plant. *Chin Herb Med* **15**, 369–375 (2023).
6. Dissook, S. *et al.* Luteolin-rich fraction from *Perilla frutescens* seed meal inhibits spike glycoprotein S1 of SARS-CoV-2-induced NLRP3 inflammasome lung cell inflammation via regulation of JAK1/STAT3 pathway: A potential anti-inflammatory compound against inflammation-induced long-COVID. *Front Med (Lausanne)* **9**, (2023).
7. Aochen, C. *et al.* *Perilla frutescens* L.: a dynamic food crop worthy of future challenges. *Frontiers in Nutrition* vol. 10 Preprint at <https://doi.org/10.3389/fnut.2023.1130927> (2023).
8. Zhong, J.-J., Tatsuji, S., Kinoshita, S. & Toshiomi, Y. Production of Red Pigments by *Perilla Frutescens* Cells in Bioreactors. in *Biochemical Engineering for 2001* (1992). doi:10.1007/978-4-431-68180-9_70.
9. Jeon, I. H. *et al.* Anti-inflammatory and antipruritic effects of luteolin from *perilla* (*P. frutescens* L.) leaves. *Molecules* **19**, (2014).

10. Li, W. F. *et al.* Application Potential of Luteolin in the Treatment of Viral Pneumonia. *Journal of Food Biochemistry* vol. 2023 Preprint at <https://doi.org/10.1155/2023/1810503> (2023).
11. Taheri, Y. *et al.* Paving Luteolin Therapeutic Potentialities and Agro-Food-Pharma Applications: Emphasis on in Vivo Pharmacological Effects and Bioavailability Traits. *Oxidative Medicine and Cellular Longevity* vol. 2021 Preprint at <https://doi.org/10.1155/2021/1987588> (2021).
12. Baque, M. A., Moh, S. H., Lee, E. J., Zhong, J. J. & Paek, K. Y. Production of biomass and useful compounds from adventitious roots of high-value added medicinal plants using bioreactor. *Biotechnology Advances* vol. 30 Preprint at <https://doi.org/10.1016/j.biotechadv.2011.11.004> (2012).
13. Khan, S. A., Siddiqui, M. H. & Osama, K. Bioreactors for Hairy Roots Culture: A Review. *Curr Biotechnol* **7**, (2019).
14. Korde, V. V., Dhas, S. S. & Gurave, N. A. Hairy Root Culture: A Promising Approach in Biotransformation. *Pelagia Research Library Asian Journal of Plant Science and Research* **6**, (2016).
15. Zheleznichenko, T. *et al.* Impact of different *Agrobacterium rhizogenes* strains on secondary metabolites accumulation in *Nitraria schoberi* L. hairy roots and antiviral activity of their extracts against influenza virus of subtypes A (H5N1) and A (H3N2). *In Vitro Cellular & Developmental Biology - Plant* **59**, 378–392 (2023).
16. Amini, S., Fattahi, M. & Nazemiyeh, H. Optimization of induction and hairy root culture establishment in two mullein species, *Verbascum erianthum* and *Verbascum stachydiforme*. *Sci Rep* **14**, (2024).
17. Xu, J. *et al.* Establishment of hairy roots culture of *Perilla frutescens* L. and production of phenolic acids. *Medicinal Plant Biology* **4**, 0–0 (2025).
18. Shvets, D. Yu., Berezhneva, Z. A., Musin, Kh. G., Baimukhametova, E. A. & Kuluev, B. R. rol Genes of Agrobacteria: Possible Biological Functions. *Biology Bulletin Reviews* **13**, (2023).
19. Veremeichik, G. N. *et al.* Agropine-type rolA modulates ROS homeostasis in an auxin-dependent manner in rolA-expressing cell cultures of *Rubia cordifolia* L. *Planta* **261**, 20 (2024).
20. Thakore, D. & Srivastava, A. K. Mass Scale Hairy Root Cultivation of *Catharanthus roseus* in Bioreactor for Indole Alkaloid Production. in *Plant Cell and Tissue Differentiation and Secondary Metabolites: Fundamentals and Applications* (eds. Ramawat, K. G., Ekiert, H. M. & Goyal, S.) 487–502 (Springer International Publishing, Cham, 2021). doi:10.1007/978-3-030-30185-9_21.
21. Kozłowska, W. *et al.* Upscaling biomass production of rosmarinic acid-rich hairy root cultures of *Agastache rugosa* (Fisch. & C.A.Mey.) Kuntze. *Plant Cell Tissue Organ Cult* **156**, (2024).
22. Sivakumar, G., Liu, C., Towler, M. J. & Weathers, P. J. Biomass production of hairy roots of *Artemisia annua* and *Arachis hypogaea* in a scaled-up mist bioreactor. *Biotechnol Bioeng* **107**, 802–813 (2010).
23. Mehrotra, S., Mishra, S. & Srivastava, V. Bioreactor Technology for Hairy Roots Cultivation. in *Bioprocessing of Plant In Vitro Systems* (eds. Pavlov, A. & Bley, T.) 483–506 (Springer International Publishing, Cham, 2018). doi:10.1007/978-3-319-54600-1_10.

24. Mirzabe, A. H., Hajiahmad, A., Fadavi, A. & Rafiee, S. Temporary immersion systems (TISs): A comprehensive review. *Journal of Biotechnology* vol. 357 Preprint at <https://doi.org/10.1016/j.jbiotec.2022.08.003> (2022).
25. De Carlo, A., Tarraf, W., Lambardi, M. & Benelli, C. Temporary immersion system for production of biomass and bioactive compounds from medicinal plants. *Agronomy* **11**, 2414 (2021).
26. Veena, V. & Taylor, C. G. *Agrobacterium rhizogenes*: Recent developments and promising applications. *In Vitro Cellular and Developmental Biology - Plant* vol. 43 Preprint at <https://doi.org/10.1007/s11627-007-9096-8> (2007).
27. Ron, M. *et al.* Hairy root transformation using *Agrobacterium rhizogenes* as a tool for exploring cell type-specific gene expression and function using tomato as a model. *Plant Physiol* **166**, (2014).
28. Shakeran, Z., Keyhanfar, M., Asghari, G. & Ghanadian, M. Improvement of atropine production by different biotic and abiotic elicitors in hairy root cultures of *Datura metel*. *Turkish Journal of Biology* **39**, (2015).
29. Sudha, C. G. *et al.* *Agrobacterium rhizogenes* mediated transformation of the medicinal plant *Decalepis arayalpathra* and production of 2-hydroxy-4-methoxy benzaldehyde. *Plant Cell Tissue Organ Cult* **112**, (2013).
30. Asande, L. K., Omwoyo, R. O., Oduor, R. O. & Nyaboga, E. N. A simple and fast *Agrobacterium*-mediated transformation system for passion fruit KPF4 (*Passiflora edulis* f. *edulis* × *Passiflora edulis* f. *flavicarpa*). *Plant Methods* **16**, (2020).
31. Magdum, S. S. Effect of *Agrobacterium* Induced Necrosis, Antibiotic Induced Phytotoxicity and Other Factors in Successful Plant Transformation. *Journal of Stress Physiology & Biochemistry* **9**, (2013).
32. Liu, S. *et al.* *Agrobacterium rhizogenes*-mediated transformation of *Arachis hypogaea*: an efficient tool for functional study of genes. *Biotechnology and Biotechnological Equipment* **30**, (2016).
33. Saravanakumar, A., Aslam, A. & Shajahan, A. Development and optimization of hairy root culture systems in *Withania somnifera* (L.) Dunal for withaferin-A production. *Afr J Biotechnol* **11**, (2012).
34. Babich, O. *et al.* Modern trends in the in vitro production and use of callus, suspension cells and root cultures of medicinal plants. *Molecules* **25**, 5805 (2020).
35. Sarkar, J., Misra, A. & Banerjee, N. Genetic transfection, hairy root induction and solasodine accumulation in elicited hairy root clone of *Solanum erianthum* D. Don. *J Biotechnol* **323**, (2020).
36. Mohebodini, M., Fathi, R. & Mehri, N. Optimization of hairy root induction in chicory (*Cichorium intybus* L.) and effects of nanoparticles on secondary metabolites accumulation. *Iran J Genet Plant Breed* **6**, 60–68 (2017).
37. Ribas, A. F. *et al.* *Agrobacterium*-mediated genetic transformation of *Coffea arabica* (L.) is greatly enhanced by using established embryogenic callus cultures. *BMC Plant Biol* **11**, (2011).
38. Daspute, A. A. *et al.* *Agrobacterium rhizogenes*-mediated hairy roots transformation as a tool for exploring aluminum-responsive genes function. *Future Sci OA* **5**, (2019).

39. Samadi, A., Carapetian, J., Heidari, R., Jafari, M. & Gorttapeh, A. H. Hairy root induction in *Linum mucronatum* ssp. *mucronatum*, an anti-tumor lignans producing plant. *Not Bot Horti Agrobot Cluj Napoca* **40**, (2012).
40. Kuzovkina, I. N. & Schneider, B. Genetically transformed root cultures – generation, properties and application in plant sciences. in *Progress in Botany* (2006). doi:10.1007/3-540-27998-9_13.
41. Mota, A. P. Z. *et al.* Evolutionarily conserved plant genes responsive to root-knot nematodes identified by comparative genomics. *Molecular Genetics and Genomics* **295**, (2020).
42. White, F. F., Taylor, B. H., Huffman, G. A., Gordon, M. P. & Nester, E. W. Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. *J Bacteriol* **164**, (1985).
43. Kodahl, N., Müller, R. & Lütken, H. The *Agrobacterium rhizogenes* oncogenes rolB and ORF13 increase formation of generative shoots and induce dwarfism in *Arabidopsis thaliana* (L.) Heynh. *Plant Science* **252**, 22–29 (2016).
44. Christensen, B., Sriskandarajah, S., Serek, M. & Müller, R. Transformation of *Kalanchoe blossfeldiana* with rol-genes is useful in molecular breeding towards compact growth. *Plant Cell Rep* **27**, (2008).
45. Bensaddek, L., Gillet, F., Saucedo, J. E. N. & Fliniaux, M.-A. The effect of nitrate and ammonium concentrations on growth and alkaloid accumulation of *Atropa belladonna* hairy roots. *J Biotechnol* **85**, 35–40 (2001).
46. Yousefian, S. *et al.* Investigation of Nitrogen and Phosphate Effect on Growth and Rosmarinic Acid Production in Transgenic *Mentha aquatica* Hairy Root Induction. *Journal of Medicinal plants and By-products* **10**, 199–207 (2021).
47. Naderian, P., Moshtaghi, N., Bagheri, A. & Malekzadeh-Shafaroudi, S. Influence of different *Agrobacterium rhizogenes* strains on hairy roots induction and secondary metabolites production in *Datura innoxia* Mill. *Journal of Medicinal Plants* **21**, (2022).
48. Silva-Navas, J. *et al.* D-Root: A system for cultivating plants with the roots in darkness or under different light conditions. *Plant Journal* **84**, (2015).
49. Jakovljević, D., Skrzypek, E., Stanković, M. & Warchoń, M. Phytochemical Diversity and Biological Activity of Basil (*Ocimum* L.) Secondary Metabolites Produced In Vitro. in (2023). doi:10.1007/978-3-031-35221-8_16.
50. Chen, H. & Chen, F. Induction of phytoalexin formation in crown gall and hairy root culture of *Salvia miltiorrhiza* by methyl viologen. *Biotechnol Lett* **22**, (2000).
51. Lee, S. Y., Xu, H., Kim, Y. K. & Park, S. U. Rosmarinic acid production in hairy root cultures of *Agastache rugosa* Kuntze. *World J Microbiol Biotechnol* **24**, (2008).
52. Park, N. Il, Li, X., Uddin, M. R. & Park, S. U. Phenolic compound production by different morphological phenotypes in hairy root cultures of *Fagopyrum tataricum* gaertn. *Arch Biol Sci* **63**, (2011).
53. Lee, S. Y. *et al.* Growth and rutin production in hairy root cultures of buckwheat (*Fagopyrum esculentum* M.). *Prep Biochem Biotechnol* **37**, (2007).

54. Bahmankar, M., Rahn timer, H., Salehi, M. & Noori, S. A. S. Somatic embryogenesis and genetic fidelity in camelina by RAPD markers and flow cytometry. *Plant Cell, Tissue and Organ Culture (PCTOC)* **156**, 67 (2024).
55. Mohammadpour Barough, A., Dianati Daylami, S., Fadavi, A. & Vahdati, K. Enhancing Phalaenopsis orchid production: a comparative study of permanent and temporary immersion bioreactors. *In Vitro Cellular & Developmental Biology - Plant* **60**, 523–537 (2024).
56. Alizadeh, Z. *et al.* Assessment of Molecular Variation in Pea Germplasm Using RAPD Markers. *Electronic Journal of Biology Electronic J Biol* **12**, (2016).
57. Kwon, D. Y., Kim, Y. B., Kim, J. K. & Park, S. U. Production of rosmarinic acid and correlated gene expression in hairy root cultures of green and purple basil (*Ocimum basilicum* L.). *Prep Biochem Biotechnol* (2020) doi:10.1080/10826068.2020.1789990.

Figures

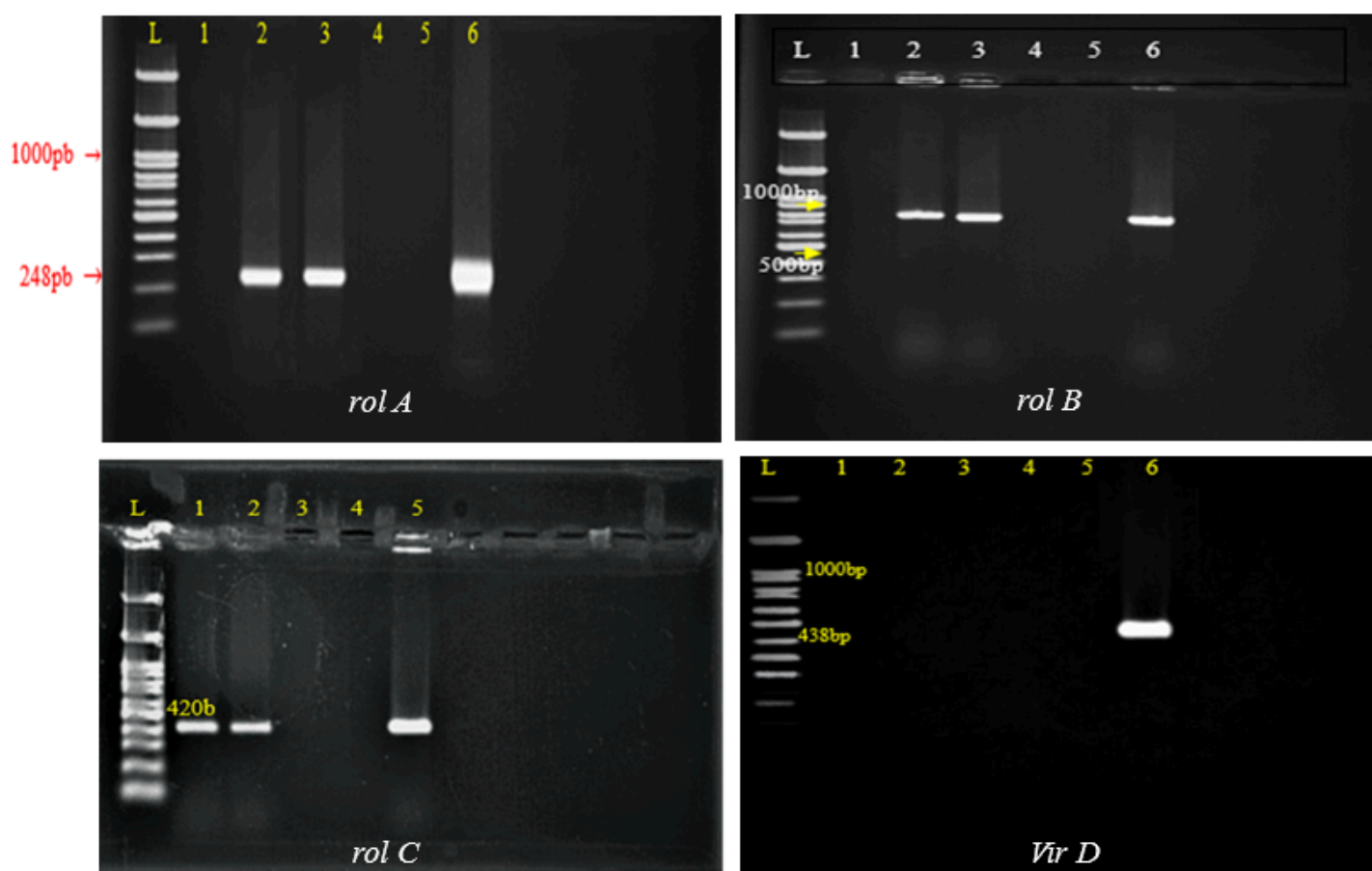


Figure 1

The *rol A*, *rol B*, *rol C* and *Vir D* genes of *A. rhizogenes* on *Perilla* hairy root DNA were amplified via PCR using particular primers. In the gels related to *rol A* and *rol B*, L: 100 bp DNA Ladder, 1: sterile distilled

water, 2 and 3: hairy root lines, 4 and 5: normal roots as a negative control, and 6: *A. rhizogenes* plasmid as a positive control. In gel related to *rol C*, L: 100 bp DNA Ladder, 1 and 2: hairy root lines, 3: normal root as a negative control, 4: sterile distilled water, 5: *A. rhizogenes* plasmid as a positive control. In gel related to *vir D*, L: 100 bp DNA Ladder, 1 and 2: hairy root lines, 3 and 4: normal roots as a negative control, 5: sterile distilled water, 6: *A. rhizogenes* plasmid as a positive control.

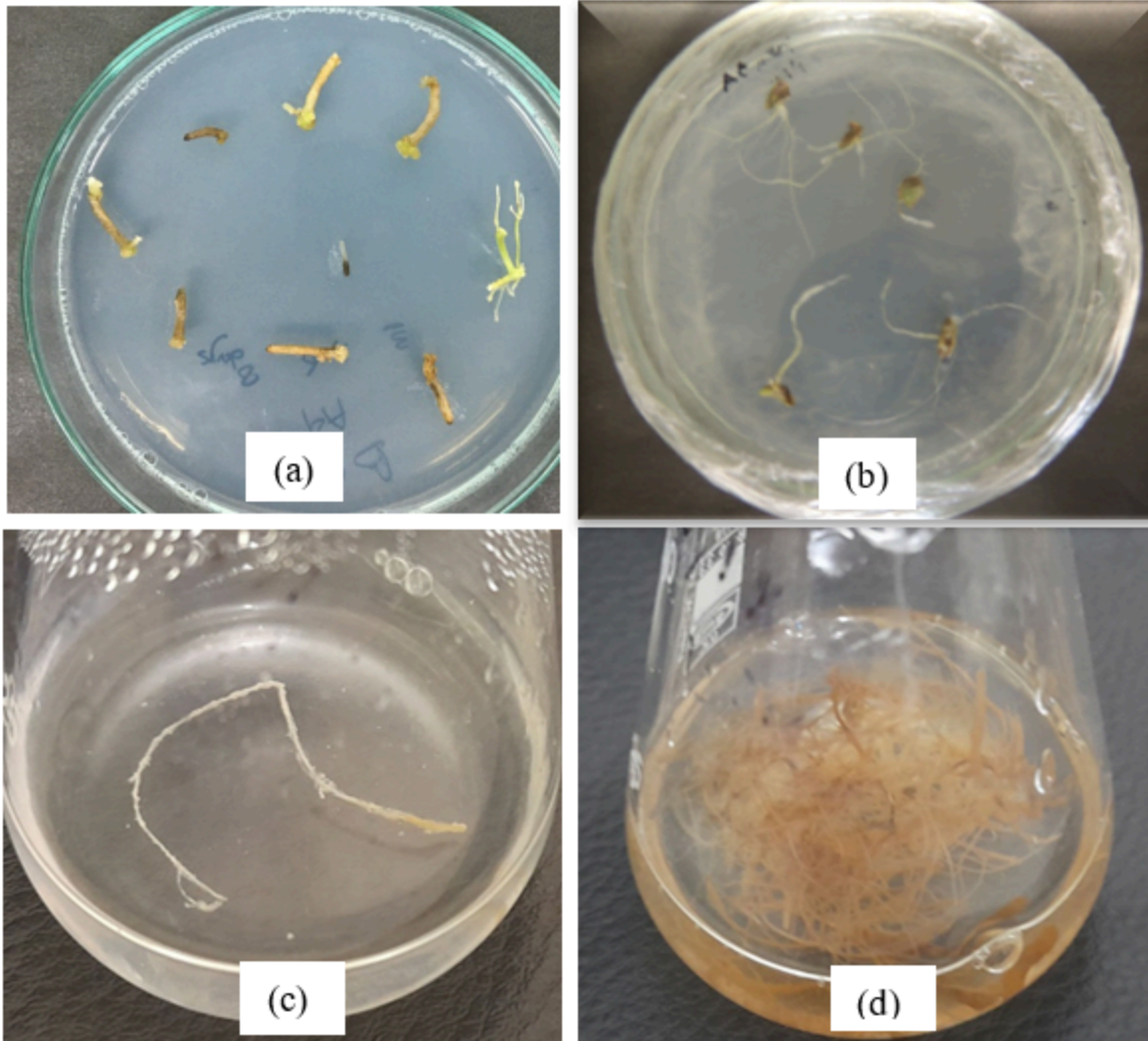


Figure 2

Perilla explants with hairy roots induced by *A. rhizogenes*(ATCC 15834 strain). (a): Hypocotyl response to hairy root induction, (b): Cotyledon response to hairy root induction, (c): Selected hairy root line, (d): Proliferated hairy root.

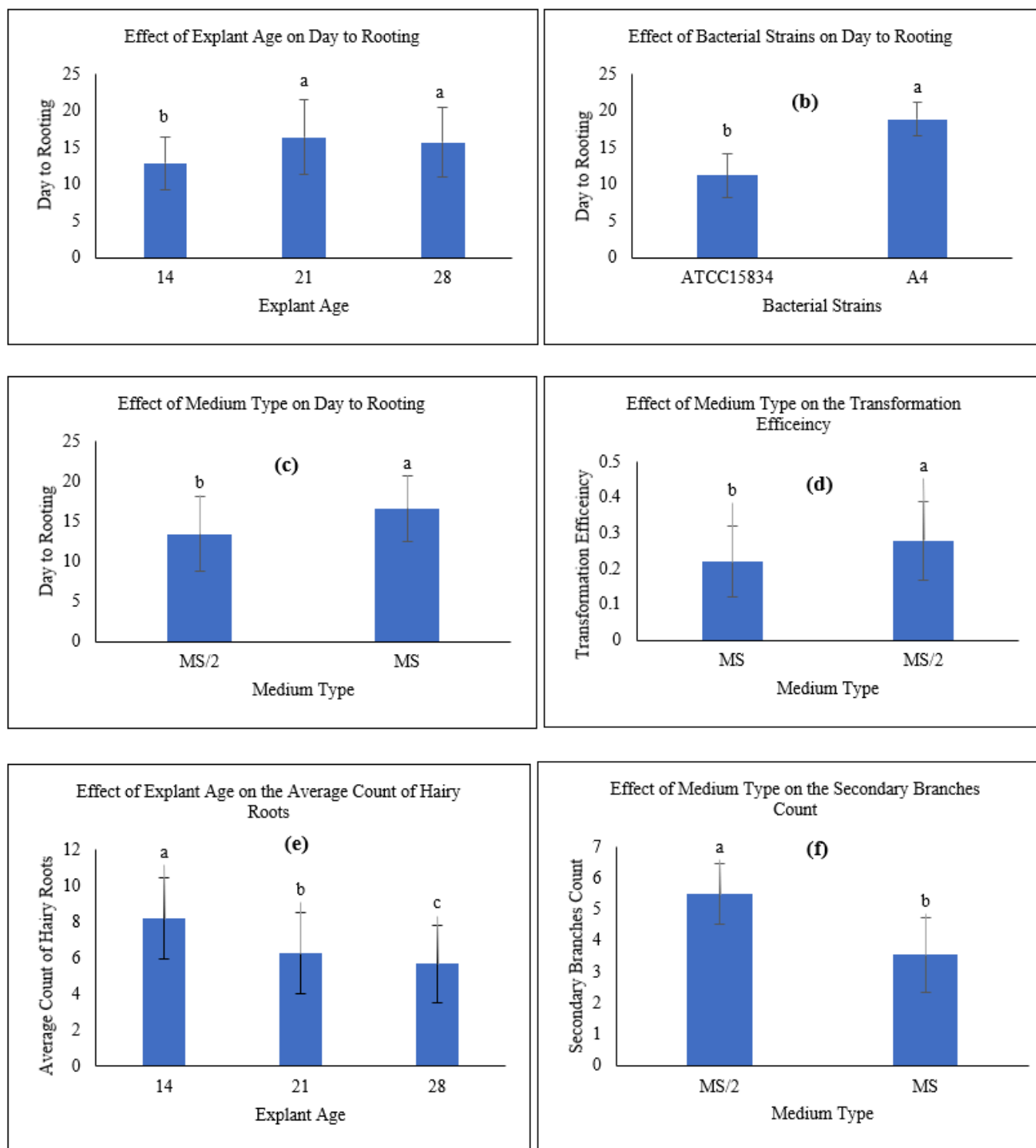


Figure 3

Effect of explant age, medium type, and bacterial strain on the studied traits. According to the LSD test, treatment with the same letter in each chart do not differ significantly at the 5% level.

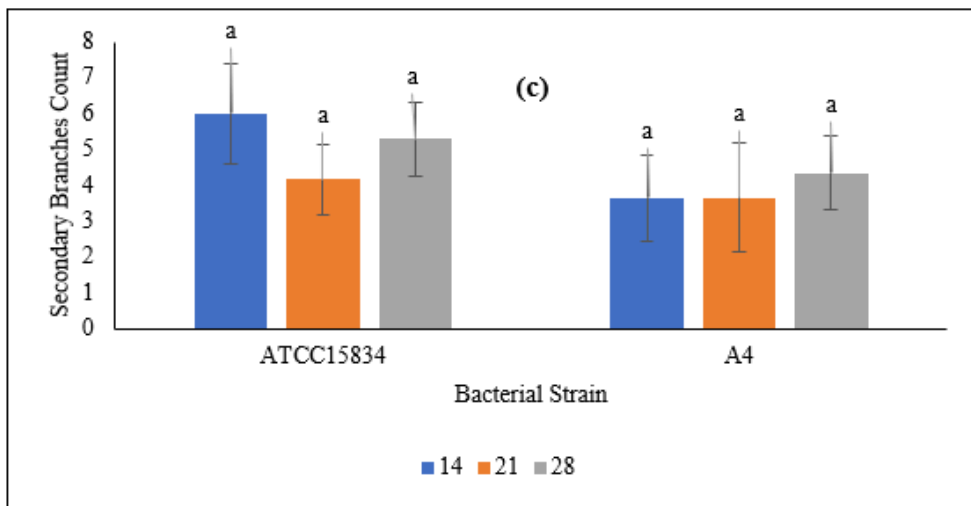
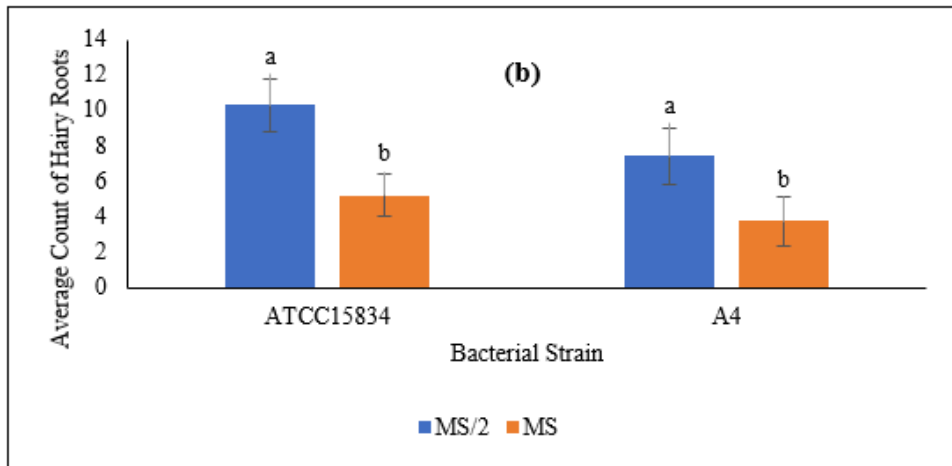
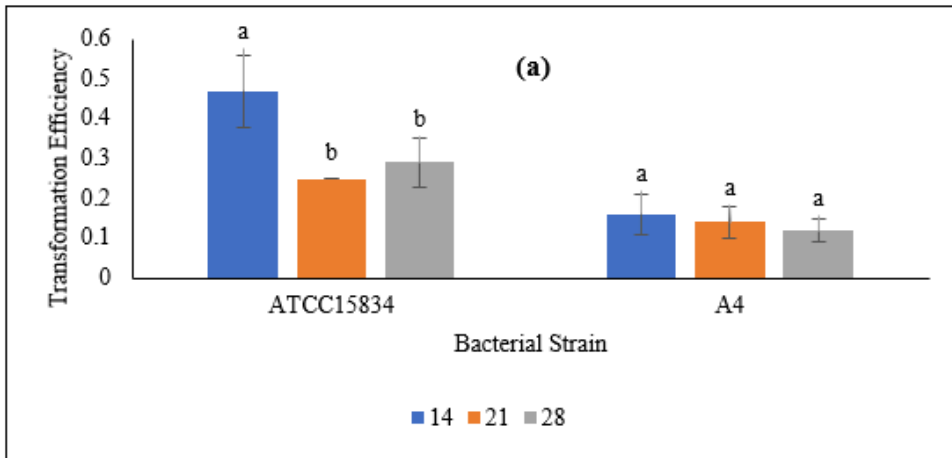


Figure 4

Comparison of double interaction effects on the studied traits. Combination treatments which have the identical letter in each chart do no longer differ substantially at the 5% level, in keeping with the LSD test.

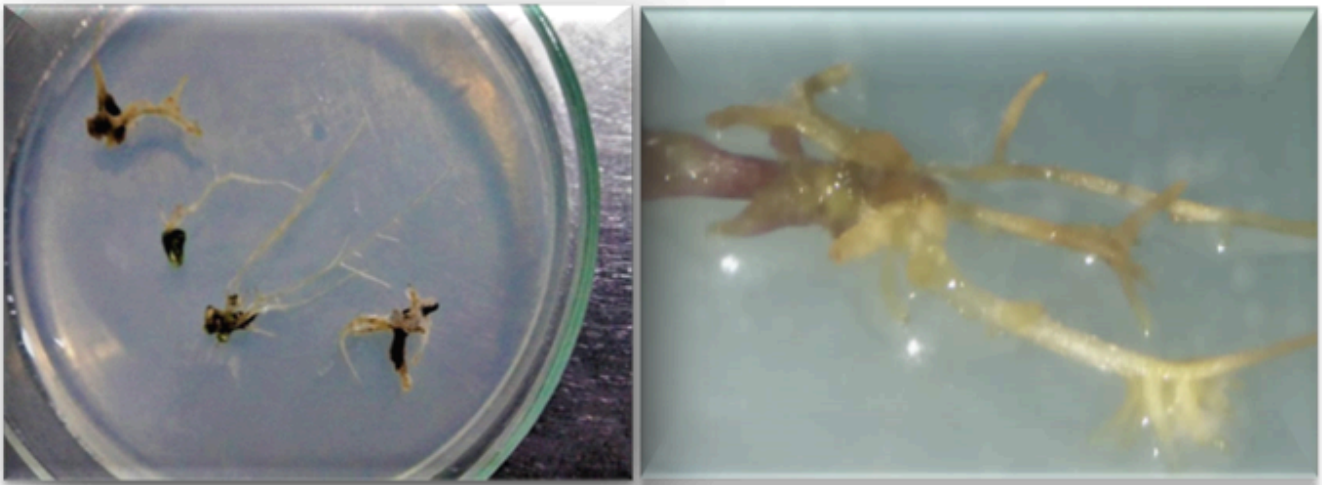


Figure 5

Secondary branches of hairy roots in 14-day-old cotyledon and ATCC 15384 strain.

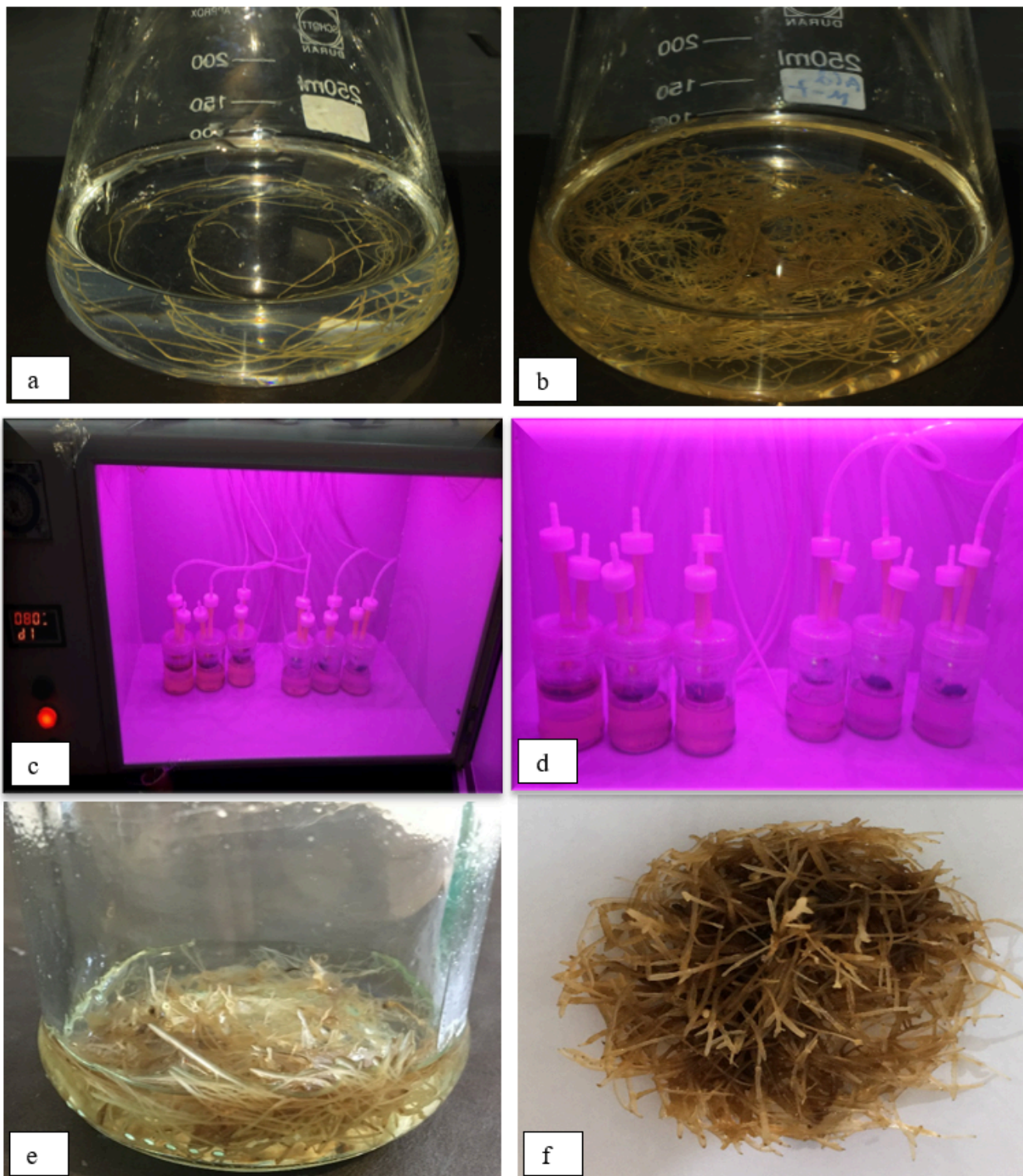


Figure 6

Hairy root biomass in shake flasks and bioreactor. (a) and (b): Hairy roots in shake flasks, (c) and (d): Temporary immersion bioreactor, (e) and (f): Proliferated hairy roots in bioreactor.

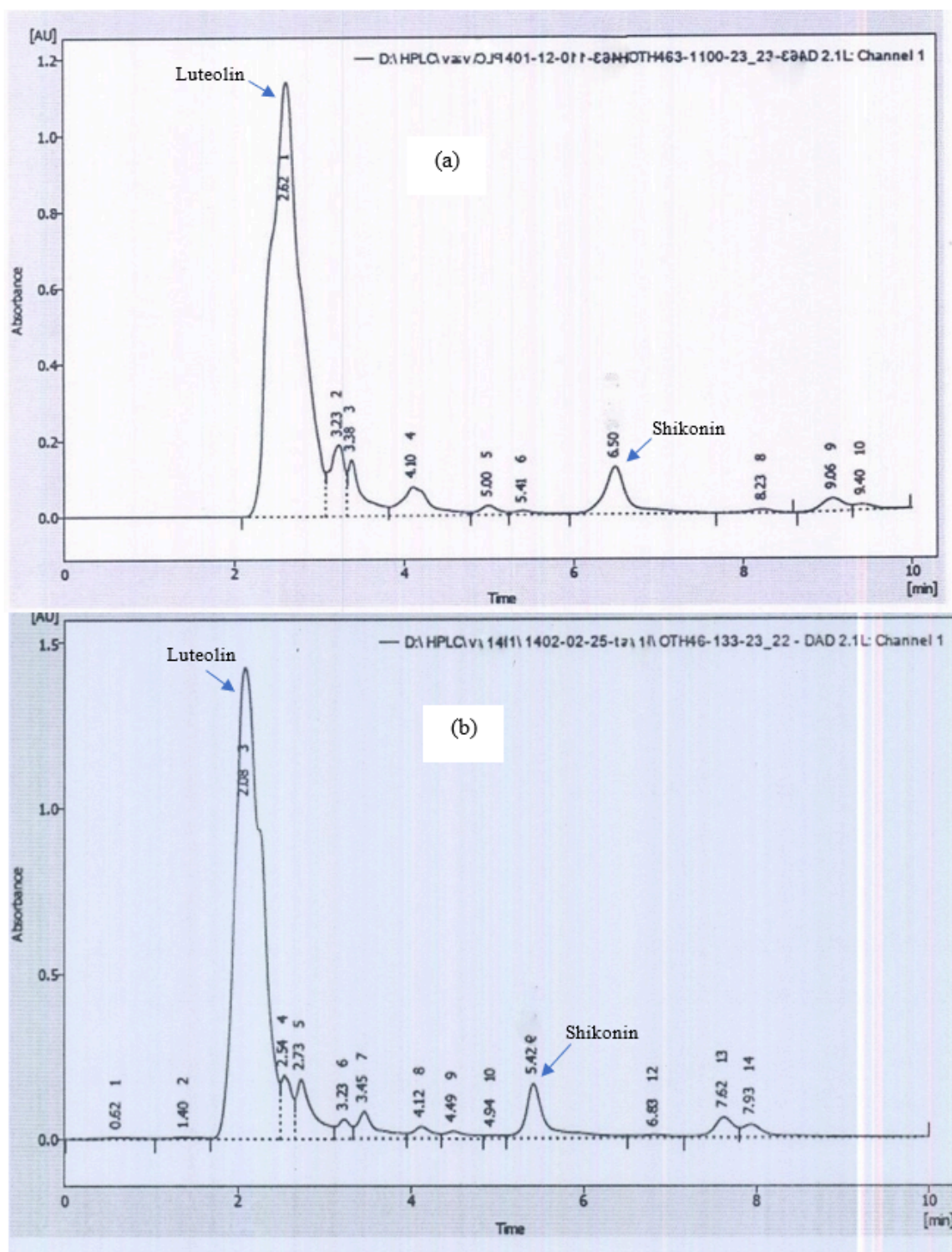


Figure 7

High-performance liquid chromatography profile of shikonin and luteolin production in *Perilla frutescens* (L.). (a) leaf; (b) hairy roots.

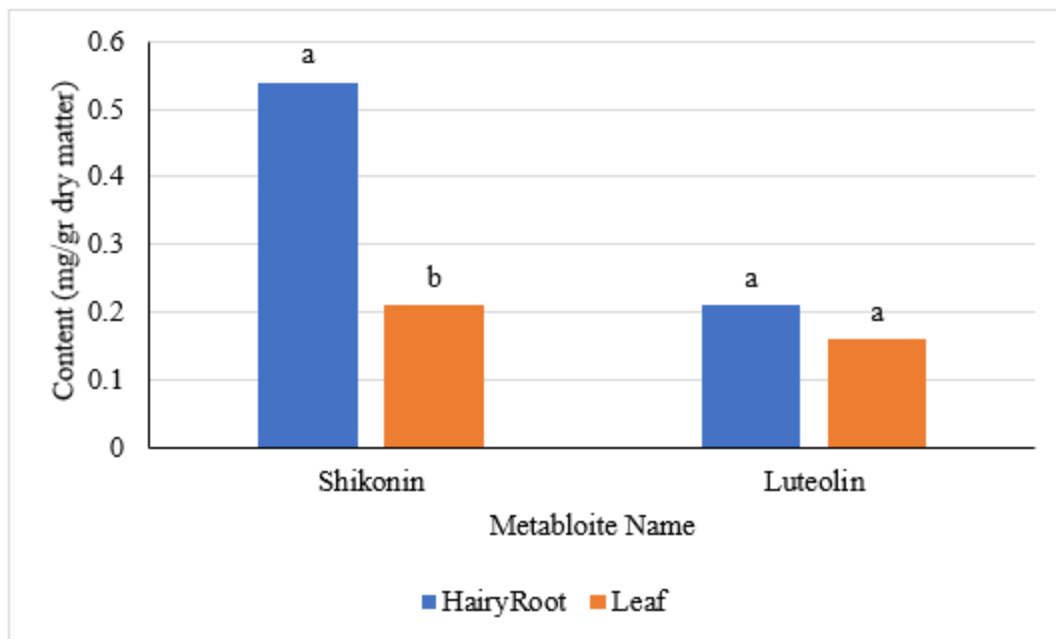


Figure 8

Mean Comparison of shikonin and luteolin content (mg/gr dry matter) extracted from hairy roots, and leaf of perilla. The presence of as a minimum one common letter in every variable indicates that there's no significant difference at the 5% level primarily based at the t check.

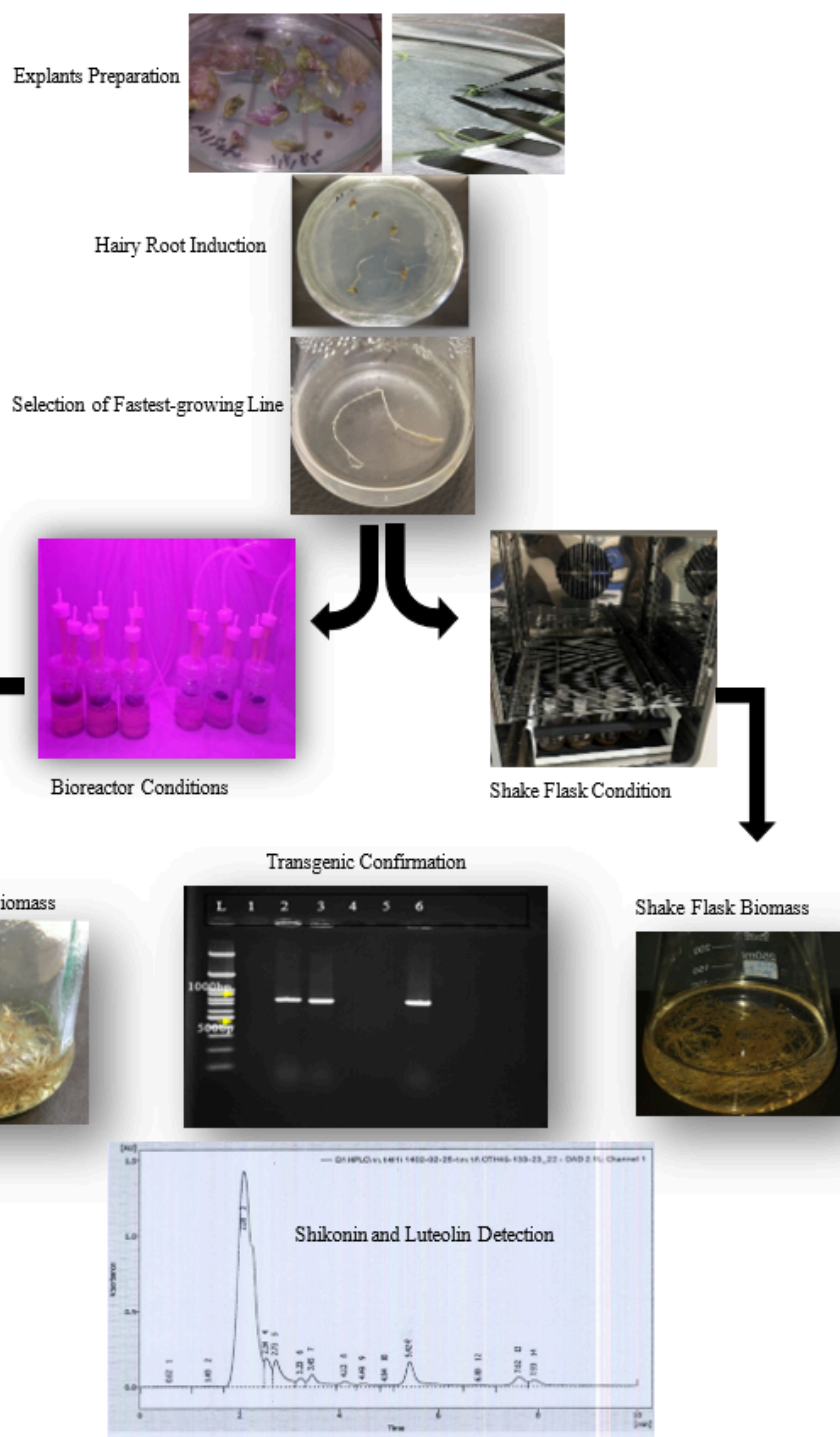


Figure 9

Workflow to optimize of growth condition and detection of shikonin and luteolin in perilla hairy root.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementaryfile.docx](#)