Hairy Root Cultures of *Hypericum perforatum* L.; A Promising Method for The High Scale Production of Hypericin

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Abstract

Background: Biotechnologic methods are common for secondary metabolites production from the plants and other sources in pharmaceutical sciences. Hairy root cell lines as the biotechnologic method have been used for *in vitro* production of major plant metabolites.

Objective: In this study, hairy roots of *Hypericum Perforatum* have been prepared using the seeds and bio transformed by bacteria. Finally, the hypericin have been producted by the hairy roots.

Methods: First, the seeds have been incubated in the plant media to hairy roots produced. Then, the hairy roots have been dipped in *Rhizobium rizogenes* suspension for biotransformation of bacterial genes. Morphological and phytochemical features of hairy roots have been determined in order to select the *H. perforatum* genotypes with higher hypericin contents. The fresh and dry weight of ten lines clones were measured after 30 days. In addition to, methanolic extracts of final hairy roots have been prepared and hypericin has been isolated and assayed by High Performance Liquid Chromatography.

Results: According to the observations, one of ten hairy root lines shows 75-fold higher hypericin content (339.27 ppm) compared to non-transformed *H. perforatum* (4.58 ppm) in the same of body weight of roots. The clone with the most content of hypericin had significant development of biomass of hairy roots and increase the hypericin production. Transformed clones were varied in morphology, growth, and metabolite productivity.

Conclusion: The mentioned methods induce the production of hairy root secondary metabolites in high scale to improve the quality and the quantity of pharmaceutical compounds.

Keywords: ***Hypericum perforatum*, *Rhizobium rhizogenes,* Hairy root, HPLC, Hypericin**

Introduction

Many plants have been recommended for the medicinal and non-medicinal usages. In addition to, these natural resources due to different phytochemical compounds have been important [1, 2].

Hypericum perforatum L. (Hypericaceae) is one of the best-studied medicinal plants in the world [3]. *H. perforatum* is native to Europe, West Asia and North Africa. In addition to, this plant distributed in temperate regions of Iran such as Mazandaran, Golestan and Gilan provinces [4, 5]. Aerial part of *H. perforatum* included buds, young stems, sepals, leaves and especially flowers have several secretary structures and unique specialized glands. These components contain secondary metabolites especially antraquinones and antraquinone glycosides such as hypericin and pseudohypericin have been biosynthetized by polyketide pathway in the plant (Figure 1) [6]. The compounds have been used in numerous analytical and pharmacological purposes in medical sciences [7, 8]. In addition to, *H. perforatum* produce the secondary metabolites with limited capacity and high amounts of the plant should be gathered to achive the significant percentages of hypericin and pseudohypericin. Thus, laboratory methods for the secondary metabolites production in high scale are needed.

Hence, researchers almost have been discovered the methods for achieve to commercial production of these valuable compounds due to their high demand. Hairy root bioreactors, cell suspension technology and immobilized plant cell bioreactors are the modern and applicable methods for high scale production of secondary metabolites [7-11].

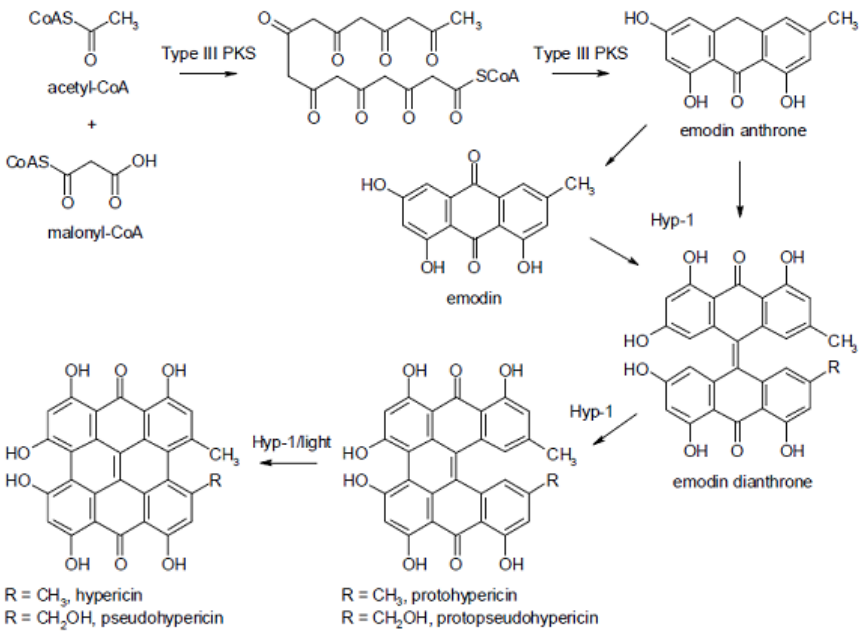


Figure 1- Biosynthesis pathway of hypericin in the plants

Hairy roots have a wide range capability to produce the secondary metabolite similar to the parent plants as well as novel compounds, are a potential systems for secondary metabolites production in large scale [12]. The roots have organized nature, fast growth rate, sustainability in metabolite production, lower sensitivity to shear stress than cell suspension cultures, and also their clones are genetically and biosynthetically stable for long periods. In addition to, secondary metabolite production induces using gene transformation by some suitable bacterial strains especially *R. rhizogenes* [13-17].

R. rhizogenes is a gram-negative soil bacterium which transfers its gene information into genetic content of host plants. Actually, host hairy roots have been modified in type and scale of their secondary metabolites production. The transformed hairy roots capable to produce the secondary metabolites in large amounts [18].

The aim of this study was evaluation of production in large quantities of hypricin through hairy root lines of *H. perforatum*. In the study, *R. rhizogenes* bacteria have been employed for gene transformation into hairy root lines. Actually, these processes induce the production of hypericin in high scales.

Materials and methods

This study was conducted to access hypericin in high amounts using hairy roots technology. The seeds of *H. perforatum*were prepared from "Isfahan Pakan Bazr" Institute (Voucher's number: E214411). The seeds have been disinfected by dipping in 70% ethanol solution for 15 seconds and washing with pure distilled water. In addition to, the seeds were exposed in stoke of aqueous sodium hypochlorite solution 1.5 % with a drop of Tween 80 for 10 minutes. The seeds have been washed with distilled water 4 times and were transferd to Murashige and Skoog (MS) medium supplemented by soluble vitamins (according to B_5 and B_{12}), 0.8% agar and 2% sucrose at pH 5.8 before autoclaving (20 minutes at 120°C) to germination [19, 20]. Then, the sterilized seeds and the germinated plants were placed in growth chamber (25 ±2°C+16 hours light photoperiod, 60-70% relative humidity).

Inoculation, Polymerase Chain Reaction (PCR) and hairy root production

Hairy root (HR) clones have been obtained using A4 strains of *R. rizogenes* from sterile explants leaves, buds and stems by optimized method.

Actually, direction inoculations have been transferred using *Rhizobium* strains in LB broth and MYA liquid containing 100 mg/L rifampin. Then, the medium was shaked and incubat at 28 °C with speed 160 rpm for twenty-four hours. Two mls of this culture medium was transferred to 100 ml of new bacteria culture medium with the same conditions. The bacterial suspension precipitated at 5000 rpm and 4°C after reaching 0.4-0.6 optical density (OD) in 660 nm. In addition to, sedimented bacteria were dispersed slowly in Murashige and Skoog ($^{1/2}$MS) liquid medium with 100 mg/l acetosyringone (AS) at pH 7.5.

The explants of germinated seeds mentioned above, have been dipped in *Rhizobium* suspension without acetosyringone

and with acetosyringone (in OD 660: 0.4-0.6), separately in 45 minutes, and have been kept for 72 hours in co-culture medium at 25±2 °C and darkness to inoculation of the bacterial DNA was completed [21].

For *Rhizobium* removing, subculturing of explants was done in the culture media MS ،½ MS, B5 and ½ B5 that containing 500 mg/l cefotaxime antibiotic.

Polymerase chain reaction has been done using primers of genes rolB and rolC for determination of inoculation of T-DNA into *H. perforatum* cells. Length of duplicated pieces of sequence of the rolB and rolC genes which is available in the bacterial T-DNA are 420 bp and 612 bp, respectively. Genomic DNA of hairy roots and control samples (Non-transgenic roots) have been extracted using CTAB method and plasmid DNA of *Rhizobium* strains extracted using alkaline lysis method. In addition to, quality and quantity of the extracted DNA was studied using the results of spectrophotometry. The cycle of polymerase chain reaction was performed according to cited process. In addition to, the initial denaturing 4 min at 95°C, followed by 35 cycles with 45s at 95°C, 45s at 55°C for annealing rol BC, extension 1:30s at 72°C and final extension (5min at 72°C). Reaction mixture was prepared with 50 ng of genomic DNA, 2.0 ml of each primer (5 pmol), 0.5 ml of dNTP mix (2.5 mM each), 2.5 ml of PCR buffer, and 0.25 ml of Taq DNA polymerase (DyNazyme^{TM} Company, 5 U/ml) and final 25 µl volume have been obtained using parenteral sterile water. Finally, electrophoresis has been carried out using 1% gel and 5 V/cm voltage and biotransformed hairy roots were determined.

Then, the obtained biotransformed clones were exposed at the same condition in a shaker incubator with 78 rpm. After the growth in darkness for 30 days and reaching to the suitable biomass, they harvested from the culture medium for extraction and evaluation of the clones morphology. The clones washed using distilled water and their fresh weight measured and maintained in oven at 40°C until reach to the constant weight and their dry weight recorded [22, 23].

Extraction of Hypericum from hairy roots

0.1 gram of various HR clones were dried in oven with 40°C temperature and crushed. The obtained powder has been dissolved in methanol and rotated using shaker incubator with 60 °C and 140 rpm for 24 hours. Then, the solution was filtered in dark condition and was evaporate by rotary evaporator with 40°C. Dried extracts of *Hypericum* hairy roots have been weighed and dissolved in 1 ml methanol. Finally, the solution was filtered through a membrane 0.2 µM (Gelman, USA) and injected and analyzed using HPLC.

Hypericin assay

The HPLC-UV method have been used for this study (Shimadzu system, Duisburg from Germany). HPLC conducted on a Hypersil reversed-phase column (RP C18, 100 Å) (LC 150 × 4.6 mm, 5 µm). In addition to, the programs and conditions have been conducted for injection contains Isocratic Elution Type, in 25°C, 1.5 ml/min flow rate and the peak was detected using UV-VIS in 590 nm.

The mobile phase was prepared with combination of 50% acetonitrile, 30% methanol and 20% buffer solution 0.1N Ammonium Acetate with pH 9.7. In addition to, total procedure time with injection 20 µl of the extract solution of each the clones were 20 min, separately. A calibration curve was obtained using standard methanolic solutions of hypericin (Sigma-Aldrich) with various concentrations (0-100 µg / ml)[24, 25]. Finally, retention time values (RT) for hypericin was observed at 15.38 min (Figures 2, 3).

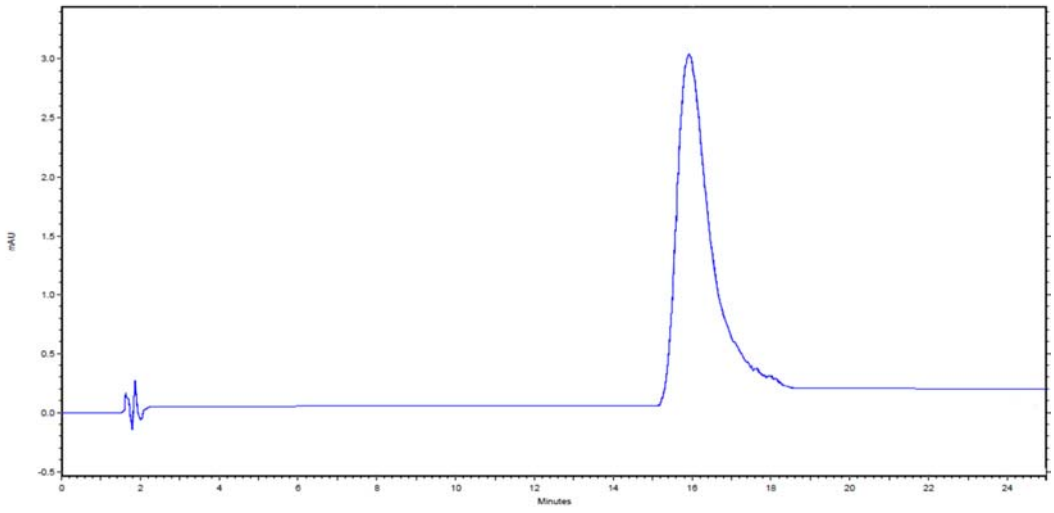


Figure 2- HPLC Chromatogram of standard solution of hypricin in concentration of 10 ppm

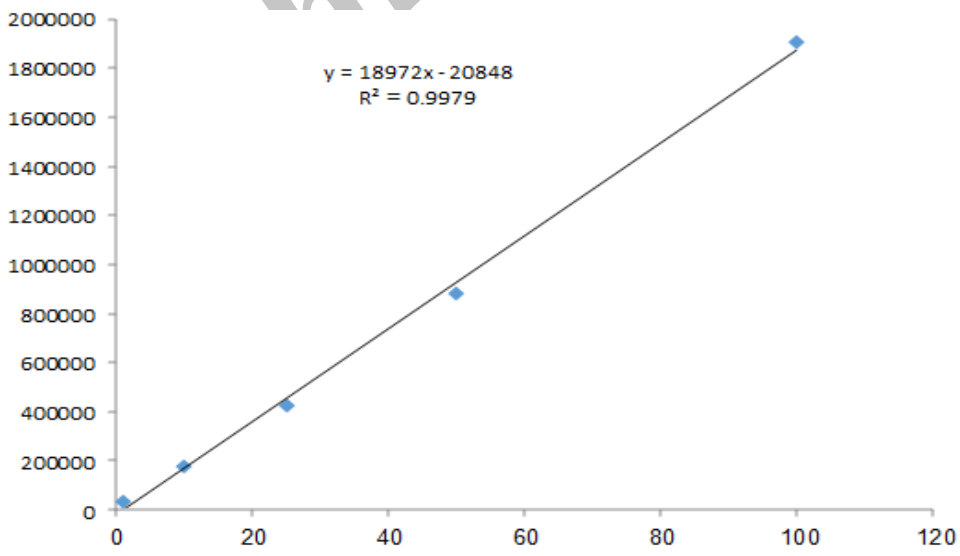


Figure 3- Standard curve of Hypericin

Data analysis

Integration of T-DNA in the genome of hairy roots with polymerase chain reaction was evaluated. In addition to, the hypericin amount in the hairy roots of *Hypericumperforatum*L. was measured. After collection of information as mean±SD, data analysis and charts drawing were performed using SPSS 15 and Excel 2007.

Results

Electrophoresis data of PCR products have been showed T-DNA integration into the host genome of *H. perforatum* cells, certainly (Figure 4, lane 7 and 9 have been received the T-DNA of *R. rizogenes*).

This case has been used in the next steps. Unfortunately, in the hairy roots production process, explants of *Hypericum* despite the several turning subculture in media with different cefotaxime concentrations became afflicted to severe necrosis. Primary hairy roots have been showed in Figure 5.

Comparison of four kinds of culture media in the same conditions showed that *R. rhizogenes* at ½MS medium had the highest efficiency (89.58%) for hairy roots induction, so was placed in (a) class. *Rhizobium* along with acetosyringone at B_5 medium also had the lowest percentage (1.35%) of induced hairy roots, so was placed in (d) class. In addition to, the leaf area, number and density of dark glands in the leaves were different even among the clones.

After 30 days of growth, fresh and dry weight was measured in ½ MS hormone-free medium. Comparisons of mean fresh and dry weight of clones at level of 99% have shown significant differences (Figure 6).

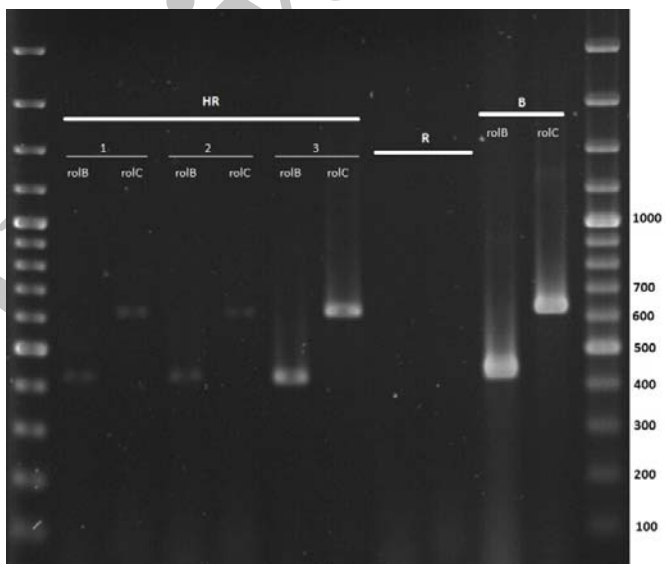
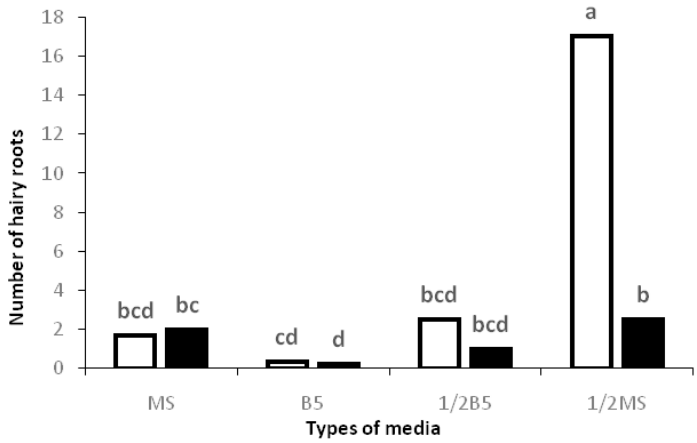


Figure 4- PCR analysis of *H. perforatum* hairy root cultures

Figure 5- Primary hairy roots

A



B

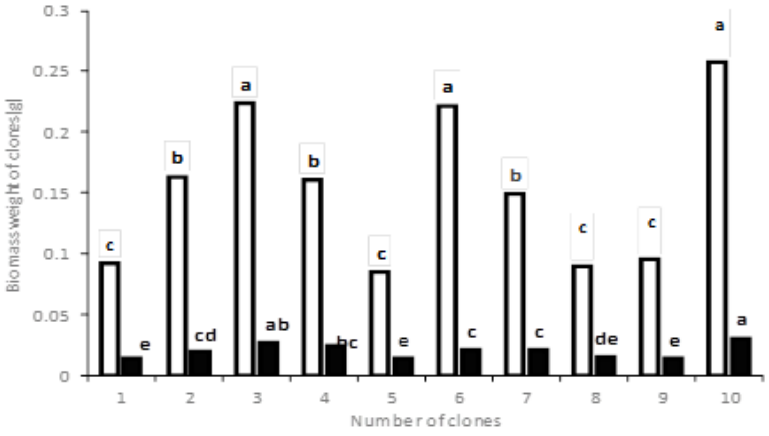


Figure 6- (A) The effect of different culture medium on induction of hairy roots (□: Inductional hairy roots with A4 strains, ■: Inductional hairy roots without A4 strains). (B) Figure 6- Comparison of average fresh and dry weight of hairy roots clones (□: Fresh Weight, ■: Dry Weight).

The clone's numbers 3, 6 and 10 with the highest fresh weight were placed in (a) class. The clone's number 10 (with highest dry weight) and clone 3 (with dry weight less than clone 10) were placed in (a) and (ab) class, respectively (Table 1).

In addition to, perfect plant with flowers spontaneously was regeneratedin the majority of clones at hormone-free medium. The transgenic roots had regeneration to complete plants capability. The numerous morphological and physiological changes such as wizened and small leaves, decrease fertility, reduce apical dominance, shorter internodes; more picks of gland were created in them but as same as original plants are stable, genetically.

HPLC screening of methanol extracts showed a large variation in hypericin content of clones (Figure 7). In the best case (Line 3) was observed 75-fold higher hypericin content (339/27 ppm) compared to nontransformed *H. perforatum* (4.58 ppm) in the same of concentration (Figure 8). Fresh and dry weight of 10 lines clones were measured after 30 days. Furthermore, the line 3, with highest hypericin, had significant development of biomass. This clone had significant production of hypericin compound towards before researches.

Table 1- Phenotypic comparison of the hairy roots clones

Number of Clones	ST.ERROR	ST.DEV	Dry weight (g)	ST.ERROR	ST.DEV	Wet weight (g)
Clone 1	0.002	0.0015	e0.0145	0.014	0.0045	**c0.0928**
Clone 2	0.002	0.00057	cd0.0203	0.014	0.0255	**b0.1633**
Clone3	0.002	0.0061	ab0.0266	0.014	0.002	**a0.224**
Clone 4	0.002	0.00152	bc0.0243	0.014	0.0065	**b0.1613**
Clone 5	0.002	0.00332	e0.0141	0.014	0.0035	**c0.0855**
Clone 6	0.002	0.0034	c0.0211	0.014	0.0397	**a0.2216**
Clone 7	0.002	0.0036	c0.021	0.014	0.019	**b0.149**
Clone 8	0.002	0.00264	de0.015	0.014	0.012	**c0.09**
Clone 9	0.002	0.0002	e0.014	0.014	0.015	**c0.095**
Clone 10	0.002	0.00378	a0.0306	0.014	0.0548	**a0.258**

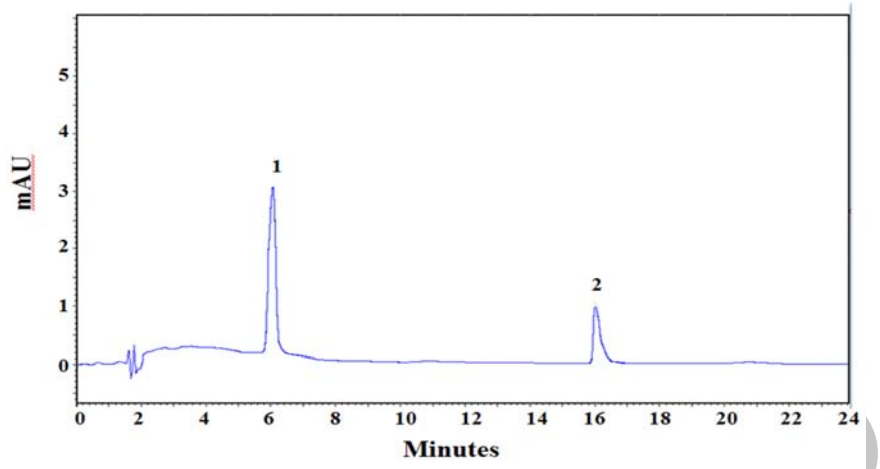


Figure 7- HPLC chromatogram of *H. perforatum* hairy root extract (1 = pseudohypericin, 2 = hypericin)

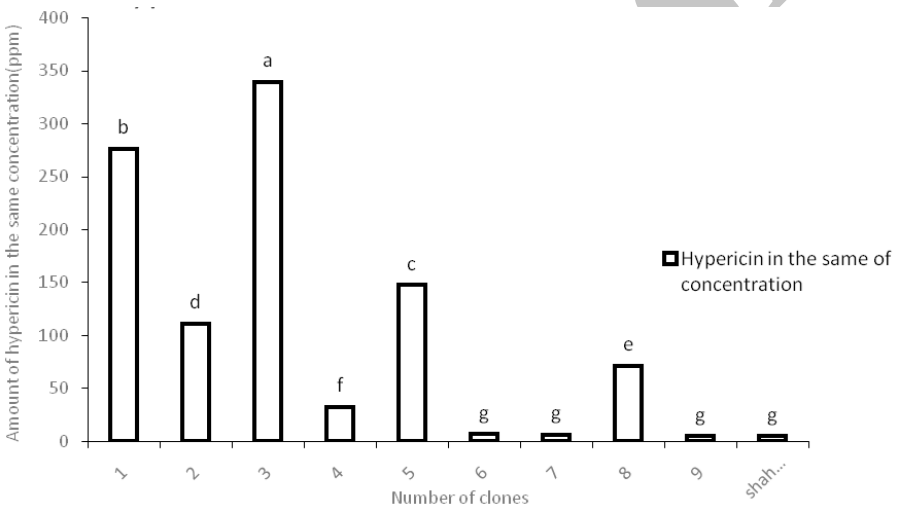


Figure 8- Comparison between amounts of hypericin in extract clones and in the same concentration of hypericin at control sample.

Discussion

Different factors have been influenced in hairy roots preparation included types of co-cultivation medium, exposure time with co-cultivation medium, insemination methods, types of *Rhizobium* strains, different explants and select of appropriate antibiotics to remove bacteria [26]. In addition to, several experiments have been performed for optimizing induction conditions and establishment of plant hairy roots in the world.

Bivadi et al. (2014) ave been optimized the culture conditions of hairy roots of *H. perforatum* which contains three strains of *Rhizobium* (A4, ATCC15834 ,11325) and three types of explants including leaves, stems and buds [21].

In this study, the maximum number of the hairy roots have been obtained by treatment of *H. perforatum* explants with A4 strains of *R. rizogenes* for 45 minutes inoculation in 72 hours co-cultivation using $^{1/2}$MS co-cultivation medium.

Various spieces of the plants have different sensitivity to infection with *R. rhizogenes*, *R. tumefaciens* and other bacterial species [27]. In the different studies, inoculation methods immersion in *R. rhizogenes* suspension was compared with direct injection of *R. rhizogenes* in the same conditions. Direct injection was more efficient than immersion method in *Rhizobium* suspension. Among leaves, stems and terminal buds explants, stem was the most suitable explant in the both insemination methods. In addition to, hairy roots almostly have been induced in the stem of explant [27].

Different tissues of *H. perforatum* such as leaves, stems, flowers and roots generally are containing secondary metabolites such as hypericin and hyperforin [28].

Onelli et al. during his studies showed that hypericin will accumulate in glandular structures of *Hypericum* leaves [29]. In addition to, Zobayed et al. Studies showed that concentrations of hypericin and pseudohypericin in the species are dependent on the number, size and surface area of dark glands [23].

In addition to, Kornfeld et al. have been explained that may be other different parts of the plant such as the peripheral cells of leaves were related to the production of hypericin using electron microscope techniques (SEM). In cases where secondary metabolites accumulate only in aerial parts of a plant, hairy roots culture of them is able to synthesize and accumulate of metabolites [30]. For example hairy roots of Artemisia, like its aerial parts,can produce artemisinin [31].

Bertoli et al. (2008) are studied morphological and phytochemical features of hairy roots lines in order to select *H. perforatum* genotypes with higher valuable bioactive compounds [32]. In our study at the same conditions and free of any environmental changes (culture medium and light, temperature, oxygenation and purveyance speed at shaker incubator), hypericin contents were different in all clones. Numerous studies in transgenic plants or hairy roots have been shown the diversity in metabolite production, for example, these observations were accordance with the results obtained by other studies. In the Bertoli study, one of the twelve lines of hairy root, have been contained 10-fold higher hypericin content (0.25 mg/g DW) compared to the quantities that reported in literature [32]. The results of Koperda'kova indicated that plants obtained from clone B had the most number and density of dark glands on their leaves and its hypericin has been the amount of 1/5 up to 3 fold higher than other studies [22].

In our research, clones have been obtained from various strains or one strain have significantly difference in the rate of growth, development of biomass, the amount of regeneration and hypericin production among the clones. This phenomenon could be due to the diversity in transform process. For example, the presence different doses of T-DNA in primary transgenic cells of each

clone and different sites insertion of foreign T-DNA in plant genome or in other cases, gene silencing involved at the secondary metabolism that causes blocking and inhibiting production of specific compounds [33].

Cui et al. Investigate adventitious roots of *H. perforatum* in balloon type airlift bioreactors (3l capacity) and after 6 weeks of culture, an approximately 50-fold increase in biomass was recorded at an inoculum density of 3 g l-1 and an aeration volume of 0.1 V/Vm [14, 15]. Plant roots can synthesize, store, and secrete a vast array of compounds and transformed root cultures have a wide range of biosynthetic capacities. The use of high potential of hairy roots between different methods that are stimulants biosynthetic pathway of these compounds considerable success provides in the commercialization of valuable compounds. In order to gaining economic interest recommended its cultivation in the optimal condition of bioreactors.

Conclusion

We attempted to produce the hypericin in high scale using transformed hairy root cell lines. Finally, the methods have been used in the study suggest high potential of hairy roots to improve the quality and the quantity of pharmaceutical compounds.

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