Short Communication

Silymarin production by hairy root culture of *Silybum marianum* (L.) Gaertn

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Abstract
Silymarin production by hairy root culture of Milk thistle (*Silybum marianum* L. Gaertn) was investigated using *Agrobacterium rhizogenes AR15834*. Hairy roots were induced by injection or inoculation of explants with *A. rhizogenes*. One month old hairy roots were dissected from the explants and grown in Murashing and Skoog (MS) liquid medium. Polymerase Chain Reaction (PCR) using the *B* gene and the B-glucoronidase (GUS) assays were used for identification of the transformed hairy roots. Flavonolignan levels in the hairy roots were determined by high-performance liquid chromatography (HPLC). Five different components were isolated; taxifolin, silychristin, silydianin, silybin and isosilybin, with the following quantities, 0.009, 0.041, 0.042, 0.007 and 0.011 mg g^{-1} dry weihht, respectively. Silybin was the major flavonolignan. Produced by hairy roots culture may serve as a useful system for producing silymarin or studying its biosynthetic pathways.
Keywords: *Silybum marianum; Agrobacterium rhizogene*s; Hairy root; Flavonolignans.

Silymarin is an antihepatotoxic polyphenolic substance isolated from the milk thistle plant, *Silybum marianum*, (Kvasnicka *et al*., 2003). Silymarin consists of a large number of flavonolignans, including silybin (SBN), isosilybin (ISBN), silydianin (SDN), silychristin (SCN) and taxifolin (TXF) as precursor of silymarin (Sanchez-sampedro *et al*., 2005a and Hasanloo *et al*., 2005b). Silymarin and silybin have been so far mostly used as hepatoprotectants (Sonnenbichler *et al*., 1999). These metabolites have also been shown to have other interesting functions, such as anticancer and cancero-protective properties, neurodegenerative and neurotoxic repressing activities. They are also associated with the treatment and prevention of gastrointestinal problems, nephropathy, cardio-pulmonary problems and skin protection (Van Erp *et al*., 2005; Veladimir *et al*., 2005; Katiar, 2002). Silymarin compounds are usually extracted from dried fruits of field grown plants that often require months to years to obtain.

The *in vitro* production of plant secondary metabolites can be possible through plant cell culture under controlled conditions and free from environmental fluctuations. However, the major limitations of cell cultures are their instability during long-term culture and low product yields (Bonhomme *et al*., 2000).

Therefore great efforts have been focused on transformed hairy roots (Kim *et al*., 2002). Hairy roots, the results of genetic transformation by *Agrobacterium rhizogenes* (Hu and Du, 2006) have attractive properties for secondary metabolite production, as compared to differentiated cell cultures (Dhakulkar *et al*., 2005; Kim *et al*., 2002). Root induction is due to the integration and subsequent expression of a portion of bacterial DNA (T- DNA) from the bacterial Ri (Root inducing) plasmid in plant genom. Four loci involved in root formation have been identified in the T- DNA of the Ri plasmids and designated root loci (*rol*) A, B, C and D. (Ayala-Silva *et al*., 2007). Hairy roots are genetically stable and not repressed during the growth phase of the

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it's culture (Bourgaud *et al*., 1999). They often grow as fast as or faster than plant cell cultures (Srivastava and Srivastava, 2007). The greatest advantage of hairy roots is that their cultures often exhibit approximately the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Kim *et al*., 2002).

Hairy root culture of *S. marianum* could therefore be an alternative method for the production of flavonolignanas, however, untill now, few studies have been conducted in this field, with very low production observed in the fruits of this plant (Alikaridis *et al*., 2000). In this study, we describe an efficient protocol for development of the hairy root culture of *S. marianum* and production of silymarin, by using *A. rhizogenes*.

Dried fruits (Hungarian seeds) derived from milk thistle were supplied by the Institute of Medical Plants (Karaj, Iran) and the Iranian Academic Center for Education, Culture and Research (ACECR). The seeds were cultured in hormone-free MS (Murashige and Skoog, 1962) medium and incubated in the dark at 26 ± 1ºC for a photoperiod of 16 h light (Hasanloo *et al*., 2008). The bacterial strain *A. rhizogenes* AR15834 (a gift from Dr P. Noroozi, the Sugar Beet Seed Institute, Karaj, Iran) harbouring (UK) or devoid of the binary vector pBI121 (Clontech, Canada) were used in hairy root induction. The binary vector pBI121 carries genes such as *nptll* coding for neomycin phosphotransferaseII driven by the nopaline synthase promoter, and an *uidA* gene coding for β-glucoronidase (Gus) driven by a cauli flower mosaic virus (CaMV) 35S prompter. Various excised explants such as the hypocotyl, leaf and cotyledons were isolated from *in vitro* grown seedlings. All explants were precultured for 3 days on hormone-free medium containing MS salts, vitamins and 3% (w/v) sucrose. The medium was adjusted to pH 5.8 before adding agar (7 g/l). The precultured explants were immersed in overnight cultures of bacterial suspension (optical density at 600 nm, (OD_{600} = 0.7) for 10 min and then blotted dry on sterile filter paper and incubated under light in the same medium. After three days, they were transferred to a medium containing MS salts and vitamins, 3% (w/v) sucrose, 250 mg/l cefotaxime and 7 g/l agar. Within 4- 5 weeks, roots emerged from the wounded sites. In all cases where *A. rhizogenes* harboring pBI121was used, the medium was supplemented with 50 mg/l kanamycin. In the second approach, the hairy roots were induced by injection of the overnight culture of *A. rhizogenes* suspension culture (OD_{600} = 0.7) into the hypocotyls of the whole seedling using an insulin syringe. Hairy roots which arose mainly from the cut surface of the explants, after reaching a length of 4-5 cm, were separated and subcultured in the dark on the same media explained above. Wild type root cultures were established in hormone-free MS liquid medium as control. Hairy root lines were maintained by transfer of 3-4 long root pieces to hormone- free liquid medium containing MS salts, vitamins and 3% (w/v) sucrose at 25ºC on a rotary shaker (130 rpm) in complete darkness and subcultured every 2 weeks. Root genomic DNA was extracted as described by Khan *et al*. (2007). Polymerase chain reaction (PCR) was performed in 35 thermal cycles (denaturation at 94ºC for 1 min, primer annealing at 53ºC for 1 min, and primer extension at 72ºC for 1 min) for *rol*B (Forward primer 5′-ATGGATCCCAAATTGC-TATTCCCCACGA-3′ and Reverse primer 5′- TTAG-GCTTCTTTCATTCGGTTTACTGCAGC-3′) and in 35 thermal cycles (denaturition at 94ºC for 1 min, primer annealing at 61ºC for 1 min, and extension at 72ºC for 2 min) for GUS (Forward primer 5′-GGTGGGAAAGCGCGTTACAAG-3′ and Reverse primer 5′-TGGATTCCGGCATAGTTAAA-3′) gene specific primers. The β-glucoronidase histochemical assay was performed according to the method by Jefferson *et al*. (1987) using root segments. Roots were immersed in sodium phosphate buffer (pH 7.0) containing 2 mM 5- bromo-4-chloro-3- indolyl-β-glucoronic acid. The reaction was allowed to proceed for 20 h in the dark at 37ºC (Jefferson *et al*., 1987). The one month old hairy roots were harvested from the liquid medium and placed between the folds of a blotting paper to remove excess water and were then freeze-dried. Root growth was measured in terms of dry weight (DW). The samples were treated with ethyl acetate to remove fats. The flavonolignans were extracted from the dried residue with 10 ml of methanol at 40ºC for 8 h. The methanolic solution was concentrated under vacuum to a dry residue and re-dissolved in 2 ml of methanol and kept at 4ºC in the dark (Cacho *et al*., 1999). Flavonolignans levels were determined by high-performance liquid chromatography (HPLC) according to the method by Hasanloo *et al*. (2005a). This procedure involved the use of a Knauer, Germany liquid chromatography system equipped with a Knauer injector consisting of a 20 µl loop, a Eurosphere (Knauer, Germany) C185 µ (250 × 4.6

mm) column, a Knauer K2600A UV detector with Chromgate software for peak integration. The mobile phase consisted of the solvents; acetonitrile: water (40:60) with 10% (v/v) H_3PO_4, (pH 2.6). All solvents and chemicals were of HPLC grade (Sigma, Aldrich, Germany). The elution time and flow rate were 30 min and 1 ml/min respectively, and the resulting peaks were detected at 288 nm. Identification was achieved by comparison of the sample retention times (R_t) with those of the standards SCN, SDN, SBN, TXF and a standard mixture of SLM.

The flavonolignan content of the root was expressed as mg/g (roots DW) and derived using a known concentration of standard and sample peak areas. The data obtained from the analysis of each sample allowed the plotting of a calibration curve showing good linearity (a correlation coefficient of 0.999). The data were displayed as the mean of at least three replicates. Statistical significance was calculated using the Duncan test for unpaired data ($\alpha' \geq 0.05$) and the Analysis of variance (ANOVA) method was used for comparisons of the means. Statistical analysis was made by Statistical Analysis Software (SAS) software (Version 6.2). Standards of SLM, SBN and TXF were purchased from Sigma (Germany); SCN and SDN were obtained from Phytolab (Germany).

Hairy root cultures are usually able to produce the same compounds that can be found *in vivo*, without the loss of concentration frequently observed with callus or cell suspension cultures. A few studies addressing the possibility of flavonolignan production in "*in vitro*" cultures have been carried out and in all cases the production has been found to be very low and even disappearing during prolonged cultures (Namdeo, 2007; Tumova *et al*., 2004).

In the present study, we observed that hairy root induction in *S. marianum* can be made by inoculation

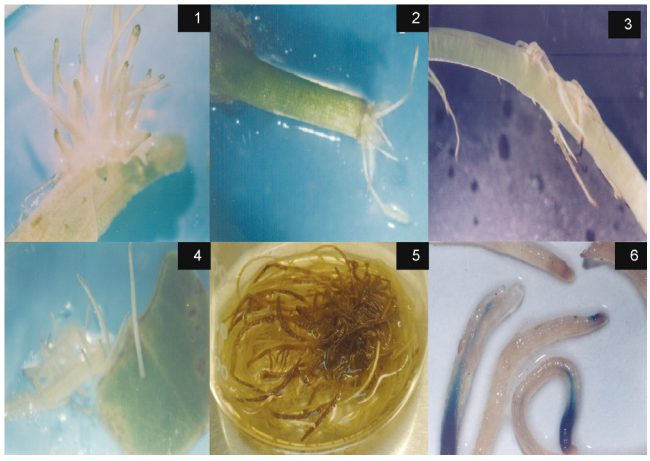

Figure 1. Hairy root induction of *S. marianum*. leaf (1), hypocotyl (2), whole plant *via* the injection method (3), and cotyledon (4), Hairy roots in liquid culture (5). GUS staining in hairy roots indicates successful integration of β-glucoronidase transgene (6).

of hypocotyl, cotyledon and leaf explants with *A. rhizogenes AR15834*. In all the explants (cases), hairy roots were induced in 7-10 days after inoculation, emerging on the wounded side of the explants (Fig. 1). In the first experiment for optimizing hairy root transformation, the efficiency of transgenic root selection based on screening of hairy-roots for GUS activity was compared in explants of *S. marianum*. Of 150 cotyledon explants inoculated with *A. rhizogenese* containing the pBI121 vector, 48 roots were produced after 4 weeks. Subsequent histochemical GUS staining of root tissues confirmed GUS activity in 45 (30%) of the hairy root clones. Transformation efficiencies were 7.9% for hypocotyls, 21.6% for cotyledons and 20% for whole plants by using the injection method. All of the Gus positive hairy roots as tested by histochemical analyses were confirmed by PCR analyses of the *rolB* and *gus* transgenes (Fig. 2).

Because of simple handling, possibility of less con-

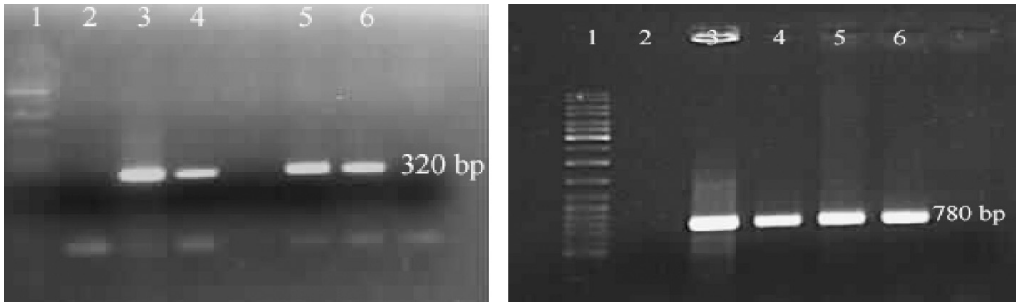

Figure 2. PCR analysis of hairy roots for *gus* (left) and *rolb* (right) transgenes. 1: Molecular weight marker, 2: Non transgenic root, 3: Positive control, 4- 6: Transgenic hairy roots.

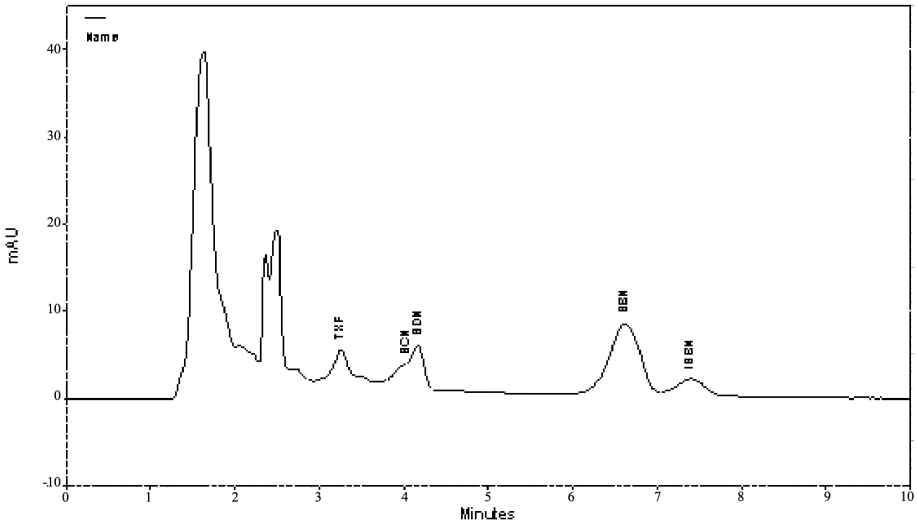

Figure 3. Chromatogram (HPLC) of methanolic extract of hairy roots of *S. marianum.*

tamination and higher transformation efficiency, we therefore, propose inoculation of cotyledon and leaf explants for induction of hairy roots in *S. marianum*. In the second experiment cotyledon explants were transformed using *A. rhizogenes* without the reporter gene, for induction of hairy roots. Transformed hairy roots were selected via PCR analysis of the *rol*B gene.

Eight different hairy root lines were established on liquid MS medium and compared analytically with non-transformed roots. Significant statistical differences were observed among the lines. The highest biomass production was found in line 7 (about 1 g). The

Table 1. Dry weight (DW) and flavonolignans contents (mg/g DW) (± SD) produced by *S. marianum* hairy root lines after 4 weeks in culture. The flavonolignans were determined by HPLC method.

D W (g)	Taxifolin	Silydianin	Silychristin	Silybin	Isosilybin
0.953 ± 0.008 ^{b}	0.015 ± 0.001 ^{b}	0.019 ± 0.001 ^{d}	0.012 ± 0.001 ^{g}	0.002 ± 0.001 ^{de}	0.001 ± 0.000 ^{c}
0.367 ± 0.010 ^{f}	0.019 ± 0.001 ^{a}	0.091 ± 0.001 ^{a}	0.030 ± 0.001 ^{d}	0.004 ± 0.002 ^{bcd}	0.005 ± 0.003 ^{bc}
0.178 ± 0.025 ^{g}	0.004 ± 0.000 ^{ef}	0.067 ± 0.002 ^{b}	0.072 ± 0.004 ^{b}	0.006 ± 0.001 ^{ab}	0.008 ± 0.001 ^{b}
0.057 ± 0.006 ^{h}	0.002 ± 0.000 ^{e}	0.086 ± 0.003 ^{a}	0.141 ± 0.005 ^{a}	ND	ND
0.352 ± 0.014 ^{f}	0.009 ± 0.000 ^{c}	0.042 ± 0.004 ^{c}	0.041 ± 0.002 ^{c}	0.007 ± 0.001 ^{a}	0.011 ± 0.001 ^{a}
0.638 ± 0.006 ^{d}	0.012 ± 0.07 ^{b}	0.026 ± 0.002 ^{d}	0.029 ± 0.002 ^{d}	0.005 ± 0.000 ^{abc}	0.006 ± 0.001 ^{bc}
1.000 ± 0.011 ^{a}	0.015 ± 0.005 ^{b}	0.022 ± 0.002 ^{d}	0.022 ± 0.003 ^{ef}	0.004 ± 0.001 ^{bcd}	0.003 ± 0.001 ^{bc}
0.853 ± 0.011 ^{c}	0.013 ± 0.001 ^{b}	0.022 ± 0.002 ^{d}	0.024 ± 0.004 ^{g}	0.003 ± 0.002 ^{dc}	0.002 ± 0.000 ^{bc}
0.554 ± 0.056 ^{e}	0.006 ± 0.000 ^{cd}	0.019 ± 0.003 ^{d}	0.018 ± 0.002 ^{f}	0.002 ± 0.001 ^{de}	0.003 ± 0.001 ^{bc}

Three flasks were harvested for each root line; 1-8: Hairy root lines; 9: untransformed roots; ND: non-detectable;. S.D: standard deviation. The superscript letters following the calculated means and standard deviations are an indication of similarity in the data. Values sharing a letter within a column are not significantly different at $p< 0.05$.

lines were also assessed for the production of flavonolignans. The flavonolignan content varied greatly from one line to another. The presence of silybin and isosilybin were detected by HPLC analysis of the methanolic extract of the hairy root culture sample (Fig. 3). Further experiments will be conducted on the single hairy root line showing the highest biomass and flavonolignans production.

We have shown that such roots developed on *S. marianum* produced SBN (0.007 mg/g DW), ISBN (0.011), SCN (0.041), SDN (0.042) and TXF (0.009) similar to those produced by dried fruits of this plant (Table 1, line 5). In this case, while accumulating in the seeds, silymarin has also been shown to accumulate in the hairy root cultures. This is in agreement with some previously reported results in other plants (Kim *et al*., 2002), although the yield percentage is lower than what is generally found in dried fruits of *S. marianum*. The major flavonolignans in the untransformed root culture were SBN (0.002 mg/g DW), ISBN (0.003), SCN (0.018), SDN (0.019) and TXF (0.006).

The hairy root cultures of *S. marianum* can be a promising source for continuous and standardized production of silymarin under controlled conditions. Only one report has been published regarding hairy root induction and flavonolignan production in *S. marianum* cultures. In that report silymarin production was demonstrated to be very low and the accumulation of silybin was not even detected (Alikaridis *et al*., 2000). Transformed root cultures of several other species have been evaluated for their content of secondary metabolites relative to other wild type plants. The secondary product profiles have often been observed to be conserved. For example hairy roots of ginseng (*Panax ginseng* Meyer) produced the same saponins and ginsenoides as the wild type roots. (Yoshikawa *et al*., 1987). Hairy root culture has been used as a model system to study various aspects of the metabolic and molecular regulation of several secondary metabolites. For example, Chen *et al*. (2000) have shown that methyl viologen, a generator of the superoxide anion triggers the formation of cryptotanshione in hairy root cultures of *Salvia miltiorrhiza*. Savita *et al*. (2006) have studied the effects of several biotic and abiotic elicitors in hairy root cultures of *Beta vulgaris* in the shake-flask and bioreactor. They were able to show a good level of betalanin production (1.2% or 88.4 mg/l) using the hairy root culture system.

The availability of this protocol for the production of silymarin similar to those described above provides a powerful system to study various aspects of the metabolic and molecular regulation of silymarin biosynthesis. It is also necessary to evaluate and screen the effects of various elicitors with different mechanisms on the production and accumulation of silymarin for pharmaceutical industries.

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