Improvement of silymarin production in hairy root cultures of *Silybum marianum* (L.) Gaertn using fungal elicitors

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**Abstract**

The objective of the present study was to enhance silymarin production in hairy root cultures of *Silybum marianum* (L.) Gaertn using fungal elicitors. The effects of different concentrations of the fungal elicitors (0, 10 and 20 mg/50 ml culture), including *Fusarium proliferatum*, *Aspergillus niger*, and *Rhizoctonia solani*, were studied on silymarin production in the *S. marianum* hairy root cultures. The hairy roots were harvested 0, 24, 48 and 72 h after inoculations. Detection and identification of flavonolignans was carried out by high performance liquid chromatography method. The maximum silymarin production for each of the fungi treatments were as follow: 10 mg *A. niger* / 50 ml culture (0.18 mg/g DW) after 48 h, 20 mg/ 50 ml culture *F. proliferatum* (0.34 mg/g DW) and 20 mg *R. solani* (0.22 mg/g DW) after 72 h. The flavonolignans of hairy roots treated with *F. proliferatum* included taxifoline (0.068 mg/g DW), silydianin (0.11 mg/g DW), silybin (0.021 mg/g DW) and isosilybin (0.015 mg/g DW) which were respectively 1.94, 1.19, 2.62 and 1.25 fold greater than that the untreated cultures. The results indicated that the type of fungi, their concentrations and exposure time have significant effect on the stimulation of silymarin production.

**Keywords**: *Aspergillus niger*, *Fusarium proliferatum*; Hairy root culture, *Rhizoctonia solani*; *Silybum marianum*, Silymarin.

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1. **Introduction**

The dried fruits of *Silybum marianum* L. (milk thistle) have been used for the treatment of hepatic and biliary diseases since ancient times [1]. Its active compounds (silymarin) have been identified from 1952 [1, 2]. Silymarin is composed of an mixture of the flavonolignans, silychristin (SC), isosilychristin (ISC), silydianin (SD), silybin (SB) A and B, and isosilybin (ISB) A and B with the precursor flavonoid taxifolin (TXF) [3, 4].

Plant cells have been considered to be producers of secondary metabolites. Recently, the transformed root cultures have been offered additional advantages such as rapid growth, uniformity, genetic stability and high biosynthetic capacity. Commonly, hairy roots are formed by genetic transformation of plant cell using *Agrobacterium rhizogenes* [5, 6].

Usually the production of secondary metabolites remained low in tissue cultures, so, elicitation techniques have been employed to improve the production of bioactive molecules. Elicitors are compounds of mainly biotic or abiotic origin, which upon contact with higher plant cells and trigger the increased production of bioactive molecules and other defense related compounds [7].
Previously, we showed that cell and hairy root cultures of *S. marianum* synthesize flavonolignanes, but the amount of these produced compounds is less than that obtained from the intact plant. Therefore elicitation strategies have been investigated to try and enhance the production of flavonolignanes in *S. marianum* cell and hairy root cultures (8, 9, 10, and 11).

Previous studies have reported improvement of metabolite production by different fungal elicitors *Phytophthora megasperma* [2] and *Aspergillus niger* [13] in hairy root cultures.

The production of phytoalexin was enhanced in suspension cultures of carrot by *A. niger* and *F. moniliforme* [14]. The suspension cultures of periwinkle treated with either *A. niger* and *Rhizopus* or *Trichoderma viride* increased the intracellular accumulation of tryptamine [15]. Chitosan stimulation of anthraquinone synthesis in *Rubia tinctorum* reported by Vasconsuelo et al., (1996) (16). Liu et al., (1997) showed that artemisinin production was increased to 550 mg / l when the cultures of *Artemisia annua* L. hairy root were elicited with a homogenate of *A. oryzae* [17]. An increase of 150% in tabersonine specific yield was observed upon addition of 72 units of pectinase in *Catharanthus roseus* hairy root cultures by Rijhwani and Shanks (1998) [18]. Liu et al. (1999) reported that elicitors derived from mycelia extracts of *Penicillium chrysogenum* enhanced artemisinin production in hairy roots of *Artemisia annua* 1.2-fold higher than that of the control experiment [19]. Wang et al., (2002) demonstrated that artemisinin content in hairy roots of *Artemisia annua* increased from 0.8 mg/g DW to 1 mg/g DW by using elicitor treatment of mycelial extracts from the endophytic fungus *Colletotrichum sp* [20]. Zhao et al (2001) studied production of a novel antimicrobial tropolone, beta-thujaplicin, in *Cupressus lusitanica* suspension cultures. Significantly improved beta-thujaplicin production (187 mg/L) was obtained using fungal elicitor treatment in a production medium [21]. Upon elicitation with fungal cell wall elicitors from *P. cinnamoni*, the production of rosmarinic acid (RA) was enhanced 2.67-fold compared with the untreated control [22]. The elicitation of the *opium poppy* cell cultures by fungal preparation lead to a nine-fold increase in the content of sanguinarine (23). A crude extract from *F. oxyprum* caused significant cell apoptosis in suspension cultures of *Taxus chinesis* var. mairei. The maximum concentration of taxol was three times higher than that of the control. Cell apoptosis and taxol production are closely relevant in elicitor-treated culture of *T. chinensis* var. mairei [24]. Hairy root cultures of *Cichorium intybus* L. produced volatile aromatic compounds under the influence of fungal elicitors. It was observed that the intensity of the production of volatile aromatic compounds in the hairy root cultures of *C. intybus* with 10 ml L\(^{-1}\) media filtrate (MF) of *P. parasitica var nicotiana* reached a maximum on the 21st day [25]. Lu et al (2003) showed when, on the 15th day of growth, an elicitor from *F. solani* was added at 40 mg/Lt to *Cistanche deserticola* cell suspension cultures, the contents of echinacoside, acteoside and total phenylethanol glycosides (PeGs) in cultured cells all increased over the next 27 days by over 100% to 15 mg/g DW, 9 mg/g DW and 57 mg/g DW, respectively. The final biomass (1.3 mg DW/ml) was not affected (26). The accumulation of baicalin in transformed hairy roots was enhanced through exposure to various elicitors. Elicitation was attained by the addition of methyl jasmonate, salicylic acid and various concentrations of fungal cell wall elicitors to the medium. The accumulation of baicalin in the elicited cultures ranged from 10.5 to 18.3 mg/g DW of the roots, which was 1.5- to 3-fold the amount attained in controls [27]. Artemisinin production by hairy roots of *Artemisia annua* was increased 6-fold to 1.8 μg/mg DW over 6 days by adding 150 / Lchitosan [28]. Hasanloo et al., (2009) suggested that reactive oxygen species may mediate yeast extract elicitor signals to jasmonate pathway that lead to the production of silymarin in *S. marianum* hairy root cultures [10]. Ajungla et al., (2009) established root cultures of *Datura metel* and studied the influence of biotic (*A. niger, Alternaria sp.*, *F. moniliforme* and yeast extract) and abiotic 8222
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(salicylic acid, AlCl₃, CaCl₂, NaCl and Na₂SO₄) elicitors on the growth and production of hyoscyamine and scopolamine. The highest hyoscyamine (4.35 mg/g DW) and scopolamine (0.28 mg/g DW) accumulation was obtained in cultures treated with 500 μM salicylic acid, followed by treatment with 0.75 g / L yeast extract (3.17 mg/g DW hyoscyamine & 0.16 mg/g DW scopolamine) (29). Zheng et al., (2009) reported multiple responses of *Inonotus obliquus* cells in media supplemented by fungal elicitor prepared from the cell debris of the plant-pathogenic ascomycete *Alternaria alternata*. Nitric oxide (NO) mediates an elicitor-induced increase in production of antioxidant polyphenols in *I. obliquus* via a signaling pathway independent of oxylipins or jasmonic acid (JA), a mechanism which differs from those in some higher plants [30]. Lu et al (2011) demonstrated that nitrate reductase (NR) is involved in the fungal elicitor-triggered NO generation and the fungal elicitor induces camptothecin production of *Camptotheca acuminata* cells dependently on NR-mediated NO generation [31].

To the best of our knowledge, no previous study has investigated the effects of different fungal elicitors on enhancement of silymarin productivity. Therefore, the objective of the present study was to evaluate effects of different types and concentrations of fungal extracts on silymarin accumulation in *S. marianum* hairy root cultures.

## 2. Material and methods

### 2.1. Hairy root cultures

Hairy root culture of *S. marianum* was transformed by *A. rhizogenes* (AR15834), and the genetic transformation of these hairy roots was confirmed by polymerase chain reaction (PCR) according to the method described by Rahnama et al. (2008) [8]. PCR was performed for 35 thermal cycles (denaturation at 94 °C for 1 min, primer annealing at 53 °C for 1 min, and primer extension at 72 °C for 1 min) for rolB (forward primer 5´-ATGGATCCCAAATTGCTATTCCCCACGA-3’ and reverse primer 5’-TTAGGCCCTTCTTCAATTCCGGTTTACTGCAGC-3’). Hairy roots cultures were induced by transferring six 1cm roots to 50 ml of Murashige and Skoog liquid medium (MS) supplemented with 30 g / L sucrose in 150 ml flaks (32). All the experiment were carried out on orbital shaker set at 150 rpm and incubated at 25 °C in the dark.

### 2.2. Preparation of elicitors and elicitation

Three fungi, including *A. niger*, *F. proliferatum* and *R. solani* were received from Microbial Gene Bank of Microbial Biotechnology and Biosafety Department of Agricultural Biotechnology Research Institute of Iran. The fungus elicitors were prepared according to the method previously described by Chong et al., (2005) [33]. The fungi were grown in liquid potato/dextrose medium incubated on a rotary shaker (150 rpm) at 28 °C and sub-cultured every 2 weeks. After 15 days the mycelia were collected by filtration and homogenized. The homogenates were autoclaved and used as elicitor at different concentrations (0, 10 and 20 mg/ 50 ml culture media). The elicitors were added to 30-day-old hairy root cultures. For a time course study, untreated and elicited hairy roots were harvested at different time intervals (0, 24, 48 and 72 h) and then frozen immediately at -80 °C for the next biochemical assays. Biomass was quantified by dry weight.

### 2.3. Analytical procedures

Silymarin were quantified by high performance liquid chromatography (HPLC) analysis as described by Hasanloo et al. (2009) (10) on a Knauer liquid chromatography equipped with a injector with a 20 μl loop, a Nucleosil C18 5 μ (250 × 4.6 mm) column, K2600A UV detector and Chromgate software for peak integration. Hairy roots were
harvested from the shake-flasks and dried by tissue paper. Lyophilized powdered hairy root samples were measured in terms of DW. The samples were defatted with petroleum ether. The flavonolignans were extracted from the dried residue with 10 ml of methanol at 40°C for 8h. The methanolic solution was concentrated to a dry residue. The extract was dissolved in 2 ml of methanol and kept at 4°C in darkness [8].

2.4. **Statistical analysis**

The data were given as the mean of at least three replicates. Statistical analysis was performed with SAS software (Version 6.2) using ANOVA method with Duncan test set at \( \alpha \leq 0.05 \).

3. **Results and Discussion**

3.1. **Growth and silymarin production**

Previously, we had optimized the process of biomass accumulation and silymarin production in the hairy root cultures of *S. marianum* in a 35 days period (11). The highest biomass was obtained at the end of the growth period (from 28 to 35 days after culture). Silymarin content was analyzed over the culture period. The highest silymarin content was observed after 28 days. Therefore, treatments were done 30 days after culture, when hairy roots were in the maximum active growth phase.

3.2. **Influences of fungal elicitors on culture biomass and silymarin production**

3.2.1. *F. proliferatum*

Hairy root cultures (30 days old), were treated with three different concentrations of *F. proliferatum* biomass, including 0, 10 and 20 mg/ 50 ml culture. A significant increase in dry weight (DW) of the cultures was observed in media supplemented with 10 mg *F. proliferatum/ 50 ml culture and the highest biomass production (0.51 mg) was obtained 72 h after elicitation was significantly higher than the control (0.33 mg) (Fig. 1A). A significant reduction in DW was observed by the addition of 20 mg/ 50 ml culture *F. proliferatum/ 50 ml culture after 24, 48 and 72 h (0.29, 0.35 and 0.43 mg, respectively) compared with media treated with 10 mg/ 50 ml culture. The DW of non- treated hairy roots was not significantly changed after 24, 48 and 72 h (0.28, 0.30 and 0.33 mg, respectively). The time course of the effect of *F. proliferatum* on silymarin accumulation is presented in (Fig. 1B). Silymarin accumulation in non- treated hairy roots showed no significant changes after 24, 48 and 72 h (0.19, 0.17 and 0.21 mg, respectively). The timing of feeding had significant effect on the silymarin accumulation in media treated with 20 mg/ 50 ml culture *F. proliferatum*. The silymarin accumulation was increased and reached an extremely high level (0.34 mg/g DW) after 72 h of treatment that was 1.61- fold higher than the control. The changes of the accumulated silymarin after 48 h of treatment was similar to those of silymarin accumulation in the period of 72 h time- course study. The production of silymarin (0.27 mg/g DW) was also increased after 48 h of treatment in media supplemented with 20 mg/ 50 ml culture *F. proliferatum*, being 1.58- fold higher than the control (0.17 mg/g DW). The silymarin production of the treated hairy roots with 10 or 20 mg/ 50 ml culture (0.18 and 0.19 mg, respectively) was not significantly changed after 24 h in compare with control (0.19 mg).

To determine which flavonolignans changes in the treated hairy root cultures, the extracted silymarin quantified by HPLC analysis. The presence of silybin and isosilybin were detected. The results showed that the hairy roots produced silybin, isosilybin, silychristin, silydianin and taxifolin, which were similar to the compound reported by the dried fruits of *S. marianum*. A significantly higher content of taxifolin (0.1 mg/g DW) was achieved after 72 h in media treated with 10 mg/ 50 ml culture *F. proliferatum* that was 3.33- fold higher than control. Our results showed that silychristin and isosilybin content increased after 72 h. There
was a gradual decline in silydianin accumulation after 72 h. Silybin production was slightly enhanced over the culture period (Table 1). The flavonolignans analysis was also carried out on hairy roots treated with 20 mg/50 ml culture \emph{F. proliferatum}. Taxifolin and silydianin content rise dramatically from 24 to 48 h (0.072 and 0.117 mg/g DW, respectively), but then stabilized. Silycristin, silybin and isosilybin accumulation showed no significant changes within the culture period.

A         B

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{The effects of different concentrations of \emph{F. proliferatum} (0, 10 and 20 mg/50 ml culture) and exposure time (0, 24, 48 and 72h) on biomass (A) and silymarin (B) production in hairy root cultures of \emph{S. marianum}. Data show means ± SD from triplicate experiments.}
\end{figure}

3.2.2. \emph{A. niger}

The time course of the effect of \emph{A. niger} on the hairy root growth and silymarin content are presented in Fig. 2A and B. The DW of non-treated hairy roots was slightly increased after 24 h that reached to 0.38 g after 72 h. An enhancement in DW was observed in media treated with 10 mg \emph{A. niger} / 50 ml culture after 48 h (0.39 g), hitting a peak after 72 h (0.41 g). No significant difference was found between DW in media supplemented with 20 mg \emph{A. niger} / 50 ml culture (0.37 g) after 24 h and in the control (0.22 g). Fig. 2A shows silymarin content in hairy root cultures treated with 0, 10 and 20 mg/50 ml culture \emph{A. niger}. There was a slight increase in silymarin content of non-treated hairy roots but then stabilized. As it can be clearly seen the highest content of silymarin (0.18 mg/g DW) was obtained in media treated with 10 mg/50 ml culture after 48 h which was 1.51-fold higher than the control but the trend was not upward and a dramatic fall observed in silymarin accumulation (0.14 mg/g DW) after 72 h.

Table 2 compare the results obtained from the HPLC analysis of \emph{A. niger} treated hairy root cultures. It can be seen from the data in Table 2 that 48 h feeding time in media treated with 10 mg \emph{A. niger} / 50 ml culture reported significantly more taxifolin (0.071 mg/g DW) than the other two groups. The most striking result to emerge from the data is that silybin and isosilybin content (0.019 and 0.016 mg/g DW, respectively) had higher significant accumulation in media supplemented with 10 mg \emph{A. niger} / 50 ml culture after 48 h that were 1.9 and 1.6-times more than that of the control (0.01 mg/g DW). No significant differences were observed between \emph{A. niger} treated and non-treated hairy root cultures in silycristin and silydianin content for different exposure times (24, 48 and 72 h).

3.2.3. \emph{R. solani}

Fig. 3A presents the results obtained from the DW of \emph{R. solani} treated and non-treated hairy roots. From this data we can see that the highest DW (0.5 g) was obtained from cultures treated with 10 mg/50 ml culture \emph{R. solani} after 72 h that was 1.42-fold higher than the control (0.35 g). No significant differences were found between DW of cultures treated with 10 and 20 mg/50 ml culture \emph{R. solani} after 24 h. The mean score for DW was 0.45 g that
was 1.5-fold higher than the control. A rapid increase in DW of cultures elicited with 10 mg/50 ml culture was seen after 48 h (0.42 g) that was 1.57-times that of the control (0.28 g). The DW content in 20 mg/50 ml culture treated media after 48 h was 0.407 g that was 1.45-times that of the control (0.28 g).

![Figure 2. The effects of different concentrations of A. niger (0, 10 and 20 mg/50 ml culture) and exposure time (0, 24, 48 and 72h) on biomass (A) and silymarin production (B) in hairy root cultures of S. marianum. Data show means ± SD from triplicate experiments.]

No significant differences were found between interactions of concentration and feeding time of elicitation in total silymarin and flavonolignan content (Fig. 3B). There were significant differences among media treated with different concentration of R. solani in silybin and total silymarin. From this data we can see that feeding time had significant effect in total silymarin, silybin, silychristin and taxifolin. The highest content of silymarin was observed in media containing 20 mg/50 ml culture R. solani after 72 h.

The results obtained from the HPLC analysis of silymarin (flavonolignans) extracted from R. solani treated hairy roots are shown in table 3. As can be seen from the table 3, no significant increase in taxifolin production was detected in treated hairy roots after 24 and 48 h. The more surprising enhancement is with the R. solani treated hairy roots (10 and 20 mg/50 ml culture) after 72 h that were 1.79-fold higher than the control (0.034 mg/g DW). It is apparent from this table that very few changes were observed in silychristin content in R. solani treated and non-treated hairy roots. None of these differences were statistically...
significant. As table 3 shows, there is a significant difference between the two groups of treatments (0 and 20 mg/50 ml culture) in different exposure times for silydianin production. Silydianin content in cultures treated with 20 mg \( R. \) solani /50 ml culture was 0.037, 0.04 and 0.051 mg/g DW that were 1.1, 1.25 and 1.13- times that of the control, respectively. The highest content of silydianin (0.051 mg/g DW) was observed in media elicited with 20 mg \( R. \) solani /50 ml culture after 72 h.

Elicitors induced accumulation of secondary metabolites have received wide acceptance because of its ability to improve productivity of the plant tissue cultures significantly (20, 34, and 35). Elicitors can stimulate the synthesis of plant metabolites or induce accumulation of new secondary metabolite compounds; a specific eliciting effect of fungal homogenates and fractions therefore has been widely documented (36, 21).

The effects of fungal elicitors dose and exposure time on the silymarin accumulation of hairy root cultures of \( S. \) marianum were examined. Our results showed that the accumulation of total silymarin and flavonolignans can be stimulated by a fungal elicitor. These findings raise a possibility that some fungus could affect the secondary metabolism of cultured tissues. From a biotechnological point of view, the increase of productivity after elicitor treatment is of great practical value.

At the present study, it was shown that \( S. \) marianum hairy root cultures accumulated considerable amounts of silymarin in response to treatment with \( F. \) proliferatum. For maximum production of silymarin, the optimum period of elicitation was 72 h and the optimum concentration of this fungal elicitor was 20 mg/50 ml culture. Similar results were obtained after the addition of fungal elicitor (\( F. \) moniliforme) to cell cultures of \( C. \) roseus [37]. In the present study, exposure time plays an important role for the production of flavonolignans. The optimum exposure time favored silymarin production. Thus, it may be concluded that there is an optimum exposure time for maximum formation and accumulation of desired bioactive molecules. This finding is in agreement with the results of Namdeo et al (2002) who showed the exposure time of 48 h resulted in a significant accumulation of ajmalicine in \( C. \) roseus cells elicited with \( A. \) niger, \( F. \) moniliforme, and \( T. \) viride. Khalili et al (2009) have reported that the production of silymarin was 2.52-fold higher than the control after 24 h and a further increase in the exposure time inhibited the synthesis of silymarin in media treated with salicylic acid [11]. Previously, we observed that the accumulation of silymarin had a 4.9-fold increase when hairy roots were treated for 72 h with yeast extract (2.5 mg/50 ml culture) while content of this compound decreased by 96 and 120 h after treatment(10). Our results showed that silymarin production decreased between 48 h (when it reached maximum production) and 72 h after addition of 10 mg/50 ml culture \( A. \) niger. These results seem to be related to toxic effects of elicitor. The toxic effect of elicitors may be related to their mechanism of action. It has previously been suggested that cellular damage is caused by elicitors, especially to membranes [10].

Wiktorowska et al (2010) were investigated the effects of elicitors on cell growth and oleanolic acid (OA) accumulation in shaken cell suspension cultures of \( Calendula \) officinalis. Elicitors were added individually at various concentrations to 5-day-old cell cultures and their effects monitored at 24 h intervals for 4 days. After 72 h of treatment with 100 M Jasmonic acid, the intracellular content of OA reached its maximum value (0.84 mg/g DW), which was 9.4-fold greater than that recorded in an untreated control cultures [38].
Table 1. The flavonolignan content (mg/g DW) in *F. proliferatum* treated (0, 10 and 20 mg/50 ml culture) and non-treated (control) hairy root cultures of *S. marianum* 24, 48 and 72 h after elicitation. The flavonolignans were analyzed with HPLC. Data show means ± SD from triplicate experiments.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Concentration of fungi</th>
<th>TXF SC</th>
<th>SD ISB SB</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.043±0.113</td>
<td>0.036±0.000</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0.047±0.006</td>
<td>0.034±0.001</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0.042±0.011</td>
<td>0.03±0.001</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
<td>0.033±0.004</td>
<td>0.038±0.006</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0.046±0.006</td>
<td>0.035±0.000</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0.072±0.014</td>
<td>0.036±0.003</td>
</tr>
<tr>
<td>72</td>
<td>0</td>
<td>0.039±0.011</td>
<td>0.035±0.005</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0.10±0.017</td>
<td>0.061±0.006</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0.068±0.002</td>
<td>0.034±0.001</td>
</tr>
</tbody>
</table>

A possible explanation for this might be that, the fungal extracts included chitin oligomers (oligochitin, N-acetylchitooligosaccharides) and Chitosan (the deacetylated form of chitin), which can be generated from fungal cell walls. The main component of the cell wall is chitin, which is a polysaccharide composed of beta-1,4-linked N-acetylglucosamine. Chitosan is produced by deacetylation of chitin, which is a common process in fungi. Chitosan has various applications in biotechnology due to its biocompatibility and biodegradability. However, the exact mechanism by which the fungi act on the plant cells is not fully understood.
walls of some fungal species can be inducing defense-related cellular responses in plants [39]. Chitin oligomers have been shown to induce various defense-related cellular responses and increased production of secondary metabolites in plant tissue cultures [38, 40]. The production of anthraquinone colorants in madder (Rubia akane Nakai) cell culture elicited with 25 mg/ L chitosan, was increased approximately two times in a seven-day culture as compared to that in the unelicited cells [41]. The positive effects of chitosan elicitation on xanthone biosynthesis in calli and in cell suspension cultures of Hypericum perforatum subsp. angustifolium was reported by Tocci et al (2010) [42]. The addition of chitosan at 50 mg/ L produced a 5-fold enhancement of OA accumulation (0.37mg/g DW) after 48 h of treatment in cell suspension cultures of Calendula officinalis L.. Chitosan induces cell wall lignification [43]. Production of phytoalexins and the generation of hydrogen peroxide, a reactive oxygen species, are also responses of plants elicited with chitosan [44, 45]. There is scarce evidence on the signal transduction pathways involved in the elicitor actions. It has been reported that chitosan stimulates the accumulation of jasmonic acid, a signal molecule related to defense-gene regulation (34). Vasconsuelo et al (2003) recently provided evidence on the participation of the PLC/PKC pathway in the anthraquinones response elicited by chitosan in Rubia tinctorum (46). Additionally, they searched for interactions of this pathway with PKC, phosphoinositide 3-OH-kinase (PI3K) and adenylyl cyclase (AC)/cAMP/PKA messenger systems. The transduction of elicitor signals in plant cells may utilize a mechanism similar to the process reported in animal cells in response to extracellular stimuli, where second messengers are generated, leading to the activation of protein kinase cascades which may activate the biosynthetic ability for specific plant products (47).

Another possible explanation for this is that, H2O2 from the oxidative burst link to biosynthesis of second metabolites that was reported by many researchers. Hydrogen peroxide (H2O2), the most stable compound among reactive oxygen species (ROS), has been implicated in plant disease resistance and in a number of plant pathogen interactions at the early stage. However, it is not clear whether inhibition or enhancement of H2O2 would affect second metabolites biosynthesis. Treatment of Taxus chinensis cultures with fungal elicitor stimulated the H2O2 accumulation (48), which agrees with the observations by Yuan et al. [49, 50] and Yu et al. (2002) (24). Lan et al (2003) reported that elicitor-induced taxol production was not accorded with the amount of H2O2 production. In cell suspension cultures of Taxus chinensis, fungal elicitor (A. niger) and HgCl2 elicited taxol, which was a 9-fold and 5-fold increase compared with the control. The fungal elicitor induced hydrogen peroxide (H2O2) accumulation but HgCl2 did not, indicating that H2O2 was not necessary for enhancement of taxol induced by elicitor. These results showed that elicitor-induced taxol production did not depend on the intensity of H2O2 from oxidative burst, which is in contrast to the observations of Yuan et al. (2001 and 2002) [49, 50]. Therefore, further investigations are required to identify fungal elicited silymarin production in S. marianum hairy root cultures is or not related to oxidative burst.

The obtained results indicated that synthesis of silymarin in hairy root cultures of S. marianum was elicited by fungal elicitation. Further research should be done to investigate the JA as a part of signal transduction pathway that mediates the induction of defensive genes and study of the activated defensive genes that has been proposed to occur via the JA pathway. Chitosan is the deacetylated form of chitin, which is the main component of the cell walls of some fungal species, a further study with more focus on chitosan elicitation and other fungal cell wall parts, is therefore suggested.
4. Conclusions

1. Both the type and concentration of fungal elicitors are very important in determining the enhancement of silymarin accumulation in the *S. marianum* hairy root culture.

2. The *F. proliferatum* elicitors (20 mg/ 50 ml culture) were identified as the most effective elicitor to enhance silymarin accumulation (0.34 mg/g DW), regardless to its concentration.

3. The application of elicitors to *S. marianum* hairy root culture could be a useful tool for studying the regulation of plant cell metabolism in responses to various stress factors.

4. Further investigations are required to characterize the mechanisms underlying the intracellular accumulation of silymarin in elicited *S. marianum* hairy root cultures medium, and also to identify key steps participating in the signaling network activated by the elicitor. Such knowledge may be useful for manipulating the biosynthesis of silymarin in *S. marianum*.

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References


