

Evaluation of the yeast-extract signaling pathway leading to silymarin biosynthesis in milk thistle hairy root culture

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Received: 11 March 2009 / Accepted: 10 June 2009 / Published online: 26 June 2009
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Abstract The biosynthesis of silymarin, a potent antihepatotoxic compound, from the dried fruits of *Silybum marianum* L. Gaertn in hairy root cultures can be stimulated by a yeast extract elicitor. These results correlated with culture time, and the biosynthesis reached a maximum of $0.47 \text{ mg g}^{-1} \text{ DW}$ by 72 h after culture (2-fold higher than the control). Lipxygenase activity and linoleic acid content were stimulated by this treatment, suggesting that the jasmonate pathway may mediate the elicitor-induced accumulation of silymarin. The H_2O_2 content increased 24 h after elicitation and did not have marked changes between 48 and 72 h. In addition, the tocopherol content (especially α - and δ -tocopherols) increased 72 h after elicitation in comparison with non-treated cultures. Ascorbate had trace changes during feeding time and was lower than the control. The antioxidant activity was assayed by the 1-1-diphenyl-2-picrylhydrazyl stable free radical method and results were calculated base on an IC_{50} that increased upon treatment, especially 24 h after treatment, with changes related to H_2O_2 content. These observations suggested that reactive oxygen species may mediate elicitor signals to the jasmonate pathway that lead to the production of silymarin.

Keywords *Silybum marianum* · Hairy root · Yeast extract · Silymarin

Introduction

Milk thistle (*Silybum marianum* L.) seeds have been extracted for their flavonolignans, including silychristin, silydianin, silybin, isosilybin and taxifolin, which are generally known as the silymarins (Subramaniam et al. 2008). This plant has been used in medicine for more than 2,000 years. These flavonolignans are used for treatment of toxic liver damage, for therapy of chronic inflammatory liver diseases and liver cirrhosis, and for their anticancer and chemopreventive effects (Flora et al. 1998; Kren and Walterová 2005).

Cell cultures have been established from this plant and are able to produce silymarin, but often less than the amount that accumulates in the fruits (Sanchez-Sampedro et al. 2005). Recent work in improving production of plant secondary metabolites has mainly focused on the following aspects: (1) manipulation of plant cell culture to improve productivity of target compounds, (2) studying signal transduction pathways, (3) studying transcription factors, (4) cloning of secondary metabolite biosynthetic genes, (5) studying metabolic flux and (6) studying gene transcripts to understand the regulation of plant secondary metabolites (Zhao et al. 2005). However, in many cases, the production of silymarin can be enhanced by the treatment of undifferentiated cells with elicitors. Elicitors can trigger an array of defense or stress responses and activate specific genes for the enzymes involved in secondary metabolite biosynthesis, thus improving the production of plant secondary metabolites (Rajendran et al. 1994). Yeast extract (YE) has been reported to stimulate or decrease different secondary

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metabolites in many species such as syringin, valeportriates and podophyllotoxin (Xu et al. 2007; Kittipongpatana et al. 2002; Shams-Ardakani et al. 2005). A number of elicitors such as YE and methyl jasmonate have been investigated for the enhancement of silymarin production in the cell suspension cultures of *S. marianum*. Sanchez-Sampedro et al. (2005) and Hasanloo et al. (2008) reported that the treatment of *S. marianum* cell cultures with methyl jasmonate strongly increases silymarin accumulation both in cells and in the culture medium. Addition of YE to *S. marianum* cultures also improved the production of silymarin to a level about three-fold higher than that of the control (Sánchez-Sampedro et al. 2005). They proposed that YE may cause a complex stress response in the cultures in addition to creating an accumulation of flavonolignans. Antioxidants in plant cells mainly include ascorbate, tocopherol and others, which can protect the cells from oxidative damage by scavenging reactive oxygen species (ROS).

We have recently reported the production of silymarin in hairy root cultures of *S. marianum* (Rahnama et al. 2008). Hairy root is a plant disease caused by *Agrobacterium rhizogenes*, a gram-negative soil bacterium. Hairy roots grow rapidly, and are highly branched in culture media. The transformed roots are highly differentiated and stable. They can produce high amounts of secondary metabolites, whereas plant cell cultures have a strong tendency to be genetically and biochemically unstable and often synthesize very low levels of secondary metabolites (Kittipongpatana et al. 2002; Hu and Du 2006). The aim of this work was to investigate the role of elicitors in the production of silymarin in hairy root cultures. For this reason, different concentrations of yeast extract were added to hairy root cultures to investigate the possible signaling pathway that may be involved in yeast elicitor-induced accumulation of silymarin.

Materials and methods

Hairy root culture

Hairy roots were established according to Rahnama et al. (2008). These roots were maintained by subculture of three 1 cm pieces in Murashige and Skoog liquid medium (MS) on a rotary shaker (150 rpm) in complete darkness (Murashige and Skoog 1962). The roots were subcultured every 4 weeks. Various concentrations (0.1, 0.5 and 1 mg l⁻¹) of the auxin analogues, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA), were tested. Growth rates were determined by measuring the fresh weight of cultured roots at a 4-week interval. The samples with the highest growth index were selected and extracted for determination of silymarin content.

Elicitation study

Yeast extract was dissolved in MS media, prepared as a concentrated stock solution and added to cultures after autoclaving. The control received an equivalent volume of culture media. Five concentrations (0, 0.5, 1, 2.5 and 5 mg/50 ml culture) of yeast extract were added to the 30-day-old hairy roots cultures and the hairy roots were allowed to grow for 72 h before being harvested and analyzed. Since, maximum silymarin production was obtained in the media supplemented with 2.5 mg/50 ml culture YE, to study the effects of the duration of exposure, 2.5 mg/50 ml culture YE were added to 30-day-old hairy roots cultures. Treated and non-treated hairy roots were harvested after 24, 48, 72, 96 and 120 h of treatment and then frozen immediately at -80°C for biochemical assay.

Extraction and determination of silymarin

The silymarin was extracted and quantified by high-performance liquid chromatography (HPLC) analysis as described by Cacho et al. (1999) and Hasanloo et al. (2005) respectively.

Lipoxygenase activity assay

For the lipoxygenase activity assay, the roots were homogenized in an ice bath with 0.1 M Tris-HCl buffer (pH 8.5) containing 1% PVP (w/v), 1 mM CaCl₂, 5 mM DTT, and 10% (v/v) glycerol (Zhao and Sakai 2003). The homogenate was centrifuged at 11,000g for 20 min at 4°C, and the supernatant was used as the enzyme extract. Lipoxygenase was assayed according to Axelroad et al. (1981). About 50 mg of linoleic acid was added to 50 mg Tween 20 and mixed with 10 ml of Na₂HPO₄ buffer (0.1 M, pH 8.7) by stirring and ultrasonic dispersion. The solution was cleared by addition of 250 µl of 1 M NaOH and diluted to 25 ml with the buffer. One ml of the enzyme reaction mixture contained 50 µl enzyme extract, 0.95 ml Na₂HPO₄ buffer and 5 µl of substrate solution. The increase in absorbance was monitored at 234 nm. Total protein was assayed according to Bradford (1976) and the results reported base on Δ OD mg⁻¹ protein min⁻¹.

Fatty acid analysis

Three individual 0.1 g dried hairy roots of *S. marianum* were refluxed with 10 ml of petroleum benzene. The oils were recovered at 40°C. The total lipids were dissolved in petroleum benzene and kept at -20°C until analysis.

For total Fatty acid methyl esters (FAME) analysis, 0.25 ml (10–12 mg) of each lipid extract was methylated with 1.5 ml 5% anhydrous HCl/methanol (w/v) into a 2 ml

vial at 80°C for 1 h. After cooling to room temperature, 2 ml *n*-hexane was added and mixed with a vibration mixer. The hexane layer was collected. The layers were allowed to separate and the *n*-hexane fraction was injected to GC-FID for analysis. Linoleic acid content of the hairy roots of *S. marianum* was determined by GC (Varian, CP-3800, Australia and CP-Sil 88 column and temperature program was from 150°C in 5 min to 240°C in 32 min) (Yi et al. 2009).

Determination of H₂O₂ content

Hydrogen peroxide content was determined according to Velikova et al. (2000). Frozen hairy roots were homogenized in an ice bath with 5 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000g for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the supernatant was measured at 390 nm. The content of H₂O₂ was calculated by comparison with a standard calibration curve previously made by using different concentrations of H₂O₂.

DPPH radical scavenging activity

The scavenging effect of each extract was estimated according to the procedure established by Brand-Williams et al. (1995). The 1-1-diphenyl-2-picrylhydrazyl DPPH concentration (C_{DPPH} as mg ml⁻¹) in the reaction medium was calculated. The parameter IC₅₀ (mg DPPH/ml of extract) was calculated graphically. A lower IC₅₀ value indicates greater antioxidant activity.

α-, γ- and δ-tocopherol analysis

Total α-, γ- and δ-tocopherol were determined as described by Sanchez-Machado et al. (2002). The separation of tocopherols was carried out on an HPLC instrument (all from Knauer, Germany), using a Eurosphere C₁₈ 5 μm (250 × 4.6 mm) column, with a methanol-acetonitrile (50:50 V/V) mobile phase and a flow rate of 1 ml min⁻¹. Detection was carried out at an excitation wavelength of 295 nm and an emission wavelength of 325 nm at room temperature.

Extraction and measurement of ascorbate

Ascorbate was measured as described by Asai et al. (2004). About 0.1 g of fine powdered frozen hairy roots were homogenized in a 20-fold volume of cold 5% (w/v) metaphosphoric acid. The total ascorbate pool was quantified by the 2,4-dinitrophenyl hydrazine method. The amount of ascorbate was calculated by subtracting the

amount of dehydroascorbate from that of the total ascorbate pool.

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 $\alpha \leq 0.05$.

Results

Optimization of culture medium for hairy root growth

The growth rates of hairy root cultures were improved by the addition of auxin to the culture medium. Although rapid growth rates were induced by 1 mg l⁻¹ IBA or NAA, the highest growth rates (3.04 g) and silymarin content (0.15 mg g⁻¹DW, threefold higher than control) were observed by addition of 0.1 mg l⁻¹ NAA (Fig. 1 a, b). Exogenous application of auxin was also reported to stimulate growth in hairy cultures of *Lippia dulcis* Trev. (Sauervin et al. 1991), *Opium poppy* and *California poppy* (Park and Facchini 2000).

By HPLC analysis of the methanolic extract of the hairy root culture sample, the presence of silybin and isosilybin

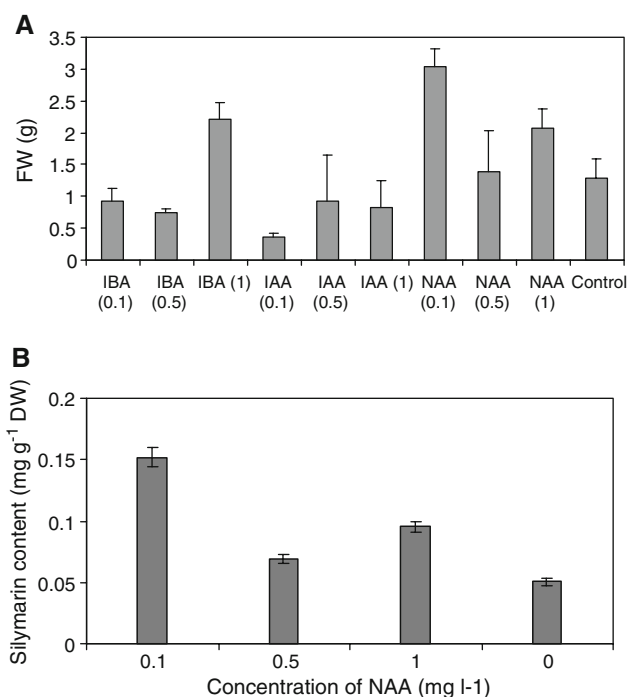


Fig. 1 Effects of auxins on the growth (a) and silymarin accumulation (b) of hairy roots culture of *S. marianum*. Values represent the mean \pm SD from triplicate experiments

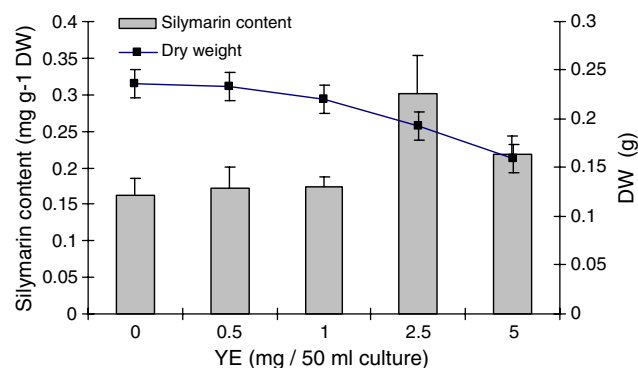


Fig. 2 Effect of different concentrations of YE on silymarin accumulation and DW of *S. marianu* hairy root culture. 30-day-old cultures were treated with 2.5 mg/50 ml culture YE and hairy roots were analyzed for DW and silymarin accumulation after 72 h treatment. Data are the average of three experiments, each performed in triplicate (means \pm SD)

were detected. We have shown that the hairy roots produced silybin ($0.009 \text{ mg g}^{-1}\text{DW}$), isosilybin ($0.005 \text{ mg g}^{-1}\text{DW}$), silychristin ($0.030 \text{ mg g}^{-1}\text{DW}$), silydianin ($0.011 \text{ mg g}^{-1}\text{DW}$) and taxifolin ($0.102 \text{ mg g}^{-1}\text{DW}$), which were similar to the compounds produced by the dried fruits of *S. marianum*. Based on the results, media supplemented with 0.1 mg l^{-1} NAA were chosen for further experiments.

Effects of different concentrations of YE

Hairy root cultures (30 days old), supplemented with NAA (0.1 mg l^{-1}), were treated with four different concentrations (0.5, 1, 2.5 and 5 mg/50 ml culture) of YE. Different concentrations of YE did not stimulate any increase in dry weight, even at higher doses. The amount of silymarin accumulation was significantly increased ($0.3 \text{ mg g}^{-1}\text{DW}$) in hairy roots after 72 h treatment at 2.5 mg/50 ml culture of YE (Fig. 2). Based on the results obtained, the concentration of 2.5 mg/50 ml culture was chosen for further experiments.

Effects of feeding time on growth index and silymarin production

The next experiment was based on the above conditions. Time course for the induction of silymarin and growth index in culture treated with 2.5 mg/50 ml culture medium of YE are presented in Fig. 3a, b. The yeast extract had a positive effect on the biomass after 72 h, which was higher than the control (Fig. 3a). Also, the production of silymarin was higher than the control after 48 and 72 h (0.33 and $0.47 \text{ mg g}^{-1}\text{DW}$, respectively). The yeast extract not only increased the growth index but also induced the production of silymarin, however, the silymarin content decreased after 96 h. The highest content of taxifolin was obtained

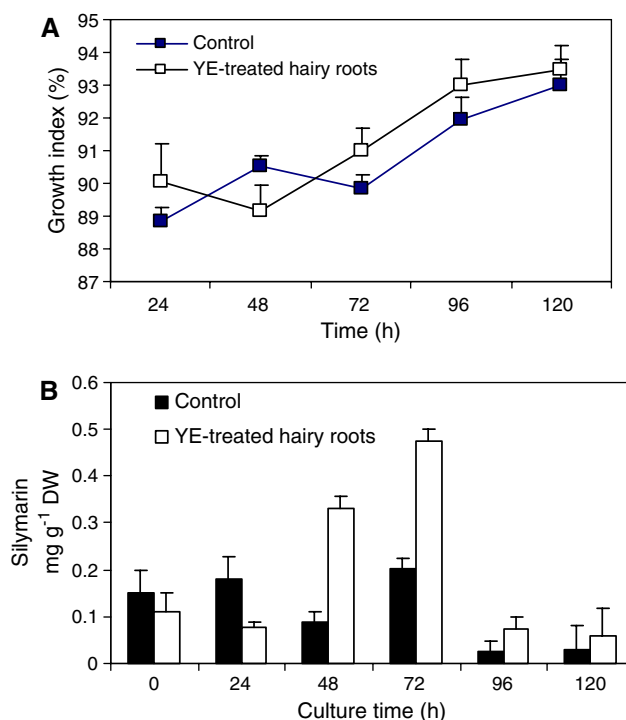


Fig. 3 Time course of the YE-induced growth index (a) and silymarin accumulation (b) of *S. marianu* treated and non-treated (control) hairy root culture. 30-day-old cultures were treated with 2.5 mg 50 ml⁻¹ culture YE and hairy roots were analyzed for growth index (DW) and silymarin (SCN, SDN, SBN and Tax). Data are the average of three experiments; each in triplicate (means \pm SD)

after 48 and 72 h in YE treated media with yeast extract, and was higher than in the control.

The highest silychristin and silydianin production were observed after 72 and 48 h, respectively (Table 1). Our results showed that silybin and isosilybin production after 72 h were about 0.056 and $0.023 \text{ mg g}^{-1}\text{DW}$, respectively and silybin production increased with time of culture.

Lipoxygenase activity and linoleic acid content was stimulated by YE

To determine how YE stimulates silymarin accumulation, and how the JA signaling pathway is also an integral part of the elicitor signaling pathway leading to the accumulation of silymarin, the activity of lipoxygenase was assayed. Lipoxygenase is an important enzyme in the octadecanoid pathway to the biosynthesis of JA. As shown in Fig. 4, lipoxygenase was activated by YE and reached in an extremely high level after 72 h of treatment, and then decreased to the primary level.

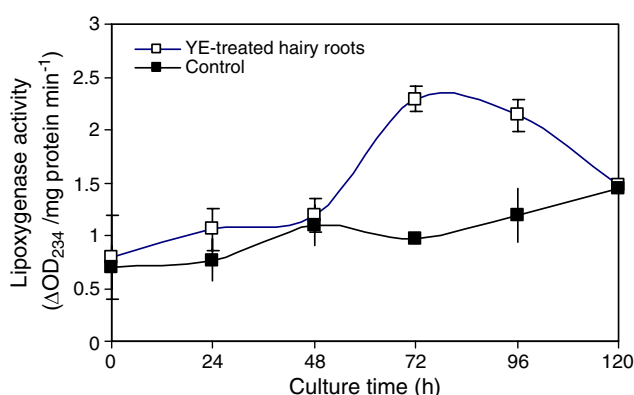
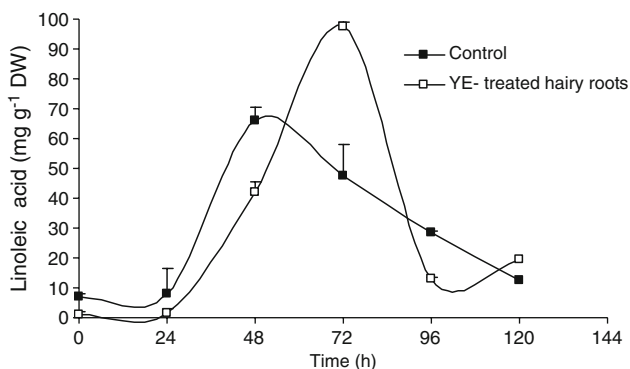
Figure 5 indicates the linoleic acid content in treated and non-treated hairy roots within the period of 120 h. As an overall trend, it is quite obvious that the content of linoleic acid dramatically rose, hitting a peak ($97.62 \text{ mg g}^{-1}\text{DW}$)

Table 1 Flavonolignan content (mg g^{-1} DW) in YE-treated (2.5 mg l^{-1}) and non-treated (Control) hairy root cultures of *S. marianum* for exposure given time periods

Time (h)		Taxifolin	Silychristin	Silydianin	Silybin	Isosilybin
24	Treated	0.0766 ± 0.08^a	0.0578 ± 0.04^b	0.0097 ± 0.00^c	0.0079 ± 0.00^{cd}	0.0105 ± 0.00^b
	Control	0.0486 ± 0.06^b	0.0825 ± 0.01^a	0.0333 ± 0.03^{ab}	0.0065 ± 0.07^c	0.0086 ± 0.05^b
48	Treated	0.02072 ± 0.18^d	0.0685 ± 0.04^b	0.0555 ± 0.00^a	0.0137 ± 0.01^{bc}	0.0030 ± 0.00^c
	Control	0.08712 ± 0.18^a	0.0877 ± 0.14^a	0.0453 ± 0.06^a	0.0196 ± 0.11^b	0.00230 ± 0.08^a
72	Treated	0.0575 ± 0.00^b	0.2935 ± 0.25^a	0.0453 ± 0.02^b	0.0566 ± 0.07^a	0.023 ± 0.03^a
	Control	0.090 ± 0.01^a	0.045 ± 0.12^b	0.0177 ± 0.02^c	0.0439 ± 0.02^a	0.0031 ± 0.13^c
96	Treated	0.0393 ± 0.01^c	0.0067 ± 0.00^c	0.008 ± 0.00^{cd}	0.0215 ± 0.00^b	0.002 ± 0.03^d
	Control	0.0141 ± 0.03^c	0.0031 ± 0.00^{bc}	0.004 ± 0.01^d	0.002 ± 0.10^{cd}	0.0021 ± 0.02^c

Data show means \pm SD from triplicate experiments

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 $p < 0.05$

**Fig. 4** Time-course of lipoxygenase activity in *S. marianum* hairy root cultures treated with YE ($2.5 \text{ mg } 50 \text{ ml}^{-1}$ culture). The control received only MS medium. Data show means \pm SD from triplicate experiments**Fig. 5** Time-course of linoleic acid content in *S. marianum* hairy root cultures treated with YE ($2.5 \text{ mg } 50 \text{ ml}^{-1}$ culture). The control received only MS medium. Data show means \pm SD from triplicate experiments

from 24 to 72 h that was 2-times that of the control (47.64 mg g^{-1} DW). There was a gradual decline in linoleic acid content from 72 to 96 h in treated hairy roots (12.86 mg g^{-1} DW). The linoleic acid content in non-treated

hairy roots reaching a peak after 48 h (65.88 mg g^{-1} DW) but then was on the decline from 48 to 120 h dropping to 12.29 mg g^{-1} DW.

H_2O_2 content and radical scavenging activity

H_2O_2 content increased in both treated and non-treated hairy root cultures of *S. marianum* during incubation times with YE. H_2O_2 content increased upon stimulation by YE after 24 h treatment and reached a maximum content (1.81 mM g^{-1} FW) 48 h after treatment, about twofold higher than the control (Fig. 6a). No marked change was observed between 48 and 72 h, but thereafter the H_2O_2 content increased.

Antioxidant activity of *S. marianum* methanolic extract was determined by the DPPH assay. The DPPH stable free radical method is an easy, rapid and sensitive way to evaluate the antioxidants that scavenge free radicals (Yu et al. 2002). As shown in Fig. 6b, the IC_{50} value was changed significantly after 72 h treatment. The IC_{50} increased upon stimulation by YE after 24 h treatment and reached a maximum at about 24 h after treatment. No marked change in IC_{50} was observed between 48 and 72 h, but it was thereafter increased when the elicitor treatment time was between 72 and 96 h. After 96 h treatment, the IC_{50} significantly decreased, but was still higher than that of the control. As we know, a lower IC_{50} value indicates greater antioxidant activity. Indeed, the antioxidant activity was much higher than the control 24 h after elicitation and did not change between 48 and 72 h after treatment, but thereafter decreased. The antioxidant activity changes were related to H_2O_2 accumulation.

Ascorbate (vitamin C) and tocopherols (vitamin E)

Ascorbate had trace changes between 24 and 48 h after treatment and declined drastically thereafter. The content

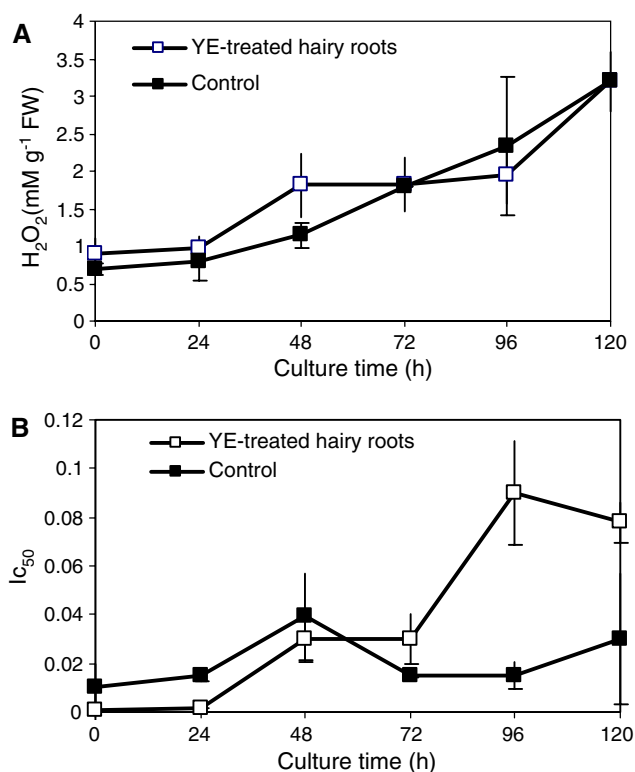


Fig. 6 Time-course of H₂O₂ variations (a) and IC₅₀ variations (b) in *S. marianum* hairy root cultures treated with YE (2.5 mg 50 ml⁻¹ culture). The control received only MS medium. Data show means ± SD from triplicate experiments

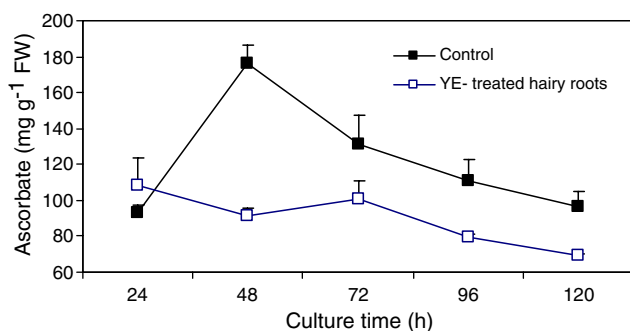


Fig. 7 Time-course of vitamin C content in *S. marianum* hairy root cultures treated with YE (2.5 mg 50 ml⁻¹ culture). The control received only MS medium. Data show means ± SD from triplicate experiments

of ascorbate was lower than the control during the incubation period (Fig. 7).

The changes of total tocopherol content under YE treatment were similar to that of control experiments. However, addition of elicitor induced the increase of α -tocopherols significantly after 72 h treatment, and reached a maximum, but thereafter decreased. When the treatment time was 72 h, hairy root cultures without elicitor treatment showed a significant change and the α -tocopherol

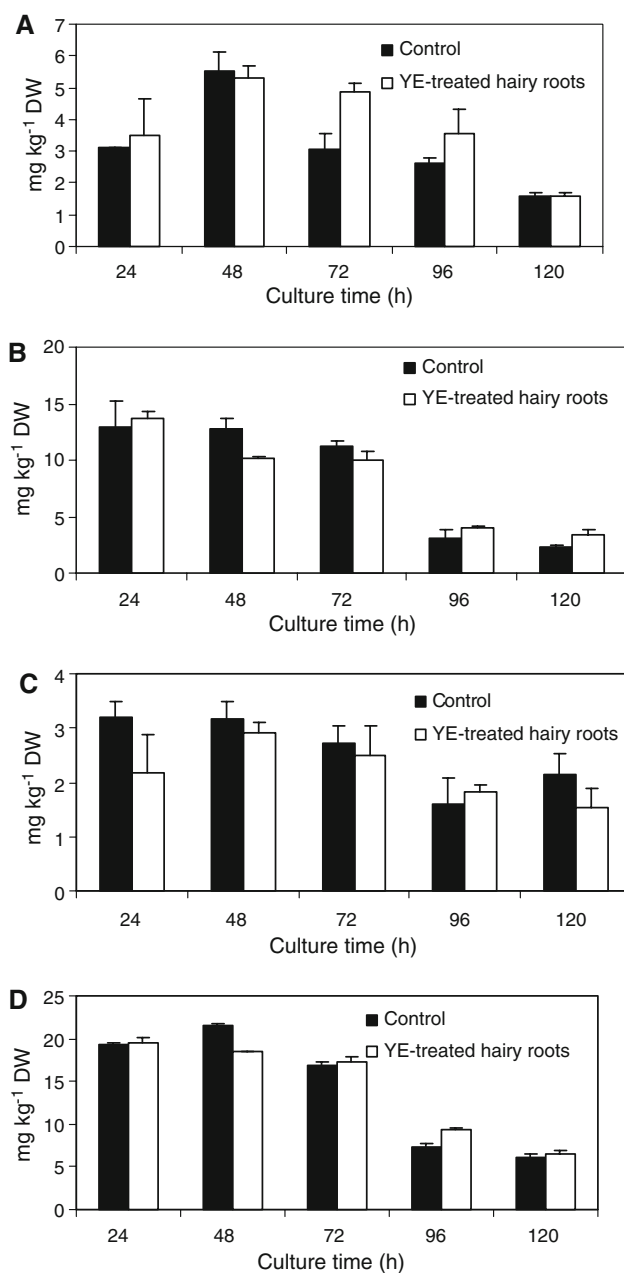


Fig. 8 Time course of vitamin E [α (a), γ (b), δ (c) and total (d) tocopherols] production in *S. marianum* hairy root cultures treated with YE (2.5 mg 50 ml⁻¹ culture). The control received only MS medium. Data show means ± SD from triplicate experiments

content reached 5 mg kg⁻¹ DW, almost 1.6-fold of that in the control (Fig. 8).

Discussion

Treatment of hairy root culture with YE has improved production of silymarin to a level about two-fold higher than that of the control (72 h after treatment), while content

of this compound decreased by 96 and 120 h after treatment. As previously reported, these results seem to be related to toxic effects of YE (Sanchez-Sampedro et al. 2005a). The toxic effects 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 (Kittipongpatana et al. 2002). This paper presents evidence for the signaling pathway acting as an integral signal and elicitor signal transducer for silymarin production.

The role of jasmonic acid (JA) in the plant defense system has been widely studied. It was found that this compound accumulates following pathogenic or environmental stress. The most significant and complicated research results have been able to show the relationship between elicitor and JA signaling pathways. More recent studies show that JA-stimulated accumulation of plant secondary metabolites does not fully overlap with elicitor-induced production of secondary metabolites (Zhao et al. 2005). For the first time, this study demonstrates that the JA signaling pathway is an integral part of elicitor signal transduction leading to silymarin accumulation in hairy root cultures of *S. marianum*. Jasmonate production ubiquitously takes place in plants as a signal to alter gene expression in response to biotic and abiotic stresses. In many elicited plant cells, a transient increase of endogenous jasmonate is followed by defense gene expression and the accumulation of secondary metabolites (Zhao and Sakai 2003; Subramaniam et al. 2008; Sanchez-Sampedro et al. 2005a, b).

The present results support the hypothesis that the jasmonate signaling pathway is involved in the YE-induced production of silymarin by activation of lipoxygenase activity and linoleic acid content. Phospholipases hydrolyze phospholipids, such as fatty acid that can be a precursor for biosynthesis of jasmonic acid (JA) and related oxylipins via the octadecanoid pathway. The JA biosynthesis pathway is believed to start with the oxygenation of free linoleic acid (Schaller 2001; Zhao et al. 2005).

The current study also suggests the presence of H_2O_2 or an oxidative burst induced by YE. Different stress conditions of both biotic and abiotic natures enhance the cellular production of reactive oxygen species (ROS) (Paradiso et al. 2005). H_2O_2 is an important toxic intermediate that also induces expression of many defense genes and secondary metabolites, such as syringin, sesquiterpene cyclases and phenylalanine ammonia lyase (Mehdy 1994; Xu et al. 2007). Because of the highly cytotoxic and reactive nature of ROS, their accumulation must be under tight control. Higher plant cells have very efficient enzymatic and non-enzymatic antioxidant defense systems that allow scavenging of ROS and protection of plant cells from oxidative damage (Shao et al. 2008). Shao et al. (2008) reported approximate redox potential and intracellular concentrations of main redox

couples in plant cells. High intracellular concentrations of antioxidants prevent indiscriminate oxidation of key cellular components by maintaining low oxidant concentrations. In addition to the abundant pools of ascorbate and glutathione and the battery of peroxide processing enzymes, numerous other compounds can act as chemical antioxidants, including tocopherols and flavonoids.

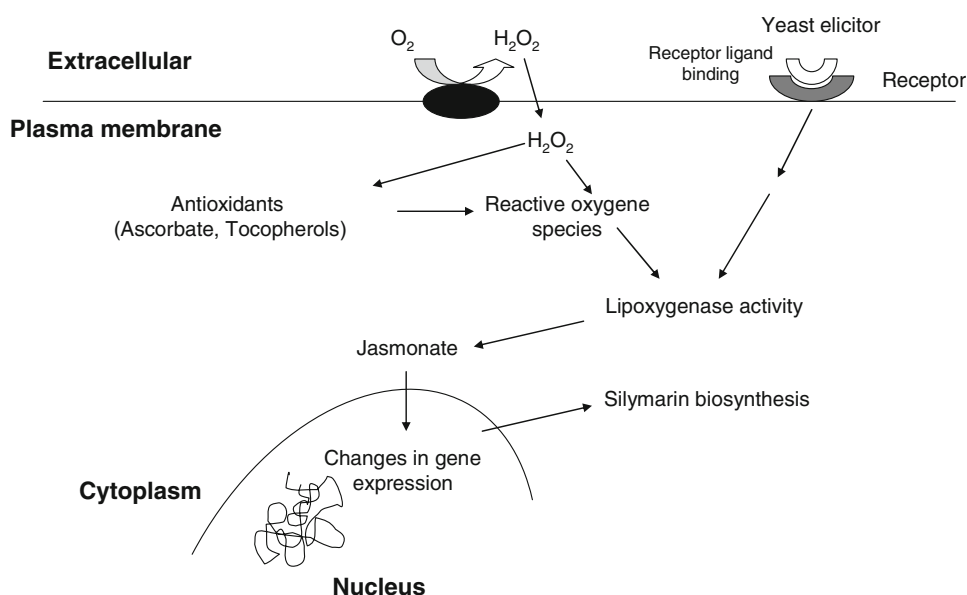
When YE treatment was applied to the hairy root cultures for 72 h, production of α - and δ -tocopherols increased. α -tocopherol (vitamin E) scavenges lipid peroxy radicals through the concerted action of other antioxidants (Kiffin et al. 2006; Hare et al. 1998). Furthermore, tocopherols were also known to protect lipids and other membrane components. Researchers have reported a two-fold increase in α -tocopherol content in drought-stressed plants (Kiddle et al. 2003; Ledford and Niyogi 2005; Shao et al. 2005). α -tocopherols are lipophilic antioxidants synthesized by all plants. α -tocopherols interact with the polyunsaturated acyl groups of lipids, stabilize membranes, and scavenge and quench various reactive oxygen species (ROS) and lipid-soluble byproducts of oxidative stress (Fath et al. 2002; Cvetkovska et al. 2005). A single α -tocopherol molecule can neutralize up to 120 singlet oxygen molecules in vitro before being degraded (Wu and Tang 2004).

Ascorbate (vitamin C) (AA) is the low-molecular mass antioxidant in plants (Noctor and Foyer 1998; Horling et al. 2003). The level of ROS and the cellular redox homeostasis are regulated by different antioxidant systems where ascorbate plays a pivotal role. It can be a scavenger of ROS and the electron donor of ascorbate peroxidase, a key enzyme for scavenging hydrogen peroxide in plant cells (Paradiso et al. 2005). AA reacts not only with H_2O_2 but also with O_2^- , OH and lipid hydroperoxidase. Moreover, AA has an additional role in protecting tocopherols (Shao et al. 2005, 2006). Therefore, AA has effects on many physiological processes including the regulation of metabolism of plants.

Ascorbate is a protective substance of plant cells that works against a variety of stresses by regulating the H_2O_2 concentration. $H_2O_2^-$ mediated non-enzymatic or enzymatic lipid peroxidation can initiate the octadecanoid pathway leading to biosynthesis of jasmonic acid and related compounds, and other oxylipins, which have an effective function in the induction of plant secondary metabolites (Thoma et al. 2003). The decrease in ascorbate seems to be related to consumption of this metabolite and it is one of the first alterations in the redox regulating systems induced in the YE treated hairy root cultures (Paradiso et al. 2005).

The present results show that it is possible to enhance silymarin production in hairy root cultures of *S. marianum* by addition of a biotic elicitor (Fig. 9). YE treatment induces the activity of lipoxygenase to allow for the production of jasmonate. Therefore, jasmonate signaling is an

Fig. 9 Schematic overview of the elicitor signal transduction pathway leading to silymarin biosynthesis in *S. marianum* hairy root cultures



integral part of the YE signal transduction for the production of silymarin. These results indicate that the H_2O_2 content can be controlled during the elicitation and that the ascorbate and tocopherols are the major antioxidants in treated hairy roots in these conditions. These results will lead to a more profound understanding of synthesis and regulation of silymarin in hairy root cultures.

Acknowledgments This research was funded (No. 1-05-05-8702) by Agricultural Biotechnology Research Institute of Iran (ABRII).

References

- Asai N, Matsuyama T, Tamaoki M, Nakajima N, Kuba A, Aono M, Kato T, Tabata S, Shirano Y, Shibata D, Hayashi H, Mullineaux PM, Saji H (2004) Compensation for lack of a cytosolic ascorbate peroxidase in an *Arabidopsis* mutant by activation of multiple antioxidative systems. *Plant Sci* 166:1547–1554
- Axelroad B, Cheesbrough TM, Laakso S (1981) Lipoxygenase from soybeans. *Methods Enzymol* 17:441–451
- Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical to evaluate antioxidant activity. *Lebensm-Wiss Technol* 28:25–30
- Cacho M, Moran M, Corchete P, Fernandez-Tarrago J (1999) Influence of medium composition on the accumulation of flavonolignans in cultured cells of *Silybum marianum* (L.) Gaertn. *Plant Sci* 144:63–68
- Cvetkovska M, Rampitsch C, Bykova N, Xing T (2005) Genomic analysis of MAP kinase cascades in *Arabidopsis* defense responses. *Plant Mol Biol Rep* 23:331–343
- Fath A, Bethke P, Beligni V, Jones R (2002) Active oxygen and cell death in cereal aleurone cells. *J Exp Bot* 53:1273–1282
- Flora K, Hahn M, Benner K (1998) Milk thistle (*Silybum marianum*) for the therapy of liver disease. *Am J Gastroenterol* 93:139–143
- Hare PD, Cress WA, Staden JV (1998) Dissecting the roles of osmolyte accumulation during stress. *Plant Cell Environ* 21:535–553
- Hasanloo T, Khavari-Nejad RA, Majidi E, Shams-Ardakani MR (2005) Analysis of flavonolignans in dried fruits of *Silybum marianum* (L.) Gaertn from Iran. *Pak J Biol Sci* 8:1778–1782
- Hasanloo T, Khavari-Nejad RA, Majidi E, Shams-Ardakani MR (2008) Flavonolignan production in cell suspension culture of *Silybum marianum* (L.) Gaertn. *Pharm Biol* 46:876–882
- Horling F, Lamkemeyer P, König J, Finkemeier I, Kandlbinder A, Baier M, Dietz KJ (2003) Divergent light-, ascorbate-, and oxidative stress-dependent regulation of expression of the peroxiredoxin gene family in *Arabidopsis*. *Plant Physiol* 131:317–325
- Hu ZB, Du M (2006) Hairy root and its application in plant genetic engineering. *J Integr Plant Biol* 48:121–127
- Kiddle G, Pastori GM, Bernard B, Pignocchi C, Antoniow J, Verrier PJ, Foyer CH (2003) Effects of leaf ascorbate content on defense and photosynthesis gene expression in *Arabidopsis thaliana*. *Antioxid Redox Signal* 5:23–32
- Kiffin R, Bandyopadhyay U, Cuervo AM (2006) Oxidative stress and autophagy. *Antioxid Redox Signal* 8:152–162
- Kittipongpatana N, Davis DL, Porter JR (2002) Methyl jasmonate increases the production of valepotriates by transformed root cultures of *Valerianella locusta*. *Plant Cell Tissue Organ Cult* 71:65–75
- Křen V, Walterová D (2005) Silybin and silymarin—New effects and applications. *Biomed Pap* 149:29–41
- Ledford HK, Niyogi KK (2005) Singlet oxygen and photo-oxidative stress management in plants and algae. *Plant Cell Environ* 28:1037–1045
- Mehdy MC (1994) Active oxygen species in plant defense against pathogens. *Plant Physiol* 105:467–472
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* 49:249–279
- Paradiso A, Tommasi F, Gara LD, Pinto MC (2005) Alteration in ascorbate and ascorbate peroxidase in programmed cell death and oxidative stress. *BMC Plant Biol* 5:S28
- Park S, Facchini PJ (2000) Agrobacterium rhizogenes-mediated transformation of *Opium poppy*, *Papaver somniferum* L., and

- California poppy, Eschscholzia California* Cham. Root cultures. J Exp Bot 51:1005–1016
- Rahnama H, Hasanloo T, Shams MR, Sepehrifar R (2008) Silymarin production in hairy root culture of *Silybum marianum* (L.) Gaertn. Iran J Biotechnol 6:113–118
- Rajendran L, Suvarnalatha G, Ravishankar GA, Venkataraman LV (1994) Enhancement of anthocyanin production in callus cultures of *Daucus canota* L. under the influence of fungal elicitors. Appl Microbiol Biotechnol 42:227–231
- Sanchez-Machado DI, Lopez-Hernandez J, Paseiro-Losada P (2002) High performance liquid chromatographic determination of α -tocopherol in macroalgae. J Chromatogr A 976:277–284
- Sanchez-Sampedro MA, Fernandez-Tarago J, Corchete P (2005) Yeast extract and methyl jasmonate induced silymarin production in cell culture of *Silybum marianum* L. Gaertn. J Biotechnol 119:60–69
- Sánchez-Sampedro MA, Fernández-Tárrago J, Corchete P (2005) Enhanced silymarin accumulation is related to calcium deprivation in cell suspension cultures of *Silybum marianum* (L.) Gaertn. J Plant Physiol 162:1177–1182
- Sauervin M, Yamazaki T, Shimomura K (1991) Hernandulcin in hairy root cultures of *Lippia dulcis*. Plant Cell Rep 9:579–581
- Schaller F (2001) Enzymes of biosynthesis of octadecanoid-derived signaling molecules. J Exp Bot 354:11–23
- Shams-Ardakani MR, Hemmati S, Mohagheghzadeh A (2005) Effect of elicitors on the enhancement of podophylotoxin biosynthesis in suspension cultures of *Linum album*. Daru 13:56–60
- Shao HB, Liang ZS, Shao MA, Sun Q, Hu ZM (2005) Investigation on dynamic changes of photosynthetic characteristics of 10 wheat (*Triticum aestivum* L.) genotypes during two vegetative-growth stages at water deficits. Biointerfaces 43:221–227
- Shao HB, Chu LY, Zhao CX, Guo QJ, Liu XA, Ribaut JM (2006) Plant gene regulatory network system under abiotic stress. Acta Biol Szeged 50:1–9
- Shao HB, Chu LY, Lu ZH, Kang CM (2008) Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. Int J Biol Sci 4:8–14
- Subramaniam S, Vaughn K, Carrier DJ, Clausen EC (2008) Pretreatment of milk thistle seed to increase the silymarin yield: an alternative to petroleum ether defatting. Bioresour Technol 99:2501–2506
- Thoma I, Loeffler C, Sinha AK, Gupta M, Krischke M, Steffan B, Roitsch T, Mueller MJ (2003) Cyclopentenone isoprostanes induced by reactive oxygen species trigger defense gene activation and phytoalexin accumulation in plants. Plant J 34:363–375
- Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants. Protective role of exogenous polyamines. Plant Sci 151:59–66
- Wu YS, Tang KX (2004) MAP Kinase cascades responding to environmental stress in plants. Acta Bot Sin 46:127–136
- Xu C, Zhu B, Ou Y, Wang X, Yung X, Wang Y (2007) Elicitor-enhanced syringin production in suspension cultures of *Sussurea medusa*. World J Microb Biotechnol 23:965–970
- Yi C, Kramer J, Xue S, Jiang Y, Zhang M, Ma Y, Pohorly J (2009) Fatty acid composition and phenolic antioxidants of winemaking pomace powder. Food Chem 114:570–576
- Yu L, Haley S, Perret J, Harris M, Wilson J, Qian M (2002) Free radical scavenging properties of wheat extracts. J Agric Food Chem 50:1619–1624
- Zhao J, Sakai K (2003) Multiple signalling pathways mediate fungal elicitor-induced β -thujaplicin biosynthesis in *Cupressus lusitana* cell cultures. J Exp Bot 54:647–656
- Zhao J, Davis LCT, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol 23:283–333