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Original article

Salicylic acid increases flavonolignans accumulation in the fruits of hydroponically cultured *Silybum marianum*

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ABSTRACT

Silybum marianum (L.) Gaertn. (Asteraceae) was hydroponically cultured using a nutrient film technique system. Silibinin, isosilibinin and silychristin were detected in the fruits of the cultured plants. The effect of salicylic acid on the improvement of flavonolignans production by the fruits of the hydroponically cultured *S. marianum* was investigated. Salicylic acid was added to the nutrient solution at different concentrations (100, 200 and 400 μM) and the mature fruits of the plant were collected five days after elicitor addition. The fruits were then analyzed for their total flavonolignans contents and individual components using quantitative proton nuclear magnetic resonance spectroscopy (qHNMR) and high-performance liquid chromatography (HPLC). The results showed that elicitation with salicylic acid at 200 μM for five days increased production of total flavonolignans (1.7-fold by qHNMR and 1.6-fold by HPLC) higher than the control cultures and (1.4-fold by qHNMR and 1.1-fold by HPLC) higher than the cultivated plants. Silychristin was the major flavonolignan produced by the cultured plant. Elicitation by 200 μM salicylic acid increased silychristin production (1.6-fold by qHNMR and HPLC) higher than the control cultures and (1.3-fold by qHNMR and 1.0-fold by HPLC) higher than the cultivated plants. The present study provides a chance to improve secondary metabolite yield, serves as a useful tool for studying the biosynthesis of these medicinally valuable compounds and its regulation in plant and spots more light on hydroponic system as an important agricultural technique.

1. Introduction

The milk thistle *Silybum marianum* (L.) Gaertn., synonymous *Carduus marianus* L., (Asteraceae) is an annual or biennial plant. The plant is native to the Mediterranean region; however, it was naturalized elsewhere in the world (Morazzoni, 1995). The fruits of this plant accumulate isomeric mixture of flavonolignans in their outer shells. Silibinins, isosilibinins, silydianin and silychristin are the major flavonolignans produced by *S. marianum* fruits. The standardized extract of the fruits is known as silymarin, which is an important pharmaceutical raw material used for oral treatment of liver disorders. This plant was placed in 2016 at the sixth position among the top-selling herbal dietary supplements in the natural and health food market and the sixteenth position in the mainstream multi-outlet channel market in U.S.A., at about $9.968 million and $17.077 million, respectively (Smith et al., 2017).

In consideration of the market requirements for a standardized product with a high content of bioactive principles, several efforts are directed to the setup of suitable growing conditions for stimulation of plant secondary metabolite production. Open-field culture does not allow a strict control neither of the growing conditions nor the secondary metabolism. Therefore, the development of an alternative growing system could be an effective tool to overcome the drawbacks linked to open-field cultivation (Maggini et al., 2014).

Hydroponics is a growing system, in which the nutrient elements that are normally found in the soil are dissolved in a proper quantity of the irrigation water supplied to the plants. Hydroponic is also known as 'soilless culture', because the plants are cultivated in pure nutrient solutions (water culture) or in artificial growing media (substrate culture) instead of the common agricultural soil (Pardossi et al., 2005). With more isolated condition and well-

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defined composition in this technique, the management of important growing parameters such as surrounding climate or nutrition represents a significant tool for the regulation of secondary metabolism. In particular, a proper change in the composition of the nutrient solution could stimulate the secondary metabolism and favor the accumulation of bioactive compounds inside the tissues (Briskin, 2000). A further major advantage of hydroponics is the possibility to expose the plants to stress factors that can elicit an increase in the concentrations of secondary metabolites (Brechner et al., 2007, Rahimi et al., 2012a). Among the most remarkable representatives of elicitors is salicylic acid which is a natural plant stress mediator that can highly improve the production of pharmaceutically active compounds in plants (Kuzel et al., 2009). It successfully enhanced silymarin production in *S. marianum in vitro* cultured cells in MS liquid medium (Elwekeel et al., 2012) but not previously used in hydroponic culture.

Nutrient film technique is one of the hydroponic types in which the plants are housed in net pots that are placed on channels having the nutrient solution. The nutrient solution is pumped though the channels and constantly running along the bottom of the channel and the roots are not completely submerged (Kaul et al., 2017). Hydroponic culture of medicinal plants aiming at production of important secondary metabolites is rarely reported (Kaul et al., 2017).

Nuclear magnetic resonance spectroscopy (NMR) can provide useful qualitative and quantitative information in analysis of complex mixtures such as plant extracts. Quantitative proton nuclear magnetic resonance spectroscopy (qHNMR) can offer an overview of the sample composition through quantification of multiple metabolites without the need for chromatographic separation. This method, orthogonal to the high-performance liquid chromatography (HPLC) analysis, allows quantification of targeted compounds without the need of reference materials (AbouZid et al., 2016a).

So, the objectives of the present study were to establish a hydroponic culture protocol for *S. marianum* as one of the medicinally valuable plant species using nutrient film technique; enhance plant capacity to accumulate flavonolignans using salicylic acid as elicitor and determine the influence of the hydroponic system, as a different cultivation technique and elicitation on seed productivity and silymarin yield which was achieved by comparing the flavonolignan content in different cultures using qHNMR and HPLC techniques. In addition to, provide a demonstration of the effect of salicylic acid on biosynthetic pathway leading to flavonolignan production.

2. Material and methods

2.1. Plant material and experimental design

2.1.1. Open field-grown S. marianum

Seeds were collected from plants located beside the Cairo-Alexandria dessert road, Egypt (31°0′8.45″N, 29°48′20.91″E) in March 2014. The plant was botanically authenticated by Dr. Abdel Halim Mohamed, Flora and Phytotaxonomy Department, Agricultural Research Center, Cairo, Egypt. About 25 seeds were randomly cultivated under open field conditions in a completely randomized design in the middle of August 2016 at the Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University, Egypt. Flowering for a total of 15 plants took place at the beginning of December at growth stage 69 according to *Biologische Bundesantalt, Bundessortenamt, and Chemische Industrie* [BBCH] scale (Martinelli et al., 2015). Fruits were harvested during the first half of February 2017 from completely mature flower heads at the beginning of seed dispersal process at BBCH growth stage 88. Ripe fruits were manually separated from the heads, freed from the pappus, and kept at −20 °C until use.

2.1.2. Hydroponic culture of S. marianum

Hydroponic culture of *S. marianum* was established from the seedlings and a single Nutrient film technique system was used on which the plants were randomized. Plants at 4-6 leaves at BBCH growth stage 14 were transplanted into the culture system supplied with the nutrient solution pumped through them and running along the bottom of the channel. The nutrient solution drops into a reservoir and sent back through a submerged pump to the beginning the system. The nutrient solution consists of solution A ($Ca(NO_3)_2$ 1003 g/l and iron chelate 79 g/l) and solution B (K_2PO_4 263 g/l, KNO_3 583 g/l, $MgSO_4$ 513 g/l, $MnSO_4$ 6.1 g/l, $CuSO_4$ 0.39 g/l, boric acid 1.7 g/l, NH_4Mo 0.37 g/l and $ZnSO_4$ 0.44 g/l). The two solutions A + B are diluted 100 times with water upon mixing. *S. marianum* plants are kept in the channels using net pots and their roots are not completely submerged in the system. The plants were kept on the system for three months till full ripening and maturation of their fruits (Fig. 1). The fully developed fruits at BBCH growth stage 88 were collected. Ripe fruits were manually separated from the heads, freed from the pappus, and kept at −20 °C until use.

2.2. Elicitor addition

Different salicylic acid treatments were supplied to each single plant and the plants were randomized on one nutrient film apparatus so that each single plant was a biological repetition. Salicylic acid was added at concentrations of 100, 200 and 400 μM, into the nutrient medium when the plant is at BBCH growth stage 75. Non-elicitor treatment was considered as the control culture. The mature fruits of the cultured *S. marianum* plants were collected at BBCH growth stage 88, 5 d after the elicitor addition. Sampling was performed individually from each single plant and further fruit analysis was performed at single plant level.

Fig. 1. *Silybum marianum* plant growing on hydroponic culturing system.

2.3. Fruit productivity

To study the influence of the hydroponic culture and elicitation on fruit productivity of the milk thistle, weight of each flower-head and seed number in each flower-head were measured.

2.4. Flavonolignans extraction

The whole fruits (1.00 g) was used for the extraction of silymarin. The pericarp was separated from the kernel using the reported procedure (AbouZid et al., 2016a). The pericarp represents 41%, and the kernels 52% of the weight of the fruits. The pericarp was air dried, ground, and extracted with 75% methanol (×3, 10 ml) to yield crude extract. The combined extracts were dried and subjected to analysis.

2.5. Quantitative nuclear magnetic resonance (qNMR) spectroscopic analysis

qNMR is a non-destructive method that needs only limited sample preparations steps, can be readily automated and provides both qualitative and quantitative analyte information without the need of chromatographic separation or additional analytical devices (Pauli et al., 2005, Pauli et al., 2007). The NMR spectrometer used was a Bruker model AVANCE III HD (Fällanden, Switzerland) equipped with a BBFO Smart Probe and Bruker 400 AEON Nitrogen-Free Magnet, and operating at a ^{1}H frequency of 400.13 MHz (O1). The following conditions were used for acquisition of the ^{1}H NMR spectra: 30°pulse experiment, excitation pulse 10 μs corresponding to an angle of xyz; acquisition time of 4.1 s; sweep width 15.1 ppm (8012 Hz); temperature 295.1 K. Free induction decays were Fourier transformed after applying a line broadening factor (LB) of 0.1 Hz and zero filling to 256 k. DMSO d_5 was used for sample preparation. Mnova software was used for post-acquisition processing. Silibinin (Sigma) was used as a reference standard. The ^{1}H NMR data of the compounds under investigation in this study has been previously published (AbouZid et al., 2016b, Kim et al., 2003). In silibinin ^{1}H NMR spectrum, the doublet at 5.07 ppm (J = 11.2 Hz) assigned for H-2 was chosen for establishing the calibration curve (Fig. 2). The calibration curve was established by plotting integration values for this signal versus the molar concentration. The regression equation was y = 0.5699x − 0.733 (R^2 = 0.9987). Flavonolignans contents in the extracts were quantified using broad singlet at 2.73 for silydianin, doublet at 5.47 for silychristin and the two double doublets around 7.10 for silibinins/isosilibinins.

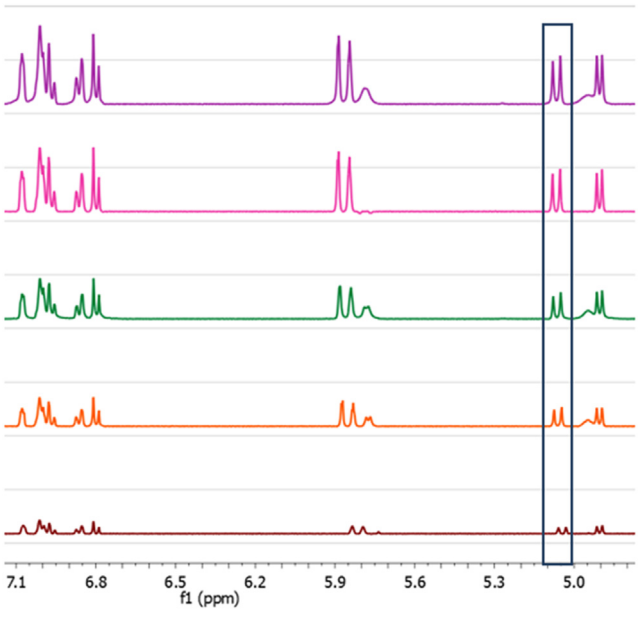

Fig. 2. Expanded region (4.8–7.1 ppm) of ^{1}H NMR spectra of silibinin reference standard using different concentrations (0.4–2.0 mg/ml) used for preparation of standard curve (400 MHz, DMSO).

2.6. Chromatographic analysis

An Agilent 1260 Infinity High Performance Liquid Chromatography (HPLC) system equipped with an Agilent 1260 Infinity pump (G1361A), Agilent 1260 diode array detector VL (G1315D), Agilent ZORBAX SB-C18 and Agilent 1260 Infinity autosampler (G2260A) were used to analyze the content of individual silymarin components according to the reported method by (AbouZid et al., 2016b). The mobile phase was composed of methanol/0.1% formic acid (Phase A) and water/0.1% formic acid (Phase B); 0–5 min gradient 40–45% phase A; 5–10 min isocratic 45% phase A; 10–20 min gradient 45–50% phase A; 20–25 min isocratic 50% phase A. The flow rate was 1.5 ml/min, and ambient temperature was used. The auto sampler was adjusted to inject 10 μl and quantified at 280 nm. The method offered the following retention times (minutes) for the major silymarin compounds: silychristin A (R_t = 8.9), silydianin (R_t = 10.6), silybin A (R_t = 19.6), silybin B (R_t = 21.3), isosilybin A (R_t = 25.4), and isosilybin B (R_t = 26.6). Flavonolignans identification and quantification was obtained using reference standards (Sigma-Aldrich). Total flavonolignan content is the result of the sum of the single constituents derived from HPLC analysis.

2.7. Statistical analysis

The data of fruit productivity and chromatographic analysis are the mean of triplicate measurements. The results are expressed as mean ± standard error. Statistical significance was determined by Tukey's post hoc comparison test with $P < 0.05$ considered significant.

3. Results and discussion

3.1. Hydroponic culture of S. marianum and fruit productivity

In the past 40 years, numerous strategies have been developed to improve plant productivity using *in vitro* culture techniques such as elicitation (Hasanloo et al., 2008). However, there are no reports for the use of these strategies in hydroponic system. In the present study, hydroponic culture was successfully established from the seedlings at BBCH growth stage 14 using nutrient film technique system (Fig. 1). The results of measuring different factors for flower-head and seed proved that milk thistle productivity significantly affected by hydroponic system and elicitation treatments (Table 1). Considerable increase in these factors was achieved by elicitation of the hydroponically cultured plant using 200 μM salicylic acid than field-grown plant (from 6.90 to 9.46, 167 to 219 for flower-head weight, no of seeds/flower-head respectively). The most interesting observations for the hydroponic culture was its ability to produce fast plant growth with high biomass. In addition to, the feasibility to control the growing environment and to change the composition of the nutrient solution, when it was necessary to treat the nutrient solution with elicitors, because the nutrient elements are readily available at the root zone and can be easily taken up by the plants.

The flavonolignans contents in the fruits of different cultures of *S. marianum* were analyzed using both qHNMR and HPLC. Both

Table 1
Flavonolignans contents in different cultures of *Silybum marianum*.

	Control culture	Salicylic acid (μM)			Field grown plant
		100	200	400	
Fruit productivity ± SE					
Flower-head wt (g)	10.77 ± 2.16	11.60 ± 2.36	9.46 ± 1.22	8.32 ± 0.35	6.90 ± 0.70
Seed No./flower-head	195.83 ± 27.89	179.25 ± 29.29	219.00 ± 30.03	192.2 ± 11.81	167.00 ± 16.55
Flavonolignan content (mg/g DW) using qHNMR					
Silychristin	3.72	5.05	5.93	4.80	4.52
Silibinin/Isosilibinin	7.38	10.44	13.38	9.74	9.11
Total Silymarin	11.1	15.49	19.31	14.54	13.63
Flavonolignan content (mg/g DW) using HPLC ± SE					
Silychristin	3.32 ± 0.07	3.96 ± 0.09	5.23 ± 0.14* [a]	4.90 ± 0.12*	5.07 ± 0.48*
Silydianin	1.02 ± 0.03	1.05 ± 0.02	1.18 ± 0.01	1.18 ± 0.01	1.24 ± 0.02
Silybin A	2.49 ± 0.08	2.76 ± 0.06	3.74 ± 0.16*	3.62 ± 0.08*	3.44 ± 0.46
Silybin B	3.78 ± 0.10	4.34 ± 0.11	5.96 ± 0.32*	5.66 ± 0.14*	5.47 ± 0.77
Isosilybin A	1.26 ± 0.02	1.34 ± 0.01	1.59 ± 0.01*	1.59 ± 0.07*	1.59 ± 0.12*
Isosilybin B	n. d.	n. d.	0.91 ± 0.03	0.93 ± 0.01	0.93 ± 0.08
Silibinin/Isosilibinin	8.15	8.44	11.89	11.48	11.13
Total silymarin	11.87	13.45	18.69	17.87	17.75

wt is the weight, SE is the standard error and n.d. means not detected.
Statistics were carried out by one way analysis of variance (ANOVA) followed by Tukey's post hoc comparison test.
[a] Significantly different from 100 μM salicylic acid at $p < 0.05$.
* Significantly different from control culture at $p < 0.05$.

techniques were reported to be used interchangeably to quantitate flavonolignans in the silymarin complex (Cheilari et al., 2016).

3.2. Flavonolignan analysis by qHNMR

Quantitative analysis in NMR depends on the fact that the integrated intensity of a signal due to the analyte nuclei is directly proportional to its molar concentration and to the number of nuclei that give rise to this signal. The direct proportionality of the analytical response and molar concentration is one of the main advantages of qNMR as a method for quantification over other spectroscopic methods. In UV spectroscopy, for example, the concentration is related to the molar absorptivity which is different for each molecule. Therefore, we have to obtain pure reference standard for each compound of interest. In qNMR, a single standard can be used to quantify many components in a mixture, which can be even structurally unrelated to the analytes under investigation, contains the nucleus of interest and has a resonance that does not overlap those of our target analytes. Silibinin (Sigma) was used as a reference standard. The ^{1}H NMR data of the compounds under investigation in this study has been previously published (AbouZid et al., 2016b, Kim et al., 2003). The chemical structures of flavonolignans under investigation in the present study are shown in Fig. 3. The flavonolignan contents in the fruits of hydroponically cultured plant are shown in Table 1. Silychristin, silibinin and isosilibinin were the major flavonolignans detected in the fruits of the cultured plants while silydianin was not detected. (AbouZid et al., 2016b) estimated the silymarin content in the field-grown fruits collected from Cairo – Alexandria Road between 11.02 and 15.54 mg/g DW in which silychristin and silibinin/isosilibinin were 2.77–3.17 and 3.03–6.38 mg/g DW respectively. Taking in consideration the variation due to re-cultivation in different habitat in Beni-Suef governorate.

Elicitation of the hydroponic culture with salicylic acid at different concentrations (100, 200 and 400 μM) enchanced the production of these flavonolignans in the fruits. A dose of 100 μM salicylic acid added to the hydroponic cultures of *S. marianum* for 5 d increased total silymarin content 1.4-fold higher than the control. The highest content of total silymarin (19.31 mg/g DW) was observed after addition of 200 μM salicylic acid for 5 d. This is considered as 1.7-fold higher than in the control. For individual flavonolignans, there was a 1.6-fold increase in silychristin content and 1.8-fold increase in silibinin/isosilibinin contents at 200 μM salicylic acid compared to control cultures. The addition of 400 μM salicylic acid improved total silymarin content 1.3-fold higher than the control. This clearly shows that 200 μM salicylic acid is considered as an optimum dose for the elicitor under the established culturing conditions.

3.3. Flavonolignan analysis by HPLC

Although NMR results comparing silymarin content in different cultures of *S. marianum* could prove the ability of salicylic acid to achieve an increase in the production of the flavonolignans and showed good peak resolution for silychristin, but silydianin was not detected and peak resolution for individual components of silibinin and isosilibinin was insufficient. To provide more detailed information about the silymarin composition, chromatographic analysis was used. HPLC has the advantage of separation along with quantitation of the major silymarin components (AbouZid et al., 2016a). Fruits of different cultures of *S. marianum* were subjected to identical extraction procedures for comparison. HPLC analysis (Table 1) confirmed the results obtained by qHNMR where 200 μM salicylic acid is the optimum dose. The cultures elicited with 100, 200 and 400 μM salicylic acid produced total silymarin content (13.45, 18.69 and 17.87 mg/g DW respectively) in comparison with that produced by the fruits of the non-elicited and field-grown plants (11.87 and 17.75 mg/g DW respectively). Silychristin and silybin B were the major flavonolignans in all cultures of this silychristin-rich plant. Interestingly, the cultures elicited with 200 μM salicylic acid produced silychristin and silybin B (5.23 and 5.96 mg/g DW respectively) which were higher than that produced by the fruits of the non-treated (3.32 and 3.78 mg/g DW respectively) and field-grown plants (5.07 and 5.47 mg/g DW respectively). Isosilybin B was the minor flavonolignan produced by all cultures. It was not detected in non-treated and 100 μM elicited-cultures while increased to 0.91 mg/g DW in 200 μM-elicited cultures.

Although hydroponic culture of Milk thistle is capable of producing silymarin, amounts produced are lower than those produced in the field grown plants. But the reduced volumes and closed system of the hydroponic technique were easier to work

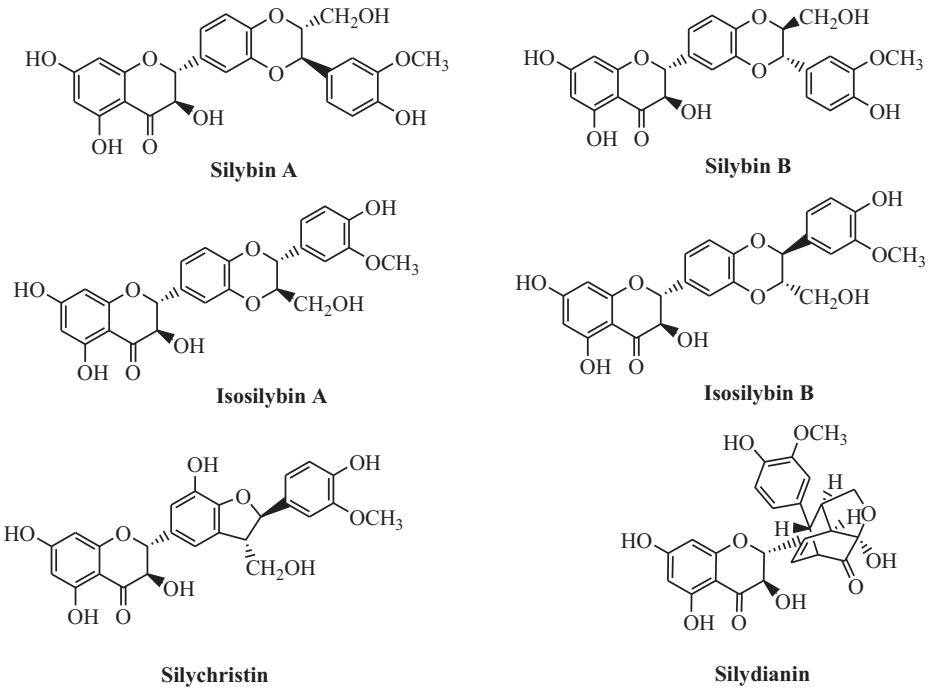

Fig. 3. Chemical structures of different components of silymarin complex.

with when it was necessary to treat the nutrient solution with elicitors. It was found that feeding the medium with elicitor such as salicylic acid could improve the production of silymarin particularly at concentration of 200 μM for 5 d. This positive effect would make the assigned treatment an interesting candidate for improving silymarin productivity on large scale processes. In this respect, the use of different elicitor offers the possibility to enhance the content of silymarin complex.

3.4. Effect of salicylic acid on silymarin biosynthetic pathway

Production of flavonolignans by cell and tissue cultures established from *S. marianum* was reported in many studies (Alikaridis et al., 2000, El Sherif et al., 2013, Torres and Corchete, 2016). The major obstacle was low productivity due to lack of differentiation and/or organization. Salicylic acid was able to stimulate flavonolignans production in *S. marianum* cultures. For example, in hairy roots of *S. marianum*, addition of salicylic acid (6 mg/50 ml culture) for 24 h increased flavonolignans content 2.42 times higher than the control (Khalili et al., 2009). Silibinin showed improvement in cell cultures of *S. marianum* elicited with 50 and 100 μg/ml salicylic acid (Elwekeel et al., 2012). Salicylic acid, a small molecule with a vital role in plant defense regulatory systems, is known to induce systemic acquired resistance to many pathogens. During the plant-pathogen interaction, a rapid salicylic acid accumulation in the infection site triggers a hypersensitive response. The signal then spreads to other parts of the plant to induce a wide range of defense responses, including the production of plant secondary metabolites, which is why salicylic acid is widely applied as a secondary metabolism elicitor (Ramirez-Estrada et al., 2016). Moreover, treatment with salicylic acid regulates the jasmonate pathway, which in turn mediates the elicitor-induced accumulation of silymarin (Khalili et al., 2009). Methyl jasmonate is known as a wide-spectrum elicitor due to the well-known involvement of jasmonate in a part of the signal transduction pathway that induces particular enzymes to catalyze biochemical reactions to form defense compounds of low molecular weight like silymarin in plants (Rahimi et al., 2012b). The use of different yield-improving strategies to increase flavonolignans production in cell and tissue cultures of *S. marianum* did not achieve the same level of accumulation in the fruits of the plant (AbouZid, 2014).

4. Conclusions

S. marianum was hydroponically cultured using a nutrient film technique system. The cultured plant accumulated silychristin, silibinin and isosilibinin flavonolignans. The production of these compounds was enhanced by elicitation with salicylic acid. Treatment with salicylic acid at 200 μM for five days was the most effective concentration to enhance silymarin accumulation. The successful production of flavonolignans from hydroponic cultures may serve as a useful system for studying the biosynthesis of these compounds and its regulation in plant cells. Finally, a lot of molecules of pharmaceutical interest can be obtained from hydroponically grown medicinal plants.

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