



Article

Salicylic Acid Foliar Spray Enhanced *Silybum marianum* Growth and Yield, as Well as Its Chemical Constituents and Chalcone Synthase Gene Activity

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Abstract: Silymarin, a secondary metabolite found mainly in the *Silybum marianum* L. fruits, has been associated with the hepatoprotective activity of the plant. Among various elicitors, salicylic acid, a “Generally Regarded As Safe” compound recognized by the Food and Drug Administration, is one of those being used in the induction and enhancement of valuable plant secondary metabolite production in various plant species. In this study, two concentrations (10^{-4} and 10^{-3} molar) of salicylic acid have been applied to the *S. marianum* plants as foliar spray to investigate their effects on plant growth and yield, as well as the production of its bioactive compound, silymarin. Our results indicated that both concentrations of salicylic acid increased the plant height, the number of branches, leaves, and capitula, as well as the dried weight of roots, aerial parts, and fruits. The enhancement effects in plant growth and yield were accompanied by an increase in photosynthetic pigments such as chlorophyll-a, b, and carotenoids as well as element contents such as nitrogen, phosphorus, and potassium. The potential of salicylic acid as an elicitor for the enhancement of secondary metabolites in *S. marianum* was supported by the increase in silymarin’s major components, silybin (A + B), in the salicylic acid-treated plants. Concomitant expressions of *CHS 1*, *2*, and *3* genes that have been associated with the production of silymarin in *S. marianum* were also observed in the salicylic acid-treated plants. A lower concentration (10^{-4} M) of salicylic acid was found to be a better elicitor as compared with the 10^{-3} M salicylic acid. An increase of 3.4 times in capitula number and fruit dried weight as well as 2.6 times in silybin (A + B) contents were observed in plants sprayed with 10^{-4} M of salicylic acid as compared with the control.

Keywords: biostimulant; gene expression; *milk thistle*; Silybin A and B



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1. Introduction

Human liver illnesses have become one of the most prominent diseases in the modern era. Traditionally, medicinal plants have been used to cure or prevent a variety of disorders, including liver diseases [1]. *Silybum marianum* L. is one of the medicinal plants that has been used as a hepatoprotective herb. It is an annual or biennial plant commonly found in the Middle East and Mediterranean regions [2]. Silymarin, a mixture of flavonolignan predominantly found in the fruits of *S. marianum* has been implicated in the hepatoprotective activity of this plant. In addition to its hepatoprotective effect, silymarin also demonstrated antiviral, neurodegenerative-, and cancer-preventive characteristics, as well as renal- and cardioprotective capabilities [3]. Silybin A and B contribute 50 to 70% of silymarin composition and have been associated with silymarin biological activities [4,5]. Plants increase the production of secondary metabolites, such as silymarin, as a response to both biotic and abiotic stresses. Therefore, one of the strategies to enhance the hepatoprotective compound, silymarin, is by applying abiotic (such as NaCl) or biotic (such as fungal extract or chitosan) elicitors to *S. marianum* plants [6–8].

Salicylic acid is a nonenzymatic antioxidant and plant growth regulator that regulates a variety of physiological processes, including the generation of various secondary metabolites as a response to alleviate plant biotic and abiotic stresses [9–12].

Exogenous application of salicylic acid has been shown to accelerate plant growth and development, as well as to initiate or boost secondary metabolite production in various plant species, such as *Mentha piperita* L., *Achillea millefolium* L., *Ocimum basilicum* L., *Thymus vulgaris* L., and others [13–17]. The Food and Drug Administration recognizes salicylic acid as a “Generally Regarded As Safe” compound to apply to medicinal species cultivated for the phytomedicine industry [18]. The effect of exogenously applied salicylic acid on plant development varies depending on the plant species and its developmental stage, as well as the salicylic acid concentration [16].

The allosteric enzyme chalcone synthase (CHS) is an essential enzyme for flavonoid synthesis in various plant species [19]. In *S. marianum*, CHS is involved in the synthesis of taxifolin, the precursor that forms the flavonolignan mixture of silymarin. [20]. Three *CHS* genes, namely *CHS 1*, *CHS 2*, and *CHS 3*, have been identified and their expressions were associated with the accumulation of silymarin in *S. marianum* [21,22]. Salicylic acid, as a plant elicitor, has been shown in multiple studies to elevate the activity of the CHS enzyme, which is involved in flavonoid production [13,23,24].

The successful induction of various secondary metabolites by salicylic acid in different medicinal plant species has prompted us to conduct the present study. We have selected 10^{-4} and 10^{-3} molar (M) salicylic acid concentrations to apply as foliar spray to the *S. marianum* plants as these two concentrations have been tested in several plant species [17,25–27]. To determine the efficiency of salicylic acid, the growth and yield, as well as the contents of photosynthetic pigments, elements, and silybin A and B (A + B) of plants from various treatment groups were measured and recorded. In addition, the expressions of *CHS* genes were also monitored to establish the correlation between silybin (A + B) production and *CHS* gene expressions.

2. Materials and Methods

2.1. Cultivation of *S. marianum* L.

S. marianum seeds were obtained from the Institute for Horticultural Science, Agricultural Research Center (ARC), Department of Medicinal and Aromatic Plants, Giza, Egypt. These seeds were cultivated in a greenhouse at the Agricultural-Veterinarian Training and Research Station, King Faisal University, Al-Asha, Saudi Arabia. During the cultivation period, temperatures ranged from 32 to 36 degrees Celsius, and the relative humidity ranged from 47 to 56 percent. The average daylight hours were 14 h per day. Seedlings of *S. marianum* (5 cm in length, with 3 pairs of leaves) were transplanted on 1 November 2020, a month after seed sowing, into plastic pots of 37.5 cm in diameter and 27.5 cm in depth. Each pot was filled with 25 kg of a 1:1 ratio of moist sand and peat moss mixture. The pots were then arranged in a completely randomized pattern that was repeated 20 times. The plants in the various treatment groups were sprayed with salicylic acid solutions at concentrations of 0 (control, distilled water), 10^{-4} , and 10^{-3} Molar (M) every two months (1st-Dec, -Feb, and -April) during the vegetative growth stage of the *S. marianum*. Foliar spray of salicylic acid was performed around 8 am in the morning. Approximately 50 mL of salicylic acid solutions (10^{-4} or 10^{-3} M) or distilled water (control) was sprayed throughout the individual plant. The salicylic acid powder (cat. no. 247588, ACS grade, 99+%) was purchased from Sigma Aldrich, Darmstadt, Germany. Distilled water was used as a diluent for the salicylic acid. The mineral contents and chemical properties of the irrigation water are shown in Table 1.

Table 1. The chemical composition and properties of the irrigation water.

Salinity Level (ppm)	Cations (meq L ⁻¹)				Anions (meq L ⁻¹)				Sodium Absorption Ratio
	Ca ²⁺	Mg ²⁺	Na ⁺	K ⁺	CO ₃ ²⁻	HCO ₃ ⁻	SO ₄ ²⁻	Cl ⁻	
864	Ca ²⁺	Mg ²⁺	Na ⁺	K ⁺	CO ₃ ²⁻	HCO ₃ ⁻	SO ₄ ²⁻	Cl ⁻	3.43
	5.72	2.02	7.27	0.38	0.28	2.68	4.03	8.4	

2.2. Measurement of *S. marianum* Growth and Yield

The growth data of ten randomly selected plants from each treatment group were recorded at 270 days after sowing; these included the plant height (cm), number (n) of leaves and branches, and the dried weights (DW) (g) of the root and aerial parts. All plants in each treatment group had mature fruits that were collected on a regular basis and dried at room temperature. The number of capitula (n) per plant as well as the DW (g) of the fruits were recorded.

2.3. Measurement of Photosynthetic Pigments in *S. marianum* Leaves

The 3rd leaf from the top of the plant was collected from the ten plants harvested at 270 days after sowing. The photosynthetic pigments from each of these leaves were extracted with 80% acetone. The absorbances of 80% acetone solutions containing the leaf pigments were then measured at wavelengths of 444, 644, and 662 nm using the Agilent 8453 UV-Visible spectrophotometer (Agilent, Santa Clara, CA, USA). The contents of chlorophyll a (Chl-a), chlorophyll b (Chl-b), and carotenoids per fresh weight (FW) of leaves were quantified according to the formulas described by [28].

2.4. Quantification of Nitrogen, Phosphorus, and Potassium Contents in *S. marianum* Leaves

All leaves from each individual plant harvested at 270 days after planting were grouped together and dried for 48 h at 60 °C. Collections of dried leaves from each of these plants were crushed and digested separately by sulfuric acid [29]. The modified micro-Kjedahl technique was used to determine nitrogen content [30]. Calorimetry was used to measure the percentage of phosphorus, as reported in [31], and atomic absorption flame photometry was used to determine the potassium (K) content, as described in [32]. In each treatment, ten sulfuric acid digested samples were prepared, and their element contents were analyzed as mentioned above.

2.5. Quantification of Silybin (A + B) in *S. marianum* Fruits by High-Performance Liquid Chromatography (HPLC)

2.5.1. Preparation of Standard Solutions and Standard Curve

The stock solution (1 mg/mL) was prepared in methanol using authentic 93.06% silybin (A + B) standard (Santa Cruz Biotechnology, Dallas, TX, USA, cat. no. sc-473918). Standard solutions of various known concentrations (750, 500, 250, 100, and 50 µg/mL) of silybin (A + B) were prepared and filtered with a syringe filter (0.22 µm). The standard curve was constructed by plotting the calibration values obtained from 10 µL of each of the filtered standard solutions.

2.5.2. Preparation of Methanolic Extracts from Ground *S. marianum* Dried Fruits

Dried fruits from individual plants were grouped together and ground with a coffee grinder to become a fine powder. A known amount of ground air-dried fruits from a single plant was mixed with 50 mL of methanol in a conical flask, and the content was sonicated for 30 min. On the subsequent day, the solvent was collected and kept aside. Then, 50 mL of fresh methanol was added to the ground fruit residue, followed by sonication. The methanol replacement process was repeated on the following day. The dried methanolic extract for each individual plant consisted of methanol solvent collected from the same conical flask on three consecutive days and dried using a rotary evaporator at 40 °C. For

each treatment group, ten methanolic extracts were prepared from ten individual plants. The dried methanolic extract was then resuspended in 5 mL of methanol and filtered with a 0.22 µm syringe filter before loading 10 µL of the filtered solution into the HPLC system.

2.5.3. Instrumentation and Analysis Conditions

High-performance liquid chromatography (HPLC) was performed using the Waters 2690 Alliance HPLC system (USA), containing a C18 Kromasil column (4.6 × 150 mm, 5 µm) and a Waters 996 photodiode array detector. A gradient mobile phase was generated by systematically varying the mixture percentage of two solvents, which consisted of water: methanol: phosphoric acid at the ratio of 80:20:0.5 and 20:80:0.5, respectively. The flow rate for the elution was maintained at 1 mL/min. The eluted compounds were monitored at 288 nm for a period of 50 min.

2.6. Gene Expression Analyses of *CHS* 1, 2, and 3 by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

2.6.1. Extraction of Total RNA and Synthesis of Complementary DNA (cDNA)

For each treatment group, petals from the inflorescence of *S. marianum* from six individual plants were collected during the early flowering stage (240 days after sowing) and stored in the −80 °C freezer until RNA extraction was performed. RNA extraction was performed using an RNeasy Mini Kit (Cat. no. 74104, Qiagen, Germantown, MD, USA). The cDNA was synthesized with the ReadyScript™ cDNA synthesis mix (Cat. no. RDRT, Sigma Aldrich, Darmstadt, Germany) using 1 µg of total RNA. RNA extraction and cDNA synthesis were executed according to the reagent manufacturer protocols.

2.6.2. Analysis of *CHS* Genes Expression by Quantitative Polymerase Chain Reaction

The quantitative polymerase chain reaction was performed with the Applied Biosystem 7500 Real-time PCR system (ThermoFisher Scientific, Germantown, MD, USA). A 25 µL PCR mixture containing 2 µL of cDNA reaction mixture was set up according to the manufacturer's (QuantiTech SYBR Green PCR Kit, Cat. no. 204143, Qiagen, Germantown, MD, USA) protocol. The PCR was executed under the following conditions: 95 °C initial heating for 10 min and 45 cycles of 95 °C (20 s) and 60 °C (1 min) temperature alteration. The forward and reverse primers for the *CHS* 1, 2, 3 (target), and *NADH dehydrogenase* (reference) genes were designed according to [22]. Two quantitative polymerase chain reactions were performed for each cDNA sample. The expression of each target gene (*CHS* 1, 2, and 3) was normalized with that of the reference gene (*NADH dehydrogenase*) and quantified using the $2^{-\Delta\Delta CT}$ method [33]. In each treatment group, the mean expression of each *CHS* gene was calculated from the average of two quantitative chain reactions performed with six cDNA samples that were prepared from the total RNA isolated from six individual plants. In summary, the mean of each treatment group was calculated from 12 replicates (six biological replicates with two technical replicates). The mean expressions of the three *CHS* genes of the 10^{-4} and 10^{-3} M salicylic acid treatments were then expressed as relative folds of those of the control treatment.

2.7. Statistical Analysis

The Statistica 6 program one-way ANOVA/MANOVA [34] was employed to evaluate the mean differences among treatment groups at a probability level of $p = 0.05$.

3. Results

3.1. Effect of Salicylic Acid on *S. marianum* Vegetative Growth and Yield

Salicylic acid at both 10^{-4} and 10^{-3} M concentrations increased the *S. marianum* plant height (cm), number (n) of leaves and branches, and the DW of root and aerial parts as compared with the control. Lower concentration of salicylic acid (10^{-4} M) produced plants with significantly highest means in all growth parameters. A further increase in salicylic

acid concentration to 10^{-3} M reduced the above-mentioned parameters as compared with that of 10^{-4} M treatment (Table 2).

Table 2. Effect of different concentrations of salicylic acid on plant height (cm), number of leaves (n), branch number (n), DW of root and aerial parts (g) of *S. marianum* L.

Salicylic Acid (M)	Plant Height (cm)	Number of Leaves (n)	Branch Number (n)	Root DW (g)	Aerial Part DW (g)
0	50.75 b *	50.50 b	3.25 b	6.56 b	38.83 b
10^{-4}	92.00 a	96.25 a	5.75 a	17.49 a	132.53 a
10^{-3}	72.25 a	52.50 b	3.50 b	9.03 b	59.49 b

* Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to LSD test.

Both concentrations of salicylic acid were found to produce plants with more capitulas and higher fruit DW as compared with the control plants (Table 3). The highest capitula number (17.0) and fruit DW (56.95 g) were obtained from the plants sprayed with salicylic acid at 10^{-4} M. These increases were statistically significant as compared with the control and salicylic acid at 10^{-3} M (Table 3).

Table 3. Effect of different concentrations of salicylic acid on capitula number (n) and fruit DW (g) of *S. marianum* L.

Salicylic Acid (M)	Capitula Number (n)	Fruits DW (g)
0	5.00 b *	16.65 b
10^{-4}	17.00 a	56.95 a
10^{-3}	7.25 b	20.53 b

* Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to LSD test.

3.2. Photosynthetic Pigments and Element Contents in *S. marianum* Leaves

Data in Table 4 showed that the foliar spray with both salicylic acid concentrations produced plants with higher contents of Chl-a, Chl-b, and carotenoids in the *S. marianum* leaves, as compared with the control treatment. Consistent with the growth and yield parameters, salicylic acid at 10^{-4} M was found to produce *S. marianum* plants with the highest Chl-a, Chl-b, and carotenoid contents, and these increases were statistically significant as compared with the control and 10^{-3} M salicylic acid treatment (Table 4).

Table 4. Effect of different concentrations of salicylic acid on Chl-a, Chl-b, and carotenoids (mg/100 g FW) pigment contents of *S. marianum* L.

Salicylic Acid (M)	Chl-a (mg/100g FW)	Chl-b (mg/100g FW)	Carotenoids (mg/100g FW)
0	28.28 b *	6.43 b	38.88 b
10^{-4}	49.98 a	12.45 a	48.07 a
10^{-3}	29.18 b	6.86 b	36.82 b

* Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to LSD test.

The data delineated in Table 5 show that both salicylic acid concentrations increased the nitrogen (N), phosphorus (P), and potassium (K) contents in the leaves of *S. marianum* as compared with the control plants. Salicylic acid at 10^{-4} M produced significantly higher N, P, and K contents as compared to the control and salicylic acid at 10^{-3} M treatments (Table 5).

Table 5. Effect of different concentrations of salicylic acid on Nitrogen (N), Phosphorus (P), and Potassium (K) percentage in *S. marianum* L dried leaves.

Salicylic Acid (M)	N%/DW	P%/DW	K%/DW
0	1.035 b *	0.305 b	1.52 b
10^{-4}	1.145 a	0.698 a	2.84 a
10^{-3}	1.115 a	0.415 b	1.73 b

* Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to LSD test.

3.3. Effect of Foliar Application of Salicylic Acid on Silybin (A + B) Contents

The silybin (A + B) contents in the fruits of *S. marianum* were analyzed using high-performance liquid chromatography (HPLC). Our results revealed that salicylic acid at the concentrations of 10^{-4} M and 10^{-3} M significantly increased the mean silybin (A + B) contents to 85.223 and 71.585 mg/100 g of fruit DW, respectively, as compared to the average content of 32.562 mg/100 g fruit D.W. for the control group (Figure 1).

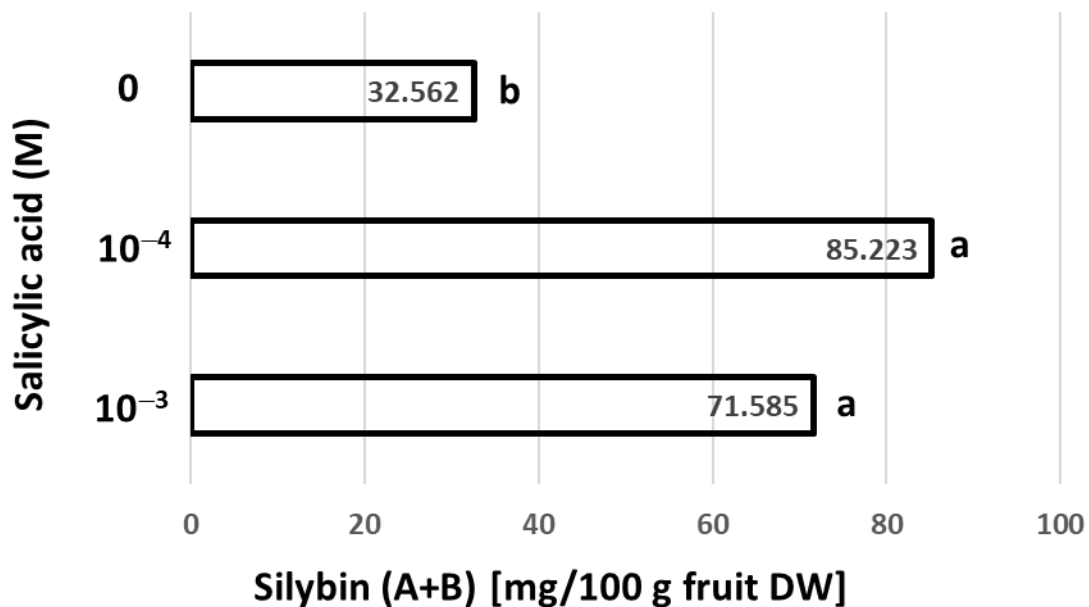


Figure 1. The contents of silybin (A + B) (mg/100 g fruit DW) in *S. marianum* L fruit. The means with the same letter are not statistically different at $p \leq 0.05$.

3.4. The Effect of Foliar Spray of Salicylic Acid on the Expression of CHS 1, 2, and 3 Genes in *S. marianum* Petals

The qRT-PCR was employed to measure the expression levels of the *CHS 1*, *2*, and *3* genes in the *S. marianum* petals. All *CHS* genes (*CHS 1*, *2*, and *3*) were found to be upregulated in plants sprayed with salicylic acid, with a higher expression recorded in plants in the 10^{-4} M salicylic acid treatment group (Table 6). In summary, consistent associations of the *chalcone synthase* gene expressions with the silybin (A + B) contents were observed in all the treatment groups (Table 6 and Figure 1).

Table 6. Fold difference of *CHS 1*, *2*, and *3* gene expressions as a result of salicylic acid treatments. In each treatment group, the mean was calculated from duplicate qRT-PCR data from six cDNA samples. Each cDNA sample was amplified from a total RNA sample isolated from a single plant.

Salicylic Acid (M)	<i>CHS 1</i> (Fold)	<i>CHS 2</i> (Fold)	<i>CHS 3</i> (Fold)
0	1.000 c * ± 0.439	1.000 c ± 0.168	1.000 c ± 0.147
10 ⁻⁴	2.984 a ± 0.990	1.682 a ± 0.343	2.479 a ± 0.063
10 ⁻³	1.912 b ± 0.104	1.449 b ± 0.249	1.425 b ± 0.020

* Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to LSD test.

4. Discussion

In this study, the effects of the foliar spraying of *S. marianum* plants with two concentrations (10⁻⁴ and 10⁻³ M) of salicylic acid on the development, yield, and silybin (A + B) accumulation in the fruits, as well as the expression of *chalcone synthase* genes in the petals, were investigated. Our results indicated that 10⁻⁴ M salicylic acid has higher enhancement effects on the plant growth parameters (plant height, number of leaves and branches, and the DW of root and aerial parts) and yield (capitula number and fruit DW) even though both concentrations (10⁻⁴ and 10⁻³ M) of salicylic acid have increased *S. marianum* plant growth and yield as compared with the control. Increased growth and yield of medicinal plants resulting from exogenously applied salicylic acid were previously reported by numerous research groups [11,15,17,27,35]. Salicylic acid is a phenolic plant hormone, which induces both cell division and elongation, resulting in greater plant growth [36]. Furthermore, exogenously applied salicylic acid has been shown to increase the levels of chlorophyll and carotenoids [37,38]. An increased level of the photosynthetic pigments, the physiological indicators linked to the plant photosynthetic capability [11,15,39], has been associated with salicylic acid's positive effects in plant nutrient uptake [39]. In the present study, consistent with the observed *S. marianum* plant growth and yield enhancement trend, the highest contents of photosynthetic pigments (Chl-a, Chl-b, and carotenoids) and elements (nitrogen, phosphorus, and potassium) were also observed in the 10⁻⁴ M salicylic acid treatment as compared with 10⁻³ M and control treatments. These observations implied that the increase in *S. marianum* plant growth and yield were the result of increased levels of photosynthetic pigments resulting from plant nutrients (nitrogen, phosphorus, and potassium) uptake.

The great potential of *S. marianum* plants' hepatoprotective effects on liver cancer and Alzheimer's disease have been attributed to the silybin compound presented in the silymarin, which was extracted from *S. marianum* dried fruits [40,41]. The present study demonstrated that exogenous application of salicylic acid was effective in eliciting production of silybin (A + B), in addition to its positive impacts on *S. marianum* growth and yield. Notably, salicylic acid at the concentration of 10⁻⁴ M gave the highest increase in silybin (A + B) composition, which was consistent with the enhancement trend in plant growth and yield. In a separate study, salicylic acid (100, 200, 400 µM) added into the nutrient media has been implicated in the increase in flavonolignan (predominantly, silychristin, and silybin B) content in the fruits of the hydroponically cultivated *S. marianum* [42]. In the corresponding study, the authors found that 200 µM (2 × 10⁻⁴ M) was the most effective concentration for the enhancement of silymarin production in the hydroponic culture system. Taken together, the results from our present study and that of the hydroponic system suggested that the optimal salicylic acid concentration for enhancement of silymarin in greenhouse cultivated *S. marianum* might be higher than 100 µM (10⁻⁴ M) but lower than 400 µM (4 × 10⁻⁴ M). This hypothesis will be further examined in future research. Indeed, salicylic acid-induced flavonoid accumulation has been reported in a variety of plant species, including tea, cucumber, and satsuma [26,43,44]. Besides flavonoid compounds, the induction of various other secondary metabolites by salicylic acid has also been reported in a variety of plants such as coriander, fennel, achillea, marigold, fenugreek, onion, and *Salvia officinalis* [11,15,25,27,35,45,46].

Exogenous salicylic acid treatment has been shown to affect flavonoid biosynthesis in plants through increased expression of flavonoid biosynthesis-related genes, such as *CHS* genes [13,23,24,44,47]. In the present study, expression of all three *chalcone synthase* genes (*CHS 1*, *CHS 2*, and *CHS 3*) was increased by the salicylic acid treatments, and these were consistent with the increase in silybin (A + B) contents.

5. Conclusions

Our study indicated that both concentrations (10^{-3} and 10^{-4} M) of salicylic acid have positively impacted the growth, yield, photosynthetic pigments, and silybin (A + B) contents in *S. marianum*. The 10^{-4} M salicylic acid has a higher impact and resulted in an increase of 3.4 times in the capitula number and fruit DW as well as 2.6 times higher silybin (A + B) contents as compared with the control. The content of silybin (A + B) was shown to be closely associated with the expression profiles of *CHS* genes. Our results suggested that salicylic acid foliar spray could be a great strategy for promoting the growth and yield of the *S. marianum* plants as well as increasing its medicinal value by raising its silybin (A + B) contents.

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