# Effect of Salicylic Acid on Antioxidant Activity in Milk Thistle Hairy Root Cultures

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## Abstract

**Background: Elicitors can trigger defense responses and activate specific genes involved in secondary metabolite biosynthesis.**

**Objective: For understanding SA-treatment signaling pathway we determined defense-related compounds and enzymes in *S. marianum* hairy root cultures.**

**Methods: 28 day old hairy roots were treated by salicylic acid (6 mg/50 ml culture) for different times (24, 48, 72, 96 and 120 h). The antioxidant activity was assayed by the 1-diphenyl-2-picrylhydrazyl stable free radical method and results were calculated based on the $IC_{50}$. $H_2O_2$, peroxidase and ascorbate peroxidase activity and total tocopherol content was measured in treated and non-treated hairy root cultures.**

**Results: The highest accumulation of $H_2O_2$ was 86.60 µmol $g^{-1}$ DW that was obtained 120 h after elicitation (2-times of non-treated hairy roots). $IC_{50}$ increased upon treatment and was 1.15- times higher than the control 24h after elicitation. The content of total tocopherol content under SA treatment was lower than the control experiments. Maximum activity of peroxidase and ascorbate peroxidase were 0.17 and 1.50 µmol $min^{-1}$ $mg^{-1}$ protein, 96 and 72 h after elicitation, respectively.**

**Conclusion: Oxidative stress defends system can be activated by salicylic acid in *Silybum marianum* hairy root cultures. This study suggested that exogenous SA can increase $H_2O_2$ content of hairy roots, and induce the expression of antioxidant enzymes and increase secondary metabolite accumulation.**

**Keywords: Hairy roots, Salicylic acid, Antioxidant activity, Peroxidase, Ascorbate peroxidase**

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## Introduction

Plants are a rich source of various phytochemicals, proteins, enzymes and other secondary products of immense biotechnological applications. The secondary products and the respective enzymes, particularly those of phenylpropanoid pathway are significantly enhanced under the influence of elicitors [1, 2]. Elicitors can trigger an array of defense or stress responses and activate specific genes for the enzymes involved in secondary metabolite biosynthesis, for improving the production of plant secondary metabolites [3]. Thus, successful application of elicitation requires extensive screening. The exogenous application of elicitor's *in vitro* cultures is useful for studying plant responses to potential microbe/insect attack as well as for enhanced biotechnological production of value-added secondary metabolites in fermentation systems. Elicitors including biotic and abiotic are known to stimulate production of secondary metabolites in plants [4, 5].

Reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$), superoxide, and the hydroxyl radical are inevitably generated by various physicochemical and biochemical reactions, including photosynthesis and elicitation. These AOS potentially oxidize and damage cellular components, resulting in oxidative stress [6]. Plants protect cell and sub cellular systems from the cytotoxic effects of these ROS with antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), catalase (CAT) and nonenzymatic substances such as glutathione, ascorbic acid, α-tocopherol and carotenoids [7-10].

Peroxidase and ascorbate peroxidase can oxidise various substrates due to its ability to perform a number of different types of reactions, such as peroxidative oxidation or oxidative and catalytic hydroxylation [11, 12].

We reported the effect of SA on silymarin, linoleic acid content and lipoxigenase activity in hairy root cultures of *S. marianum* (treated with SA) [13]. We treated hairy roots of *S. marianum* with different concentration of SA (0, 1, 2, 4, 6 and 8 mg/50 ml culture); the optimal feeding was 6 mg/50 ml culture of SA. Then the hairy roots of *S. marianum* was treated with 6 mg/50 ml culture of SA for different times (24, 48, 72, 96 and 120 h). The high content of silymarin was obtained 24h after elicitation. The lipoxygenase activity increased 24 h after treatment. The maximum content of linoleic acid was obtained 24 h after elicitation. Our results showed that elicitation with SA can be regulated the jasmonate pathway that may mediate the elicitor- induced accumulation of silymarin [13]. Therefore, to understand SA-treatment signaling pathway we determined POD, APX activity, DPPH, $H_2O_2$ and tocopherols content in hairy roots of *S. marianum*.

## Materials and Methods

### Hairy root culture

*S. marianum* hairy roots induced by *A. rhizogenes* (AR15834) were used as hairy roots source. Induction of hairy roots and their maintenance conditions have been explained by Rahnama et al (2008) [14].

The hairy roots were maintained in Murashige and Skoog liquid medium (MS) supplemented with 30 g $l^{-1}$ sucrose [15]. For experiments 100 mL flasks containing 50 mL medium were inoculated with six 1 cm pieces



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of hairy roots from 4-week-old subculture. The cultures were shaken at 150 rpm and 25 $^{o}$C in darkness [14]. Treatments were done 28 days after transfer, when hairy roots were in the active growth phase.

**Elicitation**

A total of 400 mg of SA acid was dissolved in NaOH (1 N) and then concentrated stock solution (40 ml) was prepared with distilled water. These solutions were sterilized by autoclaving at 120°C and 1 atm over 20 min and used as an elicitor solution. 600 µl of elicitor solution of SA was added to the cultures. Control was received equivalent volume of culture media. The treated and non treated hairy roots were harvested at 28-day old cultures after 24, 48, 72, 96 and 120 h of treatment.

**Determination of $H_2O_2$ content**

Hydrogen peroxide content was determined according to Velikova et al. (2000) [16]. 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 12000g 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 $H_2O_2$ was calculated by comparison with a standard calibration curve previously made by using different concentrations of $H_2O_2$.

**DPPH radical scavenging activity**

The scavenging effect of each extract was estimated according to the procedure established by Brand-Williams et al. (1995) [17]. The 1-diphenyl-2-picrylhydrazyl DPPH con- centration (CDPPH as mg ml-1) in the reaction medium was calculated. The parameter $IC_{50}$ (mg DPPH/ml of extract) was calculated graphically. A lower $IC_{50}$ value indicates greater antioxidant activity.

**δ- and γ -tocopherol analysis**

Total, δ- and γ -tocopherol were determined as described by Sanchez-Machado et al. (2002) [18]. The separation of toc- opherols was carried out on an HPLC instrument (all from Knauer, Germany), using a Eurosphere $C_{18}$ 5 µm (250 9 4.6 mm) µm 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.

**Peroxidase (POD) activity assay**

The POD assay was carried out using the method of Chance and Maehly (1955) [19], which measures the absorbance at 420 nm resulting from the oxidation of Pyrogallol to purpurogallin catalyzed by POD in a 100 mM potassium phosphate buffer solution at a pH 6.0. The absorbance of both blank and sample was measured in a 10 mm glass cuvette over a 5 min period. The final concentrations of the reagents in the cuvette were 14 mM potassium phosphate, 0.027% (w/w) hydrogen peroxide, 0.5% (w/v) pyrogallol and 0.04–0.07 units of peroxidase. One unit of POD activity was defined as the change in absorbance (420 nm) over time (min) per ml of enzymatic extract. One unit of POD was defined as the formation of 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0.

**Ascorbateperoxidase (APX) activity assay**

APX activity was determined by estimating the rate of ascorbate oxidation as reported by Nakano and Asada (1987) [20]. The reaction mixture consiste of 50 mM potassium



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phosphate buffer (pH 7.0), 0.5 mM sodium ascorbate, 0.1 mM $H_2O_2$ and enzyme extract. Decrease in absorbance at 290 nm was measured at 25 °C for 3 min (E = 2.8 $mM^{-1}$ $cm^{-1}$).

**Statistical analyses**

All analytical values represent the means of three analytical replications. Statistical significance was calculated using Duncan test for unpaired data (ά≤ 0.05) and ANOVA method was used for comparisons of means. Statistical analysis was made by SAS software (Version 6.2).

# Results

**Response of antioxidant enzymes to SA elicitation**

Fig. 1 shows $H_2O_2$ content in treated and non-treated hairy roots within the period of 120 h. The content of $H_2O_2$ gradually increased hitting a peak (60.71 µmol $g^{-1}$ FW) after 48 h that was 1.72-times that of control (35.16 µmol $g^{-1}$ FW). The content of $H_2O_2$ declined from 48 to 72 h in treated hairy roots rolling to 55.27 µmol $g^{-1}$ FW. Then, the content of $H_2O_2$ increased from 72 h to 120 h (86.60 µmol $g^{-1}$ FW) that was 2.01-times that of the control.

Antioxidant activity of *S. marianum* hairy root cultures metabolic 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 [21]. As shown in Fig. 2, the $IC_{50}$ value was changed significantly after 72 h treatment. The $IC_{50}$ increased upon stimulation by SA after 24 h treatment and reached a maximum at about 24 h after treatment. The $IC_{50}$ was decreased between 48 to 96 h, thereafter increased when the elicitor treatment time was 120 h.

The content of total tocopherol content under SA treatment was lower than the control treatment (Fig. 3C). However, addition of elicitor induced the decrease of δ-tocopherols (Fig. 3A). The γ- tocopherol was lower than the control and decreased dramatically (Fig. 3B).

Fig. 4 presents POD activity of treated and non-treated hairy roots from the first day of elicitation to 120 h after elicitation. As it can be seen, the POD activity in non-treated hairy roots remained stable over 120 h periods. The POD activity in SA treated cultures had one pick, after 24 h (0.12 µmol $min^{-1}$ $mg^{-1}$ protein) that was 3.08-fold that of the control. The POD activity increased 96 h after elicitation (0.17 µmol $min^{-1}$ $mg^{-1}$ protein) that was 13.46-times that of the control (0.013 µmol $min^{-1}$ $mg^{-1}$ protein).

Fig. 5 indicates APX activity in treated and non-treated hairy roots within the period of 120 h. The APX activity in non-treated hairy roots was increased after 48 h (1.19 µmol $min^{-1}$ $mg^{-1}$ protein) that was 1.45- fold of treated hairy roots (0.82 µmol $min^{-1}$ $mg^{-1}$ protein) after that APX activity decreased. APX activity dramatically increased after 72 h and reached to 1.50 µmol $min^{-1}$ $mg^{-1}$ protein. There was a dramatic decrease in APX activity in treated hairy roots from 72 to 120 h (0.91 µmol $min^{-1}$ $mg^{-1}$ proteins).

# Discussion

Hairy root cultures described in this paper contain a low level of silymarin under normal growth conditions and are sensitive to the elicitation with salicylic acid in silymarin formation. These cultures provide a very simple and useful system for the study of various elicitors and signal molecules on the regulation of silymarin biosynthesis.

Elicitors are biological or non-biological compounds, which upon contact with plant



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cells; trigger defense-related compounds through over-expression of relevant enzymes [1, 22]. Treatment of hairy root culture with SA has improved production of silymarin to a level about 1.6-fold higher than that of the control (24 h after treatment), the highest linoleic acid content and Lipoxygenase activity was obtained 24h after elicitation [13]. SA is one of the special elicitor in the study of elicitation of secondary compounds through physiologically pathways. SA, like methyl jasmonate, has been known as a major initiator of tow pathogen-related plant defense reactions [23]. SA can act not only as an inducer but also as an inhibitor of secondary compound production [24]. In hairy root cultures of *S. marianum* treated with SA (6 mg/ 50 ml culture) production of tocopherols decreased.

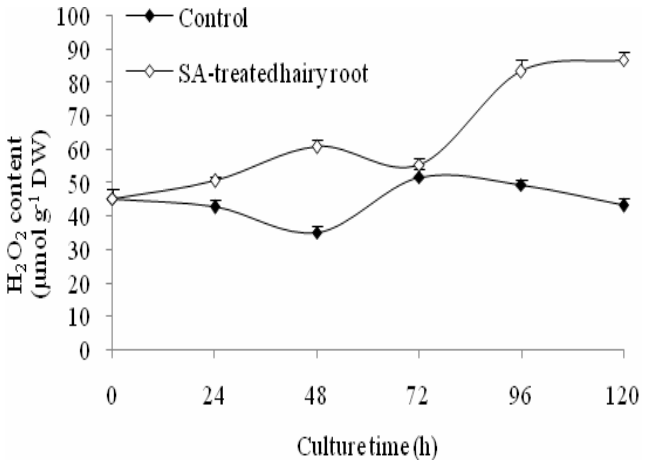


**Fig. 1- Time-course of $H_2O_2$ content in *S. marianum* hairy root cultures treated with SA (6 mg / 50 ml culture). The control received only MS medium. Data show means ± SD from triplicate experiments**

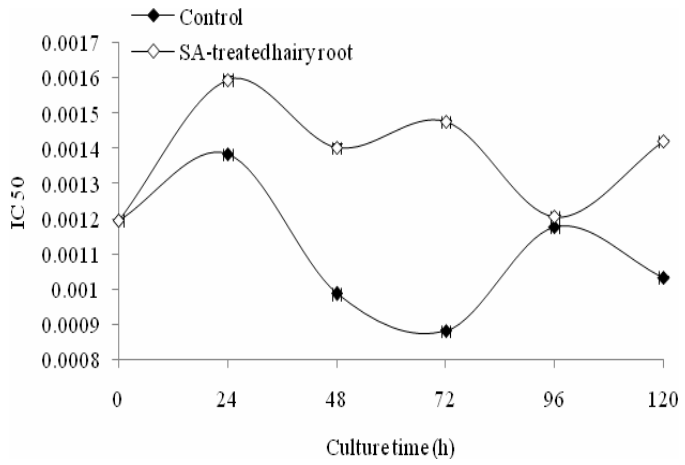


**Fig. 2- Time-course of $IC_{50}$ variations in *S. marianum* hairy root cultures treated with SA (6 mg / 50 ml culture). The control received only MS medium. Data show means ± SD from triplicate experiments**



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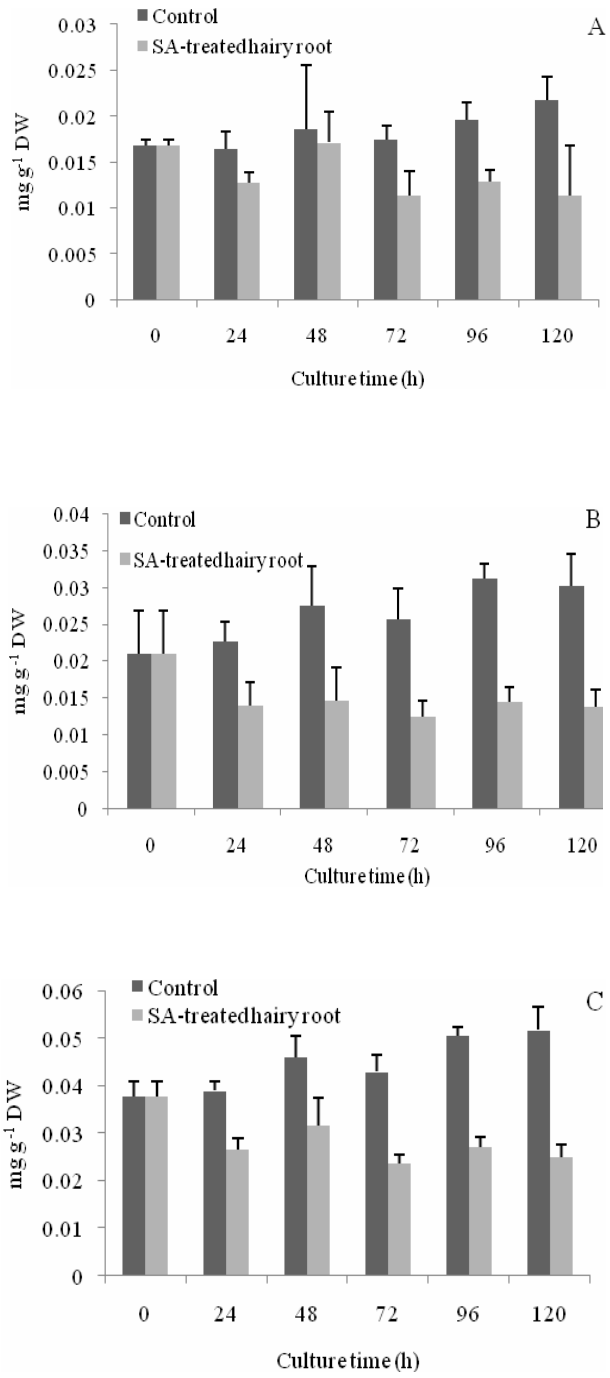


**Fig. 3 - Time course of vitamin E [δ (A), γ (B) and total (C) tocopherols] production in *S. marianum* hairy root cultures treated with SA (6 mg/50 ml culture). The control received only MS medium. Data shows means ± SD from triplicate experiments**

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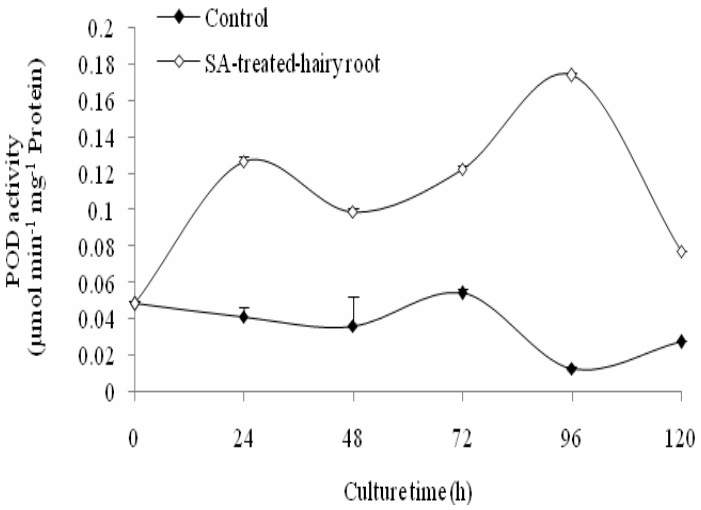


**Fig. 4 - Time-course of POD activity in *S. marianum* hairy root cultures treated with SA (6 mg / 50 ml culture). The control received only MS medium. Data shows means ± SD from triplicate experiments**

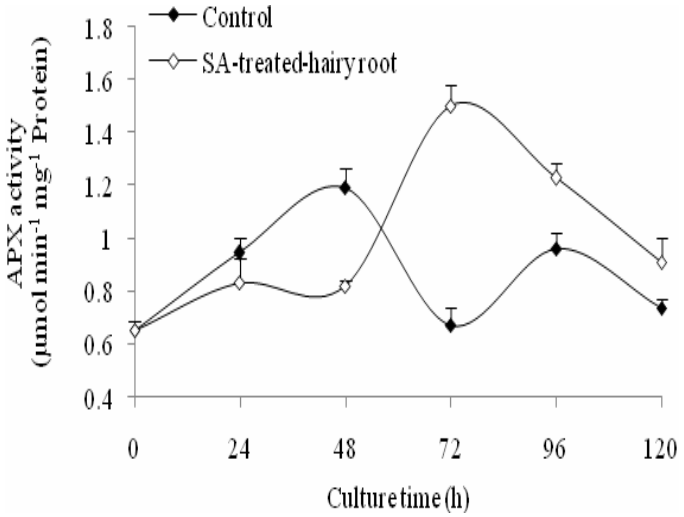


**Fig. 5 – Time-course of APX activity in *S. marianum* hairy root cultures treated with SA (6 mg/50 ml culture). The control received only MS medium. Data shows means ± SD from triplicate experiments**

Tocopherols scavenge lipid peroxy radicals. They were also known to protect lipids and other membrane components. α- tocopherols interact with the polyunsaturated acyl groups of lipids, stabilize membranes, and scavenge various ROS. The decrease in tocopherols seem to be related to consumption of this metabolite and is one of the alteration in the signal transduction pathway induced in treated hairy root cultures of *S. marianum*.

Our results showed that the antioxidant activity was lower than the control. Hasanloo et al. 2009 [25] showed that in hairy roots of



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*S. marianum* was treated with yeast extract antioxidant activity was increased after elicitation. Different stress conditions of both biotic and abiotic natures enhance the cellular production of reactive oxygen species (ROS) [26]. SA treatment induced the activity of POX and APX. POX is protective enzyme of plant cells against a variety of stresses by regulating the concentration of $O_2^-$ and $H_2O_2$. Protection against ROS and peroxidation reactions is provided by antioxidant enzymes such as superoxide dismutase, catalase and ascorbate peroxidase, and nonenzyme antioxidants such as glutathione and ascorbate. The current study also suggests the presence of $H_2O_2$ in SA treated hairy root cultures of *S. marianum*. The rapid over-production of reactive oxygen species (ROS) such as $H_2O_2$ is one of the earliest responses of *S. marianum* to elicitors. $H_2O_2$ is a toxic intermediate that also induces expression of many defense genes and secondary metabolites, such as syringin, sesquiterepene cyclases and phenylalanine ammonia lyase [26, 27]. In the present study, it was observed that ROS signaling is an integral part of the SA signal transduction for production of silymarin.

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