

## Original Article

## Hydrogen peroxide Acts as a Secondary Messenger for Production of Silymarin in Ag<sup>+</sup> Elicited *Silybum marianum* Hairy Root Cultures

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### Abstract

This study was performed to investigate the effect of Ag<sup>+</sup> as an elicitor on the activity of antioxidant enzymes, content of H<sub>2</sub>O<sub>2</sub>, accumulation of total tocopherol and antioxidant activity in hairy root cultures of *Silybum marianum* (L.) Gaertn. The hairy roots were treated with 2 mM Ag<sup>+</sup>. The treated and non-treated hairy roots were harvested in the same time and data on H<sub>2</sub>O<sub>2</sub> content, DPPH, tocopherol content and enzyme activity were measured. Ag<sup>+</sup> elicitation resulted in enhanced H<sub>2</sub>O<sub>2</sub> content after 24, 72 and 96 h. Peroxidase activity increased 24 h after elicitation, whereas the antioxidant activity decreased upon treatment. The overall trend of IC<sub>50</sub> changes was very similar to that of the control treatments. Tocopherol content decreased 24 and 48 h after elicitation. The results demonstrated a possible role of hydrogen peroxide as a secondary messenger for production of silymarin in Ag<sup>+</sup> elicited *Silybum marianum* hairy roots cultures.

**Key word:** *Silybum marianum*, Ag<sup>+</sup>, Antioxidant enzymes, DPPH, Tocopherol

### Introduction

*Silybum marianum* (L.) Gaertn (Asteraceae) is regarded a valuable source of useful chemicals (silymarin) isolated from the dried fruits of the plant and has been utilized for its protective effects on the liver disorders. A mixture of flavolignans (silybin, isosilybin, sylichrisin and silydianin) commonly referred to collectively as silymarin (SLM) [1]. In recent years, there has been an increasing interest in development of strategies to improve the yield of secondary metabolites through hairy root cultures, among which those concerning signal transduction pathway has been given special attention [2,3].

Elicitors have been found to enhance the production of secondary metabolites [4,5]. There are several reports on accumulation of secondary metabolites

in hairy root cultures upon Ag<sup>+</sup> application as an abiotic elicitor. Abiotic and biotic stresses irritate cell defense system, resulting in enhanced production of reactive oxygen species (ROS) [6]. ROS are toxic for plants and cause damage to DNA, proteins, lipids, chlorophyll, etc. Plant cells protect themselves from such toxic agents by engaging antioxidant enzymes [7,8]. Little is known about SLM production pathway and it is not clear what factors effect this signal transduction pathway. In our previous work, the accumulation of SLM was investigated under Ag<sup>+</sup> stress, in *S. marianum* hairy root cultures [9]. The aim of this study was investigation of cell responses to Ag<sup>+</sup> elicitation and determination of the quantity range of factors affecting SLM production to gain the highest yield.

### Material and Methods

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### Hairy root culture

Induction of *S. marianum* hairy roots and their maintenance conditions have been explained by Rahnama and used as a hairy root source [10]. Six 1 cm pieces of roots from 4-week-old shake-flask cultures were cultured in 50 mL Murashige and Skoog liquid medium (MS) in shake-flask cultures with 200-mL Erlenmeyer flasks on orbital shaker set at 150 rpm and incubated at 25 °C in the dark [11].

### Stress treatment

AgNO<sub>3</sub> treatments were in two levels (0 and 2 mM), which added to the culture media of 28-days old hairy root and harvested after 0, 24, 48, 72, 96 and 120h of elicitation [9]. The samples were kept at -80 °C for the analysis.

### Extraction and measurement of H<sub>2</sub>O<sub>2</sub>

H<sub>2</sub>O<sub>2</sub> was measured according to Velikova *et al.*, (2000) [12]. Briefly, frozen hairy roots were homogenized in an ice bath with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12000g for 15 min. Then, 0.5 mL of 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 recorded at 390 nm by spectrophotometry.

### DPPH free radical scavenging activity

For measurement of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity 2 mL of DPPH solution (100 μM) was added to 2 mL of extract. After incubating at room temperature for 15 min, the absorbance was recorded at 517 nm. [11]. The capability to scavenge the DPPH radical was calculated using the following equation:

Scavenging effect (%) =

$$\left[ \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100$$

Where A<sub>blank</sub> is the absorbance of the control reaction (containing all reagents except the test sample), and A<sub>sample</sub> is the absorbance of that extract.

The IC<sub>50</sub> was calculated and a lower IC<sub>50</sub> value indicates highest antioxidant activity.

### Tocopherol analysis

Tocopherol was determined by HPLC (all from Knauer, Germany) as described by Sanchez-Machado *et al.*, (2002) [13]. Briefly, using a Eurosphere C18 5 lm (250 9 4.6 mm) column, with

a methanol-acetonitrile (50:50 V/V) mobile phase and a flow rate of 1 mL min<sup>-1</sup>. Detection was carried out at an excitation wavelength of 295 nm and an emission wavelength of 325 nm at room temperature.

### Extraction and assay of enzymes

The peroxidase activity (POD) assay was carried out using the method described by Chance and Maehly [13] and total protein was assayed according to Bradford *et al.*, (1976) [15]. Briefly, 250 μL potassium phosphate buffer (100 mM, pH=7), 250 μL guaiacol (10 mM), 250 μL H<sub>2</sub>O<sub>2</sub> (70 mM) and 250 μL distilled water was mixed with 20 μL enzyme extract. POD activity was measured as the change in absorbance (420 nm) over 3 min. Finally the results reported base on ΔOD mg<sup>-1</sup> protein min<sup>-1</sup>.

Ascorbate peroxidase activity (APX) was determined by estimating the rate of ascorbate oxidation as reported by Nakano and Asada [16] using a reaction mixture (1 mL) containing 850 μL ascorbate (0.5 mM) in potassium phosphate buffer (100 mM) and 150 μL hydrogen peroxide (2 mM). The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm. Then, 30 μL enzyme extractions were added to reaction mixture. Decrease in absorbance at 290 nm was measured at 25 °C for 3 min

### Statistical analyses

The experimental design was Completely Random Design (CRD) and all analytical values represented the means of three biological replications. Significance was determined by analysis of variance (ANOVA) using SAS software (Version 6.2) and the means were compared by Fisher's Least Significant (LSD) test (α ≤ 0.05).

## Results

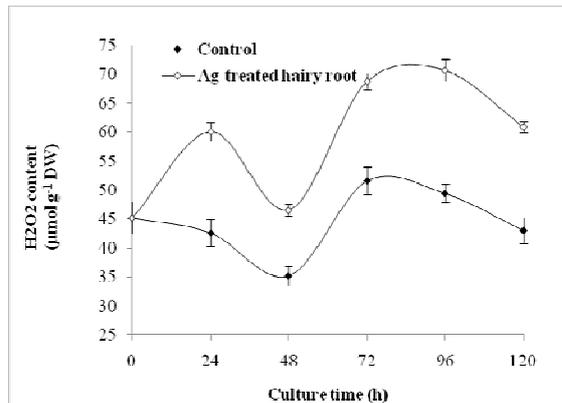
### H<sub>2</sub>O<sub>2</sub> content

The results showed that H<sub>2</sub>O<sub>2</sub> content increased 24 h after Ag<sup>+</sup> elicitation in hairy roots of *S. marianum* (60.05±0.90 μmol g<sup>-1</sup> FW), presenting up to 140.84% enhancement compared to non-treated hairy roots (Fig. 1 and Table 1). The highest content of H<sub>2</sub>O<sub>2</sub> was founded 96 h after elicitation in treated hairy roots with Ag<sup>2+</sup> (70.69±1.06 μmol g<sup>-1</sup> FW), also the content of H<sub>2</sub>O<sub>2</sub> in non-treated hairy roots was 49.457±0.91 μmol g<sup>-1</sup> FW at the same

time. The lowest content of  $H_2O_2$  was founded 48 h after elicitation ( $46.569 \pm 0.56 \mu\text{mol g}^{-1}\text{FW}$ ).

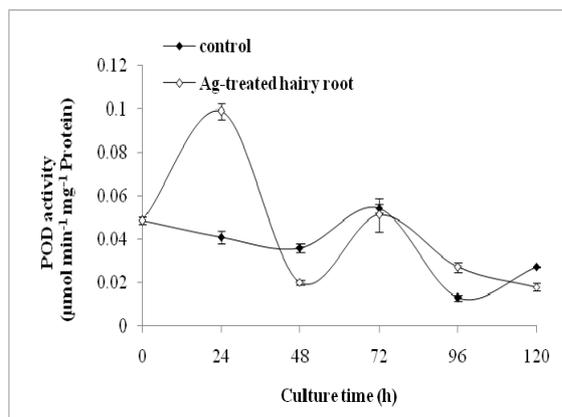
#### POD activity

The maximum activity of POD was obtained 24 h after elicitation, exhibiting approximately 2.41 fold enhanced activity compared to non-treated roots ( $0.0988 \pm 0.002$  and  $0.0410 \pm 0.002 \mu\text{mol min}^{-1} \text{mg}^{-1}$  Protein respectively) ( $P < 0.01$ ).



**Fig. 1** Time-course of  $H_2O_2$  content in *S. marianum* hairy root cultures treated with  $Ag^+$  (2 mM). The control received only MS medium. Data show means  $\pm$  SE from triplicate experiments.

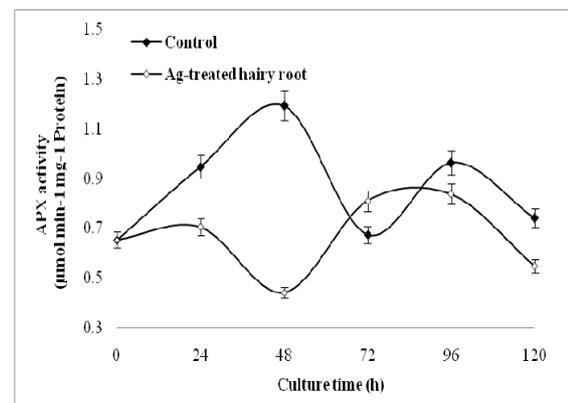
POD activity encountered an intensive decrease in 24 to 48 h after elicitation in such a way that in hour 48 ( $0.020 \pm 0.000 \mu\text{mol min}^{-1} \text{mg}^{-1}$  Protein) it was lower than the POD activity in non-treated roots ( $0.036 \pm 0.001 \mu\text{mol min}^{-1} \text{mg}^{-1}$  Protein)., the While it was always equal to or less than that in non-treated roots, POD activity was increased slightly in treated hairy roots between the 48 to 72 h. There was no significant difference at hour 72 between the POD activities of treated and non-treated hairy roots (Fig. 2 and Table 1).



**Fig. 2** Time-course of POD activity in *S. marianum* treated (with  $Ag^+$  (2 mM)) and non-treated hairy root. Data show means  $\pm$  SE from triplicate experiments.

#### APX activity

APX activity was negatively impacted by  $Ag^+$  elicitation within the first 48 hours in such a way that it was always beneath the APX activity in non-treated roots. The maximum difference in APX activities between the two groups was observed at hour 48, exhibiting the lowest APX activity in  $Ag^+$  treated hairy roots and the highest activity in non-treated hairy roots. After this period APX activity was reversely changed. It was decreased in non-treated samples and increased in treated samples so that at hour 72 the  $Ag^+$  elicited roots surpassed the non-treated root. This superiority was conserved for more than 24 hours. After 96 hours the APX activity was gradually decreased in both treated and non-treated hairy roots (Fig. 3 and Table 1).



**Fig. 3** Time-course of APX activity in *S. marianum* treated (with  $Ag^+$  (2 mM)) and non-treated hairy root. Data show means  $\pm$  SE from triplicate experiments.

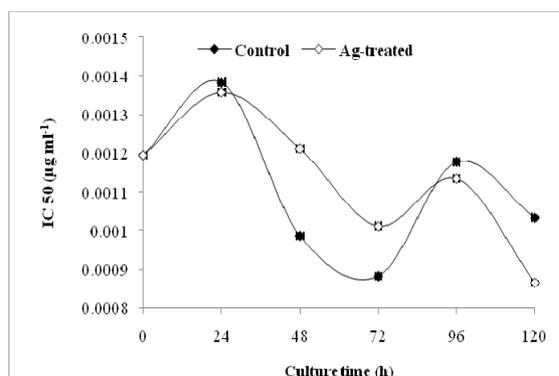
#### DPPH radical scavenging activity

The trend of  $IC_{50}$  variations in treated and non-treated hairy roots was generally similar; an increase in the first 24 hours, a gradual decrease within 24-72 h, an increase within 72-96h and finally a continuously decrease in later hours (Fig 4 and Table 1). The highest  $IC_{50}$  for both treated and non-treated hairy roots ( $0.0010$  and  $0.0013 \mu\text{g/mL}$ , respectively), was observed 24h after elicitation. There were no significant differences between these two groups, indicating that  $Ag^+$  did not influence the  $IC_{50}$  until 24 h after elicitation (Fig. 4).  $IC_{50}$  encountered a significant decrease within 24- 48 h after elicitation. As we know, a lower  $IC_{50}$  value indicates enhanced antioxidant activity.

#### Total tocopherol content

The content of total tocopherol after  $Ag^+$  addition is shown if Fig. 1. Total tocopherol content decreased 24 and 48 h after elicitation. There were no

significant differences between total tocopherol content of the control and Ag<sup>+</sup> treated hairy roots in 72, 96 and 120 h of elicitation (Fig. 5 and Table 1).



**Fig. 4** Time-course of antioxidant activity in *S. marianum* treated (with Ag<sup>+</sup> (2 mM)) and non-treated hairy root. Data show means ± SE from triplicate experiments.

## Discussion

In our previous work we showed that maximum accumulation of SLM was obtained 96 h after elicitation with 2 mM Ag<sup>+</sup> [9]. The mechanism by which secondary metabolites are produced in hairy roots has not been fully understood. Elicitors induce the accumulation of H<sub>2</sub>O<sub>2</sub> and further activation of antioxidant systems [2, 17 and 18] we reported that hydrogen peroxide from the oxidative burst is not involved in the induction of taxol biosynthesis in *Taxus chinensis* cells. Babar *et al.*, (2005) indicated that suspension culture roots of *Panax ginseng* could grow and protect themselves from O<sub>2</sub> stress by coordinated induction of various antioxidant enzymes and metabolite contents [19].

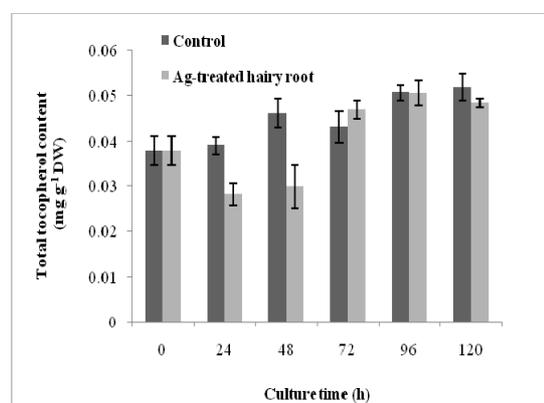
These results suggested that O<sub>2</sub> supplementation is useful for ginsenosid accumulation. Yuan *et al.*, (2002) proposed a non-linear cascade model to describe the signal transduction pathway in

**Table 1** ANOVA table of factorial completely randomized design of H<sub>2</sub>O<sub>2</sub> content, POD and APX activity, antioxidant activity and total tocopherols content treatment with 2 mM Ag<sup>+</sup> at different times (0, 24, 48, 72, 96 and 120 h) in hairy root cultures of *S. marianum*.

Variation Source	df	Mean Square				
		H <sub>2</sub> O <sub>2</sub> content	POD activity	APX activity	Antioxidant activity	Total tocopherols content
Time	5	360.998***	.002***	2.019***	2.019***	2.019***
Treatment	1	1804.621***	.000***	8.922***	8.922***	8.922***
Time * Treatment	5	87.216***	.001***	1.477***	1.477***	1.477***
Error	24	3.880	8.504×10 <sup>-6</sup>	.003	.003	.003

(ns: non-significant, \*: significant p<0.05, \*\*: significant p<0.01, \*\*\*: significant p<0.001)

suspension cultures of *Taxus chinensis* varmairei induced by oligosaccharide from *fusarium oxysporum* by taking the intensity of oxidative burst as the signal characteristic [20].



**Fig. 5** Time course of vitamin E [total tocopherols] production in *S. marianum* hairy root cultures treated with Ag<sup>+</sup> (2 mM). The control received only MS medium. Data show means ± SE from triplicate experiments

Our result showed a straightforward relationship between H<sub>2</sub>O<sub>2</sub> and SLM contents; the more H<sub>2</sub>O<sub>2</sub> content the more SLM production).

Our results also showed that the enzymatic antioxidant system was effectively activated and activity of POD was correlated with higher H<sub>2</sub>O<sub>2</sub> content. The activity of POD declined in hairy roots of *S. marianum* after 24 h as H<sub>2</sub>O<sub>2</sub> content decreased. According to the report of Chen colleagues (2007) Ag<sup>+</sup> addition increased H<sub>2</sub>O<sub>2</sub> content and APX activity significantly which led to higher echinacoside and acteoside contents in cell suspension culture of *Cistanche deserticola* [6]. As shown in their results, Ag<sup>+</sup> addition at an earlier time led to less biomass for Ag<sup>+</sup> enhanced H<sub>2</sub>O<sub>2</sub> accumulation and decreased cell viability significantly.

This also corresponds to our earlier observation, which showed that dry weight (DW) of Ag<sup>+</sup> treated hairy roots was decreased [6]. Also DW in non-treated hairy roots was higher than the treated hairy roots. Increased activation of POX has been described by Xu *et al.*, (2007) in Ag<sup>+</sup> treated cell suspension culture of *Saussurea medusa* [2]. When hairy roots were exposed to Ag<sup>+</sup> treatment, APX activity was lower than the control [2]. We did not observe an adequate increase in APX activity. Yang *et al.*, (2014) show that exogenous Sodium nitroprusside increase CAT, POD and APX activity *N.tangutorum* Bobr. Calli [20]. Gharechahi colleagues (2013) showed that MJA elicitation in hairy root culture of *S. marianum* caused accumulation of H<sub>2</sub>O<sub>2</sub> content and enhanced the activity of POD and APX [21]. These finding were unexpected and these results were different from the results of a number of previous studies which reported [2, 22] that APX activity and H<sub>2</sub>O<sub>2</sub> content increased rapidly after Ag<sup>+</sup> elicitation in cell suspension culture of *C. deserticola* and kept a constant level and decreased after that. In our previous published reports, we found the effect of SA (6 mg/50 mL culture) on SLM and antioxidant metabolites. The high content of SLM was obtained 24 h after elicitation. Maximum activity of POD was observed after 24 and 96 h. The activity level of POX in the control remained lower than those of the SA treated cultures. APX activity dramatically increased after 72 h in SA-treated hairy root cultures of *S. marianum* [5]. It can thus be suggested that production of ROS is through the action of NADH dependent POD. That is associated with external surface of plasma membrane [23]. However, further research should be done on this topic. Tocopherols are lipophilic antioxidants that are synthesized by plant cells. Tocopherol levels increase plant cell tissues in response to a variety of stresses. Tocopherols scavenge and quench various ROS and lipid peroxidation products, stabilize membranes and modulate signal transduction [24]. When Ag<sup>+</sup> was applied to the *S. marianum* hairy root cultures, total tocopherol content decreased after 24 and 48 h and total tocopherol content was similar to that of control experiments after 72, 96 and 120 h treatment. There was a clear trend of increasing the tocopherol content with increasing the time culture. The decrease in tocopherol content seems to be related to consumption of this metabolite. The DPPH is a stable radical that can be easily used for detection of antioxidant properties. Because of its rapidity and selectivity, DPPH has

been widely used in recent years for the assessment of different antioxidant [24]. Our results show that IC<sub>50</sub> decreased upon treatment, especially 48 and 72 h after treatment, while its overall trend of changes was very similar to those observed in the control treatments.

## Conclusions

Increase in SLM content following Ag<sup>+</sup> elicitation indicates that Ag<sup>+</sup> acts as a powerful inducing signal. The effectiveness of Ag<sup>+</sup> elicitation depend on time elicitation and timing of the hairy root harvesting is critical. However, there is still a lack of information about the priming of *S. marianum* hairy roots by biotic or abiotic elicitors to explain SLM production induced by elicitors. Clearly more works are needed to identify cellular target of ROS and signal transduction in Ag<sup>+</sup> treated hairy root culture of *S. marianum*.

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