Plant Omics Journal POJ

POJ 3(4):109-114 (2010) ISSN:1836-3644

Ag^+ enhanced silymarin production in hairy root cultures of *Silybum marianum* (L.) Gaertn

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Abstract

Seeds of *Silybum marianum* which have been used to treat liver diseases have an active component called silymarin. Hairy root cultures of *S. marianum* could be able to produce silymarin. There is evidence that the use of elicitors has been an important strategy for improving the production of secondary metabolites. Elicitation is an interesting topic to investigate cell signaling pathway in secondary metabolite biosynthesis. Lipoxiganase is an important enzyme in octadecanoid pathway related to the biosynthesis of jasmonic acid. The relationship between elicitors and jasmonic acid signaling pathways has been shown in previous researches. In the present study, we examined silymarin accumulation, linoleic acid content and lopoxigenas activity in hairy roots of *S. marianum* induced through the use of an abiotic elicitor, Ag^+. Hairy root cultures of *S. marianum* were fed by different concentration of Ag^+ (0.2, 0.4, 0.8, 1 and 2 mM) and harvested 72h after elicitation. Detection and identification of flavonolignans was carried out by high performance liquid chromatograph method. The highest content of silymarin was 0.56 mg g^{-1} DW that was obtained with 2 mM of Ag^+ which was 2 times of non-treated hairy roots. In the next stage, hairy roots were treated with 2 mM Ag^+ for different times (0, 24, 48, 72, 96 and 120h). The highest silymarin production reached to 1.2 mg g^{-1} DW, 96h after elicitation. The content of silybin, isosilybin, taxifolin, silycristin and silydianin were 0.069, 0.031, 0.688, 0.388 and 0.024 mg g^{-1} DW, respectively. The maximum lipoxigenase activity was obtained 72h after elicitation that was 4.33 times of non-treated hairy roots. Linoleic acid content was 19.9 mg g^{-1} DW, 96h after elicitation. Dry weight of treated hairy roots decreased, as compared to non-treated hairy roots. It is feasible that elicitation with Ag^+ changes lipoxigenase activity which mediates signal transduction pathway for production of silymarin.

Keywords: Silymarin; Lipoxigenase; Linoleic acid; Hairy root; Elicitor

Abbreviations: SLM_Silymarin; SB_silybin; ISB_isosilybin; SD_silydianin; SC_silycristin; TXF_Taxifolin; MJA_ Methyl jasmonate; SA_Salicylic acid; ET_Ethylene; MS_Murashige and Skoog; DW_Dry weights; HPLC_High-Performance Liquid Chromatograph; PVP_polyvinylpyrrolidone; DTT_dichlorodiphenyltrichloroethane; LOX_ Lipoxygenase; FAME_For total Fatty acid methyl esters ; GC_Gas chromatography ; JA_Jasmonic acid; LA_18:2– Linoleic acid; ALA_18:3–linolenic acid; AA_20: 4–arachidonic acid

Introduction

Cell and tissue culture of *S. marianum* production was silymarin (Hasanloo et al, 2009; Rahnama et al, 2008; Rahimi Ashtiani et al, 2010; Valenzuela et al, 1986). Silymarin extracted from the dried fruits of the *S. marianum* (Family: composite) widely used for therapy of liver disease and is composed of silybin (SB), isosilybin (ISB), silydianin (SD), silycristin (SC) and Txifolin (TXF). (Morazzoni and Bombardelli, 1995; Kim et al, 2003; Lee and Liu, 2003). The exploitation of transformed root cultures (hairy roots) presents a relatively novel approach to *in vitro* plant biotechnology that has received increasing attention in recent years. Hairy roots derive from the infection of wounded plant tissue by the soil bacterium, *Agrobacterium rhizogenes* (Ge and Wu, 2005; Yan et al, 2006). The hairy root cultures have several advantages, including their relatively fast growth rates, genetic stability and capacity for synthesis of metabolites (Shanks and Morgan, 1999; Sevon and Oksman-Caldentey, 2002). Recent works have focused on the fallowing method for production of secondary metabolites such as: SLM from *silybum marianum* (Rahnama et al, 2008), tanshinone from *salvia miltiorrhiza* (Ge and Wu, 2005) and valepotriates from *valerianella locusta* hairy root cultures (Kittipongpatana and Reddy, 2002). Elicitation is the induction of secondary metabolite production by biotic [methyl jasmonate (MJA), yeast extract and salicylic acid (SA)] and abiotic (Ag^+, ethylene (ET)) treatments in plant cell and hairy root cultures. Several type of secondary metabolites have been elevated by elicitation, such as terpenoids (Sudha and Ravishankar, 2002 and Bostock et al, 1982), coumarin derivatives (Conrath et al, 1989), alkaloids (Tyler et al, 1989), and flavonoids (Khalili et al, 2009). As recently published (Rahimi Ashtiani et al, 2009), treatment of *S. marianum* cell cultures with Ag^+ (0.8 mM) improved production of silymarin (56 µg g^{-1} DW) 24h after treatment which was 30-fold higher than the control; however, these results did not show the relationship between silymarin accumulation and signaling pathways in elicited cells. Also, up to now, there have been no reports on the effect of Ag^+ elicita-

Table 1. Flavonolignan and SLM content (mg g^{-1} DW) in Ag^{+}-treated (0.2, 0.4, 0.8, 1 and 2 mM) and non treated (control) hairy root cultures of *S. marianum* 72h after elicitation. The flavonolignans and SLM were analyzed with HPLC. Data show means ± SD from triplicate experiments.

Concentration of Ag^{+} (mM)	Flavonolignan (mg g^{-1} DW)					SLM (mg g^{-1} DW)
	ISBN	SBN	SDN	SCN	TXF	
Control	0.015 ± 0.000	0.031 ± 0.000	0.012 ± 0.000	0.046 ± 0.013	0.175 ± 0.009	0.281 ± 0.005
0.2	0.026 ± 0.002	0.040 ± 0.004	0.017 ± 0.001	0.085 ± 0.003	0.295 ± 0.006	0.465 ± 0.003
0.4	0.014 ± 0.000	0.027 ± 0.007	0.013 ± 0.002	0.117 ± 0.019	0.295 ± 0.019	0.466 ± 0.008
0.8	0.045 ± 0.004	0.036 ± 0.007	0.011 ± 0.001	0.102 ± 0.009	0.229 ± 0.015	0.425 ± 0.007
1	0.018 ± 0.000	0.023 ± 0.003	0.009 ± 0.001	0.053 ± 0.005	0.145 ± 0.012	0.249 ± 0.004
2	0.035 ± 0.006	0.048 ± 0.005	0.016 ± 0.001	0.112 ± 0.008	0.350 ± 0.010	0.563 ± 0.007

tion and silymarin production in *S. marianm* hairy root cultures especially the plant cell responses via signaling pathways. Therefore, the present study was designed to evaluate the role of Ag+ elicitation in *S. marianum* hairy root cultures and observe hairy roots growth and silymarn production. The signaling pathway with linoleic acid and lipoxigenase activity as major parts of jasmonic acid biosynthesis were investigated. Examination of these metabolites may allow introducing a link between sets of reactions from elicitation to silymarin biosynthesis.

Materials and methods

Plant material

Hairy root cultures derived from transformed cotyledonary leaf explants of *S. marianum* with *Agrobacteriumm rhizogenase* (AR15834) (Rahnama et al, 2008). Hairy roots cultures were induced by transferring six 1cm roots to 50ml of Murashige and Skoog liquid medium (MS) supplemented with 30 g l^{-1} sucrose in 150ml flaks. (Murashige and Skoog, 1962). All experiment were carried out on orbieal shaker set at 150 rpm and incubated at 25°C in the dark.

Elicitation

$AgNO_3$ was dissolved in water and prepared as concentration stock solutions after autoclaving. Six concentrations (0, 0.2, 0.4, 0.8, 1, and 2 mM) of Ag NO_3 were added to the 28-days old hairy root cultures. Controls received equivalent volumes of MS medium. Dry weight and SLM content of hairy roots were determined after 72h. To study the effects of the duration of exposure, 2 mM Ag^{+} was added to 28- day-old hairy root cultures and the treated and non treated hairy roots were harvested and analyzed, in triplicate for growth and silymarin content, after 0, 24, 48, 72, 96 and 120h of treatment.

Extraction and determination of silymarin

The hairy roots were harvested and dried by tissue paper. Lyophilized powdered hairy root samples were measured in terms of dry weights (DW). The samples were defatted by 10 ml petroleum benzene at 40°C for 8 h. flavonolignans were extracted from the dried residue with 10 ml of methanol at 40 °C for 8 h. The methanolic solution was concentrated to a dry residue. The extract was dissolved in 2 ml of methanol and kept at 4 °C in darkness (Hasanloo et al, 2009). The SLM were quantified by high-performance liquid chromatograph (HPLC) analysis as described by Hasanloo et al, (2005).

Lipoxygenase activity

For the lipoxygenase activity was assayed spectro photometically as described Axelroad et al, (1981), Zhao and Sakai, (2003) and Khalili et al, (2009). The protein concentrations of the samples was determined using the dye binding technique according to Bradford et al, (1976) and the results reported base on ΔOD mg^{-1} protein min^{-1}.

Fatty acid analysis

The total lipids were extracted with petroleum benzene and kept at -20°C until analysis. The total Fatty acid methyl esters (FAME) were prepared according to Yi et al, (2009). 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).

Chemicals

Standards of SLM, SB, and TXF were purchased from Sigma; SC and SD, from Phytolab.

Statistical analyses

All analytical values represent the means of three analytical replications. Significance was determined by analysis of variance (ANOVA) using SAS software (Version 6.2) And difference between the means were compared by Duncan's multi range test ($\acute{\alpha} \leq 0.05$).

Results

Effects of different concentrations of Ag^{+} on growth and silymarin accumulation

Fig.1 shows SLM content and dry weight of *S. marianum* hairy root cultures 72h after elicitation by different concentrations of Ag^{+} (0.2, 0.4, 0.8, 1 and 2

Table 2. Flavonolignan and SLM content (mg g^{-1} DW) in Ag^+ treated (2 mM) and non-treated (control) hairy root cultures of *S. marianum* for exposure of times. The flavonolignans and SLM were analyzed with HPLC. Data show means ± SD from triplicate experiments.

Time(h)		Flavonolignans (mg g^{-1} DW)					SLM (mg g^{-1} DW)
		ISBN	SBN	SDN	SCN	TXF	
0	Control	0.008 ± 0.000	0.059 ± 0.008	0.009 ± 0.000	0.176 ±0.032	0.200 ± 0.007	0.454 ± 0.009
24	Treated	0.029 ± 0.000	0.029 ± 0.000	0.009 ± 0.000	0.123 ± 0.001	0.280 ± 0.014	0.471 ± 0.003
	Control	0.011 ± 0.000	0.027 ± 0.001	0.022 ± 0.001	0.232 ± 0.005	0.453 ± 0.042	0.747 ± 0.010
48	Treated	0.028 ± 0.000	0.035 ± 0.000	0.014 ± 0.000	0.232 ± 0.007	0.338 ± 0.003	0.649 ± 0.002
	Control	0.011 ± 0.000	0.008 ± 0.000	0.030 ± 0.001	0.423 ± 0.070	0.317 ± 0.015	0.790 ± 0.017
72	Treated	0.025 ± 0.000	0.018 ± 0.000	0.016 ± 0.004	0.266 ± 0.012	0.325 ± 0.018	0.652 ± 0.007
	Control	0.003 ± 0.000	0.015 ± 0.000	0.007 ± 0.000	0.206 ± 0.025	0.157 ± 0.012	0.390 ± 0.007
96	Treated	0.031 ± 0.000	0.069 ± 0.002	0.024 ± 0.000	0.388 ± 0.009	0.688 ±0.010	1.200 ± 0.004
	Control	0.003 ± 0.002	0.024 ± 0.000	0.006 ± 0.000	0.164 ± 0.009	0.126 ± 0.007	0.326 ± 0.004
120	Treated	0.029 ± 0.000	0.033 ± 0.000	0.012 ± 0.000	0.314 ± 0.004	0.421 ± 0.005	0.811 ± 0.002
	Control	0.002 ± 0.000	0.018 ± 0.000	0.012 ± 0.000	0.158 ± 0.010	0.100 ± 0.009	0.293 ± 0.004

mM) in comparison to the control. Ag^+ at different concentration provoked the SLM synthesis. Among the concentration tested, the optimal concentration was 2 mM, and SLM production increased from 0.28 mg g^{-1} DW in control to 0.56 mg g^{-1} DW in treated hairy roots. The content of SB, ISB, SC, SD and TXF in these samples was 0.048, 0.035, 0.112, 0.016 and 0.350 mg g^{-1} DW, respectively, while in non-treated hairy roots the corresponding values were 0.031, 0.015, 0.046, 0.012 and 0.175 mg g^{-1} DW, respectively (Table 1). The highest DW was obtained (0.433 g) in non-treated hairy roots after 72h. Based on these results, a concentration of 2 mM was chosen for further experiments.

Effects of Ag^+ and culture time on growth index and silymarin production

Fig. 2 A and B show the dry weight and silymarin content in treated (2 mM Ag^+) and non-treated hairy roots (control) after different times (24, 48, 72, 96 and 120h). DW was decreased in treated hairy roots after 24 and 48 after elicitation (0.25 and 0.25 g respectively); a significant decrease was observed in the DW of cultures after 72h (021 g) and enhanced 96h after elicitation (0.34 g), the DW of cultures was hot significantly changed after 120h (0.35 g). Dry weight in non-treated hairy roots was slightly increased in time periods of 24, 48, 72, 96 and 120h which were 0.34, 0.37, 0.35, 0.4 and .042 g, respectively. Also, DW in non-treated hairy roots was higher than the treated hairy roots. Time course for the induction of silymarin in culture treated with 2 mM Ag^+ is presented in fig. 2 B. SLM content increased upon stimulation by Ag^+ after 24h and reach to 0.649 mg g^{-1} DW. No market charge was observed between 48 and 72h. Accumulation of SLM was favorably enhanced after 96h (1.2 mg g^{-1} DW) in an extremely high level which was higher than the control (0.32 mg g^{-1} DW). There was a gradual decline in SLM content from 96h to 120h (0.81 mg g^{-1} DW) but was higher than the control (0.29 mg g^{-1} DW). Table 2 presents data on the flavonolignans analysis in Ag^+ treated hairy roots. The highest content of TXF, SB and ISB were 0.68, 0.069 and 0.03 mg g^{-1}DW respectively, those were obtained 96h after

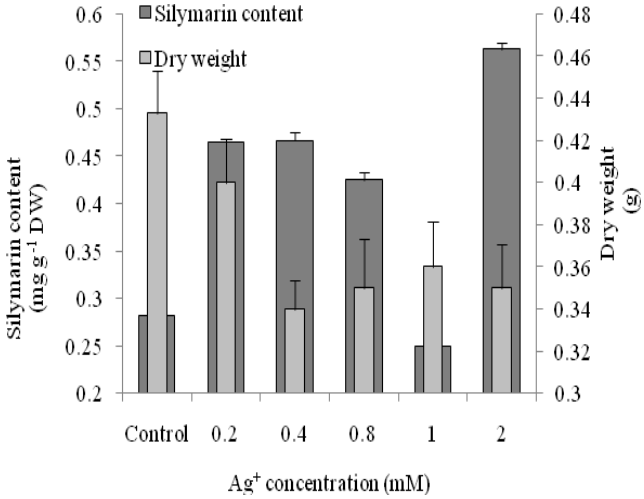

Fig 1. Effects of Ag^+ concentrations (0, 0.2, 0.4, 0.8, 1 and 2 mM) on biomass and SLM production in hairy root cultures of *S. marianum* 72h after elicitation. The flavonolignans were analyzed with HPLC. Values are means of triplicate results and error bars show standard deviations.

elicitation and that were 5.41, 2.81 and 10.23-fold of control respectively. The content of SC and SD in non-treated hairy roots reaching 48h after elicitation (0.42 and 0.30 mg g^{-1}DW respectively) and higher than of the treated hairy roots (0.23 and 0.01 mg g^{-1}DW) (Table 2).

Effect of 2 mM Ag^+ on lipoxigenase and linoleic acid

To determine how signaling leading to the accumulation of SLM, the activity of LOX and linoleic acid content in treated and non-treated hairy root cultures of *S. marianum* were assayed. The results of our observations are shown in fig 3. The activity of LOX was significantly increased after elicitation with Ag^+ to compare control. LOX activity was activities and reached on high level (0.79 ΔOD_{234} mg $^{-1}$protein min^{-1}) after 72h of treatment that was 4.33-fold that of non-treated hairy roots (0.18 ΔOD_{234} mg $^{-1}$protein min^{-1}). A reduction in LOX action was observed between 72 and 96h. The LOX activity was not significantly

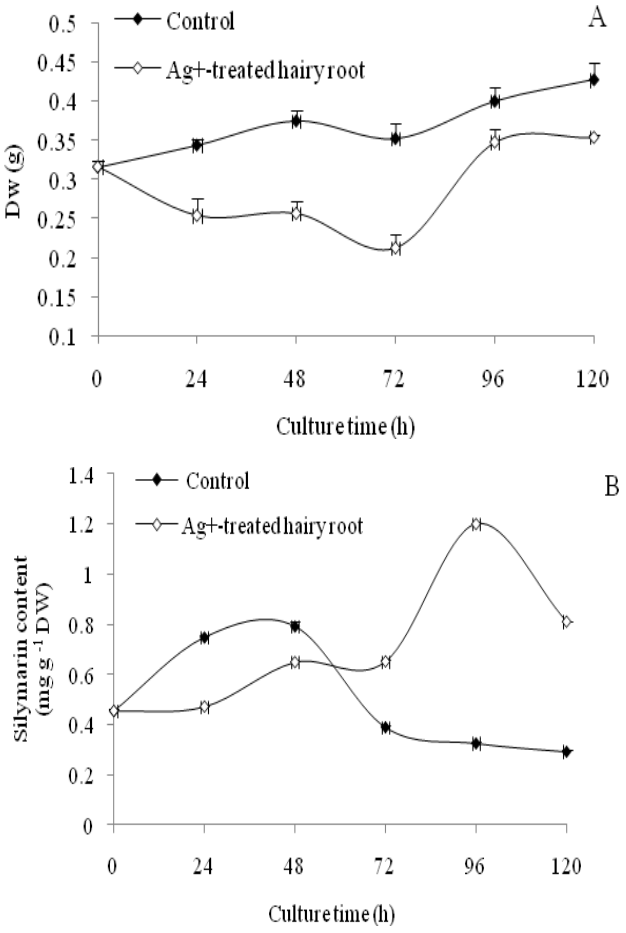

Fig 2. Time course of silymarin production (A) and biomass (B) in non-elicited (Control) and elicited (2 mM Ag^+) hairy roots of *S. marianum*. The flavonolignans were analysed with HPLC. Values are means of triplicate results and error bars show standard deviations.

changed from 96 to 120h being higher than the non treated hairy roots. The LOX activity was lower than the treated hairy roots in non treated hairy roots was no significantly changes during the incubation period (fig 3). Fig. 4 illustrates the changes of linoleic acid content in treated and non-treated hairy roots in different time after elicitation by 2 mM Ag^+. As an overall trend, it is quite obvious that the content of linoleic acid dramatically rose, hitting a peak (19.9 mg g^{-1} DW) after 96h that was 1.96- times of the control (10.12 mg g^{-1} DW). There was a decline in linoleic acid content from 96 to 120h n treated hairy roots (17.53 mg g^{-1} DW). As mentioned above, the linoleic acid content in non-treated hairy roots reaching a peak after 48h (13.89 mg g^{-1} DW) but then was on the declin from 48 to 120h dropping to 8.15 mg g^{-1} DW. Linoleic acid content in treated hairy roots was increased dramatically after elicitation. Linoleic acid content 24, 48, 72, 96 and 120h after elicitation were 12.99, 15.05, 16.37, 19.90 and 17.53 mg g^{-1} DW respectively. The highest content of linoleic acid was obtained 96h after elicitation (19.90 mg g^{-1} DW) that was 1.96-fold of non-treated hairy roots (10.12 mg g^{-1} DW).

Discussion

Our experimental results have shown that Ag^+ was an effective elicitor for the production of SLM and related flavonolignans compounds in the *S. marianum* hairy root cultures. The stimulation of SLM accumulation by biotic and abiotic elicitors such as yeast extract, SA and MJ has also been observed in the cell and hairy root cultures by Snchez-Sampedro et al, (2005) and Khalili et al, (2009). The data indicated that the optimal concentration of Ag^+ was 2 mM (Fig. 1) and the best time was 96h after elicitation (Fig. 2 B) corresponding to SLM production. The growth of hairy root cultures was inhabited by Ag^+. According our results, Ag^+ treated hairy roots grew more slowly than the non-treated hairy roots. The results of our observations are shown in fig. 2 A. DW in non-treated hairy roots was 120% higher than Ag^+ treated hairy roots (Fig. 2 A). Ag^+ stimulated the key enzyme in JA signaling pathways (Fig. 3). The high activity of LOX was 72h after elicitation that was 433% higher than the non-treated hairy roots. Also linoleic acid content was enhanced by Ag^+. The maximum content of linoleic acid in treated hairy roots was of 196% higher than non-treated hairy roots (Fig. 4). The abiotic elicitors signal transduction pathway has been studied and those results showed that the abiotic elicitors signals may be initiated by receptors on the plant plasma membrane (Savitha et al, 2006). An elicitors signal is perceived by as receptor on the surface of plasma membrane and initiates a signal transduction network and it regulates the expression of biosynthesis genes involved in plant secondary metabolism and further produces key enzymes which catalyse the biosynthesis of target secondary metabolites (Zhao and Davis, 2005)

Ag^+ has been introducing as a inhibitor of the Ethylene signal transduction pathway (Zhao and Davis, 2005 and Pan and Zhong, 2000). Ethylene is a phytohormone that regulates a wide range of plant processes, from growth and development to defense responses. Ethylene production can be inducing by various stresses, such as wounding, ozone, microbial pathogen and insect attack, as well as small molecule elicitors. While both JA and ethylene signaling pathways are essential for plant defense responses against those stresses, ethylene is not a common signal for induction of plant secondary metabolism. There are only a limited number of instances where accumulation of plant secondary metabolites can be affected by ethylene, and the effects can be either positive or negative (Zhao and Davis, 2005 and Pan and Zhong, 2000). In *Salvia miltiorrhiza* hairy root culture, treatment with Ag^+, an ethylene action inhibitor, induces production of three diterpenoid tanshinones (Zhang et al, 2004). Similarly, Ag^+ significantly increases scopolamine release and accumulation of scopolamine and hyoscyamine in the roots (Pitta-Alvarez et al, 2000). This implies that ethylene acts as an inhibitor of accumulation of the above substances. Clearly, ethylene interacts with JA or elicitor signaling to modulate the production of secondary metabolites

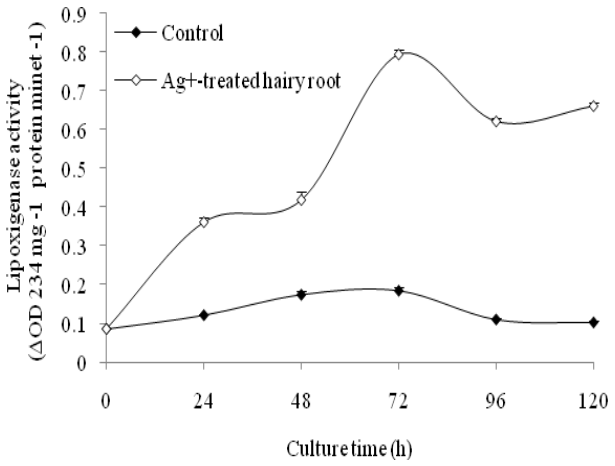

Fig 3. Time-course of lipoxygenase activity in *S. marianum* hairy root cultures treated with Ag^+ (2 mM). The control received only MS medium. Data show means ± SD from triplicate experiments.

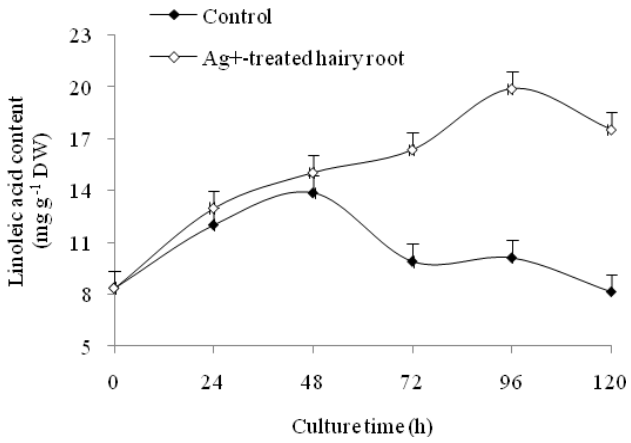

Fig 4. Time-course of linoleic acid content in *S. marianum* hairy root cultures treated with Ag^+ (2 mM). The control received only MS medium. Data show means ± SD from triplicate experiments.

induced by JA or elicitor. The role of jasmonic acid and its related compounds in plant signal transduction (Farmer et al, 2003). Jasmonates induce gene expression in response to wounding, patoden attack and formation of secondary metabolites. Our results support the hypothesis that jasminate signal pathway by activation of lipoxygenase activity was involved in Ag^+ induced production of silymarin. Lipoxygenase catalyzes the addition of molecular oxygen to polyunsaturated fatty acids (PUFAs), which present a cis, cis-1, 4-pentadiene site, as in linoleic acid (LA-18 : 2), _-linolenic acid (ALA-18 : 3) and arachidonic acid (AA-20 : 4) (Gardner, 1991; Siedow, 1991). Similar induction of LOX activity was recorded in tobacco leaves treated with cryptogein, a protein of the fungus *Phytophthora cryptogea* (Rusterucci et al, 1999). In potato tubers also, LOX activity increased upto 4 weeks following fungal infection with *Phoma exigua* (Galliard, 1978). Similar shifts towards ALA products were reported in the tobacco leaves treated with a fungal protein (Rusterucci et al, 1999). This shift is probably aimed towards the formation of traumatin and jasmonic acids, which are formed only from ALA. This may be possible with the specific release of ALA from membrane phospholipids by octadecanoid signal transduction pathway in plants in response to pathogen attack (Farmer and Ryan, 1992; Creelman and Mullet, 1995). The effectives of Ag^+ elicitation depend on elicitor concentrations and time of treatment. The timing of the hairy root harvesting is critical. It could a result of switching from primary metabolism to secondary metabolisms. Besides these consideration, the positive effect of $AgNO_3$ on production and, specifically on SLM accumulation, lipoxygenase activity and linoleic acid content, would make this treatment an interesting candidate for improving productivity in large scale processes

Acknowledgments

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

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