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Original article

Chitosan (middle-viscous) as an effective elicitor for silymarin production in *Silybum marianum* hairy root cultures

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Abstract

Elicitation with middle-viscous chitosan (30 mg/50 mL) significantly stimulated silymarin synthesis in *Silybum marianum* hairy root cultures. The root cultures established by infection with *Agrobacterium rhizogenes* AR15834 showed a potential for production of silymarin. Elicitation with medium molecular weight of chitosan (0, 5, 10, 20, and 30 mg/50 mL) was used in order to improve silymarin production. Total silymarin increased about 5.26-fold after 96 h of treatment with 30 mg/50 mL chitosan. Dry weight of the hairy roots reached the highest point (0.530 and 0.535 g) after 96 h in presence of 20 and 30 mg/50 mL chitosan, respectively. Five different flavonolignans were isolated; taxifolin, silychristin, silydianin, silybin and isosilybin (0.133, 0.200, 0.120, 0.041 and 0.056 mg/g dry weight, respectively). 30 days old hairy roots were treated by 30 mg/50 mL chitosan in different times (12, 24, 48, 72, 96 and 120 h). The amount of silymarin accumulation significantly increased (0.705 mg/gDW) in hairy roots after 96 h treatment. These observations suggested that the medium molecular weight of chitosan could be elicited by *S. marianum* hairy root cultures that lead to the higher production of silymarin. These results correlated with the culture time, and the biosynthesis which reached to a maximum of 0.705 mg/gDW by 96 h after culture (2.9-fold higher than the control).

Keywords: biotechnology, flavonolignans, *in vitro*, silymarin, *Silybum marianum*

Introduction

Milk thistle (*Silybum marianum*, Asteraceae) is a flowering medicinal plant utilized for its potentially protective effects on the liver disorders including chronic hepatitis and liver cirrhosis. Silymarin, a mixture of flavolignans is isolated from the dried fruits of the plant [1, 2]. The main active principles in milk thistle are silybin, isosilybin, silychrisitn and silydianin, commonly referred to collectively as silymarin. They are phenolic compounds known as flavonolignans, which have antioxidant, anti-inflammatory, and free radical scavenging properties [3].

Plants are the main sources for important secondary metabolites. They have been used as drugs, flavorings and fragrances, pigments, pesticides, and food additives. Plant cell and tissue culture technologies can be established for production and extraction of secondary metabolites. *In vitro* production of secondary metabolites in plant cell suspensions and hairy root cultures has been reported from various medicinal plants [4]. It is well known that elicitation is a common strategy for increasing the yield of secondary metabolites in *in vitro* plant cell cultures [5].

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For this purpose, fungal homogenates of different species have been recommended. The results depend on the fungal species employed and also on the homogenate preparation [6-8]. In order to obtain better results, using of compounds defined as a structural component such as chitosan is consequent [9]. Chitosan (polycationic polymer of β-1-4-linked D-glucosamine) is the major component of exoskeletons of insects and crustacea and can be found in the cell wall of many fungi. Improved production of different metabolites has been shown in chitosan elicited plant cell cultures [10-12].

We have recently reported the production of silymarin in hairy root cultures of *S. marianum* [13]. Hairy root is a plant disease caused by *Agrobacterium rhizogenes*, a gram-negative soil bacterium. Hairy roots grow rapidly, and are highly branched in culture media. The transformed roots are highly differentiated and stable [5].

To the best of our knowledge, no previous study has investigated the effects of chitosan on enhancement of silymarin productivity; therefore, the aim of the present study was to evaluate the effect of chitosan on silymarin production and flavonolignans profile in hairy root cultures of *S. marianum*.

Experimental

Hairy root culture

Hairy root culture of *S. marianum* was transformed by *A. rhizogenes* (AR15834), and the genetic transformation of these hairy roots was confirmed by polymerase chain reaction (PCR) according to the method described by Rahnama *et al.* [13]. PCR was performed for 35 thermal cycles (denaturation at 94 °C for 1 min, primer annealing at 53 °C for 1 min, and primer extension at 72 °C for 1 min) for rolB (forward primer 5´-ATGGATCCCAAATTGCTATTCCCCACG A-3' and reverse primer 5'-TTAGGCTTCTTTCATTCGGTTTACTGCA GC-3'). Hairy root cultures were induced by transferring six 1 cm roots to 50 mL of Murashige and Skoog liquid medium (MS) supplemented with 30 g / L sucrose in 150 mL flasks [14]. All the experiments were carried out on orbital shaker set at 150 rpm and incubated at 25 ^{o}C in the dark.

Preparation of the elicitor

Chitosan (middle-viscous) (28191, Sigma Chemical Co., Japan) was prepared according to Popp *et al.*, (1997) [15]. Chitosan was dissolved in 5% (v/v) 1N HCl through gentle heating and continuous stirring. pH of the solution was adjusted to 5 with 1N NaOH and the final concentration adjusted to 1 mg/mL by MS liquid medium. The homogenates were autoclaved for 15 min at 121 °C prior to use and used as elicitor at different concentrations (0, 10, 20 and 30 mg/50 mL culture media). The elicitors were added to 30 days-old hairy root cultures. For a time course study, untreated and elicited hairy roots were harvested at different time intervals (0, 12, 24, 48, 72, 96 and 120 h) and then frozen immediately at -80 ºC for the next biochemical assays. Biomass was quantified by dry weight.

Analytical procedures

Silymarin was quantified by high performance liquid chromatography (HPLC) analysis as described by Hasanloo *et al.* (2013) on a Knauer liquid chromatography equipped with an injector with a 20 µl loop, a Nucleosil C_{18} 5 µm (250×4.6 mm) column, K2600A UV detector and Chromgate software for peak integration [16]. Hairy roots were harvested from the shake-flasks and dried by tissue paper. Lyophilized powdered hairy root samples were measured in terms of DW. The samples were defatted with petroleum ether. The flavonolignans were extracted from the dried residue with 10 mL of methanol at 40 ºC for 8 h. The methanol solution was concentrated to a dry residue. The extract was dissolved in 2 mL of methanol and kept at 4 ºC in darkness.

Statistical analysis

The data were given as the mean of at least three replicates. Statistical analysis was performed with SAS software (Version 6.2) using ANOVA method with Duncan test set at $\acute{a} \leq 0.05$.

Results and Discussion

Effects of different concentrations of chitosan

Hairy root cultures (30 days old) were treated with four different concentrations (0, 10, 20 and 30 mg/50 mL culture) of chitosan. Dry

Table 1. Flavonolignan content (mg/gDW) in chitosan (middle-viscous) treated and non-treated hairy root cultures of *S. marianum* 96 h after elicitation.

	Concentration of chitosan (mg/50 mL)	Flavonolignan (mg/gDW)				
		Taxifolin	Silychristin	Silydianin	Isosilybin	Silybin
Control	0	0.034±0.002*	0.059±0.01	0.052±0.01	0.009±0.009	0.009±0.001
Treated hairy roots	5	0.063±0.007	0.108±0.007	0.099±0.01	0.015±0.008	0.015 ±0.005
	10	0.11±0.003	0.158±0.02	0.16±0.01	0.033±0.002	0.037±0.002
	20	0.049±0.01	0.094±0.04	0.112±0.02	0.017±0.007	0.04±0.02
	30	0.133±0.01	0.200±0.03	0.12±0.01	0.056±0.001	0.041±0.002

* Data are means±SD from triplicate experiments

weight of chitosan treated hairy roots was stimulated and chitosan had a positive effect on the biomass after 96 h. It is quite obvious that the content of dry weight dramatically rose, hitting a peak in hairy root treated with10 mg chitosan that was 3.2-times that of the control (0.15 g). There was a gradual increase in dry weight content in treated hairy roots with 20 and 30 mg chitosan (0.530 and 0.535 g, respectively). The chitosan not only increased the growth index but also induced the production of silymarin. The amount of silymarin significantly increased (0.50 mg/gDW) in hairy roots after 96 h treatment at 10 mg/50 mL culture of chitosan (figure 1). There was a gradual decline in silymarin content in treated hairy roots with 20 mg chitosan (0.33 mg/gDW). The highest silymarin content was obtained in 30 g chitosan treated hairy roots after 96 h (0.07 mg/gDW). By HPLC analysis of the methanolic extract of the hairy root culture sample, the presence of silybin and isosilybin were detected. We have shown that the hairy roots produced silybin (0.041 mg /gDW), isosilybin (0.056 mg/gDW), silychristin (0.20 mg/gDW), silydianin (0.12 mg/gDW) and taxifolin (0.13 mg/gDW), which were similar to the compounds produced by the dried fruits of *S. marianum* (table 1). Based on the results, the concentration of 30 mg/50 mL culture of chitosan was chosen for further experiments.

Effects of feeding time on dry weight and silymarin production

Time course for the induction of silymarin and dry weight in *S. marianum* hairy root cultures treated with 30 mg/50 mL culture medium of chitozan are presented in Fig. 2A and B. Chitosan did not stimulate any increase in dry weight of hairy roots between 12 and 24 h.

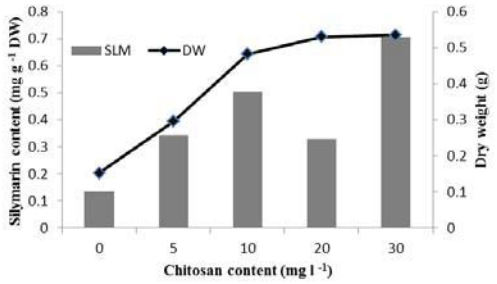

Figure 1. Effect of different concentrations of chitosan (middle-viscous) on silymarin accumulation and dry weight of *S. marianum* hairy root culture.

The dry weight of treated hairy roots significantly decreased after 24 h (0.36 g) and reached to 0.32 g after 48 h but thereafter decreased and the lowest dry weight was observed after 96 h (0.26 g). The dry weight of treated hairy roots significantly enhanced from 0.26 to 0.34 g after 96 h. No change was observed in dry weight in non-treated hairy roots after 24 h. A slight increase was observed in dry weight of non-treated hairy roots from 24 to 120 h. As shown in figure 2A, silymarin slightly increased from 12 to 48 h after elicitation with chitozan and hitting to 0.3 mg/gDW that was 1.38-times that of the control (0.18 mg/gDW). A dramatic increase (after 72 h) was observed after a slight decrease in silymarin content between 48 and 72 h, hitting a pick (0.70 mg/gDW) that was 7-times that of the control (0.15 mg /gDW).

Understanding the molecular basis and characterization of biochemical pathways affected upon elicitation could be considered as an important step in the development of our knowledge for the large-scale production of pharmaceutically valuable secondary metabolites. The mechanism of elicitation is largely unknown. To date various methods have been developed and introduced signal transduction cascades. Different authors have shown that Ca^{2+} plays an important role in the regulation of the signal transduction cascades. Vasconsuelo *et al*. showed that chitosan as an elicitor stimulated anthraquinone (Aq) synthesis in *Rubia tinctorum* L. cultures and involved activation of phospholipase C (PLC), protein kinase C (PKC), phosphoinositide 3-kinase (PI3K) which mediates protein kinase (MAPK) cascades activation [17]. Also, they showed that chitosan increased intracellular Ca^{2+} concentration in a medium devoid of calcium. They demonstrated that mobilization of intracellular Ca^{2+} participates is one of the molecular mechanism of chitosan dependent anthraquinone synthesis in *R. tinctorum* cultures [12].

Jasmonate and its methyl derivative, methyl jasmonate (MeJA), are naturally occurring compounds that mediate several plant physiological processes in response to pathogen attack, wounding, and ozone. It is well known that endogenous JA level increases rapidly and transiently in response to elicitors. It has been reported that chitosan stimulates the accumulation of jasmonic acid, a signal molecule related to defense-gene regulation [18].

Oxidative stresses are known to be an integral part of defense responses initiated by elicitors. The synthesis of many antioxidant enzymes plays a central role in defense response against oxidative stresses imposed by elicitors. Jasmonates trigger the defense responses that resemble those initiated by pathogen infection and also modulate the production of certain secondary metabolites in a variety of plant species [18]. Plant-species, class of secondary metabolite, type of elicitors, elicitor concentrations and time of elicitation in the phase of cell growth cycle are the main reasons for effectiveness of the elicitor. The timing of elicitor treatment is critical because different growth phases will show different abilities to receive and further modulate the signals in elicitation activities and various defence responses [19,20]. The observed cell growth retardation in elicited cultures in time course study could be a result of elicitor depressing primary metabolism and switching on secondary metabolisms in plant cell culture [21]. The most striking result to emerge from our data was that the dry weight of the treated hairy roots was higher than the control and had enhanced with the increase in concentration of chitosan.

Increase in silymarin content with respect to chitosan addition could be attributed to the fact that chitosan acts as a powerful inducing signal and the effectiveness of chitosan elicitation depends on time elicitation implying that 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 silymarin production induced by elicitors. Clearly more work is needed to identify cellular target and signal transduction in chitosan treated hair root culture of *S. marianm*. Finally, knowledge on the mode of action of any given elicitor might be useful to reach higher levels of silymarin which is of economic importance.

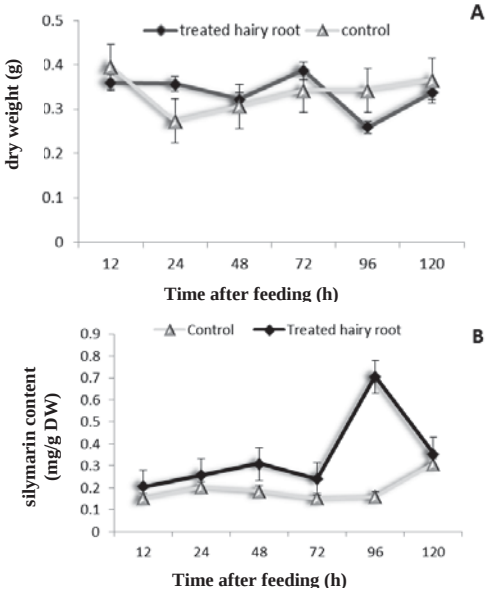

Figure 2. Time course of the chitozan-induced dry weight (A) and silymarin accumulation (B) of *S. marianu*m treated and non-treated hairy root culture

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