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ORIGINAL RESEARCH PAPER

# Elicitation of silymarin in cell cultures of *Silybum marianum*: effect of subculture and repeated addition of methyl jasmonate

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**Abstract** Production of silymarin and the effect of the elicitor, methyl jasmonate (MeJA), was monitored in cell cultures of *Silybum marianum* over 4 years. Silymarin concentrations gradually declined after prolonged subculture, making the success of elicitor strategy limited in long-term cultures. The continuous presence of MeJA in cultures for an extended period was necessary for induction of silymarin accumulation. A repeated elicitor strategy was not a good option for improving silymarin productivity in batch cultures. Removal of medium from elicited cultures and addition of fresh medium avoided the toxic effects of elicitor accumulation, allowing the system to respond to a repeated MeJA treatment without loss of productivity.

**Keywords** Cell culture · Elicitation · Secondary metabolite · Silymarin

## Introduction

The milk thistle, *Silybum marianum* (L.) Gaernt, has been used medicinally for over 2000 years. Its properties are due to the presence in fruits of silymarin, composed by an isomeric mixture of the flavonolignans silydianin, silychristin A and B, silybin A and B and isosilybin A and B (Kim et al. 2003). Silymarin is used to treat liver disorders, it also inhibits chemically-induced carcinogenesis and shows direct anti-carcinogenic activity against several human carcinoma cells; in addition, silymarin has antidiabetic, hypolipidaemic, antinflammatory, cardioprotective, neurotrophic and neuroprotective effects (Flora et al. 1998; Kren and Walterova 2005). Further studies with pure components of silymarin will extend its applications.

Elicitors have been widely employed to increase the formation of secondary metabolites in plant cell cultures and this strategy has also been effective in stimulating the production of silymarin in cell cultures derived from *S. marianum* (Sánchez-Sampedro et al. 2005). Besides improving production, elicitation allows the study of signal transduction pathways that lead to activation or de novo biosynthesis of transcription factors, which regulate the expression of biosynthetic genes involved in plant secondary metabolism (Zhao et al. 2005a, b). However, during the long-term culture of plant cells, it is difficult to maintain constant conditions, such as temperature, light irradiation, subculture cycle and inoculum size. Furthermore, small changes in growth rate between different passages of the same culture, and accumulating epigenetic changes as a function of time in culture lead to the instability of secondary metabolites (Meins 1983). These limitations could

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also account for elicitation in such a way that results from one culture batch will not necessarily extrapolate to next passages of the same culture. Therefore, to establish an experimental model system with which to study the mechanisms of elicitor-induced accumulation of secondary metabolites it is advisable to know whether cell cultures respond to exogenous application of elicitors over successive subculture cycles. For this reason, the object of this study has been to determine the extent to which long term subcultured cells of *S. marianum* retain their ability to respond to methyljasmonate (MeJA). For this, the dynamics of silymarin elicitation were evaluated in hypocotyl-derived cell cultures over 4 years of experimentation. Results concerning the duration of elicitor treatment necessary for silymarin induction and the effect of repeated addition of elicitor are also included in this work.

## Materials and methods

### Cell maintenance

Cultures of *Silybum marianum* were developed from hypocotyl-derived callus. Suspensions were routinely subcultured every 2 weeks by transferring approximately 10 ml of the previous culture, generated after mixing three parental flasks, to 40 ml Murashige and Skoog (1962) medium supplemented with 30 g sucrose $l^{-1}$, 4.5 μM 2,4-dichlorophenoxyacetic acid and 2.2 μM benzyl adenine at pH 5.6 as reported by Sánchez-Sampedro et al. (2005). Cultures were incubated in the dark and shaken at 90 rpm.

### Elicitation

For experiments, 250 ml flasks containing 50 ml medium were inoculated with cells, harvested by filtration without reduced pressure, from the previous subculture; treatments were done 3 days after transfer, when cells have already started division. Cell suspensions were aseptically treated with MeJA, prepared as a stock solution in ethanol, at 100 μM (ethanol in the cultures was at 0.1%). Controls received equivalent volumes of solvent. Silymarin was extracted separately from medium and cells and analysed by HPLC as described in previous work (Sánchez-Sampedro et al. 2005).

## Results and discussion

### Elicitation in long-term cultures

As reported previously, treatment of *S. marianum* cell cultures with MeJA strongly increased silymarin accumulation, both in cells and in the culture medium. The unelicited production of silymarin in suspended cultures fluctuated and progressively decreased between subcultures, reaching a steady state with low productivity after 2 years in culture. Cell suspensions retained their ability to respond to elicitation over prolonged subcultures, however, the intensity of elicitation declined with successive subcultures (Fig. 1), and after 4 years of culture maintenance, cells could not be longer used for elicitation of silymarin, at least with MeJA and with the same elicitation protocol. This observation contrasts with reports in other species in which elicitor's effects were studied in suspensions derived from

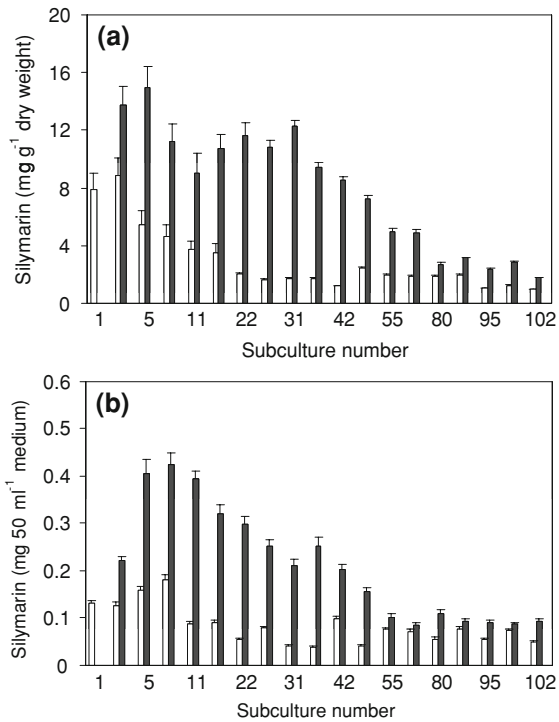


**Fig. 1** Impact of subculture cycles on the efficacy of MeJA elicitation in cell cultures of *Silybum marianum*. Silymarin was checked after 72 h of MeJA application. **a** Silymarin in cells, **b** silymarin in the culture medium. (*open square*) Control cultures, (*filled square*) elicited cultures. Results are the mean of three replicates ± SD

long-term established callus (Zhao et al. 2005a, b). Kauss et al. (1994), on the contrary, reported that parsley suspension cultures have to be initiated about twice a year from callus tissue for elicitor experimentation. In our case secondary metabolite elicitation is sensitive to prolonged subculture of suspensions and this may have been caused either by the lost of cell's capacity for recognition of the stimulus or the lost of competency factors in cell population over time.

## Elicitor contact time

MeJA treatment for up to 4 h induced no silymarin accumulation, and continuous treatment for at least 6–8 h was required to evoke a similar rate of product formation as the presence of the elicitor for 24 h (Fig. 2). The availability of active elicitor was not diminished by deactivation or binding since the culture filtrate obtained after 24 h in the presence of MeJA retained its original potency to induce silymarin formation in untreated cultures (data not shown).

Continuous recognition of elicitor for several hours was also a prerequisite for induction of a full range of defense responses in tobacco BY-2 cells (Kadota et al. 2006). In contrast, 30 min contact with an elicitor was sufficient for alkaloid formation in *Escholztia californica* cultures 24 h later and the authors suggest that a short pulse activated early events enough to trigger alkaloid biosynthesis. As reported by Sánchez-Sampedro et al. (2008), some common and rapid signal transduction events, like calcium fluxes, phosphorylation/dephosphorylation cascades or $H_2O_2$ generation, are not necessary for the elicitor-induced accumulation of silymarin in cell cultures of *S. marianum*. However, upon elicitation, a metabolic reprogramming that affected amino acid and carbohydrate metabolism was observed in the system. Changes were noticeable only after 7 h of treatment and lasted for 72 h (Sánchez-Sampedro et al. 2007). These observations suggest that silymarin elicitation is complex and a long duration of elicitor recognition might be required for full induction of a variety of responses that eventually will lead to activation of silymarin synthesis.

## Silymarin production in cultures treated repeatedly with MeJA

The effect of a second addition of 100 μM MeJA in silymarin production is shown in Fig. 3. Application of 200 μM MeJA to cultures slightly decreased production. A further elicitor contact 8 h after the first did not substantially improve silymarin accumulation. When the second addition was delayed for 24 h or 48 h an increase in silymarin production was observed, this

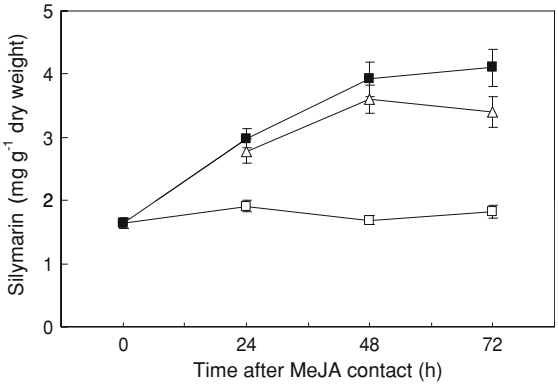


**Fig. 2** Silymarin production by *Silybum marianum* cells after permanent (*filled square*) or an 8 h single contact (*open triangle*) with 100 μM MeJA. (*open square*) Untreated cells. Elicitor was was added 3 days after subculture and was removed by washing with excess fresh growth medium at 0, 0.5, 1, 2, 4, 8, 12 and 24 h after application, followed by silymarin analyses in cells at 48 h and 72 h. Results are means of three independent experiments, each in triplicate ± SD

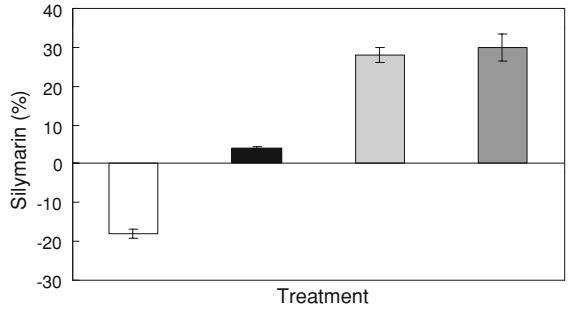


**Fig. 3** Effect of second addition of MeJA on silymarin accumulation in cell cultures (medium + cells) of *Silybum marianum*. (*open square*) A single dosage of 200 μM MeJA, (*filled square*) second addition of 100 μM MeJA given 8 h after the first, (*filled square*) second addition of 100 μM MeJA given 24 h after the first, (*filled square*) second addition of 100 μM MeJA given 48 h after the first. Silymarin was evaluated in all cases at day 3 with respect to the first dosage. Data are normalized to the silymarin production triggered by a single contact with 100 μM MeJA for 72 h which is set to 0% (3.98 mg culture$^{-1}$ ± 0.47). Each value represents the mean ± SD, $n = 3$

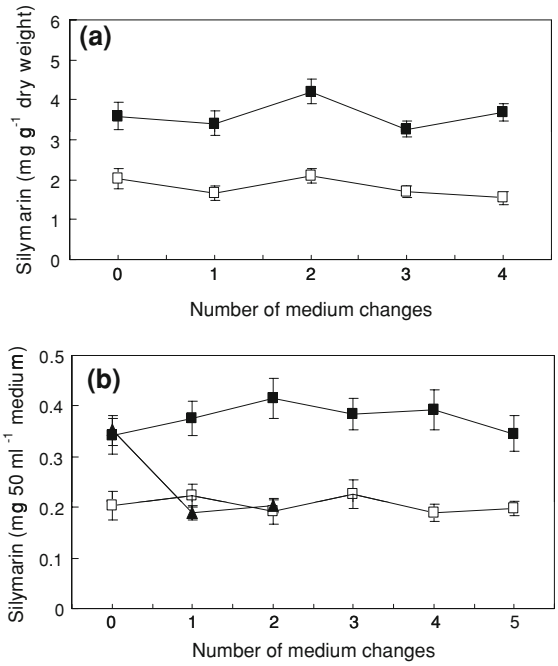


**Fig. 4** Silymarin production in cell cultures of *Silybum marianum* elicited repeatedly with 100 μM MeJA with medium changes. Cells (300 mg dry weight), obtained by filtration from 48 h elicited cultures, were re-inoculated in 50 ml fresh medium. After 48 h, a second addition of 100 μM MeJA was given; at 48 h post re-elicitation, medium recovered by filtration and a sample of cells were analysed. Cells (300 mg dry weight) were again reinoculated in new medium. The re-elicitation continued up to four times (five MeJA doses in total), controls were managed in the same way. **a** Silymarin in cells, **b** silymarin in the culture medium. (*open square*) Control cultures, (*filled square*) elicited cultures, (*filled triangle*) cultures reinoculated in fresh medium which received a single dosage of MeJA. The number 0 means production after the first MeJA addition without medium change. Each value represents the mean ± SD, $n = 3$

increase representing a 20% over those levels detected in 72 h MeJA elicited cultures. A third addition of MeJA caused the necrosis of cultures rendering silymarin production negligible (data not shown).

In another set of experiments silymarin production in response to repeated elicitor contact with medium changes was tested. It should be noted that cultures without medium changes secreted silymarin to the extracellular medium only during the first 5–7 days of culture (data not shown and as in Sánchez-Sampedro et al. 2005).

Re-elicitation with MeJA with medium changes avoided cell necrosis. Silymarin production by re-elicited cells was always higher than in controls, however, for every 48 h of elicitor treatment, no significant differences were seen among cultures which received only one MeJA dosage and cultures elicited repeatedly (Fig. 4).

A repeated elicitor strategy seems not to be a good option for improving silymarin productivity. This contrasts to reports in other plant culture systems as, for example in *Taxus chinensis* cultures in which Wang and Zhong (2002), improved taxol production by 50–60% in flasks and by about 80% in airlift bioreactors after a second dosage of MeJA. Although large increases in alkaloid production were observed by MeJA re-elicitation with medium changes in *Escholztia californica* cultures (Byun 2000), this approach was not suitable for increasing silymarin production beyond that observed by a single MeJA dosage. However, addition of fresh medium avoided the toxic effects of elicitor accumulation allowing the system to respond to a repeated MeJA treatment. This semicontinuous cultivation process may be applicable for silymarin extraction from the culture medium over a prolonged time without loss of productivity.

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