Journal of Genetic Engineering and Biotechnology (2016) xxx, xxx–xxx

HOSTED BY

ELSEVIER

Academy of Scientific Research & Technology and National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ORIGINAL ARTICLE

# Enhancement of silymarin and phenolic compound accumulation in tissue culture of Milk thistle using elicitor feeding and hairy root cultures

**Ahmed M.M. Gabr [a,b,*], Hassan Ghareeb [a], Haatem M. El Shabrawi [a], Iryna Smetanska [b], S.A. Bekheet [a]**

[a] *Plant Biotechnology Dept., National Research Centre, Bohouth Str., Dokki, Cairo, Egypt*
[b] *Department of Plant Food Processing, Agricultural Faculty, University of Applied Science Weihenstephan-Triesdorf, Markgrafenstr 16, 91746 Weidenbach, Germany*

Received 16 July 2016; revised 2 October 2016; accepted 17 October 2016

**KEYWORDS**

Milk thistle;
Hairy root;
Elicitors;
Silymarin;
Phenolic compounds;
DPPH scavenging activity

**Abstract** In the present study, the effects of the metabolite elicitors chitosan, methyl jasmonate (MeJA) and salicylic acid (SA) as well as the hairy root transformation were tested for silymarin and phenolic compound accumulation in *in vitro* cultures of Milk thistle. For callus induction, leaf explants were cultured on MS medium supplemented with 5 mg/l NAA + 2 mg/l Kin + 0.1 mg/l GA3. Chitosan, SA and MeJA were added separately in three concentrations 200, 400 and 800 mg/l; 10, 20 and 40 mg/l; 20, 40 and 80 mg/l, respectively, to hormone free B5 medium. Alternatively, cotyledons of 12 day old seedlings were transformed with *Agrobacterium rhizogenes* A4 strain. Overall, increasing the concentrations of the three elicitors dramatically increased the total silymarin content. Remarkably, the elicitors mainly enhanced the accumulation of silybine A&B that were not detected in un-treated callus culture (control). In addition, the hairy root culture triggered the accumulation of silybine A&B, and silydianin, which was not detected in the non-transgenic roots. The hairy root culture was superior in production of the phenolic compounds in comparison to the control and elicitor treatments. The hairy root cultures showed also higher antioxidant capacities than non-transformed cultures and/or chemically elicited-callus cultures. Thus hairy root provide instrumental in enhancing the production of economically valuable metabolite.



## 1. Introduction

Milk thistle (*Silybum marianum* L.) belongs to family Asteraceae. *S. marianum* contains phenolic compounds that are involved in the biosynthesis of flavonolignans. Flavonolignans

* Corresponding author at: Plant Biotechnology Dept., National Research Centre, Bohouth Str., Dokki, Cairo, Egypt.
E-mail address: a_m_gabr2@yahoo.com (A.M.M. Gabr).

Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2016.10.003

are formed by combination of coniferyl alcohol and dihydroquercetin (Taxifolin). Flavonolignans in *S. marianum* called silymarin. Silymarin is a mixture of different isomers; silibinin, isosilibinin, silicristin, silidianine and silychristine. Silymarin has a liver protective function as it acts an antioxidant and by promoting the growth of new liver cells [1]. Silymarin has been used (especially in Europe) to treat hepatitis and liver damage due to alcoholism [2]. A standardized extract should be 80% silymarin (the active ingredient). The usual dosage of milk thistle extract is between 300 and 600 mg daily. In addition, Milk thistle has been recently described as anticancer, antidepressant, antioxidant, cardio protective, demulcent, digestive tonic, hepatoprotective, hepatoregenerative, immunostimulatory and as a neuroprotective [3].

Silymarin and other active compounds of Milk thistle are usually extracted from dried fruits of field grown plants that often require months to years to be obtain. *In vitro* culture has been considered as an economic alternative for the production of silymarin [4]. Generally, there are various advantages of a cell culture system over the conventional cultivation of whole plants. Useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions, cultured cells would be free of infection from microbes and insects, the cells of any plants, tropical or alpine, could easily be multiplied to yield their specific metabolites and automated control of cell growth and rational regulation of metabolite processes would reduce labor costs and improve productivity [5]. Furthermore, elicitation is one of the most effective strategies for improving the *in vitro* productivity of plant bioactive secondary metabolites. Jasmonate has been shown to be key molecules in the elicitation process leading to de novo transcription and translation that resulted in the enhancement of secondary metabolites biosynthesis in *in vitro* plants [6]. In this context, MeJA strongly promoted the accumulation of silymarin in cell cultures of *S. marianum*. MeJA acted in a number of steps of the metabolic pathway of flavonolignans and its stimulating effect was totally dependent of "de novo" protein synthesis [7]. Otherwise, SA plays important roles in regulating photosynthesis and accumulation of phenolic acid production [8]. In this respect, *in vitro* production of silymarin from *S. marianum* was investigated using different strategies; growth regulators and carbon sources [9–13], elicitors [14–17] root and hairy root cultures [18–23].

This study aimed to investigate the effect of hairy root transformation and elicitation of *in vitro* cultures of Milk thistle using chitosan, methyl jasmonate and salicylic acid on phenolic acids and silymarin accumulation.

## 2. Materials and methods

### 2.1. Establishment of in vitro cultures

Seeds of Milk thistle were used as starting plant material for *in vitro* germinated and subsequently seedlings were used for tissue culture experiments. Seeds were washed with distilled water, then immersed in 70% ethanol for 1 min followed by 50% commercial Clorox (containing 5.25% sodium hypochlorite) for 10 min and finally washed three times with distilled sterilized water. Seeds were placed in 250 ml Erlenmeyer flasks containing 50 ml liquid free MS-basal medium and shake-incubated at 120 rpm [24]. All cultures were maintained at 25 ± 2 °C and under light regime 16/8 h light/dark. The experiment contained 20 replicates and each replicate contained 5 seeds.

Cotyledons from 12 day old sprout culture were re-cultured on fresh solidified MS medium containing 3 mg/l kinetin (Kin) to generate the starting plant material. For callus induction cotyledons were cultured on solidified MS medium containing 5.0 mg/l Kin and 0.5 mg/l IAA. Callus cultures were obtained after five weeks of incubation in darkness and they were subcultured every 4 weeks on fresh medium for callus proliferation.

### 2.2. Elicitor treatments

Methyl jasmonate (MeJA) and salicylic acid (SA) were dissolved in ethanol and added to the callus induction medium at concentrations 20, 40 and 80 mg/l and 10, and 40 mg/l, respectively. Chitosan was dissolved in 5% (v/v) 1 N HCl through gentle heating and continuous stirring and added to the callus induction medium at concentrations 200, 400 and 800 mg/l.

Sequentially, 500 mg (fresh weight) callus from 3-month-old undifferentiated hypocotyl callus was transferred to free B5 medium supplemented with different concentrations of elicitors and maintained at 25 ± 2 °C on the dark for 14 days.

### 2.3. Transformation of cotyledons explant with Agrobacterium rhizogenes

#### 2.3.1. Preparation of A. rhizogenes

Culture of *A. rhizogenes* strain A4 was initiated from glycerol stock and maintained on MYA-solid medium (5.0 g/l Yeast extract, 0.5 g/l Casamino acids, 8.0 g/l Mannitol, 2.0 g/l Ammonium sulfate, 5.0 g/l NaCl and 15 g/l agar) [25] for 48 h at 28 °C in the dark. The single clone was grown for 24 h in 20 ml MYA-liquid medium at 28 °C on a rotary shaker at 100 rpm in the dark.

### 2.4. Establishment of hairy root cultures

The transformation experiment was done according to [24]. Briefly, 12 day old cotyledons of Milk thistle was used as starting material. Each cotyledon was immersed in bacterial suspension separately for 10 min. The explants were blotted dry on sterile filter-paper to remove excess of bacteria and incubated in the dark at 28 °C in 200 ml Erlenmeyer flask with 50 ml of liquid hormone-free MS medium with 30 g/l sucrose on a rotary shaker at 100 rpm. Uninfected explants (control) were cultured under the same conditions. After 24 h of co-cultivation, the explant tissues were transferred to new growth medium (solidified MS medium supplemented with 0.2 mg/l NAA) containing 500 mg/L cefotaxime to eliminate bacteria and then incubated in growth chamber at 25 ± 2 °C and under light regime 16/8 h light/dark.

### 2.5. Sample preparation

Samples from both hairy root transformed culture and two weeks elicited cultures were harvested and immersed in liquid nitrogen to avoid any possible enzymatic degradation, the