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

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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

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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

$25 \pm 2\,°C$ 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 $25 \pm 2\,°C$ 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 $25 \pm 2\,°C$ 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

samples were freeze-dried. The lyophilized samples were ground by flint mill (Retsch, Germany) (20,000 rpm, 2 min) to a fine powder.

2.6. Extraction of flavonolignan (silymarin) compounds and HPLC analysis

The extraction was performed according to [26], flavonolignans were extracted from the lyophilized with 10 ml of methanol at 40 °C for 8 h. The methanol solution was evaporated and concentrated to a dry residue. The extract was dissolved in 1 ml of methanol and kept at 4 °C in darkness.

The content of silymarin compounds was determined by HPLC on a UNICAM CRYSTAL 200 Liquid Chromatograph. The mobile phase consisted of methanol and water (both acidified with 0.3% orthophosphoric acid p.a. - w/v). Flavonolignans were eluted with linear gradient from water to 50% methanol in 5 min, followed by isocratic elution with 50% methanol for 20 min. The flow-rate was 1.4 ml/min. Substances were detected by absorption at $\lambda = 288$ nm and their identification were carried out by the comparison of retention times and absorption spectra with standards complex of silymarins (Sigma-Aldrich). All experimental analyses were carried out in a minimum of three independent complexes for each time and each concentration of elicitor. The silymarin content was expressed as mg/g dry weight and derived using a known concentration of standard and sample peak areas.

2.7. Extraction of phenolic acids and HPLC analysis

A total of 20 mg ground dried samples was used to extract phenolic acid using 750 μL 70% methanol (v/v, pH 4, phosphoric acid) in an ultrasonic water bath (Sonorex digital 10p, Bandelin) on ice. Samples were centrifuged for 5 min at 6000 rpm. The supernatants were collected and the pellets were re-extracted twice with 500 μL 70% methanol. Samples were analyzed with HPLC (Dionex Summit P680A HPLC-System), equipped with P680 pump, ASI-100 automated sample injector, a Narrow-Bore AcclaimPA C16-column (3 μm, 2.1 × 150 mm, Dionex) and PSA-100 photodiode array detector (Dionex) and software Chromeleon 6.8 (Dionex, USA). The HPLC analysis was operated at a temperature of 35 °C. Mobile phase consisted of 0.1% (v/v) phosphoric acid in ultrapure water (eluent A) and of 40% (v/v) acetonitrile in ultrapure water (eluent B) [27]. Phenolic acid quantity was calculated from HPLC peak areas at 290 nm against the internal standard and external standards. Identification and quantification of phenolic acids present was done by comparing retention time and area of the peaks in the extracts with that of the standard phenolic acids (chlorogenic acid, caffeic acid, cinnamic acid, p-coumaric acid, rosmarinic acid and sinapic acid).

2.8. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity

The DPPH test was utilized to [28] with minor modifications. The stock reagent solution (1 mM) was prepared by dissolving 22 mg of DPPH in 50 ml of methanol and stored at −20 °C until use. The working solution (0.06 mM) was prepared by mixing 6 ml of stock solution with 100 ml of methanol to

obtain an absorbance value of 0.8 ± 0.02 at 515 nm. Extract samples of different concentrations and synthetic antioxidant (BHA) solutions (0.1 ml of each) were vortexed for 30 s with 3.9 ml of DPPH working solution. After a 30 min of incubation period at room temperature in the dark, absorbance was recorded at 515 nm. The DPPH solution without extract served as control. Scavenging activity was calculated as follows: DPPH radical-scavenging activity (%) = [($A_{control}$ − A_{sample})/$A_{control}$] × 100, where $A_{control}$ was the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound.

2.9. Statistical analysis

All analyses were performed in triplicate and data reported as mean ± standard deviation (SD). Data were subjected to analysis of variance (ANOVA) ($P < 0.05$). Results were processed by Excel (Microsoft Office 2010) and SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA).

For correlation between phenolic and silymarin production induced using the three elicitors (chitosan, MeJA and SA). Pearson's correlation coefficients were computed to study the association between quantitative variables. Differences were considered significant at $p \leqslant 0.05$.

3. Results

3.1. Effect of chitosan, MeJA and SA on growth kinetics

The effect of MeJA, SA and chitosan were tested on two month old Milk thistle callus culture derived from leaf explant. Callus was cultured on basal MS medium supplemented with different concentrations of chitosan (200, 400 and 800 mg/l), MeJA (50, 100 and 150 μM) and SA (0, 25, 50, and 100 μM).

Milk thistle callus culture showed a different proliferation response to each elicitor comparing to the control Fig. 1A and B. Chitosan has induced a protocorm-like body (PLB) in almost all callus. Some of calli were friable and differentiated into shoots under concentration of 200 mg/l Fig. 1C. While at 400 mg/l chitosan concentration the PLB were compact and later differentiate into few long roots Fig. 1D. SA had more pronounced effect on callus on differentiation than the other elicitors. The treatment of 10 mg/l of SA showed dark brown callus differentiated from each side several into long primary roots which have a lot of secondary roots Fig. 1E and F. But in case of 20 mg/l SA the dark brown callus differentiated into complete shortened shoots including its leaf trichomes Fig. 1G an H. The effect of MeJA on Milk thistle callus culture showed the least effect on callus differentiation. At 20 mg/l of MeJA very few roots were formed from the dark brown callus Fig. 1I. Also, root formation was observed on some callus cultures with high (80 mg/L) of MeJA Fig. 1J.

3.2. Effect of chitosan, MeJA, SA and hairy root on phenolic production

Callus cultures of Milk thistle were treated with different concentrations of chitosan (200, 400 and 800 mg), MeJA (20, 40 and 80 mg) and SA (10, 20 and 40 mg). Fourteen days after

treatment, samples were collected; lyophilized and phenolic compounds were extracted and quantified. The total phenolics obtained with the three elicitors were lower than that obtained from the control (Table 1). Chitosan treatments resulted in the lowest total phenolic accumulation compared to that of other elicitated cultures, in particular at the highest concentration 800 mg/l, which showed the lowest phenolic content (0.723 µg/g DW) (Table 1).

Next, we transformed shoot cultures of Milk thistle with *A. rhizogenes* A4 strain to trigger hairy root formation as previously described [24]. The transformed hairy root was confirmed by PCR amplification of the *rolb* gene as reported by [24]. In hairy root cultures, we noted higher accumulation of both p-Coumaric acid and Cinnamic acid (1.011 and 18.092 µg/g DW, respectively) compared with non-transformed root (root control) or untreated callus (callus control) (Table 1).

3.3. Effect of chitosan, MeJA, SA and hairy root on silymarin production

We also analyzed to the effects of the three elicitors (chitosan, MJ and SA) as well as hairy root culture on production of silymarin compounds, silybin A&B and silydianin. Overall, the total silymarin contents with elicitors and hairy root were higher than untreated callus (Table 2). With increasing concentrations of chitosan from 200 to 800 mg/l, the treated callus showed gradual increase in total silymarins (4.85, 5.6 and 5.77 µg/g DW) in comparison to the untreated callus (3.7 µg/g DW). Similarly, higher concentrations of SA (20 mg/l SA to 40 mg/l) triggered accumulation of total silymarin (9.63 and 11.24 µg/g DW, respectively). MeJA showed the highest silymarin accumulation compared to other two elicitors, in particular, the lowest MeJA concentration (20 mg/l) resulted in the highest total silymarin contents compared with other concentrations (13.59 µg/g DW). As presented in Table 2, the hairy root culture showed outperformance in accumulating total silymarin (20.25 µg/g DW) compared with untreated callus and other elicitors.

The analysis of silymarin compounds revealed that silydianin was produced in the untreated callus, different elicited cultures, root control and hairy root culture. However,

chitosan was the most effective in accumulating the silydianin compared to untreated callus, SA, MeJA and hairy root culture. The highest chitosan concentration (800 mg/l) showed the highest silydianin content (5.15 µg/g DW). Silybine A & B were not detected in the untreated callus as well as the lowest concentrations of chitosan and SA. MeJA at different concentrations was effective in accumulating of silybine A & B. Interestingly, the hairy root culture showed the highest production of silybine A & B (16.92 µg DW) (Table 2).

3.4. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity

DPPH assay is an effective and simple assay to evaluate antioxidant capacity of plant extracts [29]. We performed DPPH radical scavenging activity on samples treated with the three elicitors or hairy root culture. The hairy root transformed culture showed the highest antioxidant activity compared with elicitors and untreated callus (Fig. 2). In contrast, chitosan, SA and MeJA showed reduced antioxidant activity relative to the untreated callus (Fig. 2).

4. Discussion

Hairy root cultures and elicitors were applied to plants in regulating production of secondary metabolites, in which the hormone-like substance, SA, and hormone MeJA were well-reported in promoting production of phenolic acids in plants [30,31]. In this study, we investigated the effect of hairy root transformation and elicitation of *in vitro* callus cultures of Milk thistle on phenolic compounds and silymarin accumulation. To this end, we treated callus culture with various concentrations of chitosan, MeJA and SA. Overall we observed root differentiation of the callus upon application of chitosan at 400 mg/l (Fig. 1D) and SA at 10 mg/l. Intriguingly, SA concentration 20 mg/l triggered shoot differentiation. MeJA and SA have been long known to contribute to defense against plant pathogens. Our results hits to the possibility of these hormones to play a pivotal role in reprogramming plant development.

The HPLC data of total silymarin showed that the chitosan treatment (200 and 400 mg/L) slightly increased the content of

Table 1 Effect of different elicitors on of p-Coumaric and Cinnamic acid contents of callus and hairy root cultures of Milk thistle.

Treatment		p-Coumaric acid (µg/g DW)	Cinnamic acid (µg/g DW)	Total (µg/g DW)
Control (callus)	Callus	1.512 ± 0.20	14.992 ± 0.50	16.504 ± 0.45
200 mg/l Chitosan		0.929 ± 0.05	0.00 ± 0.00	0.929 ± 0.05
400 mg/l Chitosan		0.00 ± 0.00	1.68 ± 0.53	1.68 ± 0.53
800 mg/l Chitosan		0.723 ± 0.04	0.00 ± 0.00	0.723 ± 0.04
10 mg/l SA		0.926 ± 0.06	0.590 ± 0.07	1.516 ± 0.12
20 mg/l SA		0.453 ± 0.03	2.047 ± 0.13	2.500 ± 0.53
40 mg/l SA		0.189 ± 0.08	2.07 ± 0.13	2.259 ± 0.54
20 mg/l MeJA		0.611 ± 0.11	2.298 ± 0.18	2.909 ± 0.21
40 mg/l MeJA		0.565 ± 0.13	3.834 ± 0.50	4.399 ± 0.91
80 mg/l MeJA		0.00 ± 0.00	1.332 ± 0.42	1.332 ± 0.42
Control root	Non-transformed root	0.688 ± 0.04	9.741 ± 0.90	10.4291 ± 0.92
Hairy root	Transformed root	1.011 ± 0.22	18.092 ± 1.13	19.103 ± 1.50

Data shows means ± SD from 20 replicates.

Table 2 Effect of different elicitor concentrations and hairy root on silybin A & B, silydanin, and total silymarin contents of Milk thistle.

| Treatment		Silydianin (SDN) (μg/g DW)	Silybine A&B (SBN A&B) (μg/g DW)	Total silymarin (μg/g DW)
Control callus	Callus	3.70 ± 0.20	0.00 ± 0.00	3.70 ± 0.33
200 mg/l Chitosan		4.85 ± 0.12	0.00 ± 0.00	4.85 ± 0.12
400 mg/l Chitosan		4.90 ± 0.18	0.70 ± 0.08	5.60 ± 0.25
800 mg/l Chitosan		5.15 ± 0.27	0.63 ± 0.05	5.77 ± 0.30
10 mg/l SA		3.60 ± 0.31	0.00 ± 0.00	3.60 ± 0.31
20 mg/l SA		3.52 ± 0.10	6.10 ± 0.55	9.63 ± 0.67
40 mg/l SA		3.61 ± 0.23	7.63 ± 0.71	11.24 ± 0.96
20 mg/l MeJA		2.50 ± 0.11	11.10 ± 0.80	13.59 ± 0.88
40 mg/l MeJA		1.76 ± 0.35	10.45 ± 0.65	12.21 ± 0.90
80 mg/l MeJA		1.32 ± 0.30	11.45 ± 0.44	12.77 ± 0.47
Control root	Non-transformed root	0.00 ± 0.00	5.93 ± 0.50	5.93 ± 0.50
Hairy root	Transformed root	4.33 ± 0.42	16.92 ± 1.20	21.26 ± 0.95

Data shows means ± SD from 20 replicates.
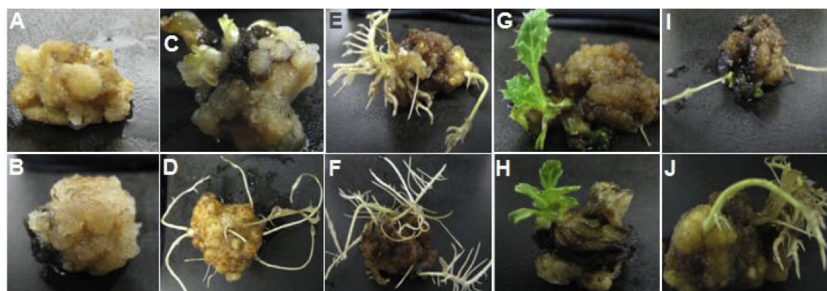
Figure 1 Five week old Milk thistle callus culture, derived from cotyledon cultured on solidified MS medium containing 5.0 mg/l Kin and 0.5 mg/l IAA supplemented with different concentrations of chitosan, MeJa and SA. (A) and (B) control, zero elicitor concentration. (C) and (D) calli response to 200 and 400 mg chitosan respectively. (E) and (F) calli response to 10 mgl/l SA, (H), (G) the response of Milk thistle callus to 20 mgl/l SA. (I), (J) Callus response to 40, 80 mgl/l MeJa, respectively.
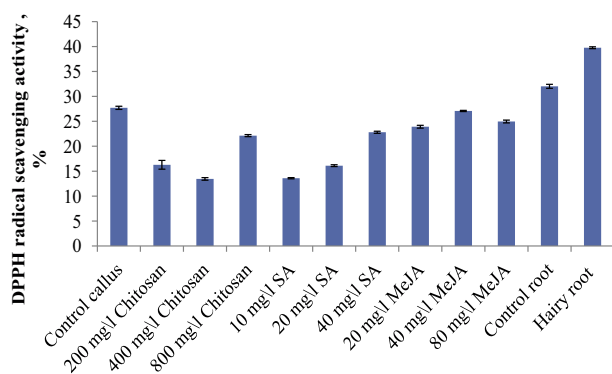

Figure 2 Effect of different elicitor concentrations on Milk thistle callus and hairy root on DPPH radical scavenging activity.

silymarin in Milk thistle callus culture (4.85 and 5.60 μg/g DW), respectively comparing to the control sample (3.70 μg/g DW). This increase of sliymarin in callus culture was associated with formation of protocorm-like bodies (PLBs) at 200 mg/l chitosan and root formation at 400 mg/l. Chitosan PLBs have proliferated into small shoots after two weeks (data not shown). Properly low concentration of produced silymarine directed shoot differentiation, and dramatic increased of silymarine concentration induced root formation. The MeJA treatment and hairy root formation via Agrobacterium produced the highest amount of total silymarin content, where callus differentiated into roots only. SA treatment triggered the lowest content of silymarine comparing to other elicitors chitosan, MeJA and hairy root A4. The 20, 10 mg/l SA treatments showed a complete shoot or root differentiation response. The hairy root A4 culture did not show any other type of differentiation except root formation. Hence, the Chitosan polymers act like phtohormones cytokinin, we can see its reduction effect when silymarine concentration goes high in callus culture [32]. MeJA revealed inhibition in root and shoot proliferations comparing to other two elicitors. The role of SA in the regulation of photosynthesis and the immune defenses has recently become clearer [33]. It was reported that the SA increases the accumulation of both ABA and IAA in plant seedlings under salt stress. However, the SA treatment did not affect the cytokinin content. So, the SA protective action in plant cells includes the development of anti-stress system and promotes the normalization of growth after removal stress factors [34].

Data presented in Fig. 3, show a significant negative correlation between total phenolic acids and total silymarin in MeJA treatment ($r = -0.9847$, $p = 0.0153$). No correlation
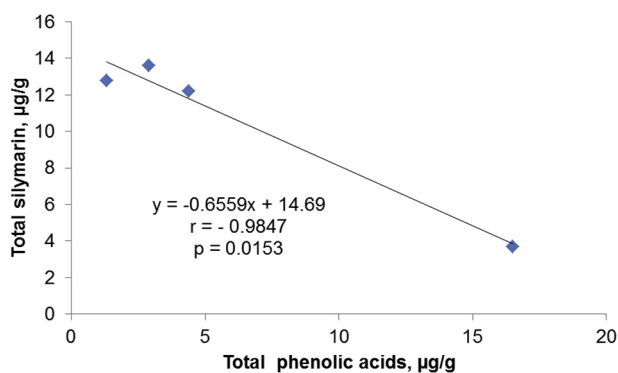
Figure 3 Correlation between total phenolics and total silymarin in MeJA-treated callus.

was seen between total phenolic acids and total silymarin in chitosan and SA treatments.

Hairy root transformation of many plant species have been widely studied for the production of useful secondary metabolites, pharmaceutical compounds, cosmetics, and food additives [35–39]. Our results of genetic transformation by *Agrobacterium rhizogenes* have attractive properties for silymarin production, as compared to differentiated cell cultures. Accumulation of total silymarin in hairy root cultures of Milk thistle was four times higher than in non- transformed root. Transformed root culture clearly showed the highest accumulation of total phenols (19.103 μg/g DW) compared to non-transformed root culture. In addition, production of different phenolic compounds in hairy root cultures was reported [40,41,27]. In our study, hairy root culture presented highest ability for silymarin production (21.26 μg/g DW) compared to non-transformed root culture (5.93 μg/g DW). Similarly [21] reported that silymarin production in transformed hairy root cultures was highly significant than non-transformed roots. Our results are in agreement with those of Khalili et al. [22] who reported that elicitation of hairy roots of Milk thistle with salicylic acid can be regulated the jasmonate pathway that may mediate the elicitor-induced accumulation of silymarin.

Although cell cultures of Milk thistle are capable of producing silymarin, amounts produced are lower than those produced in the field grown plants. It was found that the use of elicitor such as MeJA, SA, and yeast extract alone or in combination and manipulation of culture medium could improve the production of silymarin. In this respect, feeding a medium with chitosan, SA and MeJA offers the possibility to enhance the content of some compounds of silymarin complex [23]. Sánchez-Sampedro et al. [42] reported that addition of methyl jasmonate to *S. marianum* cell suspension cultures rapidly induced the activity of chalcone synthase (i.e., an enzyme in silymarin biosynthesis). The results of our study showed that elicitation with MeJA could increase the total silymarins, in particular silydanin in callus cultures of Milk thistle. The accumulation of silymarin showed a dose-dependent response to MeJA. Similar results have also been reported by Sánchez-Sampedro et al. [42], who demonstrated that addition of 50 μg/ml yeast extract and 100 μM of MeJA to Milk thistle cell suspension cultures strongly elicited accumulation of silymarin in the cells and in the culture medium. It was found that the

use of elicitor such as MeJA, SA, and yeast extract alone or in combination could improve the production of silymarin dramatically [26,43]. In this respect, cell suspension culture was used to assess the effect of phenylalanine (Phe), yeast extract (YE) and MeJA for enhancement of silymarin production in four Milk thistle genotypes [44]. Likewise, *in vitro* cell culture of Milk thistle was used to monitor the effect of elicitation by jasmonic acid (JA) on production of silymarins [45]. Silymarins content (0.413 mg g^{-1} DW) were obtained with 2 mg/l JA in darkness after 28 days. In this respect, Walker et al. [46] mentioned that JA and its methyl ester, MeJA, have been proposed to be key signaling compounds in the process of elicitation leading to the accumulation of various secondary metabolites.

Overall, chitosan, SA and MeJA had negative effects on DPPH antioxidant activity. In contrast, hairy root showed enhanced DPPH antioxidant activity. These results are in agreement with recently published data by Riasat et al. [19]. They reported that adventitious roots show high level of antioxidant activity (DPPH) compared to undifferentiated cells (callus) of Milk thistle. This study confirmed that some compounds show high level of expression in differentiated cells (root) than undifferentiated cells (callus). Previous study by [47] found higher antioxidant activity in callus cultures of Milk thistle in comparison to regenerated plantlets or wild-grown plants.

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