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RESEARCH ARTICLE

Improved silymarin content in elicited multiple shoot cultures of *Silybum marianum* L.

Fadia El Sherif · Salah Khattab · Amany K. Ibrahim · Safwat A. Ahmed

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Abstract *Silybum marianum* L. extracts are being used as antihepatotoxic therapy for liver diseases. Silymarin is a polyphenolic flavonoid mixture isolated from milk thistle which is believed to be responsible for the plant's hepatoprotective action. Regeneration of *Silybum marianum* plants from shoot tip explants and assessment of their morphogenic potential, silymarin total concentration and its major constituents upon exposure to medium composition alteration and different elicitors' application was targeted. Different concentrations of NaCl, quercetin, gamma irradiation and dried fungal extracts were used to elicit silymarin production in the cultures. The chemical composition of silymarin and its total concentration was investigated through HPLC at all the experiment stages. Multiple shoots were recorded after 3 weeks of culture on MS medium containing various concentrations of BA and/or NAA. IAA was more effective than NAA and IBA in inducing robust roots in shoot cultures. The flowering plants recorded 20 % and 40 % of the total plants number in the multiplication and rooting stages respectively. The highest total silymarin concentration reached its peak with (10 Gy) gamma-irradiation to be 6.598 % dry weight in the in vitro regenerated shoot tip explants. The in vitro grown flowers showed 1.7 times more sylimarin productivity as compared to that of the wild grown congruent.

F. El Sherif (✉) · S. Khattab
Department of Horticulture, Faculty of Agriculture,
Suez Canal University,
Ismailia 41522, Egypt
e-mail: woroofss@yahoo.com

A. K. Ibrahim · S. A. Ahmed (✉)
Department of Pharmacognosy, Faculty of Pharmacy,
Suez Canal University,
Ismailia 41522, Egypt
e-mail: safwat_aa@yahoo.com

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Silybum marianum (L.) Gaertn (milk thistle, Asteraceae) is an annual or biennial plant native to the Mediterranean area (Mossa et al. 1987). Milk thistle contains isomeric mixtures of flavonolignans, including Taxofillin (TX), silychristin (SC), silydianin (SD), silybin (SN) and isosilybin (IS) collectively known as silymarin (SM) (Kurkin et al. 2001). In nature, silybin and isosilybin have been reported to contain two groups of diastereoisomeric flavonolignans, silybin (A) and silybin (B), and isosilybin (A) and isosilybin (B), respectively (Radjabian et al. 2008). It was clearly demonstrated that silymarin exerts a strong protective effect against oxidative stress damage at the protein level which has a direct connection to its use as hepatoprotector for chronic inflammatory liver diseases and liver cirrhosis (Herman et al. 2011). Alongside with its antioxidant properties and its role in stimulating protein synthesis and cell regeneration (Tawaha et al. 2007), silymarin may also reduce incidence of certain forms of cancer (Al Mesallamy et al. 2011). Silymarin was proved to protect liver and kidney cells from toxic effects of drugs including chemotherapy and radiotherapy (Meghreji Moin et al. 2010 and Prabhaba et al. 2010). It has been revealed that silymarin may be helpful in slowing down the progression of neurodegeneration in focal cerebral ischemia. These results suggest that the neuroprotective potential of silymarin is mediated through its antioxidative and anti-apoptotic properties (Raza et al. 2011). Sylimarin has been among the most investigated plant extracts with known mechanisms of action (Abbasi et al. 2010a, b).

Silymarin compounds are usually extracted from the dried fruits of field grown plants that often require months to years to be obtained. Although cell cultures of *S. marianum* are capable of producing silymarin, amounts produced

are lower than those produced in the field grown plants (Abbasi et al. 2010a, b). The increasing worldwide demand for silymarin is endangering the sparse populations of *S. marianum* in the Mediterranean region (Ahmad et al. 2008). Moreover, knowledge of yield and chemical composition is limited (Khan et al. 2009). The efficiency of seed germination and seedling growth in some species of Asteraceae is low, inconsistent, and is highly dependent on various biological and environmental factors (Abbasi et al. 2010a, b). The in vitro production of plant secondary metabolites is possible under controlled conditions and free from environmental fluctuations because field-grown plants are susceptible to seasonal and somatic variations as well as environmental pollutants that may affect the medicinal value of the harvested tissues (Geng et al. 2001; Bonhomme et al. 2000). In addition, in vitro propagation methods offer powerful tools for germplasm conservation and mass-multiplication of many threatened plant species (Murch et al. 2000). Elicitors use for the enhancement of secondary metabolites has been well investigated. For example, the elicitor preparations from *Fusarium conglutinans* enhanced thiophene production in *Tagetes* spp., *Rhizoctonia solani* increased solavetivone production in *Hyoscyamus muticus*, and *Rhizopus oryzae* and *Aspergillus niger* enhanced the production of Shikonin. Increased levels of ajmalicine and catharanthine in *Catharanthus roseus* and capsaicin production in *Capsicum annuum* by fungal elicitors have also been reported (Ibrahim et al. 2007). There are many reports on the influence of different in vitro culture abiotic elicitation on total sylimarin yield determination in *Silybum marianum* cultures (Sánchez-Sampedro et al. 2005, 2007, 2009; Madrid and Corchete 2010; Khalili et al. 2010; Belchi-Navarro et al. 2011) but up to our knowledge; this is the first report for biotic elicitation on silymarin induction in vitro. In this study and in favor of the medicinal benefits of *Silybum marianum*, we directed our research towards the determination of the most suitable culture media composition using biotic and abiotic elicitation as well as gamma irradiation to study their action on mass production and enhancement of total silymarin production as estimated by HPLC in *Silybum marianum* shoot tip cultures.

Materials and methods

Plant material and seed germination

Seeds of *Silybum marianum* were obtained from National Research Center, Dokki, Egypt. Surface sterilization went through rinsing seeds with 70 % ethanol solution for 30 s, 0.1 % (w/v) aqueous mercuric chloride ($HgCl_2$) for 5 min and then the seeds were rinsed with 15 % (v/v) sodium hypochlorite solution for 5 min. The seeds were then washed three times with sterile tap water under laminar air-flow hood to remove all the traces of sodium hypochlorite. Surface sterilized seeds were cultured into 40 ml capacity jars containing 10 ml Murashige & Skoog (MS) medium (Murashige and Skoog 1962) containing half strength basic salts and vitamins. The medium was supplemented with 3 % (w/v) sucrose and 6.0 gl^{-1} agar and adjusted to pH 5.7, prior to autoclaving at 121 °C and 1.2–1.3 kg/cm^2 pressure for 20 min. One hundred seeds were cultured with only one seed/jar. The cultures were incubated under growth room conditions 22±2 °C, 16 h photoperiod and light intensity of 4000 Lux provided by florescent lamps (Phillips TLM 40W/33RS). After 4 weeks from seed culture, the rate of germination was determined.

Shoot multiplication

The effect of BA, 2iP and Kin on shoot induction

Four weeks-old shoot tips with the height of (0.5 cm) from in vitro culture explants were cultured onto 200 ml capacity jars containing 40 ml MS medium supplemented with 3 % (w/v) sucrose, 6.0 gl^{-1} agar and different concentrations of benzyl adenine (BA), kinetin (Kin.) and 2-isopentenyl adenine (2iP) (0.0, 0.5, 1.0 and 2.0 mgl^{-1}), medium was adjusted to pH 5.7, prior to autoclaving at 121 °C and 1.2–1.3 kg/cm^2 pressure for 20 min. Every jar contained one explants and each treatment had ten replicates. The cultures were grown for 4 weeks before data recording based on number of shoots, plant height, plant weight as well as the number of leaves, the multiplied shoots were collected and dried at room temperature before being extracted for the HPLC analysis.

The effect of BA and NAA on shoot induction

This experiment was conducted to determine the effect of BA and naphthalene acetic acid (NAA) on shoot induction in *Silybum marianum*. The MS medium (described above) was supplemented with different concentrations BA (0.0, 1.0, 2.0 and 4.0 mgl^{-1}) either alone or in combination with NAA (0.0, 0.05 and 0.1 mgl^{-1}). Sub culturing was done with the 8 weeks-old shoot tips of (0.5 cm) in height, ten culture bottles were prepared for each treatment. The cultures were grown for 4 weeks before data recording based on the number of shoots, plant height, plant weight as well as the number of leaves.

Rooting and ex vitro acclimation

For rooting, eight-weeks-old in vitro shoots (0.5–1.0 cm in length) were excised and transferred to MS medium

supplemented with different concentrations of indole 3-acetic acid (IAA), Indole buteric acid (IBA) or NAA (0.0, 0.1, 0.2 and 0.4 mgl^{-1}) each. The cultures were placed under same previously mentioned growth room conditions for root formation. After 4 weeks of culture, data were recorded based on the number of roots, root length, plant fresh weight and plant height. The rooted plantlets were transferred to greenhouse for acclimation in pots with a moist mixture of (1:1) sand and peat-moss, then, maintained inside a plant growth chamber and irrigated with a fine mist of water for 3 weeks. The percentage of survived plants was determined after 4 weeks. The samples of rooted explants cultured using different kinds of auxins as well as the acclimated plants (8 weeks old) grown under greenhouse conditions were collected and dried under room temperature before extraction for HPLC analysis.

Field grown *Silybum marianum* flowers were collected from Faculty of Agriculture Experimental Station, Suez Canal University, Ismailia, Egypt. The dried flowers were soaked in methanol (3×100 ml) before filtration and subjection to evaporation under vacuum. The dried extract (500 mg) was subjected to further quantitative HPLC analysis in order to compare its total silymarin concentration with the in vitro grown flowers.

In vitro biotic and abiotic elicitation

Preparation of the fungal elicitors

For biotic elicitation, three fungal cultures were used; *Aspergillus niger* NRRL 3, *Rhizopus stolonifer* NRRL 1472 and *Penicillium chrysogenum* ATCC 2273. The fungi were obtained from Northern Regional Research Laboratory (NRRL), Peoria, IL, USA and American Type Culture Collection (ATCC) Rockville, Maryland, USA. The fungi were cultured at 30 °C in 500-ml flasks containing 150 ml medium and were shaken at 150 rpm on a rotary shaker. The culturing medium composition was as follows in g l^{-1}: glucose, 30; NH_4NO_3, 2.5; KH_2PO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.25; and $ZnSO_4{\cdot}7H_2O$, 0.05. The cells were harvested after 7-day cultures, freeze-dried, and ground using a mortar. Dry cell powder was dissociated in water and a solution of the concentration of 10 gl^{-1} was prepared. It was then autoclaved before addition to the plant culture with the MS medium.

Elicitation experiments

Four weeks-old in vitro cultured shoot tips with the height of (0.5 cm) were cultured onto Petri dishes (92×16 mm) containing 30–35 ml of MS media basic salts and vitamins supplemented with 3 % (w/v) sucrose, 6 gl^{-1} agar 1.0 mgl^{-1} 2iP and one of the following elicitors:

Aspergillus niger NRRL3	80 mgl^{-1}
Rhizopus stolonifer NRRL1472	80 mgl^{-1}
Penicillium chrysogenum ATCC 2273	20 mgl^{-1}
NaCl	25 mM
NaCl	50 mM
Quercetin	0.5 mgl^{-1}
Quercetin	1.0 mgl^{-1}

The pH of the medium was adjusted to 5.7 with NaOH solution (0.1 N) or HCl (0.1 N) prior to autoclaving at 121 °C and 1.2–1.3 kg/cm^2 pressure for 20 min. For the abiotic elicitation using irradiation, the Petri dishes containing the shoot tip as explants were exposed to 0.0, 5,10,15 (Gy) gamma-irradiation doses using Egypt's Mega Gamma-1 type J 6600 cobalt-60 irradiation (El Sherif et al. 2011) at Cyclotron Department, Nuclear Research Center, Atomic Energy Authority, Egypt.

HPLC method

Instrumentation

Chromatographic experiments were performed with HPLC (Shimadzu, Kyoto, Japan). Separation and quantitation were made on a 250×4.6 mm (i.d) 5 μm ODS column (Luna, Phenomenex, USA). The detector was set at λ 288 nm. Data acquisition was performed on class-VP software.

Materials and reagents

Authentic standards of taxifolin (TX), silychristin (SC) and silydianin (SD) were supplied by ChromaDex Inc. (Santa Ana, CA, USA), while the authentic standards of silybin (SN) and isosilybin (IS) were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). The used methanol was HPLC grade (Fisher, Leis LE 11 5 RG UK). Phosphoric acid was of analytical grade.

HPLC conditions

The HPLC separation and quantitation were made on a 250×4.6 mm (i.d) 5 μm ODS column (Luna, Phenomenex, USA). The mobile phase was prepared by mixing methanol and 5 mM NaH_2PO_4 (pH 2.75 adjusted with phosphoric acid) in a ratio 50:50 v/v. The flow rate was 1.5 $mlmin^{-1}$. All determinations were performed at ambient temperature (at 25 °C). The mobile phase was filtered using 0.45 μm membrane filter (Millipore, Milford, MA) and

degassed by vacuum prior to use. The samples were also filtered using 0.45 μm disposable filters.

Standard solutions and calibration

Stock standard solutions were prepared by dissolving 5 mg of TX, SC, SD, SN and IS separately in 5 ml of mobile phase. The standard solutions were prepared by dilution of the stock standard solutions with the mobile phase to reach concentration ranges of 20–100. Triplicate 20 μl injections were made for each compound concentration; chromatographed under the specified conditions described above. The peak area values were plotted against corresponding concentrations to obtain the calibration graph.

Sample preparation

The extracts were prepared from the dried samples by extraction with (1:1 mixture of methanol/dichloromethane). Different identified weights of the dried samples were soaked in the extraction mixture and the solvent was replaced every day for five consequent days to insure complete extraction before drying under vacuum. Complete extraction was confirmed by thin layer chromatography and high performance liquid chromatography. For the HPLC analysis, 10 mg of each sample was dissolved in 10 ml of the mobile phase in a volumetric flask producing a final concentration of 100 mg percent. The content of each flask was shaken vigorously for 10 min, sonicated for 15 min before filtration through 0.45 μm disposable filters. A sample of 20 μl was then injected using the general procedures described under calibration and the concentrations of TX, SC, SD, SN and IS were calculated.

Statistical analysis

Experiments were set up in completely randomized design. Data were statistically analyzed using ANOVA\MANOVA of Statistica 6 software (Statsoft 2001). The significance of differences among means was carried out using the Least Significant Test (L.S.D) at $p=0.05$. For statistical analysis of HPLC results, data were presented as mean ± SE and were computed using Microsoft Excel program.

Results

Shoot multiplication

At the end of 4 weeks, seeds cultured on half-strength MS medium showed 90 % germination (Fig. 1a). *Silybum marianum* plants were efficiently regenerated from shoot tips. Explants were capable of directly developing multiple shoots on MS basal medium containing different concentrations of cytokinins. Shoots number increased with the increase of BA concentration. BA at the highest concentration (2.0 mgl^{-1}) gave the highest number of shoots and leaves per explant 4.6 and 39.6 respectively (Table 1, Fig. 1b). Of the three cytokinins [BA, Kin. and 2iP], BA was found to be more suitable for initiation and proliferation of multiple shoot buds (Table 1). Results obtained from our previous experiment (Table 1) revealed that, after 4 weeks of initial culture, shoot tip explants cultured on MS medium with BA (2.0 mgl^{-1}) developed maximum number of multiple shoots and leaves (Fig. 1b). These results suggested that bud formation in this plant was favored by cytokinins. Therefore MS medium containing various concentrations of BA (0.0, 1.0, 2.0 and 4.0 mgl^{-1}) alone and in combination with NAA (0.0, 0.05 and 0.1 mgl^{-1}) was considered as optimal for shoot proliferation. Multiple shoots were recorded on all explants after 3 weeks of culture, on MS medium containing various concentrations of BA and/or NAA (Table 2 Fig. 1c), variable numbers of shoots were recorded on all culture media. Maximum number of 25.6 shoots per explant was recorded on MS medium containing 1.0 mgl^{-1} BA in combination with 0.1 mgl^{-1} NAA, the highest shoot length and plant weight 5.5 cm and 12.9 g were determined on MS medium containing 1.0 mgl^{-1} BA in combination with 0.05 mgl^{-1} NAA. The highest number of leaves was 64.9 leaves per explant was recorded on MS medium containing 1.0 mgl^{-1} BA in combination with 0.1 mgl^{-1} NAA (Table 2, Fig. 1c).

Rooting and ex vitro acclimatization

The in vitro raised multiple shoots were excised and transferred individually to MS medium supplemented with varied concentrations of the root inducing auxins; IBA, NAA and IAA. IAA was found to be more effective than NAA and IBA in inducing robust roots in shoot cultures. Of the concentrations tested, IAA (0.2 mgl^{-1}) resulted in the maximum number of roots as well as highest root length (11.0 root/explants and 2.4 cm respectively) (Table 3). Further increase in the concentration of IAA had no effect on the number of roots (Table 3). On contrary, the enhanced level of NAA had shown a stimulating effect on the total number of roots of *Silybum marianum* (Table 3). The success of any in vitro regeneration protocol largely depends on the survival and growth performance of the propagated plantlets ex vitro (Joshi and Dhar 2003). In the present study, the acclimation procedures applied was successful using the previously mentioned experimental conditions. In vitro regenerated plantlets showed 100 % survival when transferred to soil (Fig. 1d). In vitro-derived plants did not display any phenotypic variation during subsequent vegetative development.

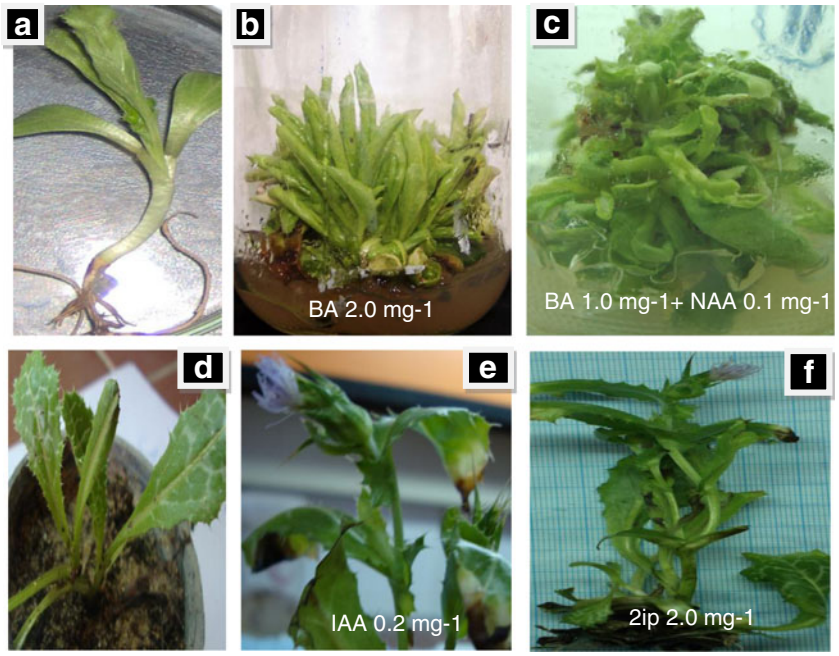

Fig. 1 In vitro culture of *Silybum marianum*. **a** germinated seed, **b** and **c** multiple shoots generated from shoot tip explant, **d** plants in greenhouse after 8 weeks of acclimatization and **e** and **f** in vitro flowering

In vitro flowering

Flowering was considered to be a complex process regulated by both internal and external factors; its induction under in vitro culture conditions is extensively rare (Stephen and Jayabalan 1998). In vitro flowering was observed on MS medium containing 2iP and BA in multiplication stage (Fig. 1f) and MS with the IAA, IBA and NAA in the rooting (Fig. 1e). The flowering plants recorded 20 % and 40 % of the plants in the multiplication and rooting stages respectively (data not shown). Flowers produced from tissue culture systems presented normal morphological aspects, the flowers were different in size, had normal petals and sepals, and open (Fig. 1e and f).

Results for the HPLC method

A new single, isocratic, selective reversed phase- liquid chromatographic method has been developed for quantification of the main constituents of SM in the extracts of different stages of the in vitro cultures of *Silybum marianum*. The method allowed good separation and quantification of the main constituent within 18 min. The average retention times ± standard deviation for TX, SC, SD, SN (A), SN (B), IS (A) and IS (B) were found to be 3.86±0.02, 4.88±0.04, 5.93±0.04, 10.95±0.05, 12.36±0.03, 16.10±0.04 and 17.75±0.03 min., respectively. The selectivity of the RP-HPLC is illustrated in (Figs. 2 and 3), where, there was a good separation of different SM components from the other components of the extract at different manipulations of

Table 1 Effects of BA, Kin and 2iP concentrations on multiple shoot induction of *Silybum marianum* L.

Growth regulator (mg/L)			No. of shoots/explant	Length of the longest shoot (cm)	Explant fresh weight (g)	No. of leaves/explant
BA	2iP	Kin				
0.0	0.0	0.0	1.0d	8.0abc	9.6a	11.5bc
0.5	0.0	0.0	3.8bc	5.0c	5.3a	24b
1.0	0.0	0.0	4.6ab	4.0c	6.8a	47a
2.0	0.0	0.0	6.7a	4.5c	11.6 a	39.6a
0.0	0.5	0.0	1.8 cd	10.6a	11.8 a	8.6c
0.0	1.0	0.0	1.0d	8.0abc	10.7 a	11.0bc
0.0	2.0	0.0	1.3d	8.8ab	8.5 a	15.0bc
0.0	0.0	0.5	1.1d	7.5bc	9.4 a	14.3bc
0.0	0.0	1.0	1.0d	7.1bc	7.0 a	15.4bc
0.0	0.0	2.0	1.0d	9.3ab	9.5 a	13.8bc

*Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test

Table 2 Effects of BA and NAA concentrations on multiple shoot induction of *Silybum marianum* L.

Growth regulator (mg/L)		No. of shoots/explant	Length of the longest shoot (cm)	Explant fresh weight (g)	No. of leaves/explant
BA	NAA				
0.0	0.0	1.0c	5.4a	3.8d	14.3d
1.0	0.0	11.2b	5.1a	7.9bc	41.7abc
2.0	0.0	13.8b	4.6ab	10.9ab	55.4a
4.0	0.0	15.5b	4.1ab	6.6 cd	43.3abc
1.0	0.05	13.7b	5.5a	12.9a	50.7ab
2.0	0.05	16.3b	4.7ab	8.9abc	40.7abc
4.0	0.05	9.9b	3.4b	6.6 cd	28.1 cd
1.0	0.1	25.6a	4.9a	7.3bc	64.9a
2.0	0.1	14.6b	4.6ab	8.3bc	34.1bcd
4.0	0.1	12.1b	4.4ab	7.1bcd	33.6bcd

*Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test

the experiment. The HPLC method was selective for SM components. It was able to detect SM components in the complex natural extract with minimal interference with other compounds in the extract. Peak purity was confirmed by comparing the response of SM components at two different wavelengths over time [288 nm (UV maximum) and 315 nm]. Only one sharp peak for each component appeared on both wavelengths. The absorbance ratio in which dividing the slice area at wavelength 288 by the slice area of wavelength 350 was found to be constant throughout all corresponding slices of the peaks (Drouen et al. 1984; Law and Das 1987; Reijenga et al. 1983).

Effect of biotic and abiotic elicitation on plant growth and silymarin accumulation

The effect of NaCl, quercetin, gamma irradiation, fungal elicitation on silymarin accumulation in *Silybum marianum* has not been previously reported. From the primary results, the shoot tips cultured on 2iP as cytokinin was able to accumulate silymarin flavonoids (Table 4). The work was continued using the medium supplemented with 2iP as cytokinin. It was obvious that the number of shoots/explants and shoot length increased with increasing NaCl and quercetin concentrations, in addition, increasing NaCl and quercetin levels had a negative effect on the number of leaves/explants and explant fresh weight (Fig. 2a and b). Doubling the NaCl concentration in the medium had a positive effect on total silymarin concentration to reach 1.808 % dry weight (Table 4). The number of leaves, number of leaves/explant and plant height decreased with increasing gamma irradiations and fungal applications (Fig. 2c and d). The highest total silymarin concentration was found to be 6.598 % dry weight in the in vitro regenerated shoot tip explants when exposed to (10 Gy) gamma-irradiation dose (Table 4). The response of *Silybum marianum* cultures towards the different elicitors was presented in Table 4.

Discussion

Although cell cultures of *S. marianum* are capable of producing silymarin, amounts produced are lower than those

Table 3 Effect of different concentrations of IAA, IBA and NAA on number of roots, root length, Plant height Fresh weight and number of leaves of *Silybum marianum* L.

Growth regulators			No. of root/explant	Length of the longest root (cm)	Plant height (cm)	Plant fresh weight (g)	No. of leaves/explant
IAA	IBA	NAA					
0.0	0.0	0.0	1.8b	1.6ab	6.5ab	2.0b	22.6ab
0.1	0.0	0.0	3.9b	1.6ab	4.9b	1.9bcd	28.2a
0.2	0.0	0.0	11.0a	2.4a	8.7a	4.5a	23.8ab
0.4	0.0	0.0	3.7b	1.25ab	8.2a	4.2a	25.5ab
0.0	0.1	0.0	0.0b	0.0b	0.0d	0.0d	0.0c
0.0	0.2	0.0	0.0b	0.0b	3.5bcd	0.9bcd	8.5bc
0.0	0.4	0.0	0.0b	0.0b	7.8bc	0.8bcd	11.5bc
0.0	0.0	0.1	0.0b	0.0b	0.0 cd	0.0 cd	0.0c
0.0	0.0	0.2	0.0b	0.0b	4.3b	1.9bc	27.7a
0.0	0.0	0.4	1.7b	2.3a	5.3b	1.8bcd	22.1ab

Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test

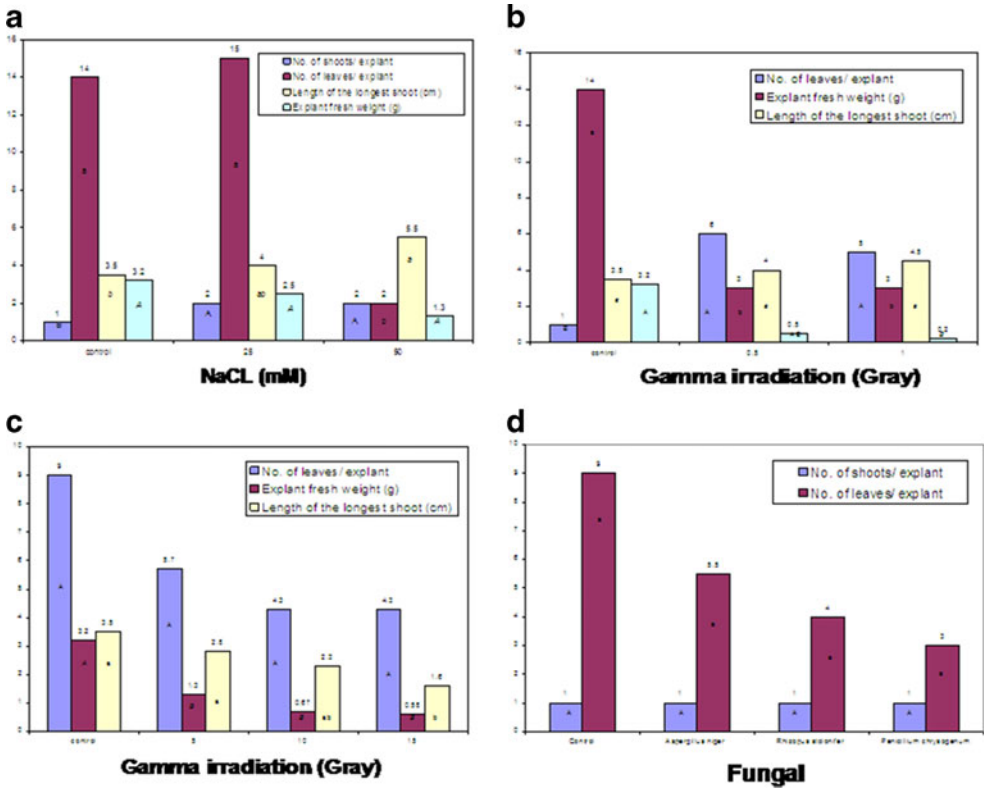

Fig. 2 Effect of NaCl (**a**), quercetin (**b**), gamma irradiation (**c**) and fungal (**d**) concentrations on multiple shoot induction of *Silybum marianum* L.

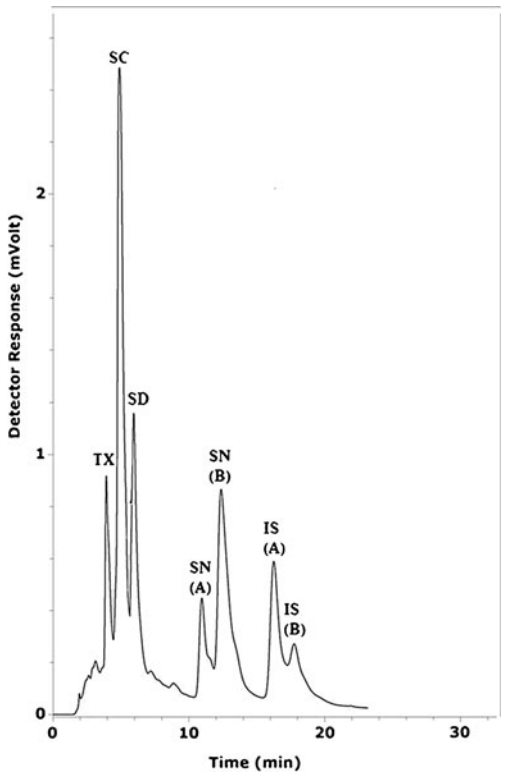

Fig. 3 HPLC chromatogram of organic extract of *Silybum marianum* exposed to (10) Gray gamma-irradiation dose showing mixture of flavonolignans, Taxofillin (TX), silychristin (SC), silydianin (SD), silybin A (SN B), silybin B (SN B), and isosilybin A (IS A) and isosilybin B (IS B)

produced in the field grown plants (Abbasi et al. 2010a, b). The increasing worldwide demand for silymarin is endangering the sparse populations of *S. marianum* in the Mediterranean region (Ahmad et al. 2008). Moreover, knowledge of yield and chemical composition is limited (Khan et al. 2009). The present study was directed towards medium components and conditions alterations wishing better silymarin yield. From the above results it was obvious that the capacity of shoot bud differentiation and shoot proliferation from shoot tip explants of *Silybum marianum* depended on hormonal variation. There was good shoot bud induction and proliferation response only in the presence of cytokinins and auxins, while no response was recorded in case of the basal medium alone. Similar results were well documented in several medicinal plants (Pattnaik and Chand 1996; Sharon and Marie 2000; Verma and Kant 1996 and Deka et al. 1999). In response to silymarin accumulation, 2iP as cytokinin was the phytohormone able to accumulate silymarin flavonoids, as a consequence it was the hormone of choice to proceed with in the later steps of the experiment. There was high percentage of flowering induction in the rooting stage which was probably due to that the differentiation to floral phase was reached its peak after two consecutive subcultures. Wang et al. (2002) stated that subculture time before flower induction could substantially affect in vitro flowering. Cytokinin is a common requirement for in vitro flowering (Scorza 1982). A number of studies reported the use of cytokinins for in vitro flowering

Table 4 The effect of different medium modifications on the % dry weight accumulation of silymarin flavonoidal content and total silymarin in the shoot tip cultures of *Silybum marianum* L.

Treatments	TX % dry wt.	SC % dry wt.	SD % dry wt.	SN (A) % dry wt.	SN (B) % dry wt.	IS (A) % dry wt.	IS (B) % dry wt.	Total sylimarin
MS	0.001±0.00005	0.006±0.0001	0.0016±0.00006	0.03±0.00015	0.015±0.00012	0.012±0.0002	0.0013±0.00005	0.0669
BA	absent	absent	absent	absent	absent	absent	absent	absent
Kin	absent	absent	absent	absent	absent	absent	absent	absent
2ip	0.004±0.00001	0.027±0.00044	0.012±0.0003	0.17±0.0009	0.18±0.0003	0.035±0.00015	0.009±0.00017	0.437
A. niger NRRL3	0.008±0.00005	0.02±0.00002	0.012±0.00014	0.46±0.00035	0.041±0.00025	0.043±0.0007	0.018±0.00025	0.602
P.chrysogenum ATCC 2273	0.013±0.0001	0.08±0.00003	0.062±0.00015	0.86±0.00024	1.65±0.0008	0.75±0.0001	0.47±0.0007	3.885
R. stolonifer NRRL1472	0.006±0.0001	0.13±0.0006	0.046±0.00034	0.25±0.0008	0.65±0.0007	0.41±0.0002	0.35±0.0005	1.842
Quercetin 0.5 mgl^{-1}	0.004±0.00005	0.03±0.00015	0.01±0.0006	0.14±0.0007	0.11±0.0006	0.045±0.0007	0.027±0.00004	0.366
Quercetin 1.0 mgl^{-1}	0.005±0.0002	0.029±0.00009	0.022±0.00056	0.13±0.0009	0.2±0.0002	0.042±0.00008	0.069±0.00007	0.497
KR-5[a]	0.006±0.00004	0.048±0.00013	0.032±0.0008	0.91±0.0002	1.21±0.0009	0.36±0.0001	0.28±0.0008	2.846
KR-10[a]	0.009±0.00005	0.077±0.00013	0.072±0.00015	0.73±0.0006	2.02±0.0009	0.98±0.0006	2.71±0.0007	6.598
KR-15[a]	0.005±0.00015	0.049±0.00016	0.012±0.0003	0.02±0.0006	0.07±0.0009	0.098±0.0007	0.063±0.0002	0.317
NaCl 25 mM	0.002±0.0007	0.039±0.00045	0.01±0.00048	0.07±0.0008	0.11±0.0009	0.095±0.0007	0.048±0.0003	0.374
NaCl 50 mM	0.004±0.0001	0.076±0.00023	0.018±0.00037	0.2±0.0001	0.51±0.0009	0.76±0.00015	0.24±0.00017	1.808
Wild flowers	0.001±0.0003	0.052±0.00045	0.01±0.0005	0.03±0.0004	0.013±0.0005	0.061±0.00012	0.039±0.0007	0.206
In vitro flowers	0.002±0.00009	0.047±0.00015	0.01±0.00035	0.24±0.00055	0.013±0.0007	0.027±0.0006	0.01±0.00015	0.349
Rooting experiment[b]	absent	absent	absent	absent	absent	absent	absent	absent

[a] (Gy) gamma-irradiation

[b] Effect of different concentrations of IAA, IBA and NAA on number of roots (Table 3)

in a number of species (Galoch et al. 2002; Pande et al. 2002; Taylor et al. 2005; Vu et al. 2006). The application of cytokinins induces molecular changes associated with the floral transition (Bernier et al. 2002). Auxins have shown opposite effects on flowering as being widely observed depending on species. Flowering plants were recorded in a ratio of 20 % and 40 % of the plants in the multiplication and rooting stages respectively using different hormonal combinations. Silymarin total concentration in the in vitro flowers was found to be 1.7 % that of the field grown flowers.

The concentrations of various secondary plant products strongly depend on the growing conditions and it is obvious that stress situations have a strong impact on the metabolic pathways responsible for the accumulation of the related natural products (Nikolova and Ivancheva 2005). Under stress, a strong oversupply of reduction equivalents is generated. In order to prevent damage by oxygen radicals, NADPH $^{+}$ H^{+} is reoxidized by photorespiration or violaxanthine cycle. Yet, the high concentration of reduction equivalents also leads to a stronger rate of synthesis of highly reduced compounds, i.e. isoprenoids, phenols or alkaloids (Selmar 2008, de Abreu and Mazzafera 2005; Ali and Abbas 2003; Gray et al. 2003, Kennedy and De Filippis 1999). In case of silymarin, medium supplementation with different concentrations of NaCl had shown significant effect on its accumulation, total silymarin concentration reach to 1.808 % % dry weight.

The resistance mechanism against diseases sometimes has a positive effect on secondary metabolism; one compound which has a central role in plant disease resistance is the flavonoid qurecetin (Terrence et al. 1994; Nakashita et al. 2002). In case of silymarin, medium supplementation with different concentrations of quercetin had shown no significant effect on its accumulation. Plants often respond to microbial infection by producing antimicrobial compounds called phytoalexins, which by its turn triggers the cellular metabolic pathways towards the production of many intracellular hormones affecting the whole secondary metabolism process. Among the supplemented fungal elicitors, *Penicilium chrysogenum* ATTC 2273 exerted the highest impact on total silymarin concentration to reach 3.885 % dry weight compared to 1.842 & 0.602 for *Aspergillus niger* NRRL 3, and *Rhizopus stolonifer* NRRL 1472 respectively. The highest total silymarin concentration was found to be 6.598 % dry weight in the in vitro regenerated shoot tip explants when exposed to (10 Gy)gamma-irradiation dose. Gamma rays belong to ionizing radiation and interact on atoms or molecules to produce free radicals in cells. These radicals can damage or modify important components of plant cells and have been reported to affect differentially the morphology, anatomy, biochemistry and physiology of plants depending on the irradiation level (Kim et al. 2004; Wi et al. 2005; El Sherif et al. 2011). This study builds a foundation for optimization of medium components and conditions as a first step towards further research for commercialization.

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