

## IMPROVEMENT OF SILYMARIN CONTENT IN CELL CULTURES OF *Silybum marianum* BY COPPER SULPHATE ELICITOR

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### ABSTRACT

*Silybum marianum* L. (Milk thistle) extracts are the main source of silymarin that is a mixture of various flavonolignan (silybin (silibinin), silydianin and silychristin). Silymarin of milk thistle has a hepatoprotective activity for liver cirrhosis and chronic inflammatory. *Silybum marianum* regeneration from hypocotyl explants and evaluation of their callogenesis, growth and total flavonolignan (silymarin) upon copper sulphate (as abiotic elicitor) elicitation was targeted. Copper sulphate (CuSO<sub>4</sub>) was applied in concentrations of 0, 3, 5, 7 and 9 μM to elicit the silymarin production in cultures. The elicitation periods used in this study were 2, 4, 7, 14 and 28 days. Half-strength MS medium recorded better results relative to full-strength MS one and seed incubation in the darkness at room temperature resulted in rapid germination and reached to the gar lid after 10 days. Callus fresh and dry weights as well as growth index were gradually increased with increasing the copper sulphate concentration till 5 μM while decreased thereafter at any elicitation period. With the increase of the elicitation period, the increase of the previous parameters was observed. Flavonolignan (silymarin) was positively correlated with CuSO<sub>4</sub> levels since all levels of copper sulphate significantly enhanced its content in relative to the control. Additionally, more silymarin was accumulated after 4 or 7 days and the accumulation significantly decreased when the elicitation period reached 14 days more. The highest silymarin (flavolignan) content (11.79 and 11.67 mg g<sup>-1</sup> DW) was obtained when 5 or 7 μM copper sulphate levels were combined with 4 days elicitation period, being about five-fold of the control.

**Key words:** *in vitro* culture, milk thistle, flavolignans, elicitation, spectrophotometric assay

### INTRODUCTION

Recently the way for a wider acceptance of plant-based medicines has been paved worldwide. Although plants as a source of bi-products have been used as medicines to treat human health diseases, the consistency and quality of these natural products are among the challenges that face the plant use in medicines [Jain and Saxena 2009]. *Silybum marianum* L. (milk thistle) belongs to Asteraceae family and is native to a narrow

area of the Mediterranean, but has been grown for centuries throughout Europe and also naturally grows in India, China, Africa and Australia [Groves and Kaye 1989]. Although various parts of this plant contain silymarin, the highest silymarin content (1.5 to 3.0 %) has been observed in seeds. Silymarin consists of a mixture of some flavonolignans, like silybin (silibinin), silydianin, and silychristin [Wichtl 2004].

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The great importance of milk thistle silymarin for medical applications results from a hepatoprotectant effect against several liver diseases such as liver chronic inflammatory [Kvasnicka et al. 2003]. Furthermore, the importance of silymarin as an anticancer drug [Davis-Searles et al. 2005, Gažák et al. 2007, Jung et al. 2009, Kim et al. 2009] and a strong antioxidant [Sánchez-Sampedro et al. 2005, Singh and Agarwal 2009, Shaker et al. 2010] has been reported.

Unfortunately, traditional cultivation of *Silybum marianum* plants meets several agricultural problems that reduce the total yield because leaves have spiny margins and the flowers are also spiny and consequently, cultural practices manipulation during growth is very difficult especially during harvest. Furthermore, using the combine machine causes crop yield damage that reaches to 40% loss during harvest stage [Hammouda et al. 1993]. Although secondary metabolites can be synthesized by chemical and microbial methods, the cost is very expensive in most cases. Otherwise, synthetic pharmaceutical metabolites may cause side effects [Luper 1998]. The promoting commercial importance of bi-products led to a great interest in using the technology of cell culture for altering the bioactive metabolites production [Vijaya et al. 2010]. Consequently, cell culture may introduce continuous source of those pharmaceutical metabolites that could be extracted on large scale by this technology. Additionally, *in vitro* culture system may be useful to overcome the previously mentioned constraints. Therefore, *in vitro* cultured cells of *S. marianum* may offer an alternative and renewable source for obtaining silymarin as a valuable natural product.

It has been accepted that milk thistle seeds were used for *in vitro* culture and posteriorly for tissue culture studies as starting material [Bekheet et al. 2013]. Otherwise, *in vitro* cultures were established from different explants include hypocotyl [Manaf et al. 2009], leaf [Abbasi et al. 2010] and cotyledon segments [Pourjabar et al. 2012]. In this regard, El Sherif et al. [2013] revealed that the germination of milk thistle seeds reached to 90% after four weeks on half-strength MS medium. *Silybum marianum* seeds incubation in darkness for two weeks and transference to 16 h light and 8 h dark positively improved the frequency of seed germination [Khan et al. 2013].

Different strategies using an *in vitro* technique have been investigated for enhancing the secondary metabolites accumulation. In order to produce silymarin in concentrations high enough for commercial manufacturing, many strategies have been followed to stimulate its productivity in cultured cells. These approaches include changes in media composition [Cacho et al. 1999], treatment with elicitors [Sánchez-Sampedro et al. 2005], calcium deprivation [Sánchez-Sampedro et al. 2009], addition of precursor [Tumova et al. 2006] and morphological differentiation. Elicitor application, which is the target of this study, has been believed as one of the most important methods for improving the secondary metabolites synthesis in medicinal plants. The accumulation of those secondary metabolites often exists in plants grown under stress conditions including several elicitors [Patel and Krishnamurthy 2013]. Furthermore, elicitors are known to qualitatively and quantitatively improve the accumulation of secondary metabolites due to higher induction of enzymatic pathways that responsible for phytochemicals synthesis [Karadi et al. 2007]. Information concerning the mechanism(s) of elicitation is required to exploit it on an industrial scale [Radman et al. 2003, Namdeo 2007].

Copper sulphate use, as an elicitor, for the improvement of secondary metabolites in various medicinal plants has been reported. Application of copper sulphate enhanced the betacyanin accumulation in suspension-cultured cells of portulaca [Bhuiyan and Adachi 2003]. The flavonoid production in suspension culture of *Trifolium pratense* L. was maximized due to copper sulphate treatment in relative to the control [Kasparova et al. 2007]. In the same direction, the flavonoid production in cell cultures of *Digitalis lanata* was improved and reached over 10 times more as compared to the control as a result of copper sulphate treatment as an abiotic elicitor [Bota and Deliu 2011]. Callus proliferation of date palm was significantly enhanced due to copper sulphate elicitor in the medium and shoot regeneration was also improved. In addition, shoot number was increased per explant and the total phenol content was higher compared with the control [AL-Mayahi 2014].

Actually, many reports concerning the effect of various abiotic elicitors on silymarin content of *Silybum marianum* *in vitro* culture have been published

[Sánchez-Sampedro et al. 2005, 2009, Madrid and Corchete 2010, El Sherif et al. 2013] however, to our knowledge; the current is the first study concerning copper sulphate elicitation on silymarin induction in vitro. Therefore, this trial aimed to study the influence of various concentrations of copper sulphate as an abiotic elicitor on callus growth and biosynthesis of silymarin in callus cultures of *Silybum marianum* after different elicitation periods.

## MATERIALS AND METHODS

**Plant materials and disinfection.** This experiment was carried out at the biotechnology laboratory, Faculty of Science, Taif University, during 2016–2017 years. The milk thistle fruits were collected from the botanical garden of Faculty of Science and seeds were exhaustively washed with tap water and pre-disinfected by soaking in 70% ethanol for 1 min, then in the laminar air flow cabinet, the seeds were sterilized by soaking in 30% Chlorox plus drops of a detergent (Tween 80) for 30 m.

**The preliminary experiments.** A preliminary experiment was conducted before each stage to select the suitable application during the tissue culture process. Factors which found ineffective or have inhibitory effect on growth, had been eliminated from the study, and the results of each preliminary experiment were considered.

**Establishment of non-contaminated cultures.** After aseptically removing the yellowish projecting apex, the remaining fruits (pericarp plus seed) were germinated on either half-strength or full-strength Murashige and Skoog (MS) medium [Murashige and Skoog 1962] (Duchefa, Haarlem, The Netherlands) supplemented with 0.8% (v/v) agar and the pH was adjusted to 5.7–5.8 with either 1N KOH or 1N HCl before agar addition. The media were autoclaved at 121°C at 1.1 kg cm<sup>-2</sup> for 20 min.

**Incubation conditions for *in vitro* cultures.** Half of jars were incubated in the incubator at 25 ±1°C and 16 h light and 8 h dark photoperiod. Illumination intensity: 1500 μ m<sup>-2</sup> s<sup>-1</sup> from fluorescent lamps while the rest of jars were incubated at room temperature (21 ±2°C) in the darkness.

**Elicitation with copper sulphate (CuSO<sub>4</sub>).** After following the previous procedures of disinfection and establishment, hypocotyl segments obtained from 10-day-old seedlings grown on half-strength Murashige and Skoog (MS) medium [Madrid and Corchete 2010, Mishra et al. 2013] incubated in the darkness at room temperature (according to the preliminary experiment) were cultured in 200 ml jars containing 25 ml of MS medium supplemented with 3% v/v sucrose, solidified with 0.8% agar, and supplemented with 1 mg cm<sup>-3</sup> 2,4-D and 0.5 mg cm<sup>-3</sup> of BA [Sánchez-Sampedro et al. 2005]. The obtained callus from this medium was subcultured on the same medium every four weeks. The three months old callus was divided and used for the elicitation experiment. Two and half grams of fresh weight of calli that was produced on the previous medium were cultured in 200 mL jars containing the callus induction medium supplemented with different concentrations of CuSO<sub>4</sub> (0, 3, 5, 7 and 9 μM). Cultures were incubated in the darkness at 25 ±1°C in the incubator for 2, 4, 7, 14 and 28 days. Callus was cultured with 2 g of callus and each treatment contained five jars. After the desired period of time, 5 jars were taken to estimate the fresh weight, dry weight, growth index and flavonolignan content.

**Growth estimation of callus tissue.** At the end of the cultivation period, callus was removed from each jar and weighted accurately on a piece of pre-weighted aluminum foil. After that, callus was dried in the hot air oven at 40°C until constant weight. Dried calli were accurately weighted to estimate the dry weight. Callus growth index was determined as:

$$\frac{\text{Final fresh weight} - \text{Initial fresh weight}}{\text{Initial fresh weight}}$$

**Extraction and determination of flavonolignans (Silymarin).** The silymarin was extracted as described by [Cacho et al. 1999]. Briefly, 0.1 g of dried callus was taken and homogenized with 15 ml of 80% v/v methanol in a mortar. The homogenate was extracted at 40°C in a water bath for 8 hours. The extract was filtered through filter paper and the filtrate was left at room temperature. The dry residue was resuspended in 3 ml of distilled water, extracted twice with 6 ml of

pure ethyl acetate, filtered and left at room temperature to dryness. The remaining material was dissolved in 5 mL methanol and kept for silymarin determination. Spectrophotometer was used for silymarin determination at 287 nm according to the method described by Meghreji Moin et al. [2010] and the values were presented as  $\text{mg g}^{-1}$  DW.

**Statistical analysis.** The treatments were arranged in Complete Randomized Block Design with five replicates. The experiment was repeated twice and observations were averaged within the experimental unit. MSTAT program, USA was used for statistical analysis of data obtained. The analysis of variance (ANOVA) was performed to compare means. Means were separated using LSD test at a significance level of 0.05.

## RESULTS

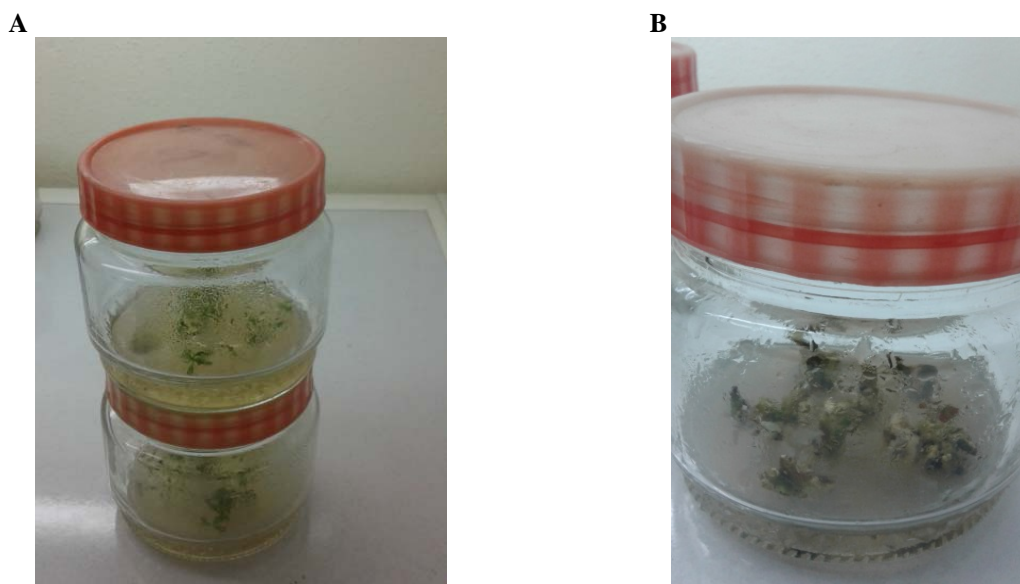
### Establishment of non-contaminated cultures.

Seeds were cultured on half-strength MS medium supplemented with 1.5% v/v sucrose or full-strength MS medium supplemented with 3% W/V sucrose. The incubation was carried out either at  $25 \pm 1^\circ\text{C}$  with

16 hours light and 8 hours dark or at room temperature ( $21 \pm 2^\circ\text{C}$ ) in the darkness. The results showed that half-strength MS medium was better than full-strength MS one. Additionally, seeds incubated at room temperature in the darkness on each media germinated rapidly and biomass reached the lid of the jar after 10 days (fig. 1). Additionally, hypocotyl segments cultured on free growth regulators MS medium produced roots and the explants became brown. However, the explants cultured on MS media supplemented with  $1 \text{ mg l}^{-1}$  2,4-D in combination with  $0.5 \text{ mg l}^{-1}$  BA did not initiate root growth and produced big creamy friable callus on every part of the explants especially on the cut ends.

**Effect of copper sulphate elicitation on callus fresh and dry weights.** Data presented in Tables 1 and 2 clearly indicate that both concentration and period of copper sulphate elicitation significantly affected the fresh and dry weight of *Silimum marianum* callus.

The dry weight results followed the same pattern as fresh weight. Increasing copper sulphate concentration gradually increased the fresh and dry weight till  $5 \mu\text{M}$



**Fig. 1.** Germination of seeds on half-Strength MS medium with 1.5% W/V sucrose incubated at room temperature in dark (A) or on full-Strength MS media with 3% W/V sucrose incubated at  $25 \pm 1^\circ\text{C}$  with 16 h light and 8 h dark (B)

**Table 1.** Influence of concentration as well as period of copper sulphate (CuSO<sub>4</sub>) elicitation and their interaction on fresh weight of *Silybum marianum* callus cultures

CuSO <sub>4</sub> concentrations (μM)	Callus fresh weight (g)					mean
	elicitation period (days)					
	2	4	7	14	28	
0.0	2.50	2.52	2.93	3.42	4.82	3.24
3.0	2.51	2.54	3.05	3.57	4.96	3.33
5.0	2.53	2.89	3.56	4.98	5.88	3.87
7.0	2.53	2.87	3.41	3.87	5.41	3.62
9.0	2.52	2.81	3.22	3.69	4.76	3.40
Mean	2.52	2.73	3.23	3.81	5.17	
LSD at 0.05						
For concentrations	0.23					
For periods	0.21					
For interaction	0.27					

**Table 2.** Influence of concentration as well as period of copper sulphate (CuSO<sub>4</sub>) elicitation and their interaction on dry weight of *Silybum marianum* callus cultures

CuSO <sub>4</sub> concentrations (μM)	Callus dry weight (g)					mean
	elicitation period (days)					
	2	4	7	14	28	
0.0	0.19	0.20	0.24	0.29	0.37	0.26
3.0	0.19	0.22	0.26	0.33	0.39	0.28
5.0	0.23	0.27	0.32	0.39	0.43	0.33
7.0	0.22	0.26	0.31	0.37	0.40	0.31
9.0	0.21	0.24	0.29	0.36	0.38	0.30
Mean	0.21	0.24	0.28	0.35	0.39	
LSD at 0.05						
For concentrations	0.013					
For periods	0.011					
For interaction	0.016					

and decreased thereafter at any elicitation period since the highest fresh and dry weight were observed at this concentration. However, the lowest values were obtained with copper sulphate free medium. Statistical analysis of results clearly shows that there were significant differences in FW and DW between the elicitation periods. The results showed also that there was a positive correlation between the elicitation period and callus fresh (5.17 g) and dry (0.39 g) weights. Cultures elicited with copper sulphate for 28 days resulted in the highest fresh and dry weights while the lowest values were obtained with the cultures were treated with the elicitor for 2 days (2.52 g FW

and 0.21 g DW). Concerning the interaction between concentration and period of copper sulphate elicitation, the maximum fresh and dry weight were recorded when the medium was supplemented with 5 μM copper sulphate after 28 days (5.88 g FW and 0.43 DW); however the minimum values in this respect were obtained with the media supplemented with elicitor at any concentration at day 2 since there were significant differences among concentrations in the second day.

**Effect of copper sulphate elicitation on growth index of *S. marianum* callus.** The growth index was gradually increased with increasing the copper sul-

phate concentration however it decreased when levels higher than 5  $\mu\text{M}$  were applied (tab. 3). Meanwhile, the lowest growth index was recorded in the control. Elicitation period with copper sulphate also affected the callus growth index and the differences among treatments were significant. With the increase of the elicitation period, the increase of the callus growth index was obtained. The cultures elicited with copper sulphate for 28 days resulted in the highest growth index however, the lowest values in this respect were observed for 2 days elicitation period (tab. 3). When elicitor levels were combined with elicitation periods the increment was very clear and higher growth index was recorded. The medium supplemented with 5  $\mu\text{M}$  copper sulphate after 28 days resulted in the highest

growth index of callus (13.2%) while the lowest value in this respect was obtained by applying elicitor at any concentration for 2 days.

**Effect of copper sulphate elicitation on flavonolignan concentration in *S. marianum* callus culture.**

The obtained results clearly show that the copper sulphate elicitation treatment had a positive effect on flavonolignan accumulation in *Silybum marianum* callus (tab. 4).  $\text{CuSO}_4$  applied in all levels significantly increased the flavonolignan content compared to the control. There were significant differences among copper sulphate treatments as well. The highest flavolignan content was observed when copper sulphate was applied at 5  $\mu\text{M}$  (10.85  $\text{mg g}^{-1}$  DW) followed by the 7  $\mu\text{M}$  level (10.17  $\text{mg g}^{-1}$  DW).

**Table 3.** Influence of concentration as well as period of copper sulphate ( $\text{CuSO}_4$ ) elicitation and their interaction on growth index of *Silybum marianum* callus cultures

CuSO <sub>4</sub> concentrations ( $\mu\text{M}$ )	Growth index					mean
	elicitation period (days)					
	2	4	7	14	28	
0.0	0.00	0.008	0.172	0.368	0.928	0.369
3.0	0.00	0.012	0.215	0.422	0.976	0.406
5.0	0.00	0.142	0.407	0.968	1.324	0.710
7.0	0.00	0.134	0.348	0.530	1.138	0.538
9.0	0.00	0.115	0.278	0.464	0.889	0.437
Mean	0.00	0.082	0.284	0.550	1.051	
LSD at 0.05						
For concentrations	0.056					
For periods	0.034					
For interaction	0.086					

**Table 4.** Influence of concentration as well as period of copper sulphate ( $\text{CuSO}_4$ ) elicitation and their interaction on flavonolignan content ( $\text{mg g}^{-1}$  DW) of *Silybum marianum* callus cultures

CuSO <sub>4</sub> concentrations ( $\mu\text{M}$ )	Flavonolignan content ( $\text{mg g}^{-1}$ DW)					mean
	elicitation period (days)					
	2	4	7	14	28	
0.0	2.43	2.62	2.57	2.52	2.33	2.49
3.0	8.56	10.36	9.77	8.38	8.13	9.04
5.0	11.24	11.79	10.64	10.60	9.98	10.85
7.0	11.22	11.67	9.54	9.33	9.11	10.17
9.0	10.97	11.13	9.88	9.26	8.66	9.98
Mean	8.88	9.51	8.48	8.01	7.64	
LSD at 0.05						
For concentrations	0.47					
For periods	0.42					
For interaction	0.54					

Otherwise, the lowest value of flavolignan was recorded on the elicitor free medium. Not only elicitor concentration but also elicitation period affected the flavonolignan content in callus. More flavonolignan was accumulated at day 4 and the accumulation was significantly decreased when the elicitation period was reached 14 days or more. Elicitation period of 4 days maximized the flavolignan content however the minimum content was observed after 28 days of elicitation period. Additionally, the combination between elicitor concentrations and periods also influenced the flavolignan accumulation in callus. The highest values in this respect were obtained when 5 or 7  $\mu\text{M}$  levels combined with 4 days elicitation period were used. On the other hand elicitor free media at any elicitation period minimized the flavonolignan content without significant differences among them.

## DISCUSSION

The target of the present study was to investigate the effect of copper sulphate elicitation on callus growth and silymarin accumulation in culture of *Silybum marianum*. At the beginning the establishment of non-contaminated cultures was investigated and the results showed that half-strength MS medium was better than full-strength one. These results may be ascribed to the osmotic pressure of the full-strength MS medium. In accordance with current results, Mishra et al. [2013] reported that seed germination of *Pterocarpus marsupium* inoculated on half strength MS medium in horizontal orientation was significantly higher than on media of other strengths. The results of incubation conditions for in vitro cultures indicated that neither the seeds cultured on the full-strength MS medium nor those cultured on half-strength one germinated during the first two weeks when incubated at  $25 \pm 1^\circ\text{C}$  with 16 hours light and 8 hours dark. These results suggest that *S. marianum* seeds did not need light during the germination period and that light inhibited the seed germination during the first days of germination. In this regard, Khan et al. [2013] found that seed incubation in complete darkness for 2 weeks significantly enhanced

seed germination frequency in *S. marianum*. However, El Sherif et al. [2013] reported that the cultures incubated under growth room conditions  $22 \pm 2^\circ\text{C}$ , 16 h photoperiod and light intensity of  $4000 \mu\text{m}^{-2} \text{s}^{-1}$  led to 90% germination when half-strength MS medium was used after four weeks from culture. The current results are suggested to be useful at commercial scale because using half-strength MS medium supplemented with 1.5% W/V sucrose plus the incubation in the darkness at room temperature reduce the production cost.

In this experiment, enhancing callus fresh and dry weight suggested that  $\text{CuSO}_4$  has no inhibitory effect on *S. marianum* callus cultures and it may have synergistic effect on the growth of callus. These results support the others of AL-Mayahi [2014] on date palm who reported that medium application with copper sulphate enhanced shoot regeneration and improved regeneration. Moreover, a positive correlation between the elicitation period and growth index was observed. Improving the growth index due to copper sulphate elicitation is logic because of increasing the fresh weight. Increment of growth index by copper sulphate elicitation has been previously reported [Bhuiyan and Adachi 2003, Hasanloo et al. 2008, AL-Mayahi 2014].

To our knowledge, the effect of copper sulphate elicitation on silymarin content in *Silybum marianum* has not been previously reported. From the current primary results,  $\text{CuSO}_4$  is suggested to have a stimulatory effect for flavonolignan accumulation in *S. marianum* callus cultures. It has been found that elicitor concentration is an important factor that strongly influences the intensity of the response and the optimum level can be moved empirically. Additionally, increasing the metabolic enzymes activities is responsible for maximizing the accumulation of secondary metabolites [Vasconsuelo and Boland 2007]. In this study, adding  $\text{CuSO}_4$  to the culture media has increased the flavonolignan content by four to five folds relative to the control.

Silymarin content has been shown to be improved due to different abiotic elicitors. In this regard Ashtiani et al. [2010] reported that the silymarin production reached to about 30-fold that of the control due

to silver elicitation treatment for 24 hours. Karadi et al. [2007] revealed that elicitors are known to alter the secondary metabolites accumulation through the enzymatic pathways induction that are responsible for phytochemicals synthesis. Such improvement of silymarin accumulation in *in vitro* cultures of *Silybum marianum* due to various elicitors has been documented [Tumova et al. 2005, Sanchez-Sampedro et al. 2009, Madrid and Corchete 2010, Tumova et al. 2011, El Sherif et al. 2013, Firouzi et al. 2013, Roostakhiz and Saboki 2016]. Otherwise, several reports concluded that copper sulphate as an abiotic elicitor improved the accumulation of secondary metabolites in *in vitro* cultures of several plants such as *Grindelia pulchella* [Hernandez et al. 2005], *Blumbago indica* [Karadi et al. 2007], *Trifolium pretense* [Kasparova et al. 2007] and *Digitalis lanata* [Bota and Deliu 2011].

## CONCLUSION

Callus fresh and dry weights and the growth index of *Silybum marianum* in *in vitro* cultures were significantly improved due to copper sulphate elicitor treatment. Moreover, a remarkable improvement in silymarin content was observed with use of 5  $\mu$ M copper sulphate as an abiotic elicitor for 4 days as the increase was observed. The current results introduce information for medium components optimization and copper sulphate elicitation conditions and further research is required for commercialization.

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