ABSTRACT
This study was carried out to investigate the effect of drought stress on in vitro growth and silymarin content of Milk thistle. Callus was induced from leaf explants using MS medium supplemented with 5 mg/l NAA + 2 mg/l Kin + 0.1 mg/l GA3. Supplementation of culture medium with mannitol slightly depressed both fresh weight and growth value of callus. However, mannitol caused increasing of dry matter and silymarin content. Addition 40 mg/l of mannitol to culture medium was the best concentration for total silymarin production. Moreover, silybin B was more responsive to osmotic stress induced by mannitol stress compared with the other measured component of silymarin. Otherwise, polyethylene glycol (PEG) caused a progressive reduction in both fresh weight and growth value. However, presence of PEG (15 %) in culture medium strongly enhanced silymarin accumulation as well as dry matter of callus. This treatment increased both silybin A and B (the most important components of silymarin) to a level more seven fold higher than that of the control.

KEYWORD: Milk thistle, mannitol, polyethylene glycol, callus, silymarin.

INTRODUCTION
Milk thistle (Silybum marianum L. Gaertn) is commonly used to treat liver diseases. The biological and pharmacological properties of Milk thistle are attributed to a flavanolignan complex, silymarin, which was first isolated from the fruits (achenes), (sometimes mistakenly called seeds) in 1968.1,2 Nowadays, Milk thistle has been commercially cultivated for the production of silymarin which has been used to treat alcoholic liver disease, acute and
chronic viral hepatitis and toxin-induced liver diseases. Silymarin also inhibits hepatitis C virus (HCV) infection and also displays antioxidant, anti-inflammatory, and immunomodulatory actions that contribute to its hepatoprotective effects. Moreover, silymarin is a promising agent for cancer prevention, adjuvant cancer treatment, and reduction of iatrogenic toxicity. The principal components of silymarin are silybin A, silybin B, isosilybin A, isosilybin B, silychristin A, silychristin B and silydianin. The silymarin content in seeds depends on the cultivated genotypes and environmental conditions. It was found that silymarin from native ecotypes had lower quantities of silybinin as compared to that of silymarin from cultivated ones, but they had higher amounts of other compounds such as silychristin, silydianin and isosilybinin.

Although, secondary metabolites of plants are fundamentally produced by genetic processing, their biosynthesis is strongly influenced by environmental factors. Abiotic stresses exert a considerable influence on the production of several secondary metabolites in plants. In this respect, drought is one of the most important abiotic stress factors affecting plant growth and leaf photosynthesis and altering biochemical properties of plants. Abundant studies have been proven that the content of secondary metabolites including cyanogenic glycosides, terpenoids, alkaloids, flavonoids and organic acids in various plant tissues rise under drought stresses. On the other hand, some of the medicinal compounds localized in morphologically specialized tissues or organs of native plants have been produced in culture systems not only by inducing specific organized cultures, but also by undifferentiated cell cultures. Callus grown in vitro has the potential to show secondary metabolites activity and can often be compared with the original plant. In this respect, silymarin synthesis in cell and tissue cultures of Milk thistle has been reported. Nonetheless, silymarin levels in in-vitro Milk thistle cultures are much lower than those detected in the seeds. Therefore, efforts should be made to develop high yielding clones with elevated silymarin level. Generally, improvement of phytopharmaceutics biosynthesis by in vitro cultured plant cells can be achieved by subjecting the cultured plant cells to stress factors. In this regard, the yeast extract and methyl jasmonate (jasmonic acid derivatives), strongly promoted the accumulation of silymarin cell cultures of Milk thistle. Hasanloo et al. reported that media supplemented with or jasmonis acid in is be useful for production of silymarin in cell suspension cultures of Milk thistle. Otherwise, Khalili et al. 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. The present work aimed to study
the growth and silymarin content of Milk thistle callus cultures affected by mannitol and polyethylene glycol.

MATERIALS and METHODS

Establishment of in vitro cultures

Seeds of Milk thistle were washed with distilled water and then surface sterilization went through rinsing seeds in 70% ethanol for 1 min followed by 50% commercial Clorox (containing 5.25% sodium hypochlorite) for 20 min. The seeds were then washed three times with sterile water under laminar air-flow hood to remove all the traces of sodium hypochlorite. The disinfected seeds were placed in jars containing 50 ml of MS-basal medium. Vigorous seedling were obtained after six weeks of culturing (Fig. 1-A). Shoot tips were isolated from the aseptic grown seedlings and re-cultured on fresh medium contained 2 mg/l kinetin (kin) + 2 mg/l benzyl adenine (BA) + 0.1 mg/l indoleacetic acid (IAA) to get stock shootlet cultures (Fig 1-B). For callus induction, leaf explants were excided from in vitro grown shootlets and cultured on MS medium supplemented with 5 mg/l NAA + 2 mg/l Kin + 0.1 mg/l gibberellic acid (GA3) according to Bekheet et al. Leaf derived callus was subcultured twice onto same freshly prepared medium (callus induction medium) to obtain stock of callus cultures (Fig. 1-C).

Effect of mannitol on callus growth

To assess the effect of mannitol on callus growth of Milk thistle, about 250 mg of callus tissue were cultured on callus growth medium (MS + 5 mg/l NAA + 2 mg/l Kin + 0.1 mg/l GA3) supplemented with 20, 40 and 60 mg/l of mannitol. Every jar contained one inoculum and each treatment had ten replicates. The cultures were grown for five weeks before data recording based on fresh weight, growth value and dry matter.

\[
\text{Final fresh weight - Initial fresh weight}
\]
\[
\text{Initial fresh weight}
\]

Growth value = 

\[
\text{Dry weight}
\]
\[
\text{Fresh weight}
\]

Dry matter = 

Effect of polyethylene glycol on callus growth

In order to examine the effect of polyethylene glycol (PEG) on growth of callus derived from leaf explants of Milk thistle, three different concentrations i.e., 5, 10 and 15 g/l of PEG (400) were added to callus culture medium. Healthy callus incula (250 mg weight) were
individually grown for five weeks on the stressed medium. Fresh weight, growth value and dry matter were recorded as mentioned before.

**Culture medium and incubation conditions**
The medium was supplemented with 3 % (w/v) sucrose and 7 g/l agar and adjusted to pH 5.7, prior to autoclaving at 121 °C and 1.2–1.3 kg/cm² pressure for 20 min. The cultures were incubated under growth room conditions 25 ± 2 °C, 16 h photoperiod and light intensity of 4000 Lux provided by fluorescent lamps (Phillips TLM 40W/33RS).

**Experimental analysis**
Each experiment was set up as a separate completely randomized design and tissue culture data were analyzed using standard error (SE) according to Snedecor and Cochran.[21] However, data of silymarin determination were expressed as mean of six reading.

**Extraction of flavonolignans**
Samples of callus grown on both mannitol and PEG containing medium in addition of control (stress-free medium) were used for flavonolignans extraction. Callus samples (one g weight) were defeated by ethyl acetate. The flavonolignans were extracted from the dried residue with 10 ml of methanol at 40 °C for 8 h. The methanol solution was concentrated to a dry residue. The extract was dissolved in 1 ml of methanol and kept at 4 °C in darkness. Silymarin content was extracted according to Cacho et al.[22]

**Determination and quantification of silymarin**
The amounts of flavonolignans were determined by high performance liquid chromatography (HPLC). The elution time and flow rate were 30 min and 1 ml min⁻¹ and peaks detected at 288 nm. Identification was achieved by comparison of retention times (Rt) of standards of silybin A, silybin B, silychristin and silydianin (Sigma-Aldrich). Quantification of these metabolites, expressed in mg g⁻¹ of dry weight, was accomplished using a known concentration of standard and peak areas.

**RESULTS AND DISCUSSION**

**Callus growth and silymarin content affected by mannitol**
Mannitol, a sugar alcohol is used as an osmotic agent for *in vitro* studies in many plant species. In this experiment, three different concentrations i.e., 20, 40 and 60 g/l of mannitol were examined for their effects as osmotic stress on callus growth and silymarin content of
Milk thistle callus cultures. Data presented in Table (1) reveal that supplementation of culture medium with mannitol generally depressed callus growth (fresh weight and growth value) compared to the control (mannitol-free medium). However, dry matter values were increased as increasing of mannitol concentration. The highest value (11.50 %) of dry matter was recorded with 40 g/l mannitol containing medium. Observation of callus during the stress period allowed us to visually distinguish a first difference in size, color and texture of the calli. Calluses from unstressed media were large, nodular and white to pale yellow. As far as the mannitol concentration increased, the calluses lost their compactness and evolved into a mucilaginous texture, their size decreased. In some cases there has been a development of a compact whitish embryogenic cell mass that lead after proliferation to the formation of a secondary callus (Fig. 1-D). In concern of the effect of mannitol on silyamrin content, data obtained revealed that the measured components of silymarin (silybin A, silybin B, silychristin and silydianin) were increased in callus of Milk thistle as increasing of mannitol in culture medium till 40 g/l and then decreased (Table 2). The highest values of silybin A (0.47 mg/g DW), silybin B (0.69 mg/g DW), silychristin (0.014 mg/g DW) and silydianin (0.31 mg/g DW) were registered with medium contained 40 g/l mannitol. It was noticed that silybin B was more responsive to mannitol stress compared with the other measured component of silymarin. Treatment of Milk thistle callus culture with 40 g/l mannitol increased production of silybin B to a level about 5 fold higher than that of the control. However, silychristin component was the less responsive to mannitol stress.

The addition of mannitol to tissue culture medium decreased the water potential of the media inducing water stress that adversely affected the callus growth. Such osmoticums reduce mineral up take by cells through differences in osmotic pressures thereby retarding plant growth.\[23\] Otherwise, plant tissue cultures accumulate a large number of metabolites namely phenylpropanoids and flavonoid compounds, osmoprotectants, sugars, polyamines and antioxidant enzymes in response to drought stress.\[24,25\] In the present study, growth parameters of fresh callus were reduced with increasing drought stress levels generated by mannitol. However, the dry matter increased during the stress period. Moreover, the production of silyamrin of the mannitol treatment was higher than that of control. Similarly, Hadi et al.\[26\] reported that increasing water stress generated by increasing mannitol concentration in caused a progressive reduction in callus fresh weight of *Ruta graveolens*. Retardation in growth in tissue-cultured grown plants exposed to osmotic stress has been reported in several species both monocotyledons and dicotyledons.\[27, 28, 29, 30\] Growth
Shawky

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inhibition under osmotic conditions might be mainly due to the reduction in cytoplasmic volume and the loss of cell turgor as result of osmotic outflow of intracellular water.\[31\] Otherwise, in a study on the effect of mannitol and sodium chloride on some secondary metabolites of Fenugreek calli, Hussein and Aqlan \[32\] stated that the lower concentration of mannitol enhanced growth while the higher concentration enhanced growth, total phenolics and total flavonoid contents in comparison to the control. El-Far and Taie.\[33\] also observed that sorbitol treatment resulted in an increase in sweat potato callus phenolics and flavonoids. In this context, Solanelli et al.\[34\] suggested that sugars play a role in regulating the genes responsible for the synthesis of secondary metabolites like polyphenolics, anthocyanins and flavonoids.

**Callus growth and silymarin content affected by PEG**

Polyethylene glycol (PEG) has been the most extensively used to stimulate drought stress in plant tissue cultures. PEG is a nontoxic, nonionic polymer which is not expected to penetrate into plant cells. This experiment was conducted to investigate the effect of PEG as drought stress on the growth and silymarin content of Milk thistle callus cultures. For this purpose, healthy callus inocula were grown on medium contained three levels i.e., 5, 10 and 15 g/l of PEG. The obtained results indicate that increasing drought stress induced by PEG caused a progressive reduction in callus fresh weight and growth value (Table 3). Sharp reduction in callus weight was observed in response to 15 g/l PEG. Moreover, calli grown on PEG containing medium became darker with strong browning and necrosis (Fig 1-E). On contrary, data presented in Table (3) show gradually increasing of dry matter as increasing of PEG in culture medium. The data pointed out that the highest value (12.50 %) of dry matter was recorded with medium contained 15 g/l PEG. It was observed that callus grown on PEG containing medium turned to dark brown color. On the other hand data of silyamrin content indicate that all components had positive response to PEG. The highest values of silybin A (0.73 mg/g DW), silybin B (0.85 mg/g DW), silychristin (0.047 mg/g DW) and silydianin (0.51 mg/g DW) were recorded when 15 g/l of PEG added to culture medium (Table 4). Otherwise, silychristin component was the most responsive to presence of PEG in culture medium. It is important here to notice that enhancement of silymarin accumulation in callus cultures of Milk thistle by PEG was higher compared with mannitol.

Stress is one of the factors that enhance the production of secondary metabolites in plants. In this respect, polyethylene glycol (PEG) has been used as osmotic stress in tissue cultures of
many plant species in order to enhance their active ingredients. In this study different concentrations of PEG were subjected for the improvement of silymarin production in callus of Milk thistle as well as callus growth. The generated data reveal a substantial reduction in fresh callus growth of Milk thistle with increasing of PEG in culture medium. Whereas both dry matter and silymarin content were strongly enhanced when culture medium was amended with PEG. Similar results for direct treatment were reported by Wani et al.\[35\] in rice, who indicated that increasing PEG in the culture medium caused a decrease in callus morphogenic capacity. The effect of water stress induced by polyethylene glycol on callus growth, callus water content, callus necrosis and regeneration was investigated on four cultivars of durum wheat.\[36\] The results showed that increasing PEG concentration in the medium causes a gradual decrease in growth and water content of calluses. High concentrations of PEG caused callus necrosis as well. In concern of enhancement of secondary in response to drought, Azhar et al.\[37\] mentioned that the total phenolic contents of Desi ajwain plant increased significantly with increasing drought stress levels. There are many reports on the influence of different in vitro culture abiotic elicitation on total sylimarin yield determination in Silybum marianum cultures.\[17, 38, 16, 39, 40\] but up to our knowledge; this is the first report on the influence of drought on silymarin production in vitro.

Table 1: Fresh weight, growth value and dry matter content of callus derived from leaf explant of Milk thistle grown for five weeks on medium contained various concentrations of mannitol.

<table>
<thead>
<tr>
<th>Mannitol (g/l)</th>
<th>Fresh weight (g)</th>
<th>Growth value</th>
<th>Dry matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.25 ± 0.07</td>
<td>0.80</td>
<td>10.10</td>
</tr>
<tr>
<td>20</td>
<td>1.15 ± 0.10</td>
<td>0.78</td>
<td>10.88</td>
</tr>
<tr>
<td>40</td>
<td>1.00 ± 0.20</td>
<td>0.75</td>
<td>11.50</td>
</tr>
<tr>
<td>60</td>
<td>0.85 ± 0.17</td>
<td>0.70</td>
<td>11.00</td>
</tr>
</tbody>
</table>

± Standard error (SE).

Table 2. Effect of different concentrations of mannitol on silymarin content of Milk thistle callus cultures.

<table>
<thead>
<tr>
<th>Mannitol (g/l)</th>
<th>Silymarin (mg/g DW)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Silybin A</td>
<td>Silybin B</td>
<td>Silychristin</td>
<td>Silydianin</td>
</tr>
<tr>
<td>0.0</td>
<td>0.10</td>
<td>0.12</td>
<td>0.005</td>
<td>0.08</td>
</tr>
<tr>
<td>20</td>
<td>0.36</td>
<td>0.48</td>
<td>0.009</td>
<td>0.19</td>
</tr>
<tr>
<td>40</td>
<td>0.45</td>
<td>0.60</td>
<td>0.014</td>
<td>0.31</td>
</tr>
<tr>
<td>60</td>
<td>0.33</td>
<td>0.55</td>
<td>0.012</td>
<td>0.21</td>
</tr>
</tbody>
</table>
Table 3. Fresh weight, growth value and dry matter content of callus derived from leaf explant of Milk thistle grown for five weeks on medium contained various concentrations of polyethylene glycol.

<table>
<thead>
<tr>
<th>Polyethylene glycol (g/l)</th>
<th>Fresh weight (g)</th>
<th>Growth value</th>
<th>Dry matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.24 ± 0.04</td>
<td>0.79</td>
<td>10.00</td>
</tr>
<tr>
<td>5</td>
<td>0.95 ± 0.03</td>
<td>0.73</td>
<td>11.90</td>
</tr>
<tr>
<td>10</td>
<td>0.90 ± 0.05</td>
<td>0.72</td>
<td>12.20</td>
</tr>
<tr>
<td>15</td>
<td>0.85 ± 0.11</td>
<td>0.70</td>
<td>12.50</td>
</tr>
</tbody>
</table>

± Standard error (SE).

Table 4. Effect of different concentrations of PEG on silymarin content of Milk thistle callus cultures.

<table>
<thead>
<tr>
<th>Polyethylene glycol (g/l)</th>
<th>Silymarin (mg/g DW)</th>
<th>Silybin A</th>
<th>Silybin B</th>
<th>Silichristin</th>
<th>Silydianin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td></td>
<td>0.10</td>
<td>0.12</td>
<td>0.005</td>
<td>0.08</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.56</td>
<td>0.58</td>
<td>0.019</td>
<td>0.29</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.67</td>
<td>0.79</td>
<td>0.034</td>
<td>0.41</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>0.73</td>
<td>0.85</td>
<td>0.047</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Fig. 1: Six weeks old seedling of Milk thistle growing on MS-basal medium (A); shootlets multiplication on MS medium + 2 mg/l kin + 2 mg/l BA + 0.1 mg/l IAA (B); callus induction using MS medium + 5 mg/l NAA + 2 mg/l Kin + 0.1 mg/l GA₃ (C); callus grown on medium supplemented with 20, 40 and 60 g/l mannitol (from left to right) (D) and callus grown on medium supplemented with 5, 10 and 15 g/l Polyethylene glycol (from left to right) (E).
REFERENCES


