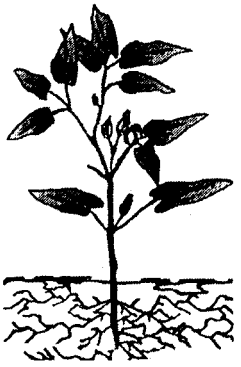

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IN VITRO CALLUS FORMATION AND PLANT REGENERATION OF *SILYBUM MARIANUM* (L.) GAERTN

[22]

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Keywords: *Silybum marianum*, Callus, Regeneration, Plant growth regulators

ABSTRACT

The present study was carried out to investigate the effect of plant growth regulators and type of explant on the callus formation and plant regeneration. Hypocotyl was the most convenient explant for callus formation. MS medium supplemented with 1 mg/l NAA + 0.1 mg/l BA showed the highest callus fresh weight in light condition (16 h/day at 6000 Lux). Direct regeneration (shoot formation) was achieved by cotyledonary node explant alone on MS medium supplemented with 2, 3, 4 mg/l BA in light condition. The best shoot formation was recorded on MS medium containing 3 mg/l BA. Root formation for regenerated shoot was superior in 1 mg/l NAA.

INTRODUCTION

The milk thistle *Silybum marianum* (L.) Gaertn is an annual or biennial plant and a member of the daisy family (Composite or Asteraceae), distributed in Eastern region (**Mossa *et al* 1987**), Mediterranean and naturalized in North and South America (**Hyam and Pankhurst, 1995**). In Egypt, it is very common at Burg El-Arab, especially along Burg El-Arab; El-hammam road, inside the cultivated fields, and along the canals (**Aly, 1998**).

Silybum marianum is recommended in traditional European and Asiatic medicine for more than 2000 years, mainly for treatment of liver disorders (**Morazzoni and Bombardelli, 1995**). It has been shown that flavonolignans inhibit leucotriene production; this inhibition explains their anti-inflammatory and antifibrotic activity (**Dehmlow *et al* 1996**). Also, silymarin may be beneficial for reducing the chances for developing certain cancers (**Katiyar *et al* 1997**) and inhibit cholesterol biosynthesis (**Krecman *et al* 1998**)

Plant tissue culture techniques have become a powerful tool for studying basic and applied problems in plant biology. Furthermore, in the last years these techniques have found wide commercial application in the propagation of plants, mainly horticulture species. Success in the technology and application of *in vitro* methods is due to a better understanding of the nutritional requirements of cultured cells and tissues (**Murashige and Skoog, 1962**). **Liu and Cai (1990)** reported that tissue culture protocols have been established for *Silybum marianum* from hypocotyl to induce callus on MS medium containing 0.8 mg/l naphthalene acetic acid (NAA) and 0.5 mg/l benzyl adenine (BA). **Hetz *et al* (1995)** isolated mesophyll protoplasts of six lines of *Silybum marianum* from young leaves. Plant regeneration experiments undertaken with the protocallus on medium containing benzyl amino purine (BAP) led to shoot formation in only two lines with regeneration frequencies of less than 1% in one and up to 7% in a second line. When the protocallus from second line were treated with thidiazuron (TDZ) in a first culture step and with BAP in a second step the shoot formation frequency rose to 22%. Shoots were rooted on hormone free MS agar medium and transferred into soil where plants grew to maturity

The objective of this work was to define the optimum conditions of *in vitro* culture and the most convenient explant for callus production and regeneration of *Silybum marianum* plant for its medical benefit.

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MATERIALS AND METHODS

Callus formation

Preparation of the explant

Achenes of *Silybum marianum* were obtained from National Research Center, Dokki, Egypt, were washed with water and sterilized by soaking in hydrogen peroxide (H_2O_2) 12% (v/v) with detergent (Tween 20) 2-3 drops/100ml for 20 min. The disinfected achenes were transferred into sterilized glass jars containing 0.7% (w/v) agar medium and placed in an incubator in the light conditions at 25°C ±1 for 7 days. Seedlings were harvested and excised hypocotyl (5 mm) and cotyledonary leaf (divided into two parts). These explants were examined for giving callus growth in order to identify the most convenient part for callus initiation.

Media

Three explants for each jar were placed on the surface of MS medium salts and vitamins **(Murashige and Skoog, 1962)**. The medium was modified by supplementation with growth regulators naphthalene acetic acid (NAA) and benzyl adenine (BA) with 16 combinations between them.

Treat.	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16
NAA	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	1.0	1.0	1.0	1.0	2.0	2.0	2.0	2.0
BA	0.0	0.1	1.0	2.0	0.0	0.1	1.0	2.0	0.0	0.1	1.0	2.0	0.0	0.1	1.0	2.0

The acidity of the media were adjusted to pH 5.6 ± 0.1 prior to adding agar (7 g/l), then media were dispensed into glass jars (100ml) containing 25 ml of tested media. Cultures were autoclaved at 121°C and 1.1Kg/cm^2 for 20 min.

Culture conditions

Cultures of all treatments were divided into two groups; the first group was maintained in light condition for 16 h/day photoperiod at intensity of 6000 Lux from white fluorescent lamps. The second group was maintained in darkness (24 h). Cultures were incubated at 25°C ±1. The experiment was repeated twice in plant tissue culture laboratory, Agric. Bot. Dept., Fac. of Agric., Ain Shams Univ. Growth was measured by fresh weight of callus after 28 days from culturing the explants.

This experiment was carried out consisting 32 treatments and five replicates for each treatment.

Plant Regeneration

I. Direct regeneration

Plant material

Achenes of *Silybum marianum* were soaking in hydrogen peroxide (H_2O_2) 12% for 20 min. The disinfected achenes were transferred into sterilized glass jars containing 0.7% (w/v) agar medium and placed in an incubator in the light conditions at 25°C ±1 for 7 days. Seedlings (7 days old) were harvested and excised hypocotyl, cotyledonary leaf (two parts) and cotyledonary node as explants. Also, foliage leaves were excised as explant (0.5 cm diameter) from 8 weeks old plants grown in the farm. These explants were used to induce direct regeneration in order to identify the most convenient organ for multiplication and plantlet initiation.

Media

MS basal medium (proliferation medium) was supplemented with different combinations of plant growth regulators to induce direct regeneration as follow

a- 0.05, 0.10 and 0.15 mg/l Indole-3- acetic acid (IAA) + 0.25, 0.5 and 0.75 mg/l Benzyl adenine (BA).
b- 0.5, 1.0, 2.0 and 5.0 mg/l NAA + 2.0 and 5.0 mg/l BA.
c- 2, 4, 5 and 10 mg/l Gibberellic acid (GA_3) + 0.1, 0.5 and 1.0 mg/l BA + 0.05, 0.1 and 1.0 mg/l 2,4-Dichlorophenoxy acetic acid (2,4-D).
d- MS solid free hormones medium for one week and transported to MS + 2, 4, 6 and 8 mg/l BA.
e- 0.25, 0.50, 0.75 and 1 mg/l Kinetin (Kin).
f- 0.1, 0.5 and 1.0 mg/l Thidiazuron (TDZ).
g- 1, 2, 3, 4 and 5 mg/l BA.

Media were adjusted to a PH of 5.7, solidified with 0.7 % (w/v) agar then media were dispensed into glass jars (100ml) containing 25 ml of tested media. Culture media were autoclaved at 121°C and 1.1 kg/cm^2 for 20 min.

Culture conditions

Cultures (five replicates for each treatment) were incubated in white light condition (16 h/day) at intensity of 6000 Lux at 25 °C ±1 for 30 days.

II. Indirect regeneration

The callus which formed in the prior step from the convenient treatment (T10) was used to induce indirect regeneration. The same MS basal medium supplemented with 0.5 mg/l BA + 0.1 mg/l NAA, 2.0 and 3.0 mg/l BA was used for one week then the callus was transported to MS solid medium free hormones. Cultures were incubated in the same condition for 30 days.

III. Somatic embryogenesis

Callus, seeds and seedling were used for embryogenesis, callus which produced from the treatment (T10) was placed in 250 ml flasks containing 50 ml liquid MS medium supplemented with 35, 40 and 45 mg/l 2,4-D for 30 days. Seeds were soaked in 0.002, 0.022, 0.221, 2.21 and 22.1 mg/l 2,4-D for 1, 2 and 3 days and cultured on MS solid free hormones medium for 30 days. Also, seedlings (7 days old) were cultured on semi-solid (0.4% w/v) agar medium with the same concentrations of 2,4-D for one day and were transported to MS solid free hormones medium for 30 days in the same condition.

Treatments which led to shoot formation were subcultured in shoot elongation MS medium supplemented with 3 mg/l GA_3 for 15 days. After that, the developed shoot were transferred to root induction MS medium supplemented with NAA 1, 2 and 3 mg/l for 7 days and were transported to free hormones MS solid medium for other 30 days. The experiments were repeated twice. Number of shoots and roots from five replicates for each treatment was recorded.

Data of these experiments were statistically analyzed using **(SAS 1996)** computer program and means were compared by LSD method according to **Snedecor and Cochran (1980).**

Plantlets acclimatization

Plantlets (6 to 8 cm in height) were washed with a flow tap water and disinfected by dipping in Benlate (fungicide) solution (1 g/l) for 15 min. The plantlets were transferred to small plastic pots (10 cm height and 10 cm diameter) containing peatmose + sand mixture (1:1) and maintained in the growth chamber. The pots were covered with plastic tunnel to maintain a high relative humidity (95%) around the plantlets, and were sprayed with water at 2-day intervals. The irrigation was applied, depending on the requirement of plantlets and a control program for pests and disease was followed. After 30 days, the plastic tunnel was removed and the plantlets were transferred to the green house.

RESULTS AND DISCUSSION

Callus Formation

1- Effect of explant type

Only hypocotyl explants recorded the best response for callus formation. The cotyledonary leaf explants showed increment in explant growth but don't formed callus. These results agree with **Liu and Cai (1990)** and **Sanchez-Sampedro *et al* (2005)**. In addition, **Mohammad and Hassan (1988)** initiated callus from sunflower hypocotyls.

2- Effect of plant growth regulator treatments

The results of incubation under light and dark conditions were illustrated in **Table (1)** and **Fig. (1)**. There was significant difference in the callus formation from hypocotyl between plant growth regulator treatments (16 combinations of NAA and BA). The treatment T10 (1 mg/l NAA + 0.1 mg/l BA) was the optimum one for growth and maintenance of friable callus than other treatments. Under this treatment (T10), light condition was higher than dark condition.

In this respect, **Liu and Cai (1990)** induced hypocotyl callus of *Silybum marianum* on MS medium containing 0.8 mg/l NAA and 0.5 mg/l BA, in addition; **Iqbal and Srivastava (2000)** cultured the leaf, shoot apex and nodal segments of *Silybum marianum* seedlings *in vivo* and *in vitro*, the explants callused within four weeks on MS medium supplemented with 0.1 mg/l NAA + 0.3 mg/l BA + 0.3 mg/l Zeatin (Zt). **Sánchez-Sampedro *et al* (2005)** formed callus from hypocotyls segments (2mm) of *Silybum marianum* cultured on MS medium supplemented with 1 mg/l 2,4-D and 0.5 mg/l BA. Callus appeared after a month of culture in darkness.

Plant Regeneration

Only cotyledonary node was achieved direct regeneration which formed multiple shoots on MS medium supplemented with different concentrations of BA in light condition. The effect of BA

Table 1. Effect of plant growth regulators treatments (mg/l) on callus fresh weight (g) from hypocotyl under light and dark conditions after 28 days incubation

T / P	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16	LSD 0.05	Mean P
Light	0.096 d	0.107 d	0.184 cd	0.068 d	0.148 cd	0.167 d	0.933 b	0.226 cd	0.385 c	3.143 a	0.143 cd	0.071 d	0.126 d	0.168 cd	0.118 d	0.111 d	0.254	0.387
Dark	0.078 f	0.537 cd	0.080 f	0.096 f	0.094 f	0.119 ef	0.751 b	0.677 bc	0.496 d	1.985 a	0.126 ef	0.256 e	0.174 ef	0.256 e	0.104 f	0.089 f	0.151	0.370
LSD (P) 0.05	N.S.	0.126	0.069	0.022	0.026	0.025	0.142	0.205	N.S.	0.748	N.S.	N.S.	N.S.	0.081	N.S.	N.S.		N.S.
Mean T	0.087 e	0.322 cd	0.132 de	0.082 e	0.121 de	0.143 de	0.842 b	0.451 c	0.441 c	2.564 a	0.134 de	0.164 de	0.150 de	0.212 de	0.111 e	0.100 e	0.203	

Means with the same letter in the same row are not significantly different
P = Photoperiod T = Treatment LSD (0.05) T x P = 0.213

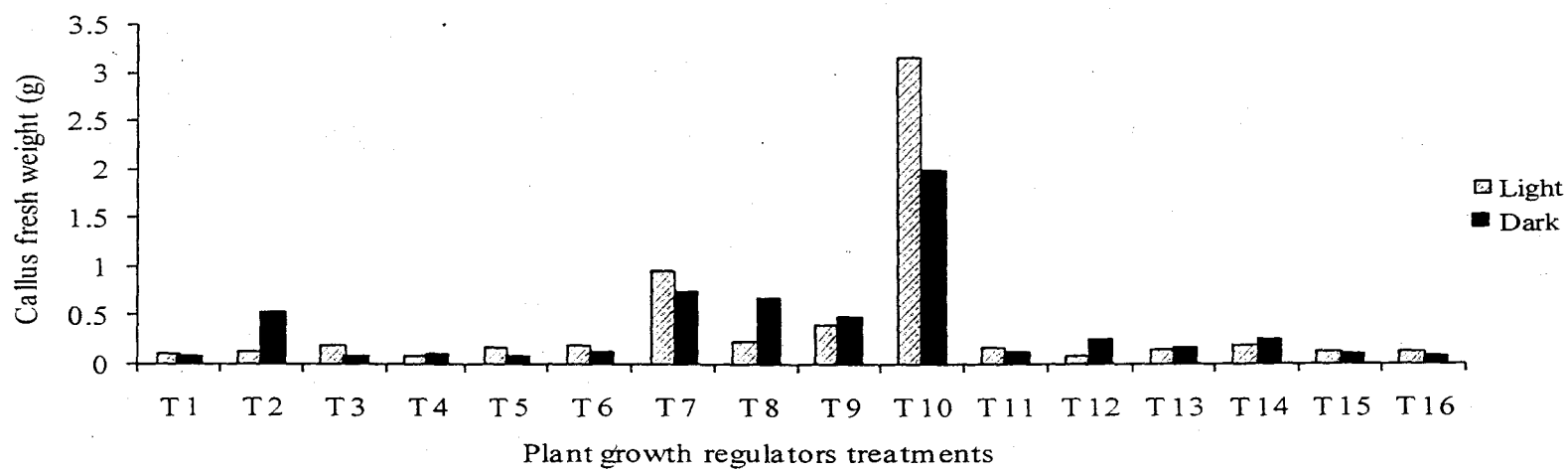

Fig. 1. Effect of plant growth regulators treatments on callus fresh weight (g) from hypocotyl under light and dark conditions after 28 day incubation.

concentration was illustrated in **Tab. and Fig. (2)**. Used of 3 mg/l BA showed the best number of shoots formation **(Tab. and Fig. 2)**. Induced rooting was achieved by transporting multiple shoots to MS medium supplemented with NAA (1 to 3 mg/l) after elongated the shoots in MS medium with GA_3. The effect of NAA concentration was illustrated in **Tab. and Fig. (3)**. The best number of roots was showed with 1 mg/l NAA which reached to 5 adventitious roots. Plantlets could be successfully transplanted to soil.

In this respect, **Liu and Cai (1990)** regenerated shoots of *Silybum marianum* at a frequency of 75% after 2 months following transfer of hypocotyl callus to MS medium (NAA 0.8 and BA 2.0 mg/l). Roots were induced on MS medium contalning 0.5 mg/l of NAA and 0.1 mg/l of IBA. **Hetz *et al* (1995)** isolated mesophyll protoplasts of six lines of *Silybum marianum* from young leaves. Plant regeneration experiments undertaken with the protocallus on medium containing BAP led to shoot formation in only two lines. Shoots were rooted on hormone free MS agar medium and transferred into soil where plants grew to maturity. Also, **Iqbal and Srivastava (2000)** transferred *Silybum marianum* explants callused within four weeks on (MS) medium supplemented with NAA (0.1 mg /l) + BAP (0.3 mg /l) + Zt (0.3 mg /l). Upon to MS + NAA (0.1 mg /l) + Zt (0.5 mg /l), the callus differentiated multiple shoots followed by rooting in 100% cultures of all the explants within ten weeks. Direct shoot regeneration could be obtained only from nodal segments after 4 weeks on MS + IAA (0.1 mg /l) + Kin (0.5 mg /l) in 100% cultures. Rooting of these shoots was achieved after 2 weeks on MS + NAA (0.1 mg /l) + Zt (0.5 mg /l). Plantlets could be successfully transplanted to soil. **Baruah and Sarma (1996)** cultured nodal segments and leaf sections of sunflowers cv. Sutton's sunbursts on MS medium supplemented with different combinations of 0 to 2.5 mg/l IBA, NAA, kinetin and BA. Shoots regenerated from nodal explants on medium supplemented with 0.5 mg/l IBA + 1.0 mg/l Kinetin and with 0.5 mg/l each of NAA, IBA and BA. In addition, **Vaknin *et al* (2007)** investigated the potential of milk thistle as a source of edible sprouts rich in antioxidants, they found that seed germination within 3-4 days was high (96%).

Table 2. Effect of BA concentrations in MS medium on shoot formation

BA conc. (mg/l)	Shoot number
1	0 d
2	6 b
3	20 a
4	3 c
5	0 d

LSD = 1.99

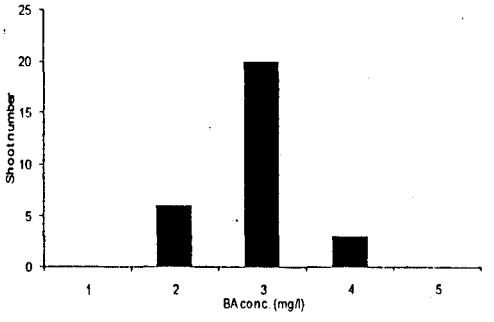

Fig. 2. Effect of BA concentrations on shoot formation

Table 3. Effect of NAA concentrations in MS medium on root formation

NAA conc. (mg/l)	Root number
1	5 a
2	3 b
3	1 c

LSD = 1.73

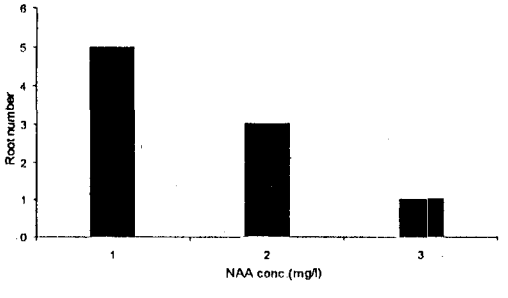

Fig. 3. Effect of NAA concentrations on root formation

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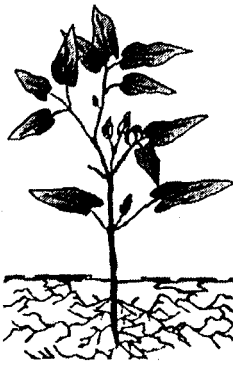
حوليـــات العلـــوم الزراعية
جامعة عين شمس ، القاهرة
مجلد(٥٤)، عدد (٢)، ٢٨٣-٢٨٩، ٢٠٠٩



تكوين الكالس وإكثار نبات الحرشف البرى معملياً

[٢٢]

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الموجـــز

أجري هذا البحث بهدف دراسة تـأثير منظمـات النمو النباتية ونوع المنفصل النبـاتى علـى تكـوين الكالس وتجديد النبات. وقد أظهرت المعـاملات أن السويقة الجنينية السفلى هـى افـضل المنفـصلات المستخدمة لتكوين الكالس ، وأن بيئـة MS مـضاف إليها ١ ملجم/لتر نفثـالين حمـض الخليـك + ٠,١ ملجم/لتر بنزيل أدنين ، أظهرت أعلـى وزن رطـب للكالس تحت ظروف الإضاءة (١٦ ساعة / اليـوم- شدة إضاءة ٦٠٠٠ لكس).

وقد حققت العقدة الفلقية فقـط التجديـد المباشـر (تكوين مجموع خضرى) على بيئة MS مضاف إليها ٢، ٣ ، ٤ ملجم/لتر بنزيل أدنين فى ظروف الإضاءة وسجل تركيز ٣ ملجم/لتر أفـضل معاملـة لتكـوين المجموع الخضرى. وقد تفوقت معاملة نفثالين حمض الخليك بتركيز املجم/لتر فى تكوين الجـذور علـى المجموع الخضرى.

تحكيـم: أ.د إبراهيم سيف الدين إبراهيـم
أ.د إبراهيم عبد المقصود إبراهيم