Journal of Applied Sciences Research, 9(1): 860-866, 2013
ISSN 1819-544X
This is a refereed journal and all articles are professionally screened and reviewed

ORIGINAL ARTICLES

Protocol for *in vitro* morphogenesis and hairy root cultures of Milk thistle (*Silybum marianum* L. Gaertn)

Bekheet, S.A., Taha, H.S. and Ahmed M.M. Gabr

Plant Biotechnology Dept., National Research Center, El-Tahrir Str., Dokki,Cairo, Egypt.

Abstract: An applicable protocol for *in vitro* morphogenesis and hairy root cultures of the medicinal plant Milk thistle is recognized. Seeds were surface sterilizes and then cultured on liquid free MS-basal medium. *In vitro* sprouted shoots (cotyledons) were taken from the cultures and re-cultured on medium contained 2 mg/l BA for shoots proliferation. Calli cultures were induced from leaf, petiole and stem explants of *in vitro* grown plantlets. The highest frequency of callus induction was observed on leaf explants cultured on MS medium supplemented with 5 mg/l kin + 0.5 mg/l IAA. Callus growth parameters revealed that medium contained 5 mg/l kin + 0.5 mg/l IAA + 0.1 mg/l GA3 gave the highest fresh weight and growth value. Concerning *in vitro* regeneration, the highest percentage of direct organogenesis (from leaf explants) was observed with medium contained 1 mg/l BA + 2 mg/l NAA. However, transferring callogenic explants to 2 mg/l kin + 2 mg/l NAA containing medium resulted in the highest percentage of indirect organogenesis presented as shoot formation. For establishment of hairy root cultures of Milk theistle, 12 days old cotyledons were used as explants.Hairy roots were induced by inoculation of explants with *Agrobacteriumrhizogenes* strain A4 within three weeks. Hairy roots were formed in high frequency on wounded regions of the young (three weeks old) leaves which subsequently used to obtain hairy root lines.

Key words: Milk thistle, organogenesis, hairy root.

Introduction

Milk thistle (*Silybum marianum* L. Gaertn) is an annual herb or biannual herbaceous plant of family Compositae. It is native to the Mediterranean, but now Milk thistle varieties are widespread throughout the world usually in dry, sunny areas. Milk thistle is recommended in traditional European and Asiatic medicine for more than 2000 years, mainly for treatment of liver disorders (Mossa *et al*., 1987). It protects and regenerates the liver in case of liver cirrhosis, jaundice, and chronic hepatitis.The active ingredients of Milk thistle are chemicals called flavonoids. The flavonoids in Milk thistle are silybin, silydianin, andsilychristin. Together, they are called silymarin (Wichtl, 1994). The parts of silymarin complex are of a considerable pharmacological interest owing to their strong anti-hepatotoxic and hepatoprotective activity (Morazoni and Bombardelli, 1995).The strong and growing demand for naturalsilymarin has refocused attention on *in vitro* plant materials as potential factories for Milk thistle secondary metabolites.

The *in vitro* production of Milk thistle medicinal compounds can be possible through plant cell culture under controlled conditions and free from environmental fluctuations. In this respect, cell cloning methods provide a promising way of selecting cell lines yielding increased levels of the product. Moreover, various tissue culture techniques are used to enhance yield of secondary metabolites by biotransformation. One of the reasons for research on various plant cell, tissue or organ cultures is the ability of these cultures to synthesize *in vitro* some of the metabolites that are found in the whole plants (Tamer and Mavituna, 1996). However, the major limitations of cell cultures are their instability during long-term culture and low product yields (Bonhomme *et al*., 2000). In this respect, tissue culture systems of *S. marianum* have been established using hypocotyls (Liu and Cai, 1990; Manaf *et al*., 2009) and Cotyledon (Cimino *et al*., 2006) explants. Otherwise, the use of hairy root cultures has revolutionized the role of plant tissue culture for secondary metabolite. Synthesis hairy root cultures are characterized by a high growth rate and are able to synthesize root derived secondary metabolites (Rao and Ravishankar, 2002). The greatest advantage of hairy roots is that their cultures often exhibit approximately the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Kim *et al*., 2002). Hairy root culture of *S. marianum* could therefore be an alternative method for the production of flavonolignanas (Alikaridis *et al*., 2000). In this connection, ilymarin production by hairy root culture of Milk thistle was investigated using *Agrobacterium rhizogenes* (Rahnama *et al*., 2008). The aim of the present work is to establish reliable protocols for various morphogenesis types and hairy root cultures of Egyptian Milk thistle (*S. marianum* L Gaertn).

Corresponding Author: Bekheet, S.A., Plant Biotechnology Dept., National Research Center, El-Tahrir Str., Dokki,Cairo, Egypt.
E-mail: shawky005@yahoo.com

J. Appl. Sci. Res., 9(1): 860-866, 2013

Materials and Methods

Establishment of in vitro sprout cultures:

Seeds of Milk theistle were used as starting plant material for *in vitro* culture and subsequently for tissue culture experiments. Seeds were washed with distilled water and then immersed in 70 % ethanol for 30 sec followed by 20 % commercial Clorox (containing 5.25 % sodium hypochlorite) for 10 min and finally washed three times with sterilized distilled water.The steps of disinfestations were took place under aseptic conditions in a laminar air-flow cabinet.

The disinfected seeds were placed in 250 ml Erlenmeyer flasks contain 50 ml liquid free MS-basal medium (Murashige and Skoog, 1962) on a rotary shaker at 110 rpm (as a sprout culture technique, Shevchenko *et al*. (2010)). All cultures were maintained at 25 ± 2°C under light irradiation 16/8 h light/dark. Cotyledons were obtained after 12 days of the sprout culturing which were taken and re-cultured on fresh medium contained 2 mg/l benzyladenine (BA) to get stock plant material.

Callus induction:

For callus induction, three types of explants i.e., leaf, petiole and stem were excided (1 cm length)from elongated plantlets and cultured on MS medium containing two combinations of BA with naphthaleneacetic acid (NAA) and two combinations of kinetin (Kin) with indoleacetic acid (IAA). Three segments per jar of each type of explants were used. Percentages of callus formation were registered after five weeks of culturing from 20 replicates.

Effect of growth regulators on callus growth:

In order to study the callus growth, about 250 mg of callus derived from leaf explant tissue were sub-cultured on MS medium supplemented with same combinations of growth regulators used for callus induction in addition of one more (5 mg/l kin + 0.5 mg/l IAA + 0.1 mg/l gibberllic acid (GA3)).Fresh weight and growth value were determined after five weeks of sub-culturing.

$$\text{*Growth value} = \frac{\text{Final fresh weight - Initial fresh weight}}{\text{Initial fresh weight}}$$

In vitro regeneration:

To investigate the ability of organogenesis and subsequent proliferation of *in vitro* plantlets, leaf explants andfresh embryogenic callus derived from leaves were cultured on MS-medium supplemented with different combinations of BA, kin and NAA. Percentages of direct (from leaves) and indirect (from callus) organogenesis were recorded after five weeks of culturing.

Culture media and incubation conditions:

Tissue culture media were solidified with 0.7 % agar, with 30 g/l sucrose and adjusted to pH 5.8 before autoclaving at 121˚C and 1.5 Ib/M² for 25 min. In all situations, the growth regulators were added to the culture medium prior to autoclaving. Cultures were maintained at 25 ± 2˚C and 16 hr photoperiod provided by white fluorescent tubes (3000 lux light intensity).

Transformation of cotyledons explant with Agrobacteriumrhizogenes:

Preparation ofAgrobacteriumrhizogenes:

Culture of *A. rhizogenes*strain A4 was initiated from glycerol stock and maintained on MYA-solid medium (5.0 g/l Yeast extract, 0.5 g/l Casamino acids, 8.0 g/l Mannitol, 2.0 g/l Ammonium sulfate, 5.0 g/l NaCl and 15 g/l agar) (Petit Tempe,1978) for 48 h at 28°C in the dark. The single clone was grown for 24 h in 20 ml MYA-liquid mediumat 28°C on a rotary shaker at 100 rpm in the dark.

J. Appl. Sci. Res., 9(1): 860-866, 2013

Establishment of hairy rootcultures:

The transformation experiment was performed using 12 days old cotyledons of Milk theistle. Each cotyledon (explant) was immersed in bacterial suspension separately for 10 min. The explants were blotted dry on sterile filter-paper to remove excess bacteria and incubated in the dark at 28^{o}C in 200 ml Erlenmeyer flask with 50 ml of liquid hormone-free MS medium with 30 g/l sucrose on a rotary shaker at 100 rpm. Uninfected explants (control) were cultured under the same conditions. After 24 h of co-cultivation, the explant tissues were transferred to new growth medium (solidified free-MS medium) containing 500 mg/L cefotaxime to eliminate bacteria and then incubated in growth chamber at 25 ± 2°C and in the dark.

Numerous hairy roots were observed emerging from the wound sites within 4-5 weeks. The hairy roots were separated from the explant tissues and sub-cultured in the dark at 25°C on solid hormone-free MS medium. After repeated transfer to fresh medium, rapidly growing hairy root cultures were obtained. Isolated roots (300 mg) were transferred to 50 ml of MS liquid medium, containing 30 g/L sucrose, in 200-mL flasks. The root cultures were maintained at 25°C on a rotary shaker at 100 rpm in growth chamber in the dark. Ten flasks were used for each culture and the experiments were repeated twice.

PCR detection:

Plant genomic DNA of Milk theistle hairy root cultures for polymerase chain reaction (PCR) analysis were extracted by using Jena Bioscience Kit (Jena Bioscience GmbH, Jena, Germany. Genomic DNA used as template on PCR was isolated from roots excised from infected and uninfected plants (control). The sequences of the primers used to amplify a fragment of the rol genes (Operon. GmbH, Germany) and the product size were as follows: rolA forward, 5´-GTTGTCGGAATGGCCCAGAC-3´, reverse 5´-CGTAGGTCTGAATATTCCGGTC-3´, product size 304 bp; rolC forward, 5´-TGTGACAAGCAGCGATGAGC3´, reverse 5´- GATTGCAAACTTGCACTCGC-3´, product size 5500 bp. The amplification cycle consisted of denaturation at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. After 30 repeats of the thermal cycle and final extension 72 °C for 5 min, amplification products were analyzed on 1.5% agarose gel.

Experimental design and statistical analysis:

Each experiment was set up as a separate completely randomized design. Data were statistically analyzed using standard error (SE) according to the method described by Snedecor and Cochran (1967).

Results and Discussion

Callus induction:

Tree different expalnts (leaf, petiole and stem) of Milk theistle plant and various combinations of growth regulators added to culture medium were investigated for their potential for callus induction. Data presented in Table (1) indicate that callus was induced in different frequency depending on growth regulators added to culture medium and type of explant within five weeks of culturing. The best results of callus frequencies induced on the three explants used were observed with 5 mg/l kin + 0.5 mg/l IAA containing medium. This composition was very effective in promoting callus induction. However, leaf segments were the most suitable expalnt for this purpose since it gave the highest percentages of callus induction with all growth regulators used. The highest value of callus induction generally was registered when leaf segments cultured on medium contained 5 mg/l kin + 0.5 mg/l IAA (Table 1).The calli were brownish to creamish in color callus and turned dark brown (Fig.1-B).

The callogenesis response of the explants is greatly influenced by the medium employed for callus induction. Moreover, callus initiation is affected by type and concentration of growth regulators in culture media. Furthermore, the response of explant on culture media also depends on the endogenous growth substances present at the time of excision. Auxins (IAA, IBA, NAA or 2,4-D) are often added to the culture medium to promote the growth of callus, cell suspensions or organs, and to regulate morphogenesis, especially in combination with cytokinin (George, 1993). In the present work, it was observed that kin in combination with IAA was more effective on callus induction compared with BA when they were combined with NAA. Responses of callus initiation were varied depending on the type of the expalnts used. Leaf explant generally registered the highest percentages of callus induction. There are some reports showed the parallel results with the present study. In their study for production of flavonolignan in cell suspension cultures of *S. marianum*, Hasanloo *et al*. (2008) used 1 mg l 2,4-D and 0.4 mg l Kin to develop callus from cotyledon, shoots and root segments. Sharma Award (2006) cultured leaves segments on MS medium supplemented with various growth

hormones like IAA, IBA, 2,4-D, NAA, BA and kin in different concentrations for callus induction of *S. marianum.* On the other hand, the present results are not compatible with those of Cimino *et al*., (2006). They cultured sections of cotyledons on B5 medium supplemented with BA and 2,4-D for callus production. Moreover, Manaf *et al*., (2009) mentioned that hypocotyl was the most convenient explant for callus formation of *S. marianum*. Otherwise, in their study on the *in vitro* morphogenic potential of *S. marianum*, John and Koperuncholan (2012) reported that 100% callus induction was obtained from leaf explants on 2, 4-D at 2.5 mg/l.

Table 1: Callus frequency induced on Milk thistle explantsi.e., leaf, petiole and stem after five weeks of culturing on MS medium contained different combinations of BA, Kin, IAA and NAA.

Growth regulators	Callus frequency(%)		
	Leaf	Petiole	Stem
1 mg/l BA + 2 mg/l NAA	30	30	10
2 mg/l BA + 5 mg/l NAA	50	40	20
2 mg/l kin + 0.5 mg/l IAA	70	50	20
5 mg/l kin + 0.5 mg/l IAA	90	60	40

Mean n= 20

Effect of growth regulators on callus growth:

To investigate the effect of growth regulators on development and growth of callus proliferated from leaf segments of Milk thistle, equal pieces of callus were cultured on MS-medium supplemented with various combinations of cytokinins, auxins and gibberellic acid. Data of callus growth presented as fresh weight and growth value indicated that combinations of Kin and IAA were more effective on growth parameters compared to combinations of BA and NAA. Medium contained 5 mg/l kin + 0.5 mg/l IAA + 0.1 mg/l GA3 gave the highest fresh weight (1.25 g) and growth value (4.0) (Table 2). Callus induced on this treatment was more healthy and suitable for establishment continuous callus cultures of Milk thistle (Fig. 1-C). However, the lowest fresh weight (0.50 g) and growth value (1.0) were registered with the medium contained 1 mg/l BA + 2 mg/l NAA.

Table 2: Fresh weights and growth values of callus derived from leaf expalnts of Milk theistle grown for five weeks on different combinations of auxins and cytokinins.

Growth regulators	Fresh weight(g)	Growth value
1 mg/l BA + 2 mg/l NAA	0.50±0.03	1.0
2 mg/l BA + 5 mg/l NAA	0.65±0.02	1.6
2 mg/l kin + 0.5 mg/l IAA	0.75±0.04	2.0
5 mg/l kin + 0.5 mg/l IAA	1.05±0.08	3.2
5 mg/l kin + 0.5 mg/l IAA + 0.1 mg/l GA3	1.25±0.07	4.0

Mean ± SE, n= 20

Data obtained revealed that Kin containing medium registered the best results of callus growth derived from leaf explants of Milk thistle. Moreover, addition of gibberellin into medium obviously enhanced callus growth.This treatment was the optimum one for growth and maintenance of friable callus than other treatments. In contrast of our results, Abbasi, *et al.,* (2010) on their study on *S. marianum* reported that the highest frequency of callus induction was observed on leaf explants cultured on MS medium supplemented with 5.0 mg BA.In this respect, Iqbal and Srivastava (2000) cultured the leaf, shoot apex and nodal segments of *S. 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). Moreover, Manaf *et al*. (2009) mentioned that highest callus fresh weight of Milk theistle was obtained with MS medium supplemented with 1 mg/l NAA + 0.1 mg/l BA.

In vitro regeneration:

The availability of efficient and long-term plant regeneration systems from callus culture is of prime importance in the application of culture for Milk theistle improvement. This part of study aimed to investigate the ability of organogenesis and subsequent proliferation of *in vitro* plantlets of Milk theistle. Data of direct and indirect shoot formation illustrated in Table (3) indicated that the two types of organogenesis were mainly varied depending on growth regulators added to culture medium. Medium contained BA in combinations with NAA was more suitable for direct organogenesis (Fig. 1-D). However, kin when combined with NAA showed best results of indirect organogenesis. At this medium calli was turned to green color and showed signs of organogenesis as pro-leaves and shoots within five weeks (Fig. 1-E).The highest percentage of direct organogenesis (60 %) was observed with medium contained 1 mg/l BA + 2 mg/l NAA. However, 2 mg/l kin + 2 mg/l NAA containing medium showed the highest percentage (50 %) of indirect organogenesis (Table 3).

In vitro morphological response in plants is influenced by various factors including initial types of explants, growth regulators, culture conditions, genotype and nutrient media composition.There are two primary morphogenic pathways leading to whole plant involve somatic embryogenesis and shoot organogenesis followed by root organogenesis. Both developmental pathways can occur either directly without a callus intermediate stage, termed adventitious; or indirectly following an unorganized callus stage (Gamborg and Phillips, 1995). A balance between auxin and cytokinin is most often required for the formation of adventitious shoots and roots. Generally, cytokinins promote cell division by activating DNA synthesis, promote the growth of lateral buds through neutralizing the effect of auxins and induce shoot formation (Pierik, 1987).The obtained results in this study reveal that BA was more effective on direct organogenesis of Milk theistle, however, kin was more effective on indirect organogenesis when they were combined with NAA. The highest percentage of direct organogenesis was registered with 1 mg/l BA + 2 mg/l NAA however, 2 mg/l kin + 2 mg/l NAA containing medium showed the highest percentage of indirect organogenesis. In this concern Abbasi *et al*. (2010) mentioned that transfer of callogenic explants of Milk thistle onto MS medium supplemented with 2.0 mg/l GA_3 + 1.0 mg/l NAA resulted in 25.5 shoots per culture flask after 30 days following culture. Otherwise, John and Koperuncholan (2012) mentioned that 100% shoot initiation was obtained from callus cultures of Milk thistle using medium contained NAA at 2 mg/l and the BA at 1.5 mg/l.

Table 3: Effect of NAA in combinations with BA and Kin on direct and indirect organogenesis of Milk thistle.

Growth regulators	Direct organogenesis (%)	Indirect organogenesis (%)
1 mg/l BA + 2 mg/l NAA	60	20
2 mg/l BA + 2 mg/l NAA	50	10
1 mg/l kin + 2 mg/l NAA	30	40
2 mg/l kin + 2 mg/l NAA.	20	50

Mean n= 20

Establishment of hairy root cultures:

For establishment of hairy root cultures of Milk thistle, cotyledons of 12 days old were used as explants. Hairy roots were induced by inoculation of explants with *A. rhizogenes* strain A4.It is well known that each *rol*genes of the Ri-plasmid in *A.rhizogenes*is responsible for the induction of hairy root from plant species. To determine the insertion of *rol*genes, PCR was performed by using *rol*A and *rol* C primers to detect the transformation. PCR amplification (Fig. 2) confirmed that the *rol*A and *rol* C genes were presented in transformed.

Our observations reveals that hairy roots were induced within four weeks after inoculation emerging on the explants. Hairy roots were formed only from wounded regions (Fig. 1-F). Each age (three, four and five weeks) of explant showed hairy root induction with varying percentage of transformation frequency.Using young leaf explants resulted in enhancing transformation frequency. The transformed hairy roots were established on liquid MS medium without growth regulators. They were subcultured in the same medium every 3 weeks and incubated at 25 ˚C, in rotary shaker at 100 rpm under dark condition to maintain hairy root lines.

In this study, hairy roots cultures of Milk thistle were obtained by infecting aseptically cotyledon explants employing simple procedures. Because of simple handling and higher transformation efficiency, we therefore, propose inoculation of fresh explants for induction of hairy roots in Milk thistle. Previous studies showed that type and age of explant have a great influence on hairy root induction since age of explant is a major factor that alters the physiological properties of the cell (Dupre *et al.*,2000). In this respect, Vergauwe *et al.* (1998) reported that to succeed in establishing a hairy root culture system, several essential conditions should be taken into consideration. These conditions include the bacterial strain of *A. rhizogenes*, an appropriate explant, a proper antibiotic to eliminate redundant bacteria after co-cultivation, and a suitable culture medium. In this concern, Rahnama *et al*. (2008) mentioned that hairy root of *S. marianum*was induced by injection or inoculation of hypocotyl, leaf and cotyledons explants with *A. rhizogenes* AR15834. However, Khalili *et al*. (2010) used cotyledonary leaf only as explants and *A.rhizogenase* (AR15834) to induced hairy roots cultures of *S. marianum*. Recently, in their study on precursor feeding in *S. marianum* hairy root cultures Rahimi *et al*. (2011) obtained *S. marianum* hairy rootculture using *A.rhizogenes* strain AR15834.

J. Appl. Sci. Res., 9(1): 860-866, 2013

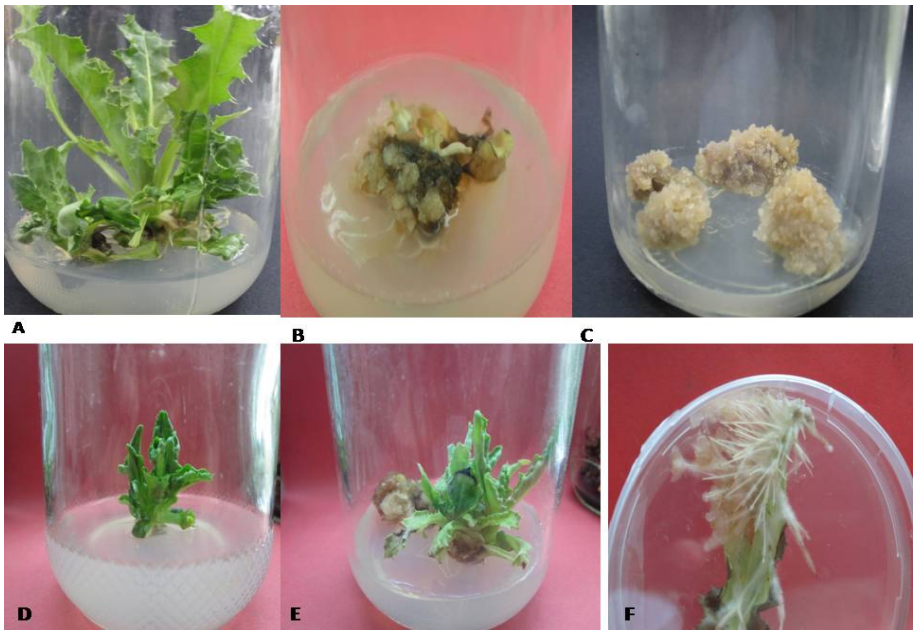

Fig. 1: Shoot cultures of Milk thistle proliferated on MS medium supplemented with 2 mg/lBA (A), callus of Milk thistle induced from leaf expalnt on medium contained 5mg/l kin + 0.5 mg/l IAA (B), five weeks old callus grown on medium contained 5 mg/l kin + 0.5 mg/l IAA + 0.1 mg/l GA3 (C), direct shoot proliferation from leaf explants on 1 mg/l BA + 2 mg/l NAA containing medium (D), indirect organogenesis (from callus) using medium contained 2 mg/l kin + 2 mg/l NAA (E) and hairy roots formed on wounded regions of Milk thistle cotyledon explant (F).

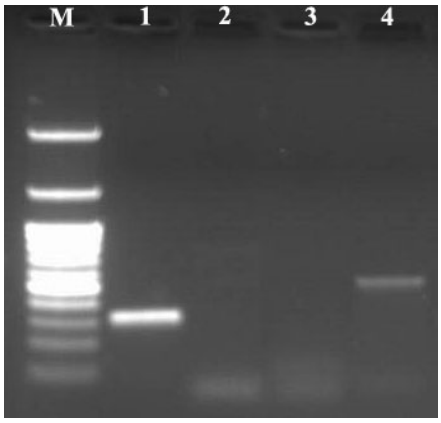

Fig. 2: PCR amplification of the rol genes (A and C) from genomic DNA isolated from wild type (lanes 2 and 3) and hairy root (lanes 1 and 4) of Milk thistle. Lane**M**: molecular size markers, Lane 1: rolA (304 bp), Lane: rolC (550 bp) genes.

References

Abbasi, B.H., M.A. Khan, T. Mahmood, M. Ahmed, M.F. Chaudhry and M.A. Khan, 2010. Shoot regeneration and free-radical scavenging activity in *Silybum marianum* L.Plant Cell, Tissue and Organ Culture, 101: 371-376.

Alikaridis, F., D. Papadakis, K. Pantelia and T. Kephalas, 2000. Flavonolignan production from *Silybum marianum* transformed and untransformed root culture. Fitotrapia, 71: 379-384.

Bonhomme, V., D. Laurain-Matter, J. Lacoux, M.A. Fliniaux and A. Jacquin-Dubreuil, 2000. Tropan alkaloid production by hairy roots of *Atropa belladona* obtained after transformation with *Agrobacterium rhizogenes*15834 and *Agrobacterium tumefaciens* containing *rol* A, B, C genes only. J Biotechnol., 81: 151-158.

Cimino, C., S.V. Cavalli, F. Spina, C. Natalucci and N. Priolo, 2006. Callus culture for biomass production of Milk thistle as a potential source of milk clotting peptidases. Electronic J. Biotechnology ISSN: 0717-3458.

Dupre, P., J. Lacoux, G. Neutelings, D. Mattar-Laurain, M.A. Fliniaux, A. David and A. Jacquin-Dubreuil, 2000. Genetic transformation of *Ginkgo biloba* by *A. tumefaciens*.PhysiologiaPlantarum, 108: 413-419.

Gamborg, O.L. and G.C. Phillips, 1995. Plant cell, tissue and organ culture-fundamental methods. Heidelberg, New York: Springer-Verlag.

George, E.F., 1993. Plant Propagation by Tissue Culture – Part 1: The technology. Edington: Exegetics, vol.1.

Hasanloo, T., R.A. Khavari-Nejad, E. Majidi and M.R. Shams Ardakani, 2008. Flavonolignan production in cell suspension culture of *Silybum marianum*. Pharmaceutical Biology, 46: 876-882.

Iqbal, S.M. and P.S. Srivastava, 2000. In *vitro* micropropagation of *Silybum marianum* L. from various explants and silybin content. J. Plant Biochem. and Biotech., 9: 81-87.

John, S.A. and M. Koperuncholan, 2012. Direct root regeneration and indirect organogenesis in *Silybum marianum* and preliminary phytochemical, antibacterial studies of its callus. Int J Pharm., 2: 392-400.

Khalili, M., T. Hasanloo, S.K. Tabar, 2010. Ag^{+} enhanced silymarin production in hairy root cultures of *Silybum marianum* (L.) Gaertn. Plant Omics. J., 3: 109-114.

Kim, Y., B. Wyslouzil and P.J. Weathers, 2002. Secondary methabolism of hairy root cultures in bioreactors. In Vitro Dev Biol Plant, 38: 1-10.

Liu, S.Q. and Q.G. Cai, 1990. Callus formation from protoplasts and plant regeneration from tissue culture of *Silybum marianum* Gaertn. Acta Botanica Sinica, 32(1): 19-25.

Manaf, H.H., A.E. Kawthar and S. Mona, 2009. *In vitro* callus formation and plant regeneration of *Silybum marianum* (L.)Gaertn.Annals Agric. Sci., Ain Shams Univ., Cairo, 54(2): 283-289.

Morazzoni, P. and E. Bombardelli, 1995. *Silybum marianum* (*Carduusmarianus*).Fitoterapia, 66: 3-42.

Mossa, J.S., M.A. El-Yahya and I.A. Al Meshal, 1987. Medicinal Plants of Saudi Arabia. King Saud Univ. Libraries Riyadh, Saudi Arabia, 1: 278.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant, 15: 473-497.

Petit, A. and J. Tempe, 1978. Isolation of Agrobacterium Ti plasmid regulatory mutants. Mol Gen Genet., 167: 147-155.

Pierik, R.L.M., 1987. *In vitro* culture of higher plants.MortinusNijhof Publisher, Dordrecht, pp: 45-82.

Rahimi, S., T. Hasanloo, F. Najafi and R. Khavari-Nejad, 2011. Enhancement of silymarin accumulation using precursor feeding in *Silybum marianum* hairy root cultures. Plant Omics J., 4: 34-39.

Rahnama, H., T. Hasanloo, M.R. Shams and R. Sepehrifar, 2008. Silymarin production by hairy root culture of *Silybum marianum* L.Gaertn. Iranian J. Biotech., 6: 113-118.

Rao, S. and G.A. Ravishankar, 2002. Plant cell cultures: Chemical factories of secondary metabolites. Biotech. Adv., 20: 101-153.

Sharma Award, V.N., 2006. Antiinflammatory activity of leaf and leaf callus of *Silybum marianum* (L.) Gaertn.in albino rats. Indian J Pharmacol., 38(3): 213-14.

Shevchenko, Y., I. Smetanska and A. Wendt, 2010. *Stevia rebaudiana Bertoni* – Überblick über die Forschung an einer verbotenen Pflanze und derer möglichen Einsätze. *Journal für Verbraucherschutz und Lebensmittelsicherheit*, *5*(2): 193-198.

Snedecor, G.W. and W.G. Cochran, 1967. Statistical methods. 6th Edition.- Iowa Stat. University Press, Iowa.

Tamer, I.M. and F. Mavituna, 1996. Protease from callus and cell suspension cultures of *Onopordum turcicum* Biotechnology Letters, 18: 361-366.

Vergauwe, A., E. Van Geldre, D. Inze, M. Van Montagu and E. Van Den Eeckhout, 1998. Factors influencing *A. tumefaciens* mediated transformation of *Artemisia annua*L. Plant Cell Reports, 18: 105-110.

Wichtl, M., 1994. Herbal Drugs and Phytopharmaceuticals. Medpharm Scientific Publishers, Stuttgart.