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Evaluation of an important flavonoid silymarin in callus cultures of *Sylibum marianum* (L.) Gaertn

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Abstract

Silybum marianum L. Gaertn is an important medicinal herb due to its active secondary metabolite, silymarin pronounced to be effective in disturbed liver function and ailments. The plant is under harvest pressure for sylimarin production and is being endangered. With the objective to produce silymarin *in vitro,* seed germination were optimized maintaining aseptic conditions. Seedlings raised from sterilized seeds *in vitro* on MS medium containing gibberellic acid, were used as explants for callus induction. Among different explants used, seed and hypocotyls were found substantially suitable explants for callus induction. Significant callus induction from hypocotyl explant was documented using MS medium added with 2, 4-D whereas seed explant produced callus under the influence of BAP along with 2, 4-D. Callus cultures from different explants were put to HPLC analysis to determine silymarin content. Since silymarin is a mixture of different components, all the callus cultures were same with respect to composition but different in amount of components, seed callus was observed to contain highest Silybinin content (89%) as compared to the callus cultures of other explants. The *in vitro* production of silymarin may prove significant source for the said valuable medicinal compound.

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Introduction

Silybum marianum (L.) Gaertn. is one of the important medicinal plants of family Asteraceae (Balian *et al.,* 2006). Since in Greco-Roman era this plant has been found to be a herbal remedy for a number of diseases due to its secondary metabolites, mostly it was used for treatment of liver, spleen, kidneys, adverse hepatitis and cirrhosis usually linked with alcohol (Ackerson, 2005). *Silybum marianum* contains silymarin, a complex of seven flavonolignans and polyphenols (Ball and Kowdley, 2005). Silymarin is primarily composed of 36.3% silibin, 5.9% silidianin, and 5.1% silichristin (Sersen *et al.,* 2006).

Silymarin, from *S. marianum,* has undergone extensive research within the last decades in view of its medicinal value for treating liver diseases and cancer. Pradhan and Girish (2006) examined the silymarin effect on hepatotoxicity and hepatobiliary ailments and proved that silymarin is really useful against liver disorders. *In vitro* and *in vivo* studies have shown that these compounds protect the liver from oxidative stress and sustained inflammatory processes. In view of the protective roles that the silymarin play, it is now widely used in clinical applications and established therapies for liver (Comelli *et al.,* 2007). Silymarin not only protects liver from certain toxins and viruses but also regenerates damaged tissue of liver (Pradhan and Girish, 2006). Its polyphenolic flavonoids, have potential for anticancer activities by reducing the proliferation of tumor cells (Malewicz *et al.,* 2006). Secondary metabolites in *S. marianum,* useful to cure many diseases, can be analyzed to separate vitamins and polyphenols by several methods such as layer chromatography (TLC), liquid chromatography (HPLC) and gas spectrometry (Helmja *et al.,* 2007).

Secondary metabolites, produced by plants as defense mechanisms (Wink, 1988; Verpoorte *et al.*, 2002) show different biological activities, and are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors and food additives. The production of secondary metabolites via field cultivation of plants has various disadvantages such as low yields, and fluctuations in concentrations due to geographical, seasonal, and environmental variations.

Subsequently, plant cell and tissue cultures with defined production systems which can result in higher yields and more consistent quality of the products (Neumann *et al.,* 2009), have appeared as attractive substitutes for the production of secondary metabolites (Ramachandra and Ravishankar 2002). The source plant is under severe harvest pressure for extraction of important flavonoid sylimarin from its seeds and is being endangered; the research work was initiated with an objective to produce silymarin through *in vitro* cultures as an alternative source for sylimarin production and to support conservation of the plant.

Materials and methods

Seed Collection, Sterilization and Germination

Seeds of *S. marianum* used to conduct the present research were collected and provided by Muhammad Ajaib, Taxonomist and Lecturer, Department of Botany. Seeds of *S. marianum* were sterilized with 0.1% $HgCl_2$ under aseptic conditions and put to germination *in vitro* in Petri plates, the optimized protocol comprised of seed soaking for 24 h, 5 seeds per Petri plate on cotton pad soaked with 20 ml distilled water, placed in growth room at 26°C ±1 temperature.

MS Medium and Explant Preparation and Culture

Murashige and Skoog (MS) medium (1962) was used as culture medium prepared and sterilized using standard protocol. Five days old seedlings were used as source of explants. Seed, cotyledonary leaf, hypocotyl and root were used as explants. The explants were transferred to culture jars containing MS medium supplemented with different concentrations and combinations of plant growth regulators such as BAP, IBA, TDZ, 2,4-D, NAA etc., under aseptic conditions. The explant containing culture jars were transferred to growth room. Observations for callus induction were recorded every week and callus cultures were sub-cultured at 15 days interval to maintain callus cultures.

Standard curve for Silymarin components through HPLC assay

Standard solution was prepared by dissolving accurately weighed 0.3 g of standard Silymarin flavonolignins (SO292 Sigma) in 100 ml of methanol of HPLC grade. Standard curve for silymarin, assayed through HPLC, was plotted to show retention time of the peaks of silymarin main components such as silybin, isosilybin, silydianin and silychristin. The analyses of silymarin standard solution was carried out using a Knauer K2600-A Liquid Chromatography equipped with a Nucleosil C18 (150 × 4.6 mm I.D, 5 µm) column. The elution was made in an isocratic mode at a flow-rate of 1ml/min and the detection made at 288 nm under the influence of UV as detector. One analysis required 15 min. the area.

Determination of Silymarin in explant tissue and callus cultures

Callus cultures obtained from different explants of *S. marianum* were used to analyze silymarin content by HPLC analysis. Explant samples and callus cultures from respective explants such as seed, cotyledonary leaf, root and hypocotyl were oven dried at 50°C for 36 h. Dried callus, 0.2 g of each sample after overnight soakeing in 5 ml of HNO_3 was boiled until reddish brown fumes appeared, 1.0 ml of H_2O_2 was added till the white fumes appeared. The mixture was shaken well during boiling and clear extract was obtained after filtration. Mobile phase was prepared by dissolving glacial acetic acid (0.5 ml/100 ml) in double distilled water (57.5 ml/100 ml), methanol (42 ml/100 ml) was added into it by filtration, this mobile phase was double filtered before use. Standard curve plotted was used to compare retention time of the peaks of silymarin and its components with explant tissues and callus culture samples for appraisal of silymarin and its main components. Identification of specific flavonoids was done by retention times and by comparing the UV spectra of the peaks with those of the available standards. The percentage content of sylimarin and its components was calculated by comparing peak areas in standard curve.

Results

Seed germination and callus formation

In vitro seed germination was achieved in 5 day with 100% germination. The germination percentage was decreased and germination duration was increased, when unsoaked seeds were put to germination. These 5 day old *in vitro* grown seedlings were used as source of explants for callus induction. Among different explants leaf and hypocotyl showed better potential for callus induction than other explants. Among different concentrations and combinations of plant growth regulators used, BAP, 2,4-D alone and in combination proved significant for callus induction in general (Fig. 1), however in case of hypocotyl, 2,4-D alone and TDZ in combination with 2,4-D both treatments induced callus effectively (Table 1).

Table 1. Response of explants to plant growth regulators for callus induction.

Explant type	PGR (mg/L)	Callus characteristics				
		Index	Weight (g)	Induction frequency (mean %)	Color	Texture
Seed	BAP 1.0	250	0.21±0.00	79±0.67	Light brown	Compact and friable
	2,4-D 0.5	165	0.08±0.00	55±0.27	Whitish brown	Compact
	2,4-D +BAP 0.5 +1.0	162	0.08±0.00	95±0.27	Whitish brown	Compact and friable
Coty- ledonary leaf	BAP 2.0	225	0.3±0.002	82±0.47	Dark green	Granular and friable
	2,4-D 1.5	230	0.14±0.001	89±0.45	Variegated brown and green	Compact
	2,4-D +BAP 1.5+2.0	169	0.09±0.00	73±0.31	Dirty green	Compact and friable
Hypo-cotyl	2,4-D 1.0	225	0.2±0.00	90±0.34	Whitish brown	Granular and friable
	2,4-D + TDZ 1+ 0.05	220	0.2±0.00	90±0.34	Whitish brown	Granular and friable
Root	2,4-D 1.0	145	0.06±0.00	80±0.24	Light brown	Granular and friable

The results included in the table are average of three replicates.

Appraisal of Silymarin in explant tissues and respective callus cultures through HPLC

Analysis of seed tissue and seed callus

The analysis of seed tissue and seed callus showed five distinct peaks for different components of silymarin. These components were detected according to their retention time. The HPLC analysis of seed callus under the influence of 0.5 mg/L 2, 4-D alone and 0.5 mg/L 2, 4-D + 1.0 mg/L BAP showed five distinct peaks for different components of silymarin. These components were detected according to their retention time. The total amount of silymarin was found to be 95.2% and 99.9% respectively, amount of silybinin (88.9%) was observed more in seed tissue and seed callus culture under the combined effect of 2, 4-D and BAP as compared to 2,4-D alone (Table 2), the amount of other components is given in the same Table.

Table 2. Silymarin and silymarin components in seed tissue and callus.

Silymarin components (%age) out of Silymarin	Seed tissue	Seed callus		Retention Time Comparison (Seed tissue and Seed callus)		
		2,4-D (0.5 mg/L)	2,4-D + BAP (0.5 +1 mg/L)	Ret. Time (Seed tissue)	Ret. Time (Seed callus)	
					2,4-D (0.5 mg/L)	2,4-D + BAP (0.5 +1 mg/L)
Taxifolin	5.0	2.5	5.9	2.4	2.093	2.1
Silybinin	89	87.6	88.9	2.70	2.71	2.69
Silydianin	0.3	0.4	1.7	4.89	4.97	4.5
Silychristin	0.3	0.1	0.8	6.34	6.04	5.2
Isosilybinin	4.4	4.6	2.6	8.2	8.4	8.9

Analysis of cotyledonary leaf tissue and callus

The chromatograph of cotyledonary leaf tissue (Fig. 2A) and cotyledonary leaf callus (Fig. 2B) showed five distinct peaks for different components of silymarin. These components were detected according to their retention time. The total amount of silymarin was found to be 97.5% in cotyledonary leaf tissue and 95.5% in cotyledonary leaf callus. The cotyledonary leaf tissue and cotyledonary leaf callus showed five distinct peaks for different components of silymarin (detected according to their retention time) under the influence of 1.5 mg/L 2, 4-D alone and 1.5 mg/L 2, 4-D with 2.0 mg/L BAP respectively. Amount of silybinin was comparable in cotyledonary leaf tissue and cotyledonary leaf callus induced under the exogenous application of 1.5 mg/L 2, 4-D with 2.0 mg/L BAP but quite low in cotyledonary leaf callus under 2,4-D alone (Table 3).

Table 3. Silymarin and silymarin components in cotyledonary leaf tissue and callus.

Percentage of Silymarin components (%age) out of Silymarin	Cotyl leaf tissue	Cotyl leaf callus		Retention Time Comparison (cotyledonary leaf tissue and callus)		
		BAP (2 mg/L)	BAP+2,4-D (2+1.5 mg/L)	Ret. Time (Cotyl leaf tissue)	Ret. Time (Cotyl leaf callus)	
					BAP (2 mg/L)	BAP + 2,4-D (2+1.5 mg/L)
Taxifolin	1.5	2.1	7.3	2.07	2.024	2.077
Silybinin	70	40.3	69.8	2.71	2.639	2.703
Silydianin	20	42.3	3.3	2.33	2.884	4.558
Silychristin	2.0	1.0	0.1	6.071	6.971	6.04
Isosilybinin	4.1	10.8	1.7	9.01	9.346	9.06

Analysis of hypocotyls tissue and callus

The analysis of hypocotyl tissue and hypocotyl callus showed five distinct peaks for different components of silymarin detected according to their retention time as was in previously mentioned sample analyses. The total amount of silymarin was found 98.4% in hypocotyl tissue from *in vitro* grown seedlings, 90.5% and 95.4% in hypocotyl callus induced under 2,4-D alone and 2,4-D with TDZ respectively.

The Table 4 shows the amounts of components of silymarin detected according to their retention time. Amount of silybinin was comparable in hypocotyl tissue and hypocotyl callus induced under the exogenous application of 1 mg/L 2, 4-D alone and 1 mg/L 2, 4-D with 0.05 TDZ (Table 4).

Table 4. Silymarin and silymarin components in hypocotyl tissue and callus.

Silymarin components (%age) out of Silymarin	Hypocotyl tissue	Hypocotyl callus		Retention Time Comparison (Hypocotyl tissue and callus)		
		2,4-D (1 mg/L)	2,4-D + TDZ (1+0.05 mg/L)	Ret. Time (Hypocotyl tissue)	Ret. Time (Hypocotyl callus)	
					2,4-D (1 mg/L)	2,4-D + TDZ (1+0.05 mg/L)
Taxifolin	4.0	1.6	4.3	2.32	2.0	2.0
Silybinin	83	82.3	84.3	2.56	2.7	2.7
Silydianin	3.0	0.6	2.9	4.6	4.7	4.5
Silychristin	2.5	0.1	2.3	5.9	6.0	5.4
Isosilybinin	6.9	5.9	1.6	8.1	8.4	8.4

Analysis of root tissue and callus

The analysis of root tissue and root callus showed the same five distinct peaks for different components of silymarin detected according to their retention time but the total amount of silymarin was found quite less in root callus induced under 2,4-D.

The amounts of components of silymarin (Table 5) were detected according to their retention time. Amount of silybinin was same in root tissue and root callus but the amount of other components of silymarin was less in root callus (Table 5).

Table 5. Silymarin and silymarin components in root tissue and callus.

Silymarin components (%age) out of Silymarin	Root tissue	Root callus 2,4-D (1 mg/L)	Retention Time Comparison (Root tissue and callus)	
		2,4-D (1 mg/L)	Ret. Time (Root tissue)	Ret. Time (Root callus) 2,4-D (1 mg/L)
				2,4-D (1 mg/L)
Taxifolin	0.9	0.3	2.0	2.0
Silybinin	6.9	6.9	2.8	2.7
Silydianin	5.5	0.8	4.6	4.5
Silychristin	1.6	1.9	8.4	5.4
Isosilybinin	6.9	5.9	-	-

Discussion

Prescription drugs and intermediate medicinal compounds derived from plants constitute 25 of pharmaceuticals. Plant tissue or organ cultures offer alternate source for the production of secondary metabolites. Many investigators have reported production of useful compounds in both callus and suspension cultures (Sanchez-Sampedro *et al.,* 2005, Sánchez-Sampedro *et al.,* 2007, Rady *et al.,* 2014). *In vitro* cultures generally require an exogenous supply of growth regulators for growth and proliferation of biomass. Different researchers through experimental results have described that the exogenous application of growth regulators influenced growth in *in vitro* cultures (Vanhala *et al.,* 1998; Weathers *et al.,* 2005; Ali *et al.,* 2010). In general, the plant growth regulator type, combination and concentration are crucial factors in cell and organ growth proliferation. The type and concentration of auxin or cytokinin, or the auxin to cytokinin ratio, altered biomass

growth in cultured cells in present study (Table 1) as has been described by many researchers, Nair *et al.,* (1992), Cimino, *et al,*. (2006), Shilpashree and Ravishankar (2009) and Tanveer *et al.,* (2012).

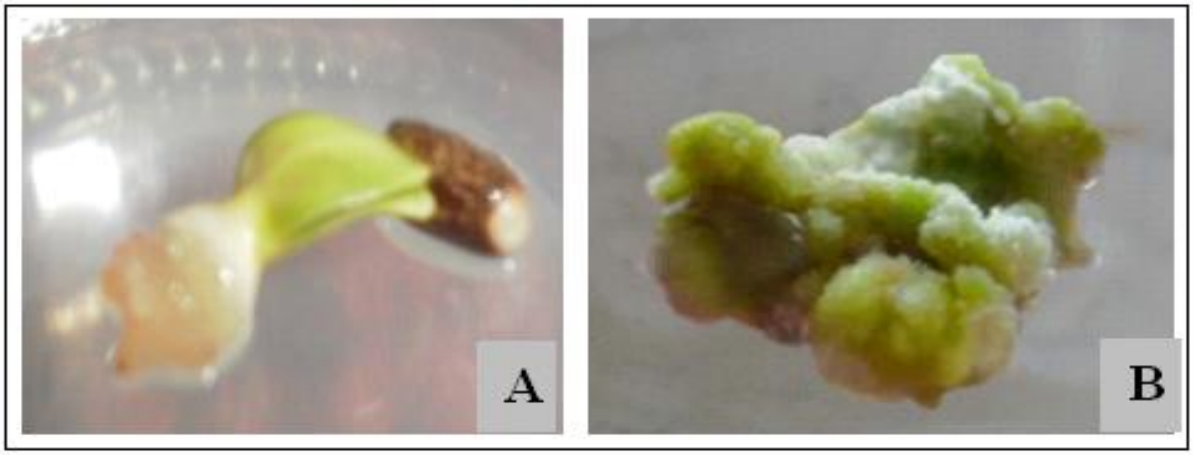

Fig. 1. Callus induction in different explants from *in vitro* grown seedlings of *Silybum marianum*. A) Callus induction from seeds on MS medium supplemented with 1.0 mg/L BAP (2x) (Culture of 7 day). B) Callus induction in hypocotyl explant on MS medium supplemented with 1.5 mg/L 2,4-D (1x) (Culture of 14 day).

Plant tissue and shoot cultures have been found promising for many medicinal plants to accumulate secondary metabolites to a greater extent than that by natural plants and secondary metabolite production can be improved by different factors Mantell and Smith (1984). For example, ginsengoside from *Panax ginseng* (27% in cell dry weight in culture: 4.4% in whole plant (Linden, 2000), shoot cultures of *Bacopa monnieri* and regenerated shoots resulted in a 3-fold increase in bacoside A, when compared to field-grown plants (Praveen *et al.,* 2009). Callus cultures produced significant amount of sylimarin in present study as shown by chemical analyses of callus cultures by HPLC and all callus samples were the same in view of the presence of silymarin components (Table 2 to 5) including silybinin (Fig. 2 & 3).

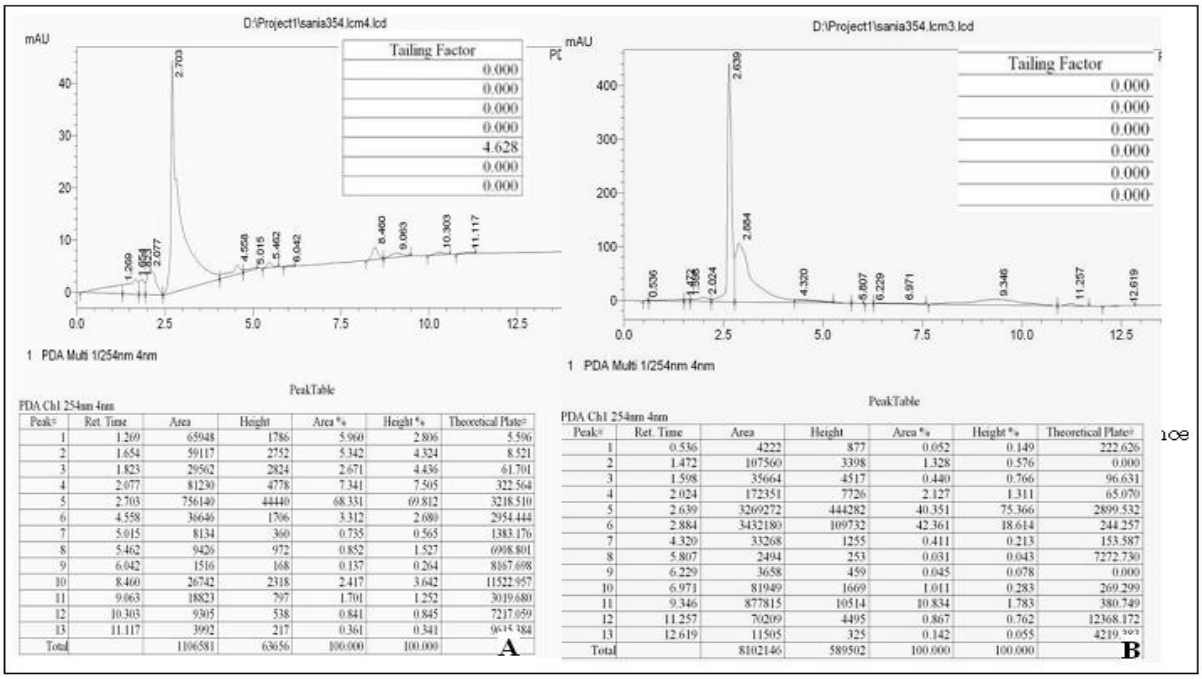

Fig. 2. HPLC chromatographs: A) Cotyledonary leaf tissue B) Cotyledonary leaf callus under the influence of 2.0 mg/L BAP.

The amount of silymarin in callus cultures was comparable to plant tissues as detection and appraisal of β-Phellandrene has been described by Ali *et al.,* (2015) in callus cultures of *Momordica charantia,* except callus samples from root of *S. marianum* had lower quantities of silybinin. Seed callus showed higher quantities of all the components as source of silymarin.

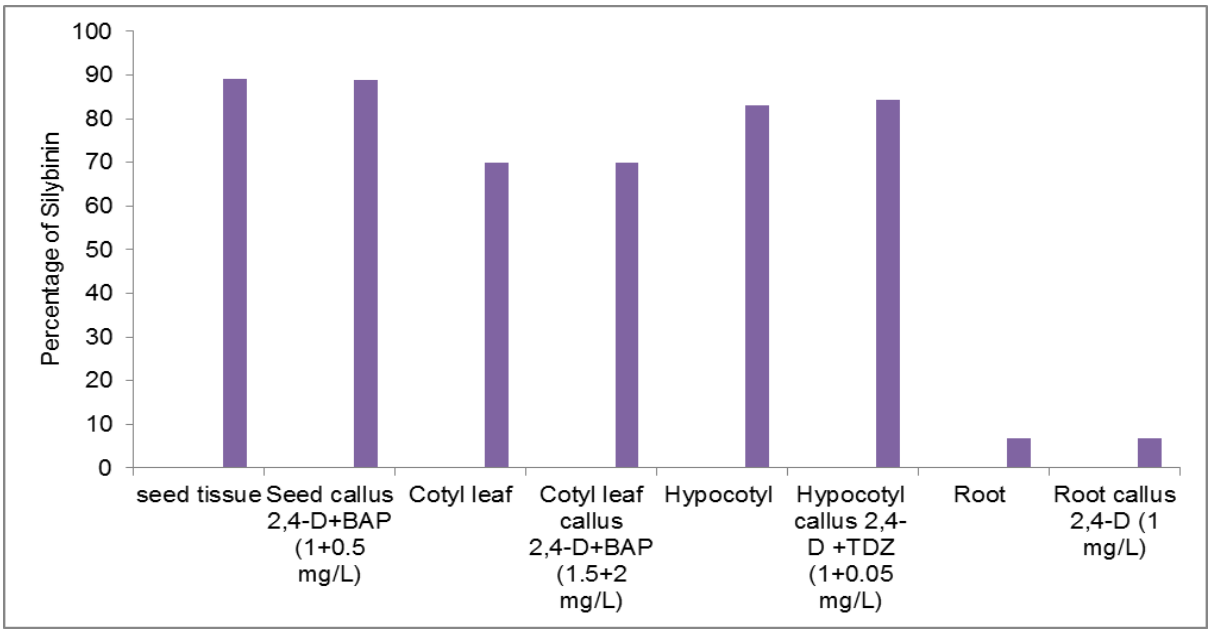

Fig. 3. Silybinin content of Silymarin in explant tissues and related callus cultures.

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