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In vitro Production and Biochemical Analysis of Silymarin from *Silybum marianum* Plant

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Abstract: *Silybum marianum* is an important medicinal plant and source of silymarine which is an antihepatotoxic polyphenolic substance isolated from the milk thistle plants. In this study, callus production of *Silybum marianum* under *in vitro* conditions was investigated with different levels of 2,4-dichlorophenox acetic acid (2, 4-D), β-naphthalene acetic acid (NAA) and 6-benzyladenine (BA). The highest callus induction, fresh weight of callus were obtained on Gamborg madium (B5) supplemented with 2.0 mg/l 2,4-D + 0.2 mg/l BA. The maximum silymarine content was observed from callus maintained on the same medium under dark condition. The highest content of silymarin, reached 8.91 mg/g dry weight comparing with the seeds content which recorded 1.51 mg/g dry weight. It was noticed that silymarin content in the callus was 6 folds that of the seeds of plant. Analysis of SDS-PAGE of soluble protein of *Silybum marainum* samples obtained from different treatments and the seeds of the plant revealed 100% similarity. Twelve isozyme systems were studied, confirmed higher polymorphism 58% among the *Silybum marianum* callus and seeds.

Key words: *Silybum marianum* · Silymarin · HPLC · Callus · *In vitro* · SDS-PAGE · Isozyme · Polymorphism

INTRODUCTION

Silymarin is a mixture of seven distinct flavonolignans namely silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin and one flavonoid taxifolin isolated from milk thistle (*Silybum marianum*, a member of the Asteraceae family) [1]. Meanwhile, this safe herbal product was used as the therapy for liver diseases, the anticancer activity and favorable effects on Hepatitis C Virus (HCV) infection and alcoholic liver disease were proved for this metabolite as well [2,3]. It is also associated with the treatment and prevention of gastrointestinal problems, nephropathy, cardio-pulmonary problems and skin protection [4, 5]. Wilasrumee *et al.* [6] found that the milk thistle also stimulates the immune system. Silymarin promotes liver regeneration ribosomal RNA synthesis, which stimulates liver regeneration [7]. *Silybum marianum* L. Gaertn distributed in Eastern region [8], Mediterranean and naturalized in North and South America [9]. 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 [10].

Plant tissue culture technique has become a powerful tool for studying basic and applied problems in plant biology. Furthermore, these techniques have found wide commercial applications, due to a better understanding of the nutritional requirements of culture cells and tissues [11]. In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches-specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites [12]. *In vitro* culture of cells and tissue culture may offer an alternative for production of silymarin but until now, few studies addressing this possibility have been carried out and in all cases, silymarin production in *in vitro* cultures is very low [13]. The manipulation of the components of the culture medium have substantially increased the production of these compounds [14]. Isozyme analysis has several

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advantages as compared not only with morphological and physiological characters, but also with other genetic markers, Isozyme analysis is mostly low expensive, detect environmental changes, co-dominant with a simple Mendelian inheritance. Isozymes are indicated as good markers, their analysis is a powerful tool for monitoring studies of plant callus cultures [15]. Long-term callus culture conditions affect constitutive and differential gene expression of some isozymes [16]. SDS-PAGE protein profile pattern could be use as biochemical marker for distinguishing the differences between *in vitro* calli and *in vivo* plant.

In this study, accumulation, of total silymarin in callus of *Silybum marianum* was assayed and the effect of growth regulators composition on silymarin production was also determined. In addition, silymarin concentration in callus and seed was compared by using HPLC and biochemical markers analyses.

MATERIALS AND METHODS

In vitro Callus Production

Plant Materials: Seeds of *Silybum marianum* were washed under tap water for 1 hour and sterilized by soaking in 100% chlorox (containing 5.25% sodium hypochlorite NaClO) and Tween 20 (0.05% v/v) for 10 min and rinsed exhaustively with distilled water. The disinfected seeds were cultured on Murashige and Skoog [11] (MS) medium free growth regulators "control", to germinate of seeds. Culture was kept in growth chambers at 27±1°C in dark for 1 week. After germination of seeds, seedlings were transferred to light for 1 week. Then, leaves from seedling were cut and uses as explants for callus production.

Callus Initiation and Production: A Basel medium (B5) [17] was used for callus initiation, supplemented with different concentrations and combinations of an auxin; 2, 4-D or NAA (β-naphthalene acetic acid 0.0, 1.0 and 2.0 mg/l) in combination with BA (benzyl adenine) (0.1, 0.2 mg/l) as a cytokinin. In addition to B5 control medium (free growth regulators). The media were supplied with 30 g/l sucrose and the pH was adjusted to 5.7-5.8, before gelling with 3 g/l phytagel. Then were dispersed into 100 ml glass jars, 30 ml per each jar. Then closed with polypropylene caps and autoclaved at 121°C under a pressure of 1.1 kg/cm^2, for 20 min. Cultures were incubated at 25±2°C in the dark till sufficient amount of calli were produced. Induced calli were transferred to fresh media of the same composition every 4 weeks. Five replicates of each treatment were used, the percentage of callus induction and mean fresh weight of callus (g/Jar) were recorded after 4 week of culture. Dry weight of fresh callus was determined after drying in a vacuum oven at 65°C until constant weight obtained.

Extraction of Silymarin for HPLC Analysis: The amount of silymarin was determined by High Performance Liquid Chromatography (HPLC) according to Wen *et al.* [18]. Appropriate weights of the grinding plant transferred to 25 ml volumetric flask and then mixed with 20 ml of MeOH. The mixtures were sonicated for 15 min at room temperature and diluted to 25 ml with MeOH. The mixtures were then filtered by a Millex-HX Nylon syringe filter (0.45 um, 25 mm; Millipore, Bed ford, MA) to remove any particles. The first 5 ml of the filtrates were discarded and the following filtrates were collected. Appropriate aliquots of the filtrates were diluted with 50% MeOH, as well as the addition of NG (final concentration of 5 μg/ml) and analyzed by the HPLC.

Biochemical Analysis

Total Protein Analysis: Callus samples (2 g) of each treatment were ground with liquid nitrogen and mixed with extraction buffer (pH7.5, 50 mM tris, 5% glycerol and 14 mMB-mercaptoethanol) in a mortar with pestle, left overnight then vortexed for 15 sec and centrifuged at 10.000 rpm at 4°C for 10 min. The supernatants were transferred to new eppendorf tubes and kept at-20°C until used for electrophoresis analysis, according to Laemmli [19].

Isozymes Detection: The Isozymes were used: α-and β-esteras (Est.), acid phosphatase (Acph.), alcohol dehydrogenase (Adh.), aldehyde oxidase (Ao.), malic enzyme (Ma) malate dehydrogenase (Mdh), SKDH (skimatic dehydrogenase), catalase (CAT), superoxide dismutase(SOD), polyphenoloxidas (pod) and peroxidase (Px). Isozymes were separated according to Stegemann *et al.* [20]. In gels staining, protocols of Scandalios [21] were used for α and β-Est.; Wendel and Wenden [22] for Ao, Acph and Adh; Jonathan and Wendell, [23] for Mal and Mdh and Heldt [24] for Px. Gels were washed two or three times with tap water; fixed in ethanol: 20% glacial acetic acid (9:11 v/v) for 24 hours; and photographed.

Statistical Analysis: All experimental analysis was carried out on a minimum of six independent samples for each treatment and four replicates were assayed. Statistical significance was calculated using Duncan test for

unpaired data ($\alpha \leq 0.05$) and ANOVA method was used for comparison of means Duncan [25] as described by Snedecor and Cochran [26].

RESULTS AND DISCUSSION

***In vitro* Callus Production:** Results presented in Table 1 showed that callus initiation was affected by concentrations of growth regulators in culture media. In this respect, 100% of leaf sections of *Silybum marinum* induced white to yellow friable callus on B5 medium containing 2, 4-D or NAA combinations with BA, except the control treatment, which gave 40% callus production. The mean fresh weight of callus after 1 month ranged between 7.09 and 31.80 g/Jar. B5 medium containing 2 mg/l 2,4-D and 0.2 mg/l BA gave the maximum fresh weight of callus, followed by 1.0 mg/l 2,4-D + 0.1 mg/l BA which gave 30.06 g callus / Jar.

It was found that the addition of 2 mg/l NAA in combination with 0.2 mg/l BA into B5 medium recorded 29.37 (g) mean fresh weight of callus. The control treatment gave the less growth parameters. Using 2, 4-D and BA were more effective in promoting callus indication than NAA and BA as recorded by Ahmed and Koperuncholan, [27]. Rasoal *et al.* [28] found that among the cytokinins, BA is more effective than kin for callus induction when used in combination with 2, 4-D. Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and usually used together for callus production [29]. It was revealed that auxins played an important role in the callus induction and different types of auxins had various effects [30] and the cytokinins facilitate the effect of auxin in callus induction [31].

Auxins and cytokinins participate in regulation of cell cycle, but auxin may regulates and even may lead to DNA replication, while cytokinin regulates event leads to mitosis, which are highly correlated to phenotype changing and cells growth in *in vitro* culture [32].

Effect of Plant Growth Regulators on Silymarin Content: To determine the effect of different treatments on the growth of callus and its silymarin content, five media (including control) under dark conditions were tested. It has previously been reported that dark conditions influence growth of callus cultures of *H. performatum* and increase production of Taxue [33]. A possible effect of light in inhibiting growth might be due to the changes in metabolism of gibberellins. Reduced amounts of active gibberellins have been found in light-grown tobacco calli and this has been associated with lower growth rates [33]. Another possibility is through the down-regulation of key regulatory enzymes responsible for mevalonate synthesis and its role in cell dividing zones, biosynthesis of gibberellins and cytokinins. It also, may be due to the stimulation of biosynthesis of phenolics by light and decrease in activity of various enzymes, or low expression of key enzyme and down regulated catabolism [33]. Data in Table 2 and Fig. 1 reveled that the maximum fresh and dry weights of callus were recorded on the medium supplemented with 2 mg/l 2,4-D + 0.2 mg/l BA comparing to the other treatment including media containing. It was noticed that fresh and dry weights increased with increasing the concentrations of 2, 4-D, NAA and BA.

The maximum amount of silymarine accumulation was noticed in callus cultures grown on B5 medium supplemented was 2 mg/l 2,4-D + 0.2 mg/l BA which gave 8.91 mg/g dry weight of callus. These results are in

Table 1: Callus induction from leaf sections of *Silybum marianum* cultured on B5 medium containing auxins with BA

Growth regulators concentrations (mg/l)				
2,4-D	NAA	BA	% Of callus induction	Mean fresh weight of callus (g/Jar)
0.0	0.0	0.0	40 b	7.09 e
1.0	0.0	0.1	100 a	30.06 b
2.0	0.0	0.2	100 a	31.80 a
0.0	1.0	0.1	100 a	23.09 d
0.0	2.0	0.2	100 a	29.37 c

Table 2: Effect of different concentrations of auxin (2, 4-D and NAA)) and cytokinin (BA) on silymarin contents in leaf calli of *Silybum marianum*

Treatments	Mean fresh weight of callus (g)	Mean dry weight of callus (g)	Silymarin content (mg/g) dry weight
Control	9.30e	0.30e	0.332
1.0 mg/l 2,4-D + 0.1mg/l BA	99.73b	4.35b	1.92
2.0 mg/l 2,4-D + 0.2mg/l BA	104.31a	5.10a	8.91
1.0 mg/l NAA + 0.1 mg/l BA	43.92d	1.73d	0.526
2.0 mg/l NAA + 0.2 mg/l BA	72.81c	2.64c	0.586
Seeds	-	-	1.51

Fig. 1: Callus of Silybum marianum on B5 madium supplement with 2 mg/l 2,4-D and 0.2 mg/l BA

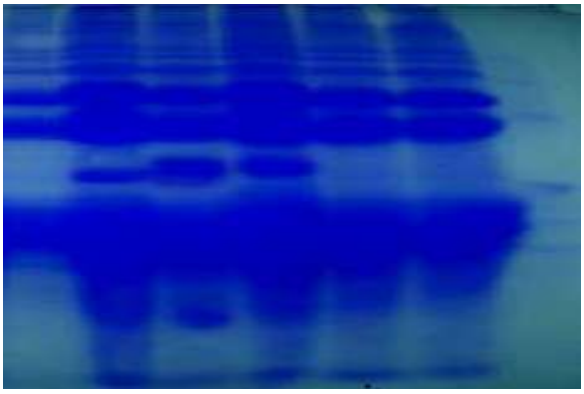

Fig. 2: SDS-PAGE profiles of soluble proteins extracted from six Silybum samples

harmony with Ceillia *et al.* [34] who noticed that the maximum callusing was obtained from leaves which cultured on B5 medium supplemented with 2, 4-D and BA. Comparing the silymarin content in callus tissue with that in seeds, it was found that callus produced the higher amounts of silymarin, reached 8.9 mg/g dry weight, than seeds, which recorded 1.51 mg/g dry weight. Silymarin content in callus tissue reached 6 folds that in the seeds of *Silybum marianum*. This result is in agreement with those obtained by Bekheet [35] who found that several products are found to be accumulating in cultured cells at a higher level than those in native plants.

Biochemical Analysis: Biochemical analysis has been conducted based on total proteins and isozyme polymorphism to distinguish between *Silybum marianum* callus cultures under different treatments and the seeds. Criterion of scoring was the absence of any given band (null phenotype) in one or more samples difference in band intensity due to either the dose effect (if present) or differential regulation of genes.

SDS-PAGE of Leaf Proteins: SDS-PAGE of total soluble proteins has been carried out on the six *Silybum marianum* samples. Electrophoretic banding patterns of total proteins for all the studied samples of *Silybum marianum* are illustrated in Fig. 2 and Table 3 the bands were detected with different molecular weights ranging from 13.5 kDa to 1.3 kDa. The resulted profile showed six polymorphic bands with a percentage of 25%, while the remainder bands were scored as common bands with different molecular weights. Concerning band intensity, there were detectable changes in bands intensity for all six samples of *Silybum marianum*.

Isozyme Analysis: Twelve isozyme systems including Acph (acid phosphatase), Adh (alcohol dehydrogenase), SKDH (skimatic dehydrogenase), Mdh (malate dehydrogenase), Ao (Alkaline phosphate), Ma (Malic acid), Prx (peroxidase), α Est and β Est (esterases), SOD (Superoxide dismutase), CAT (catalase) and POD (poly phenol oxidase) were used to test the isozyme polymorphism among the studied *Silybum marianum* samples (Fig. 3 and Table 4). The resulted acid phosphatase (Acph) banding patterns of all six studied samples revealed five bands, one was monomorphic and four bands were polymorphic. Alcohol dehydrogenase (Adh) banding pattern revealed a total of two bands, one was monomorphic and the other band was polymorphic. Shikimate dehydrogenase (SKDH) exerted two bands, one monomrphic band and the other was polymorphic, among the six studied samples. Malate dehydrogenase (Mdh) banding pattern gave a total of three bands one monomorphic and the others were polymorphic bands.

Moreover, the resulted aldehyde oxidase (Ao) patterns of all six studied samples revealed one monomophic. The Malic acid (Ma) banding patterns revealed a total of two bands, one was monomorphic and the other was polymorphic. Peroxidase (Prx) bands

Table 3: SDS-PAGE patterns of total soluble proteins extracted from six *Silybum marianum* samples

MW	1	2	3	4	5	6	Status
13.526	1	1	1	1	1	1	Monomorphic
12.702	1	1	1	1	1	1	Monomorphic
11.761	1	1	1	1	1	1	Monomorphic
10.628	1	1	1	1	1	1	Monomorphic
9.910	1	1	1	1	1	1	Monomorphic
8.800	1	1	1	1	1	1	Monomorphic
7.566	0	0	1	1	1	0	Polymorphic
6.999	0	0	1	1	1	0	Polymorphic
6.794	1	1	1	1	1	1	Monomorphic
5.908	1	1	1	1	1	1	Monomorphic
5.013	1	1	1	1	1	1	Monomorphic
4.529	1	1	1	1	1	1	Monomorphic
2.999	0	0	1	1	1	0	Polymorphic
2.159	0	0	1	1	1	0	Polymorphic
1.996	1	1	1	1	1	1	Monomorphic
1.310	1	1	1	1	1	1	Monomorphic
Total band	--	--	--	--	--	--	16
Polymorpfic bands	--	--	--	--	--	--	4
% Of poly-morphism	--	--	--	--	--	--	25

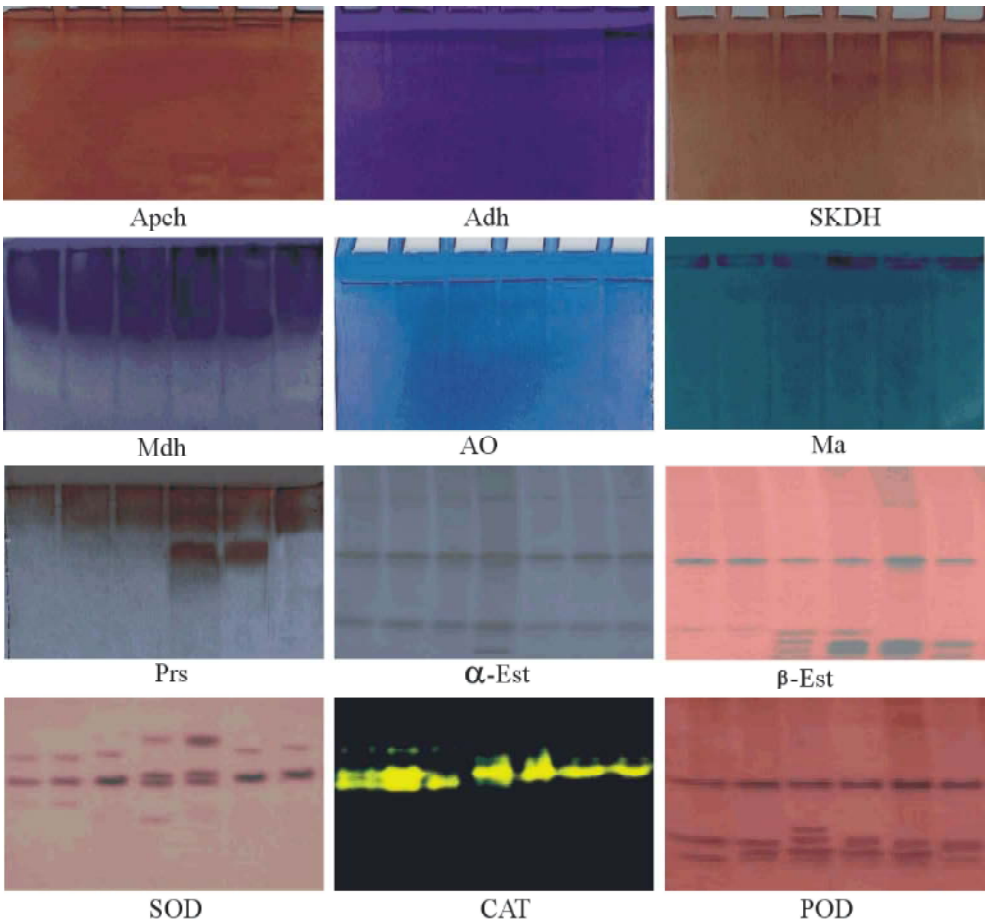

Fig. 3: Zymogram of Acph, Adh, SKDH, Mdh, AO, Ma, Prx, α-Est, β-Est, SOD, CAT, and POD, banding patterns among six *S. marianum* sample

Table 4: Polymorphism percentages generated by twelve isozymes systems among six *Silybum marianum* samples

No.	Types of isozyme	No of monomorphic bands	No of polymorphic bands	Total bands	% Polymorphism
1	Acph	1	4	5	80
2	Adh	1	1	2	50
3	SKDH	1	1	2	50
4	Mdh	1	2	3	67
5	Ao	1	0	1	00
6	Malic acid	1	1	2	50
7	Prx	1	1	2	50
8	α-est	3	1	4	75
9	β-est	2	5	7	71.4
10	SOD	3	2	5	40
11	CAT	0	3	3	100
12	POD	2	3	5	60
Total	--	17	24	41	58%

Table 5: Similarity matrix g the six *Silybum marianum* based on total analysis (protein and isozymes)

	1	2	3	4	5	6
1	100					
2	12.7	100				
3	37.5	26.3	100			
4	17.5	30.2	86.9	100		
5	37.5	32.5	85.7	60.0	100	
6	16.9	98.9	12.7	44.0	40.0	100

patterns revealed a total of two bands, one was monomorphic and one polymorphic. α Esterases (α Est), banding patterns revealed a total of four bands, three monomorphic and the other was polymorphic, β-esterase (β Est) exerted seven bands, two monomorphic bands, four polymorphic bands among the six studied samples. Superoxide dismutase (SOD) banding pattern revealed a total of five bands; three were monomorphic bands and two polymorphic bands. In addition, the resulted catalase (CAT) pattern of all six studied samples revealed 100% polymorphism.The Polyphenoloxidase (POD) banding pattern revealed a total of five bands, three polymorphic bands and two monomorphic bands. Based on isozyme markers, polymorphism percentages were estimated for the twelve isozyme systems in the six *Silybum marianum* samples as shown in Table 4. The highest polymorphism percentage was 100% for CAT and the lowest polymorphism percentage 0% for Ao. The average polymorphism using the twelve was tested isozymes was 58%among the six samples. The resulted Acph patterns, In contrast, these results are in agreement with those obtained by Saker *et al.* [36],who concluded that different Acph, Adh, AO and Esterases activity levels and isozyme banding patterns, with a variation in activity levels between the mother tree and the tissue culture clones, as well as among different tissue culture in date palm clones. The results disagree with Regla *et al.* [37], who found no polymorphism in the isoenzyme profile of Acph, Adh, AO and Esterases systems when they analyzed *in vitro* seedlings in soybean, tomato, coffee, soybean and potato with regenerating plants revealed the existence of high homogeneous enzymatic activity in the analyzed material which confirms that no genetic changes are associated to this system, due to the regeneration procedure they used. Moreover, these results are in accordance with the findings of Sang *et al.* [38] who concluded the significant increase in the activities of SOD, CAT, APX, POD and Ma under environmental stressed tissue culture calluses comparing with mother plant. In addition, these results are in agreement with those reported by Duarte *et al.* [39] who indicated polymorphism in Mdh activity levels between the mother plant and the tissue culture clones that in *Cactaceae* and *Broccoli* and SKDH in medicinal plants [40].

Combined Data of Proteins and Isozymes: Based on both protein analysis and isozyme analysis, similarity matrix was developed by SPSS computer package system as shown in Table 5. The closest relationship was scored between sample 2 and 6 with similarity %98.9. The lowest similarity was scored between sample 1 and 2with similarity 12.7%. The dendrogram Based on total

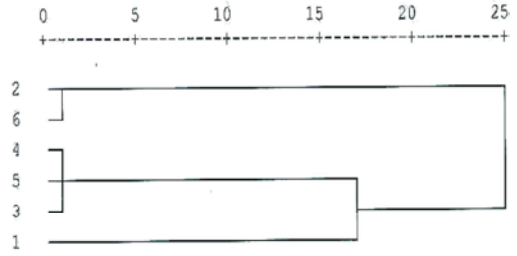

Fig. 4: Dendrogram based on isozyme and protein of six Slybum marianum samples

analysis separated the six *Silyium marianum* samples into two main clusters (Fig 4). Moreover, samples 2 and 6 were clustered together. While, the remain genotypes were clustered together in another cluster. In addition, sample 1 was separated in another cluster.

CONCLUSION

From the results obtained, the best culture medium for callus induction for biomass production of milk thistle *Silybum marianum* as a potential source of silymarine was B5 medium supplemented with 2 mg/l 2,4-D + 0.2 mg/l BA under dark incubation. Accumulation of silymarines was strong on the previous medium and gave 6 folds of active constituent content, compared with seeds in the plant. From the previous results, it is clear that tissue culture technique considered important source of secondary metabolites. Therefore, this technology are providing continuous, reliable source of plant pharmaceuticals and could be used for the large scale production under controlled condition. Biochemical genetic analysis based on SDS-PAGE of soluble proteins and twelve isozyme systems among the six *Silybum marianum* samples successfully supported tissue culture results for increasing of silymarines production under different treatments by discriminating the six *Silybum marianum* samples and helped in recommending the best treatment(B5 medium+2 mg/l 2,4-D +0.2 mg/l BA) for best production.

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