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Influence of Plant Growth Regulators on Callus Induction, Silymarin Production and Antioxidant Activity in *Silybum marianum* L. Gaertn. by Tissue Culture

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ABSTRACT

Silybum marianum (L.) is a medicinally important herb known for its liver-protecting compound, silymarin, which is an isomeric mixture of flavonolignans and taxifolin. In this study, a protocol was designed for the application of plant growth regulators at various concentrations and combinations of coconut water. This was aimed at initiating callus and silymarin production using different types of explants (root, hypoc -otyls, cotyledonary leaf) of *S. marianum*. Free radical scavenging activity of silymarin was evaluated in the extracts of callus cultures of these explants. In comparison with other plant growth regulators and explants, root explants that were cultured on MS medium and were enriched with 5.0 M NAA + 2.5 M BAP + 10% coconut water produced the most friable callus and had the highest possible level of silymarin and free radical scavenging activity. NAA + BAP + coconut water was found to be the best hormonal combination for callus initiation and mass production. This research can aid in the current understanding of the influence of plant growth regulators and different types of explants on the production of valuable secondary metabolites in *S. marianum* via *in vitro* cultures.

Introduction

Medicinal plants play a vital role in producing pharmaceutically important compounds (Owolabi et al., 2007). To explore an alternative method for producing medicinal compounds from plants, biotechnological techniques, especially plant cell and tissue cultures, are usually used as complementary traditional techniques for the industrial production of secondary metabolites (Ramachandra Rao and Ravishankar 2002). Among them, callus induction plays a major role in producing secondary plant metabolites in medicinal plants. In vitro production of PSMs is a new biotechnological technique for the large-scale production of these important medicinal compounds throughout the year (Gopi and Vatsala 2006).

The species, *Silybum marianum* (L.) Gaertn (Asteraceae) is a medicinal herb, famous for its production of a liver-protecting compound, silymarin, which is an isomeric mixture of flavonolignans and taxifolin (Hasanloo et al., 2008). Flavonolignans are formed by the combination of taxifolin (flavonoid) and coniferyl alcohol (phenylpropanoid) (Dewick, 2002). There are no reports on the correlation between free radical scavenging activity and origin of the callus, and the type of PGRs used for callus induction in *S. marianum*.

Therefore, in this study, we isolated secondary plant metabolites in callus cultures induced from different explants of *S. marianum* and evaluated

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the free radical scavenging activity.

Material and Methods

Plant material

In Petri dishes, three types of explants (root, hypocotyl, and cotyledonary leaf) were separated from seedlings that had germinated from seeds.

Callus induction

To optimize the induction conditions of soft and friable calli for cell suspension cultures as well as the production of secondary metabolites, we evaluated the effects of plant growth regulator and coconut water on callus induction. Three types of explants (root, hypocotyl, and cotyledonary leaf) were inoculated on MS (Murashige and Skoog, 1962) medium (MSM), enriched with the following:

Free hormone MSM (control)

5%, 10%, 20% coconut water.

10% coconut water in combination with different concentrations of NAA/IAA/IBA/BAP/KIN (5, 10, and 15 µM)

5, 10, 15 µM NAA + 2.5 µM BAP

5 µM NAA + 2.5 µM BAP + 10% coconut water.

Twenty-five explants were used for each treatment, and the experiments were repeated at least twice. All cultures were kept in a culture chamber at 25±2°C under a light period of 16 hours (~ 40 µmol m^{-2} s^{-1}). Callus was sub-cultured every three weeks on the same fresh medium containing the same type of plant growth regulators.

Callusing efficiency frequency was obtained using the following formula:

CEF (%) = [No. explants produced callus / Total no. of explants] X 100.

Effect of plant growth regulators and coconut water on silymarin production in callus cultures

The effects of various plant growth regulators and coconut water alone or in combination with plant growth regulators were studied. The results were used for inducing callus and for evaluating their effects on silymarin production in *S. marianum*.

Silymarin was obtained from 1 g callus obtained from root explants in MS medium with different plant growth regulators and coconut water, either by 10% coconut water, 5.0 µM NAA + 10% coconut water, 5.0 µM BAP + 10% coconut water, 5 µMNAA + 2.5 µM BAP and 5.0 µM NAA + 2.5 µM BAP + 10% coconut water.

The extracted silymarin was analyzed by HPLC and was repeated at least three times.

Effect of explant type on silymarin production in callus cultures

In this study, the effect of callus origin was evaluated on the amount of silymarin in *S. marianum*. Ten-day-old seedlings grown in vitro were harvested. Their different parts (root, hypocotyl, cotyledonary leaf) were used as explants for callus induction in enriched MSM with 5 µMNAA + 2.5 µM BAP + 10% coconut water. The silymarin content of 1.0 g of dry callus was extracted from different explants, analyzed by HPLC, and repeated at least 3 times.

Extraction and determination of silymarin

Dried powder of callus samples (1.0 gr) and treatment with ethyl acetate removed fats and then silymarin was extracted with methanol (40°C overnight). The dried crude extract was dissolved in 1 ml of methanol and stored in the dark at 4ºC (Cacho et al., 1999). The HPLC was performed according to Radjabian et al.(2008), along with a Shimadzu liquid chromatograph equipped with LC-20 AD pump, SPD-20A UV detector, C18G, 5 µm (4.6 × 250 mm) column. A mixture of methanol-water (50:50 v/v, o-phosphoric acid (0.15% W/V) was used as the mobile phase. The elution has been made in an isocratic mode (1mL/min flow rate) and wavelength of 288 nm for detection. An analysis requires 35 min.

Determination of DPPH free-radical scavenging activity

DPPH free-radical scavenging activity of dried crude extracts of calli was induced on MSM, enriched with diverse concentrations and combinations of PGRs and coconut water (5.0 µM NAA + 2.5 µM BAP + 10% coconut water;5.0 µM NAA + 10% CW;5.0 µM NAA + 2.5 µM BAP;5.0 µM BAP + 10% coconut water) as well as different explants (root, cotyledonary leaf, hypocotyl), which were examined on *S. marianum* according to Koksal et al. (2009).

Results

Callus induction

The explants, root, hypocotyl, and cotyledonary leaf of *S. marianum* were cultured on MSM without plant growth regulators and MSM enriched with different concentrations and combinations of plant growth regulators. Explants cultured on MSO failed to induce callus even after four weeks of incubation in *S. marianum*. Initially, all of these types of explants were cultured on MSM enriched with different concentrations of coconut water (5%, 10%, 20%) to standardize the coconut water concentration

for callus induction in *S. marianum*. According to our results, more callus was induced in response to 10% coconut water in all explants. In *S. marianum*, friable callus formed from all explants at different coconut water concentrations. Callus was obtained from all explants in all concentrations and was examined with PGRs + coconut water combinations (Tables 1-3). The mixture of PGRs and the type of explants influenced callus induction times, callusing efficiency, and callus weight. The absolute percentage of callus induction with friable nature was observed in root explants on MSM enriched with 5.0 µM NAA + 2.5 µM BAP + 10% coconut water, and then cotyledonary leaf explants performed similarly on the same combinations of PGRs. The mean weight of callus (6.91±0.123 gram) was higher in root explants compared to other explants (Table 1).

Table 1. Effect of NAA/IAA/IBA/BAP/KIN + CW on callus induction from root explants in *S. marianum.*
IAA= Indole-3-acetic acid **IBA**=Indole-3-butyric acid **BAP**= 6-Benzylaminopurine
KIN=kinetin **NAA**=Naphthaleneacetic acid **CW**=coconut water

The concentration of PGRs (µM)					CW (%)	No. of Days for Callus Initiation	% of callus Induction	Avg. Weight (g) of Callus (±S.E)[a]
NAA	IAA	IBA	BAP	KIN				
5.0			2.5			10	40	1.06±0.070^{h}
10.0			2.5			10	38	0.91±0.048^{hi}
15.0			2.5			10	34	0.85±0.065^{hi}
5.0					10	10	78	2.05±0.074^{ef}
10.0					10	10	72	1.86±0.068^{f}
15.0					10	10	70	1.47±0.059^{g}
	5.0				10	15	32	1.50±0.086^{g}
	10.0				10	15	34	0.60±0.043^{i}
	15.0				10	15	32	0.95±0.069^{hi}
		5.0			10	15	26	0.86±0.090^{hi}
		10.0			10	15	28	1.06±0.115^{h}
		15.0			10	15	26	1.04±0.106^{h}
			5.0		10	10	88	4.83±0.125^{b}
			10.0		10	10	78	4.18±0.126^{c}
			15.0		10	10	76	4.02±0.102^{c}
				5.0	10	20	42	2.45±0.141^{d}
				10.0	10	20	44	2.23±0.144^{de}
				15.0	10	20	48	2.26±0.085^{de}
5.0			2.5		10	7	100	6.91±0.123^{a}

a. Means sharing the same letter within columns are not significantly different ($p \leq 0.05$) using Duncan's multiple range test.
Cream-soft-friable callus was developed in all concentrations and combinations of PGRs used in *S. marianum*.

Interestingly, calluses from roots and hypocotyl explants were cream-friable, regardless of the PGRs used, whereas compact green calli was observed in all concentrations and combinations of PGRs, except in some concentrations (at 5.0 µM NAA + 10% CW, 10.0 µM NAA + 10% coconut water, 15.0 µM NAA + 10% coconut water and 5.0 µM NAA + 2.5 µM BAP + 10% CW) (Fig. 2, 3, 4). Cream-soft friable calli were produced in all explants at a concentration of 5.0µM NAA + 2.5 µM BAP + 10% CW. The correlation between the mean callus weight and callus induction percentages in all explants was significant and positive ($P \leq 0.01$).

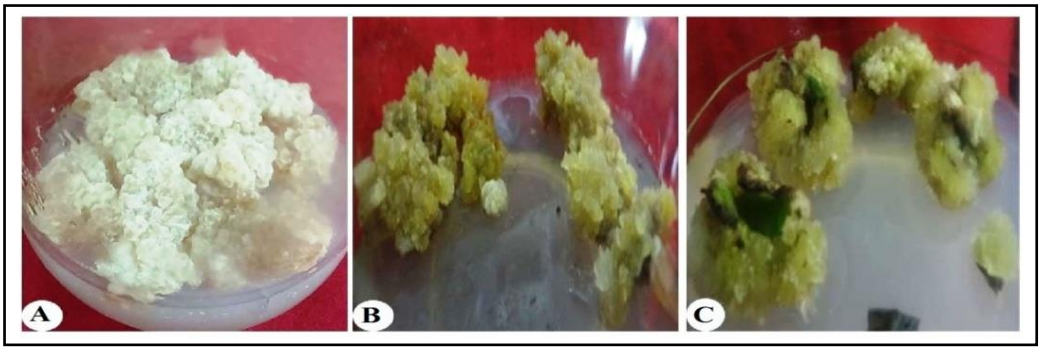

Fig. 1. Induction of callus on MS (Murashige and Skoog) medium supplemented with 5.0 µM NAA(Naphthaleneacetic acid)+ 2.5 µM BAP (6-Benzylaminopurine) + 10% CW (coconut water) using root (A), hypocotyl (B), and cotyledonary leaf (C) explants in *S. marianum.*

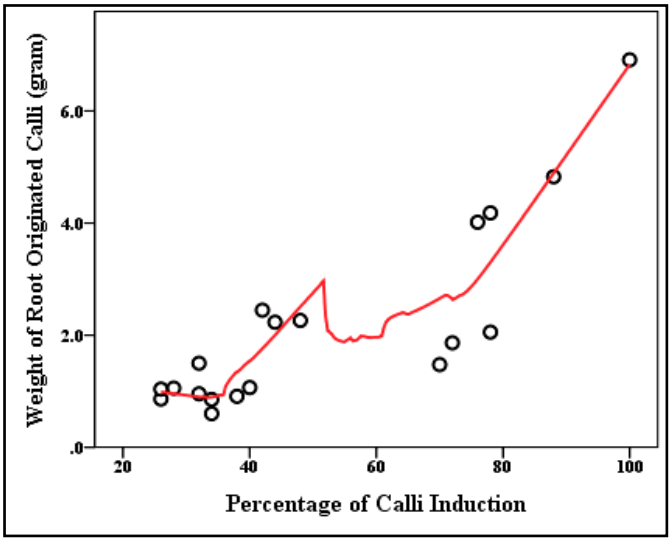

Fig. 2. Scatterplot of correlation comparison between the percentage of callus induction and weight of callus induced from root explants in *S. marianum*. [The correlation between percentage of callus induction and weight of callus was significant and positive (0.843) at the 0.01 level (2-tailed).]

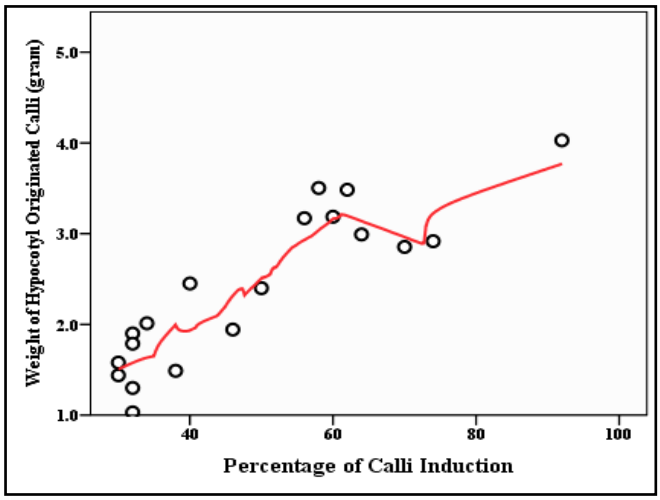

Fig. 3. Scatterplot of correlation comparison between the percentage of callus induction and weight of callus induced from hypocotyl explants in *S. marianum*. The correlation between the percentage of callus induction and weight of callus was significant and positive (0.888) at the 0.01 level

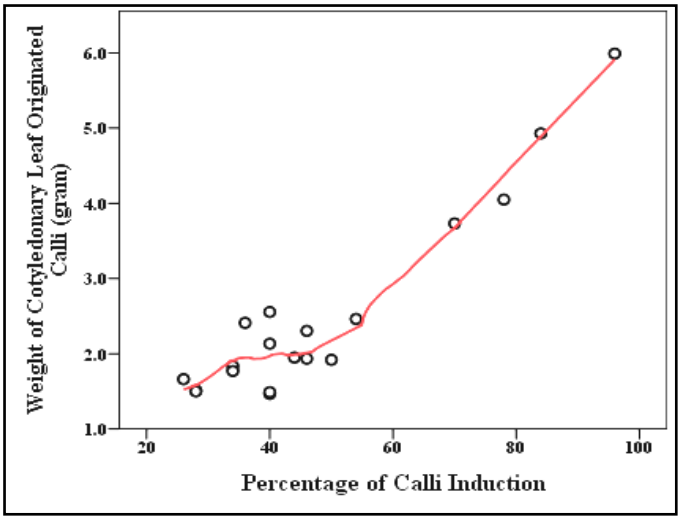

Fig. 4. Scatterplot of correlation comparison between the percentage of callus induction and weight of callus induced from cotyledonary leaf explants in *S. marianum*. [The correlation between the percentage of callus induction and weight of callus was significant and positive (0.945) at the 0.01 level (2-tailed).]

Table 2. Effect of NAA/IAA/IBA/BAP/KIN + CW on callus induction from hypocotyl explants in *S. marianum*.
IAA= Indole-3-acetic acid **IBA**=Indole-3-butyric acid **BAP**= 6-Benzylaminopurine
KIN=kinetin **NAA**=Naphthaleneacetic acid **CW**=coconut water

The concentration of PGRs (µM)					CW (%)	No. of Days for Callus Initiation	% of callus Induction	Avg. Weight (g) of Callus (±S.E)[a]
NAA	IAA	IBA	BAP	KIN				
5.0			2.5			20	60	3.19±0.096[c]
10.0			2.5			20	50	2.40±0.102[e]
15.0			2.5			20	46	1.94±0.087[f]
5.0					10	20	74	2.92±0.086[cd]
10.0					10	20	70	2.85±0.099[d]
15.0					10	20	64	2.99±0.055[cd]
	5.0				10	25	38	1.49±0.080[g]
	10.0				10	25	34	2.01±0.148[f]
	15.0				10	25	40	2.45±0.120[e]
		5.0			10	25	30	1.44±0.099[g]
		10.0			10	25	32	1.30±0.106[gh]
		15.0			10	25	32	1.90±0.116[f]
			5.0		10	20	62	3.48±0.076[b]
			10.0		10	20	58	3.50±0.085[b]
			15.0		10	20	56	3.17±0.098[c]
				5.0	10	30	32	1.79±0.077[fg]
				10.0	10	30	30	1.58±0.088[gh]
				15.0	10	30	32	1.03±0.076[h]
5.0			2.5		10	15	92	4.03±0.066[a]

Mean values sharing the same letter within columns are not significantly different ($p \leq 0.05$) using Duncan's multiple range test. Cream-soft-friable callus was developed in all concentrations and combinations of PGRs used in *S. marianum*.

Table 3. Effect of NAA/IAA/IBA/BAP/KIN + CW on callus induction from cotyledonary leaf explants in *S. marianum.*
IAA= Indole-3-acetic acid **IBA**=Indole-3-butyric acid **BAP**= 6-Benzylaminopurine
KIN=kinetin **NAA**=Naphthaleneacetic acid **CW**=coconut water

The concentration of PGRs (µM)					CW (%)	No. of Days for Callus Initiation	% of callus Induction	Avg. Weight (g) of Callus (±S.E)[a]	Texture of Callus
NAA	IAA	IBA	BAP	KIN					
5.0			2.5			15	46	2.30±0.116^{de}	Green-Compact
10.0			2.5			15	44	1.95±0.116^{fg}	Green- Compact
15.0			2.5			15	40	2.13±0.103^{ef}	Green- Compact
5.0					10	15	84	4.93±0.131^{b}	Cream-Soft-friable
10.0					10	15	78	4.05±0.090^{c}	Cream-Soft-friable
15.0					10	15	70	3.73±0.064^{c}	Cream-Soft-friable
	5.0				10	20	40	1.46±0.085^{h}	Green- Compact
	10.0				10	20	36	2.41±0.113^{de}	Green- Compact
	15.0				10	20	40	2.55±0.091^{d}	Green- Compact
		5.0			10	20	40	1.49±0.093^{h}	Green- Compact
		10.0			10	20	28	1.51±0.107^{h}	Green- Compact
		15.0			10	20	26	1.66±0.137^{gh}	Green- Compact
			5.0		10	20	54	2.46±0.117^{de}	Green- Compact
			10.0		10	20	46	1.93±0.113^{fg}	Green- Compact
			15.0		10	20	50	1.92±0.065^{fg}	Green- Compact
				5.0	10	25	34	1.83±0.110^{fgh}	Green- Compact
				10.0	10	25	34	1.77±0.058^{fgh}	Green- Compact
				15.0	10	25	28	1.50±0.127^{h}	Green- Compact
5.0			2.5		10	10	96	5.99±0.094^{a}	Cream-Soft-friable

Means sharing the same letter within columns are not significantly different ($p \leq 0.05$) using Duncan's multiple range test.

Effect of PGRs, coconut water on silymarin production in callus cultures

Silymarin extracted from the samples was measured by HPLC (Fig. 5). The callus induced on 5.0 µM NAA + 2.5 µM BAP + 10% coconut water produced the highest silymarin level, followed by 5.0 µM NAA + 10% CW. While 5.0 µM BAP + 10% coconut water showed the lowest silymarin

production (Table 4), the combination of 5.0 µM NAA + 2.5 µM BAP showed maximum taxifolin production, and 5.0 µM BAP + 10% coconut water had the least effect. Interestingly, the level of silychristin increased to a maximum of 10% coconut water, followed by the effect of 5.0 µM NAA + 2.5 µM BAP + 10% coconut water. In addition, the production of silydianin, silibinin A, silibinin B, isosilibinin A and isosilibinin B increased at 10 µM NAA + 5.0 µM BAP + 10% coconut water (Table 4). Based on our findings, the presence of NAA and coconut water in the cultured medium showed a positive effect on silymarin production. The measured flavonolignans and taxifolin correlation coefficients were always positive in *S. marianum* (Table 5).

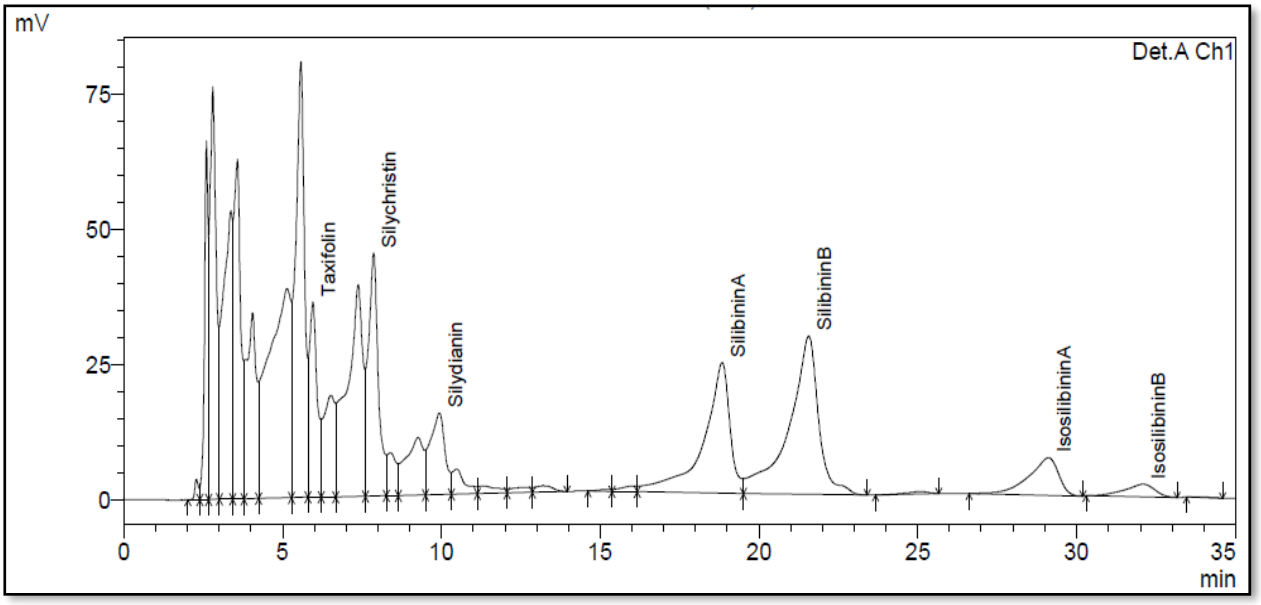

Fig. 5. HPLC of silymarin produced by root-originated callus cultures on MS medium supplemented with NAA (Naphthaleneaceticacid)+BAP (6-Benzylaminopurine) +CW (Coconut Water) in *S. marianum.*

Effect of explant type on silymarin production in callus culture

We evaluated the effects of callus origin on silymarin (flavonolignans + taxifolin) content in S. marianum in this study. After two months of callus growth on MSM, enriched with 5 MNAA + 2.5 M BAP + 10% coconut water, silymarin was extracted and analyzed using the HPLC method from 1 gm of dry powder callus. Compared to other studied explants, callus from root explants contained the highest level of silymarin (0.315 mg/g DW) (Table 6). Interestingly, taxifolin and silydianin produced approximately the same amount of callus from the root and cotyledonary leaf explants. All flavonolignans, except silydianin, are found mostly in root-originated callus. The synthesis of silydianin in roots and callus from cotyledonary leaf was nearly equal. The relationship between various flavonolignans and taxifolin was significant and positive (Table 7).

Table 4. Effect of CW, PGRs + CW on the production of silymarin from root callus cultures in S. marianum. CW=coconut waterPGR= plant growth regulators

Type of Flavonolignans	Concentration of PGRs (µM) + CW	Mean quantity (mg/g DW)	Std. Error	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Silymarin*	10% CW	.235	.007535	.20325	.26809
	5.0 NAA + 10% CW	.291	.003712	.27536	.30730
	5.0 BAP + 10% CW	.158	.004726	.13767	.17833
	5.0 NAA + 2.5 BAP	.249	.008622	.21190	.28610
	5.0 NAA + 2.5 BAP + 10% CW	.315	.003180	.30199	.32935
Taxifolin	10% CW	.028	.001528	.02143	.03457
	5.0 NAA + 10% CW	.033	.000882	.02954	.03713
	5.0 BAP + 10% CW	.027	.001155	.02203	.03197
	5.0 NAA + 2.5 BAP	.034	.001202	.02916	.03950
	5.0 NAA + 2.5 BAP + 10% CW	.032	.001453	.02608	.03858
Silychristin	10% CW	.060	.001856	.05268	.06865
	5.0 NAA + 10% CW	.048	.000882	.04487	.05246
	5.0 BAP + 10% CW	.023	.000882	.01987	.02746
	5.0 NAA + 2.5 BAP	.037	.001202	.03216	.04250
	5.0 NAA + 2.5 BAP + 10% CW	.052	.002028	.04361	.06106
Silydianin	10% CW	.021	.001453	.01542	.02792
	5.0 NAA + 10% CW	.025	.002082	.01604	.03396
	5.0 BAP + 10% CW	.016	.001000	.01170	.02030
	5.0 NAA + 2.5 BAP	.024	.000667	.02146	.02720
	5.0 NAA + 2.5 BAP + 10% CW	.026	.000333	.02523	.02810
SilibininA	10% CW	.044	.001453	.03842	.05092
	5.0 NAA + 10% CW	.065	.002186	.05626	.07507
	5.0 BAP + 10% CW	.031	.001155	.02603	.03597
	5.0 NAA + 2.5 BAP	.061	.002603	.05013	.07253
	5.0 NAA + 2.5 BAP + 10% CW	.073	.001155	.06803	.07797
SilibininB	10% CW	.061	.002186	.05226	.07107
	5.0 NAA + 10% CW	.089	.000882	.08587	.09346
	5.0 BAP + 10% CW	.041	.001528	.03443	.04757
	5.0 NAA + 2.5 BAP	.068	.001856	.06068	.07665
	5.0 NAA + 2.5 BAP + 10% CW	.098	.001202	.09316	.10350
IsosilibininA	10% CW	.013	.000882	.00954	.01713
	5.0 NAA + 10% CW	.021	.001202	.01616	.02650
	5.0 BAP + 10% CW	.015	.001732	.00755	.02245
	5.0 NAA + 2.5 BAP	.016	.001202	.01150	.02184
	5.0 NAA + 2.5 BAP + 10% CW	.025	.001453	.01908	.03158
IsosilibininB	10% CW	.005	.000667	.00280	.00854
	5.0 NAA + 10% CW	.007	.000333	.00623	.00910
	5.0 BAP + 10% CW	.004	.000333	.00290	.00577
	5.0 NAA + 2.5 BAP	.006	.000667	.00346	.00920
	5.0 NAA + 2.5 BAP + 10% CW	.007	.001764	.00008	.01526

*- Silymarin is an isomeric mixture of flavonolignans (silychristin, silydianin, silibinin, isosilibinin) and taxifolin. All experimental analyses were performed in a minimum of three independent samples for each elicitation period.

Table 5. Phenotypic correlation coefficient of the measured silymarin (flavonolignans and taxifolin) (mg/g DW) extracted from root-originated callus cultures induced by different PGRs and CW in *S. marianum*.
PGR= plant growth regulators **CW**=coconut water

	Silymarin	Taxifolin	Silychristin	Silydianin	SilibininA	SilibininB	IsosilibininA	IsosilibininB
Silymarin	1							
Taxifolin	0.689**	1						
Silychristin	0.679**	0.137	1					
Silydianin	0.902**	0.729**	0.593*	1				
Silibinin A	0.957**	0.785**	0.475	0.868**	1			
Silibinin B	0.983**	0.665**	0.959*	0.863**	0.948**	1		
Isisilibinin A	0.755**	0.522*	0.230	0.563*	0.773**	0.812**	1	
Isisilibinin B	0.735*	0.683**	0.374	0.696**	0.729**	0.721**	0.487	1

*. Correlation is significant at the 0.05 level (2-tailed) **. Correlation is significant at the 0.01 level (2-tailed)

Table 6. Effect of type of explant on silymarin production from callus cultures in *S. marianum*.

Type of Flavonolignans	Type of Explant	Mean quantity (mg/g DW)	Std. Error	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Silymarin*	Root	.315	.003180	.30199	.32935
	Hypocotyl	.203	.001528	.19643	.20957
	Cotyledonary Leaf	.290	.008544	.25324	.32676
Taxifolin	Root	.032	.001453	.02608	.03858
	Hypocotyl	.027	.001453	.02142	.03392
	Cotyledonary Leaf	.033	.001155	.02803	.03797
Silychristin	Root	.052	.002028	.04361	.06106
	Hypocotyl	.034	.001528	.02743	.04057
	Cotyledonary Leaf	.049	.001202	.04416	.05450
Silydianin	Root	.026	.000333	.02523	.02810
	Hypocotyl	.021	.001155	.01603	.02597
	Cotyledonary Leaf	.027	.002082	.01804	.03596
SilibininA	Root	.073	.001155	.06803	.07797
	Hypocotyl	.040	.002333	.03029	.05037
	Cotyledonary Leaf	.067	.002082	.05804	.07596
SilibininB	Root	.098	.001202	.09316	.10350
	Hypocotyl	.059	.003283	.04554	.07379
	Cotyledonary Leaf	.085	.002082	.07604	.09396
IsosilibininA	Root	.025	.001453	.01908	.03158
	Hypocotyl	.015	.001202	.01050	.02084
	Cotyledonary Leaf	.022	.001732	.01455	.02945
IsosilibininB	Root	.007	.001764	.00008	.01526
	Hypocotyl	.004	.000333	.00323	.00610
	Cotyledonary Leaf	.006	.000333	.00523	.00810

* Silymarin is an isomeric mixture of flavonolignans (silychristin, silydianin, silibinin, isosilibinin) and taxifolin.
All experimental analyses were carried out in a minimum of three independent samples for each elicitation period.

Table 7. Phenotype correlation coefficient of the measured silymarin (flavonolignans + taxifolin) extracted from calli originated from different explants in *S. marianum.*

	Silymarin	Taxifolin	Silychristin	Silydianin	SilibininA	SilibininB	IsosilibininA	IsosilibininB
Silymarin	1							
Taxifolin	0.745*	1						
Silychristin	0.959**	0.706*	1					
Silydianin	0.856**	0.635	0.851**	1				
Silibinin A	0.985**	0.767*	0.945**	0.819**	1			
Silibinin B	0.973**	0.648	0.904**	0.766*	0.940**	1		
Isisilibinin A	0.909**	0.450	0.883**	0.850**	0.855**	0.909**	1	
IsisilibininB	0.687*	0.786*	0.495	0.564	0.699*	0.660	0.493	1

*. Correlation is significant at the 0.05 level (2-tailed)
**. Correlation is significant at the 0.01 level (2-tailed)

Effect of PGRs on DPPH free-radical scavenging activity of callus extracts

After evaluating the free-radical scavenging activity of DPPH (FRSA), among dried crude extracts of calluses, the results varied depending on MSM, enriched with various compounds, concentrations of PGRs, and coconut water. Using cotyledonary leaf explants in *S. marianum*, the percentages of inhibition and the EC50 values of calli were different, depending on the MSM, enriched with different PGRs (Fig. 6).

Different concentrations (50-200 µg/ml) of crude extracts were used in the present study. Dilutions of callus extract, relating to 5.0 µMNAA + 2.5 µM BAP + 10% CW, showed the maximum value of FRSA, compared to other compounds of PGRs. Based on our observations, these three PGRs together affected the FRS activity in *S. marianum* (Fig. 6).

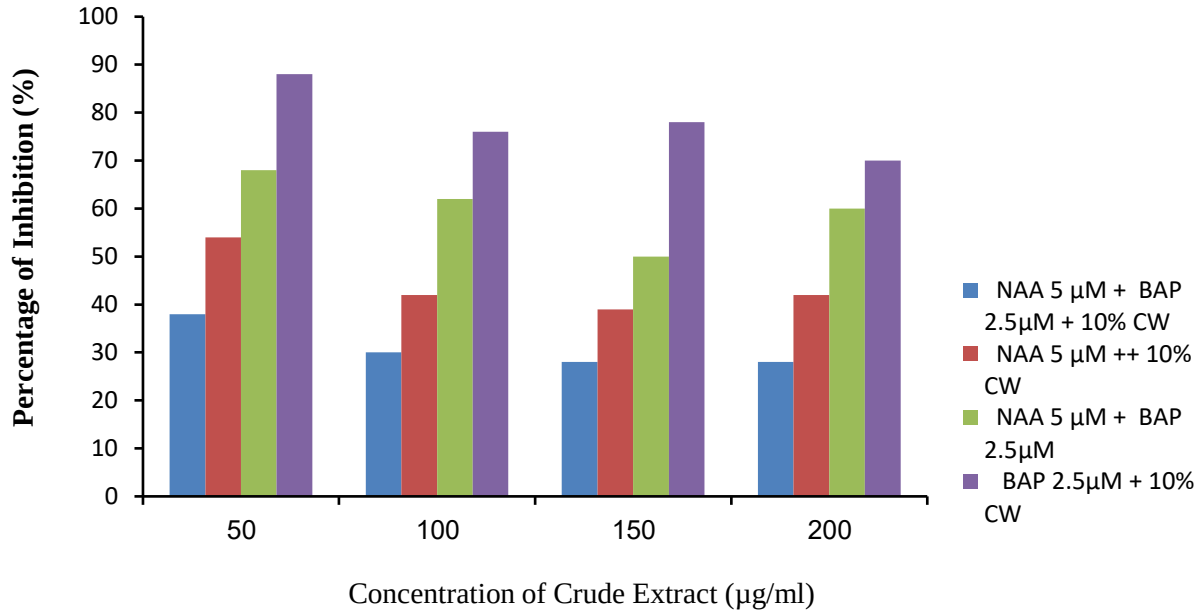

Fig. 6. Effect of PGRs (Plant Growth Regulators) on Free-Radical Scavenging Activity of callus extracts originated from cotyledonary leaf explants of *S. marianum*.

Effect of explant type on DPPH free-radical scavenging activity of the callus extract

First, the concentration and combination of PGRs on FRSA in *S. marianum* were optimized. The DPPH-FRSA dry crude extracts of MSM-induced calli enriched with 5.0 MNAA + 2.5 M BAP + 10% CW were studied herein while using root, hypocotyl, and cotyledonary leaf explants from *S. marianum* (Fig. 7). When induced calli from other tested explants were compared to calli extracted from the root, the latter type had the highest FRSA.

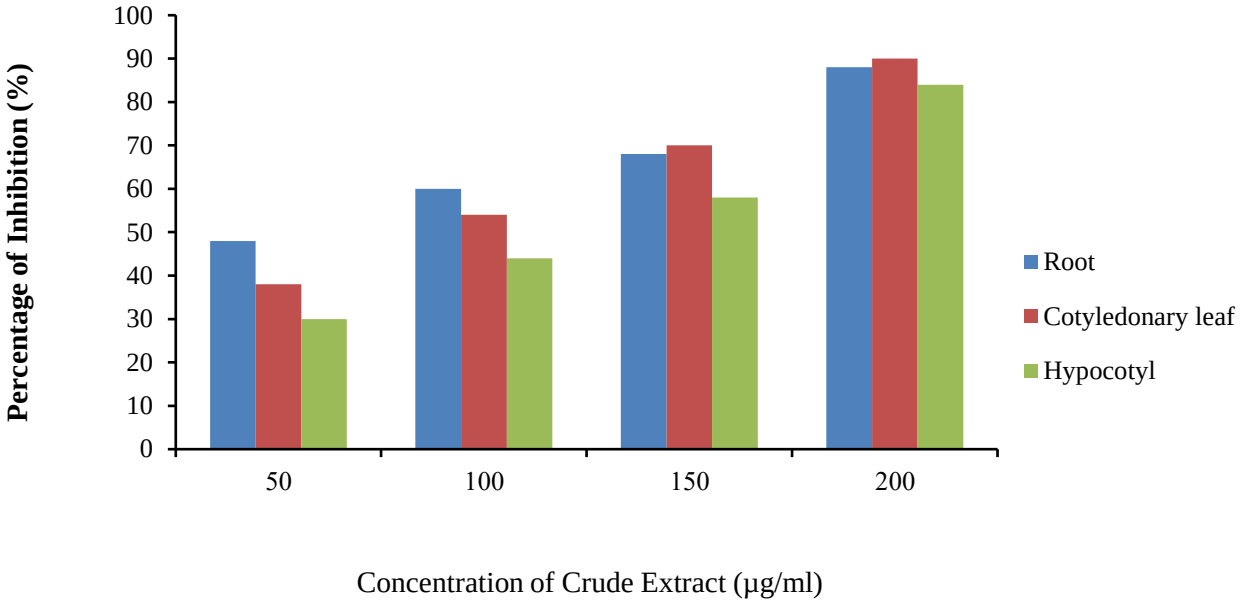

Fig. 7. Effect of type of explant on free-radical scavenging activity in *S. marianum*.

Discussion

The present study found that coconut water is the most effective PGR in assisting with the production of higher amounts of callus mass, due to its high concentrations of glucose and fructose, as well as various minerals, amino acids, and fatty acids (Santoso et al., 1995). It also contains diphenyl urea (which functions as cytokinin) and can accelerate growth by inducing plant cell division (Gnasekaran et al., 2010; Texeira et al., 2006). It was also found that 10% coconut water was most effective in producing more calli in *Phalaenopsis violacea* (Gnasekaran et al., 2010).

Growth status, as well as biotic and abiotic stress, have important effects on the production and accumulation of PSMs in medicinal plants (Nikolova and Ivancheva, 2005; Abbasi et al., 2011; Naz et al., 2013). In addition, enrichment of the medium with CW increased PSM production in callus cultures and cell suspension cultures by developing and modifying plant cell growth pathways (Orihara et al., 2002). The effect of PGRs (auxin and cytokinin) in the medium on PSM production was documented in earlier research (Sudria et al., 1999). A similar result was seen in the current study on silymarin production in the case of *S. marianum* callus cultures. Tawaha et al. (2007) discovered a correlation between total phenolic content and FRS activity in crude extracts of S. marianum. According to Wallace et al. (2008), the protective effect of silymarin on the liver is due to its free-radical scavenging activity. There are few reports on FRSA from wild and in vitro grown plants of *S. marianum* (Tawahaet al., 2007; Wojdyło et al., 2007; Ligeret et al., 2008). Based on our observations, NAA, BAP, and coconut water together affected FRS activity in *S. marianum* (Fig. 12). Farouk et al. (2010) also evaluated the effects of PGRs on DPPH free-radical scavenging activity in callus extracts. They reported that the calli which originated from leaf explants were induced on MSM enriched with 2.0 mg/L 2,4-D and 1.0 mg/L KIN, thereby showing the highest DPPH free-radical scavenging activity in Citrullus colocynthis.

Since *S. marianum* seeds are commercially important for the separation of bioactive compounds in the pharmaceutical industry, the species is vulnerable to overharvesting. Thus, developing an economic protocol using callus to grow large-scale cell culture for extracting biologically active pharmaceuticals is an attractive approach for the future.

Conclusion

According to the findings, significant effects were observed concerning PGRs concentration, coconut water, and their components, explant type, and silymarin production level of silymarin and FRSA. These parameters were effective on callus initiation, callus induction frequency, and mean callus weight ($p \leq 0.05$). According to our results, the performance of explants and calli, after cultivation on MSM without coconut water or at a concentration lower than 5 % coconut water, was insufficient to generate callus mass and higher amounts of calli formation (10-20 %). This could be because of growth factors in the higher percentage of coconut water reported by other researchers (Jean et al., 2009). As a result, further research into silymarin production using cell suspension cultures of *S. marianum* is proposed. We recommend employing the same protocol to induce soft and friable calli in S. marianum.

Conflict of interest

The authors indicate no conflict of interest in this work.

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