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Effect of Genotype, Explant Type and Growth Regulators on The Accumulation of Flavonoides of (*Silybum marianum L.*) in In vitro Culture

A. Pourjabar, S.A. Mohammadi, R. Ghahramanzadeh and Gh. Salimi

***Abstract*—**The extract of milk thistle contains a mix of flavonolignans termed silymarine.. In order to analysis influence of growth regulators, genotype, explant and subculture on the accumulation of flavonolignans, a study was carried out by using two genotype (Budakalszi and Noor abad moghan cultivars), cotyledon and hypocotyle explants, solid media of MS supplemented by different combinations of two growth regulators; Kinetin (0.1, 1 mg/l) and 2,4-D (1, 2 mg/l). Seeds of the plant were germinated in MS media whitout growth regulators in growth chamber at 26˚C and darkness condition. In order to callus induction, the culture media was supplemented whit different concentrations of 2,4-D and kinetin. Calli obtained from explants were sub-cultured four times into the fresh media of the first experiment. flavonoides was extracted from calli in four subcultures. The flavonoid components were determined by high- performance liquid choromatography (HPLC) and separated into Taxifolin, Silydianin+Silychristin, Silybin A+B and Isosilybin A+B. Results showed that with increasing callus age, increased accumulation of silybin A+B, but reduced Isosilybin A+B content. Highest accumulation of Taxifolin was observed at first calli. Calli produced from cotyledon explant of Budakalszi cultivar were superior for Silybin A+B, where calli from hypocotyl explant produced higher amount of Taxifolin and Silydianin+Silychristin. The best cultivar for Silymarin production in this study was Budakalszi cultivar. High amount of SBN A+B and TXF were obtained from hypocotil explant.

***Keywords*—**Callus culture, Flavonolignans, Silimarine

I. Introduction

THE milk thistle *Silybum marianum L*. Gaernt, a member of the Asteraceae family, is an herb whose fruits have been used medicinally for over 2000 years. It is native to the Mediterranean region and has been naturalized in Centeral Europe, North and South America and Southern Australia [1].

The extract of milk thistle contains a mix of flavonoides termed silymarine.Silymarin consist of a larg number of flavonolignans, including silybin(SBN), isosilybin(ISBN), silydianin(SDN), silychristin(SCN) and hydroflavonolignan Taxifolin(TXF). Silybin is the most biologically active component[2].

Silymarin has antioxidant effects in the liver for toxic liver disease and it is a supportive treatment for choronic inflammatory liver disease and cirrhosis of the liver.most liver toxins produce their damaging effects by free radicals mechanisms[2].

A. Pourjabar and Gh. Salimi are with the Department of Crop Production and Plant Breeding, Kermanshah Branch, Islamic Azad University, Kermanshah, Iran.(email: A_ pourjabar@yahoo.com)

S.A. Mohammadi is with theDepartment of Agronomy & Plant Breeding , Faculty of agriculture, the University of Tabriz,Tabriz, Iran.(email: mohammadi@tabrizu.ac.ir)

R. Ghahramanzadeh is with the University of Mohaghegh Ardabili, College of Agriculture (P.O. Box 179, Ardabil,Iran email:ghahramanr@uma.ac.ir)

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[3]. In vitro culture of cells and tissues 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, flavonolignans production in in vitro cultures is very low. The silymarin content in fruits depends on the milk thistle variety and geographic and climatic conditions in which they grow. The manipulation of the components of the culture medium have substantially increased the production of these compounds[4]. In the present study, we assayed accumulation of flavonoides in tissue culture of *S. marianum* (L.)Gaertn. We also determined the effect of growth regulators composition, genotype, explant and subculture on the secondary metabolites production.

II. Materials and Method

A. Plant material

In this study, seeds of *S.marianum* belonging to two genotype (Budakalszi from Hungry and Moghan ecotype) were used. The seeds were imbibed for 24 h in distilled water, sterilized by ethanol(70%) for 2-3 min, then followed by 20 min in a solution of sodium hypochlorite (2.5) and rinsed with distilled water and were germinated in MS (Murashige and Skoog) media in growth chambers at 26±1°C in darkness for 4-5 weeks. After germination of seeds, plants were transferred to light.

B. Callus cultures

In order to callus induction, cotyledon and hypocotil segments obtained from 10-day-old seedlings and cultured in MS media with 0.8% agar and supplemented with different concentrations of 2,4-D (1 and 2 mg/l) and Kin (0.1, 1 and 2 mg/l). These cultures were incubated in growth chambers at 26±1°C in darkness. Callus obtained from explants were sub-cultured four times into the fresh media of the first experiment.

C. Extraction procedure from callus

One gram (fresh weight) of calluses was homogenized with 15 ml of 80% methanol. The homogenate was filtered and concentrated under reduced pressure at 60°C .the dry residue was resuspended in 3 ml distilled water, extracted twice with 6 ml pure ethyl acetate, filtered and desiccated under reduced pressure at 60°C. The extract was dissolved in 1 ml of 1 mg/ml α- naphthol methanolic solution.

D. Flavonoeid analysis

The content of flavonoeids was determined by high-performance liquid chromatography (HPLC) system. To detect flavonoeids , HPLC system was employed utilizing a Rheodyne injector with a 20 µl loop, a Spherisorb ODS-2 (5 µm) reversed-phase 125×4 mm2 column, A varying wave-phase complementary UV detector and Spectra- Physics integrator and the mobile phase was Methanol: Acetonitryl: H2O.

E. Statistical analysis

The MSTATC and Excel software were utilized for statistical analysis. ANOVA analysis followed by Duncan multiple comparision test were used for statistic evaluations($p<0.01$).

III. Results and Discussion

Invitro propagation of medicinal plants with enriched bioactive principles and culture methodologies for selective metabolite production is found to be highly useful for commercial production of medicinally important compounds. The increased use of plant cell culture systems in recent years is perhaps due to an improved understanding of the secondary metabolite pathway in economically important plants [5].

For callus induction, two types of explants (cotyledon, hypocotil segments) of seedlings were used. Cotyledon segment was the best for callus induction because the growth of the calli was superior than hypocotil segments.

Result of analysis of variance showed that flavonolignan content of calli were not significant difference between 2 genotypes (Budakalszi and Noor abad moghan cultivars) and 2 explants (cotyledon and hypocotyle).

The effect of subcultures on the amount of TXF, SBN A+B and also ISBN A+B had significant ($P< 0.01$) difference but the amount of SDN + SCN had not significant difference in subcultures (table I).

TABEL I
The Flavonolignan Content In Different Subcultures

Subculture	Flavonolignan			
	%ISBNA+B	%SBNA+B	%SDN+SCN	%TXF
First calli	0.122 ^{ab}	2.718 ^{b}	0.471 ^{a}	0.205 ^{a}
Sub1	0.159 ^{a}	3.953 ^{b}	0.342^{b}	0.074 ^{c}
Sub 2	0.05 ^{bc}	3.595 ^{b}	0.419 ^{ab}	0.179 ^{ab}
Sub 3	0.000 ^{c}	5.418 ^{a}	0.435 ^{ab}	0.132 ^{b}
Sub 4	0.000 ^{c}	6.015 ^{a}	0.257 ^{b}	0.12 ^{bc}

Means within columns having different letters are significantly different according to LSD at p≤ 0.01.

The maximum amount of Silybin A+B obtained in the third and fourth subcultures. It seems that there was a significant difference with first and second subcultures and first callus but the amount of ISBN A+B was very low and close to zero in two last subcultures while the amount of ISBN A+B was maximum in first subculture and first callus so with increasing callus age, increases the amount of Silybin A+B but decreases the amount of ISBN A+B. Highest TXF was observed in the first callus.

Reference [6] reported that accumulation of flavonolignans in The 2-month-old calluses much lower than that was observed in fruits. This Calli lose their capacity of synthesize flavonolignans since the fourth month of culture. Reference [7] also observed similar results.

Calli produced from cotyledon explant of Budakalszi cultivar were superior for Silybin A+B, where calli from hypocotyl explant produced higher amount of Taxifolin and Silydianin+Silychristin (Fig1).

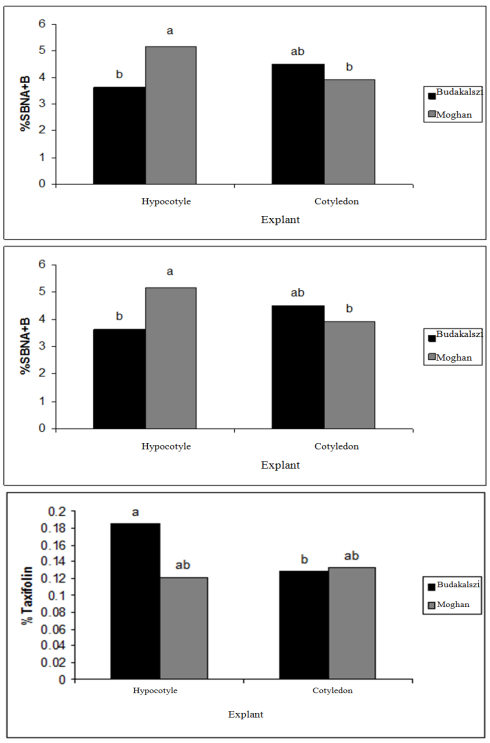

Fig. 1 The flavonolignan content in two ecotype and different explants. Means within columns having different letters are significantly different according to LSD at p≤ 0.01

To determine the effect of Kin and 2,4-D on the flavonolignan content of cell culture of *S. marianum*, four media (including treatments) (0.1, 1 or 2 mg/l kin and 1 or 2mg/l 2,4-D in dark conditions) were prepared. Refferance [8] showed that dark-grown suspensions in media were supplemented by 3 mg/l Pic and 0.4 mg/l Kin have higher dry weight (32mg).

Effect of growth regulators on total Silybin A+B was significant ($P< 1\%$) and between the other components of silymarin was not significant difference in the different concentration of growth regulators. The maximum amount of Silybin A+B was obtained from media supplemented with (2

mg/l 2, 4-D and 1 mg/l Kin) that there was no significant difference with media supplemented with (1 mg/l 2,4-D and 1 mg/l Kin)(tabel II).

TABLE II
EFFECT OF GROWTH REGULARORS ON TOTAL SILYBIN A+B

Media culture	%SBN A+B
A	4.003[b]
B	4.59[ab]
C	5.5946[b]
D	5.4174[a]

Means within columns having different letters are significantly different according to LSD at $p \leq 0.01$. A= (1mg/l 2,4-D, 0.1 mg/l kin), B= (1mg/l 2,4-D, 1mg/l kin), C= (2mg/l 2,4-D, 1mg/l kin), D= (2mg/l 2,4-D, 2mg/l kin).

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REFERENCES

[1] A. Cammie, B. B. A. Burgess and D. Pharm, "Silybum marianum (Milk Thistle)," J. Pharmacy Society Wisconsin, 2003, 2: 38-40.
[2] F. Kavasnica, B. Biba, R.Sevcik, M.Voldrich and J. Kratkta, "Analysis of active components of silymarin," J chromatogr A. 2003,990: 239-245.
[3] E. Hetz, R. Liersch, and D. Schieder, "Genetic investigations on *silybum marianum* and *S.eburneum* with respect to leaf colour, out crossing ratio and flavonolignan composition," Planta Med., 1995, 61:54-57.
[4] F. Alikaridis, D. Papadakis, K. Pantelia, and T. Kephalas, "Flavonolignan production from Silybum marianum transformed and untransformed root cultures," Fitotrapia. 2000,71: 379–384.
[5] [5] M. Vanisree, C.Y.Lee, S.F. LO, S.M. Nalawade, C.Y. Lin, and H.S. Tsay, "Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures," Rev. Bot. Bull. Acid. Sin.,2004, 45:1-22.
[6] M. Cacho, M. Moran, P. Corchete and F. ernandez-Tarrago, "Influence of medium composition on the accumulation offlavonolignans in cultured cells of *Silybum marianum* (L.) Gaertn," Plant Sci., 1999,144: 63–68.
[7] H. Becker, R. Schrall, "Tissue and suspension cultures of *Silybum marianum*: Isolation and growth of tissue and suspension cultures and the study of flavonoid content," Planta Medica 1977, 31: 185 - 92.
[8] T. Hasanloo, R.A. Khavari-Nejad and E. Majidi, "Medi optimization for flavonolignans accumulation and growth index of callus culture of *Silybum marianum* (L.) Gaertn," J. of Plant Sci. Res., 2007,1: 7–12.
[9] H. Rahnama, T. Hassanloo, M.R. Shams and R. Sepehrifar, "Silymarin production in hairy root culture of *silybum marianum* (L.) Gaetn," Iranian J. Biotechnol.,2008, 6: 113–118.