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Original Article

Effect of Genotype and Methyl Jasmonate on Silymarin Content of *Silybum marianum* (L.) Gaertn. Hairy Roots Culture

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ABSTRACT

Milk thistle is a valuable medicinal plant, and obtaining silymarin (SLM) is the main reason for the cultivation of this plant. Due to the non-agricultural properties of milk thistle, its cultivation and harvesting are accompanied by difficulties. *In vitro* producing SLM by hairy root culture can overcome these problems. Various factors such as plant genotype, *Agrobacterium rhizogenes* strain used for infection, type and concentration of elicitor and, etc., can significantly enhance SLM production. The impact of various genotypes on SLM production in milk thistle hairy root culture has yet to be investigated. In this study examined the effect of five different genotypes of milk thistle and methyl jasmonate (MeJA) on the amount of SLM in milk thistle hairy roots based on completely randomized design. HPLC was used to quantify the amount of SLM in the hairy root samples. Based on the results, SLM production showed a considerable difference among ecotypes, with levels ranging from 109.8 to 648.7 ppm. MeJA increased the amount of SLM in all genotypes except for Mashhad ecotype. Due to the approximately 5.9-fold difference in SLM levels among different genotypes and the opposite responses of genotypes to MeJA indicate a high variation in this trait among different milk thistle ecotypes. Based on these results, screening milk thistle germplasm to find ecotypes with the highest levels of SLM in hairy root culture and also considering the strong mutual effect between elicitor and ecotype can be fruitful for increasing SLM content.

INTRODUCTION

Silybum marianum (L.) Gaertn., also known as milk thistle, is a dicotyledonous plant belonging to the Asteraceae family. Milk thistle is renowned for the presence of its valuable secondary metabolites known as silymarin (SLM), which possess significant medicinal uses. Different investigations have shown that SLM is beneficial for various purposes, such as liver health [1], brain function [2], bone health [3], enhancing breast milk production [4], and, even in the treatment of cancer [5].

This wild plant is cultivated for its valuable secondary metabolite, SLM. A tall, spiny plant with multiple capsules with varying maturity periods and seed shedding. Cultivating this plant is difficult and time-consuming due to its non-agricultural attributes. Both abiotic and biotic stresses in the field can affect the content of SLM.

Hairy root culture, along with other tissue culture techniques like callus culture and cell suspension culture, can be utilized for the controlled production of secondary metabolites. Hairy root culture is preferred due to its genetic stability. The main obstacle for economically producing SLM under *in vitro* conditions, including hairy root culture, is the low quantity of SLM production in tissue culture. The quantity and composition of SLM produced in the hairy roots culture of *S. marianum* can be influenced by several factors, such as the used strain of *Agrobacterium* for transformation, the type and concentration of elicitor, the genotype of the hairy root lines, etc.

Several studies have been conducted on the production of SLM in *S. marianum* hairy roots using elicitation with different elicitor types and concentrations. Silver ion (Ag^+) [6], chitosan [7], yeast extract [8], methyl jasmonate (MeJA) [9, 10],

salicylic acid [11], and several fungal (*Fusarium proliferatum*, *Aspergillus niger*, *Rhizoctonia solani*, *Trichoderma* strains, and *Piriformospora indica*) [12- 14] were used as elicitor on *S. marianum* hairy roots culture [15].

Several studies have reported that *S. marianum* displays vast genetic diversity, encompassing both of molecular and morphological aspects [16-22]. The potential for SLM production in tissue culture has not been fully utilized despite this diversity.

MeJA stimulates or increases the biosynthesis of secondary metabolites that play a crucial role in plant adaptation to specific conditions [23]. Endogenous jasmonates are unique plant signaling molecules that regulate a diverse range of physiological and developmental processes in plants. They control root growth, pollen fertility, and also plant resistance to insects and diseases [23, 24]. The exogenous application of MeJA leads to the generation of reactive oxygen species (ROS), which in turn stimulate the synthesis of secondary metabolites [25,26]. Nouri et al. (2024) have reported a correlation between the content of H_2O_2 and SLM in *S. marianum* hairy roots [14]. MeJA was used as an elicitor in hairy root culture of several medicinal plants, leading to the enhancement of secondary metabolite production [27].

In this study, our aim was to compare the production of SLM in a hairy root culture among various genotypes of *S. marianum*. We also examined the effect of MeJA on SLM production and its potential interaction with different ecotypes.

MATERIALS AND METHODS

S. marianum Ecotypes

A total of 20 different ecotypes of *S. marianum* from Iran were examined, including Shushtar, Manjil, Shush 1, Shush 2, Ardabil, Shahediyeh, Mashhad 1, Mashhad 2, Naddafiyeh, Shirvan 1, Shirvan 2, Behbahan 1, Behbahan 2, Rostamabad, Ahvaz, P100, Isfahan, Ramhormoz, NajafAbad and Mobarakeh Isfahan. The ecotypes were named according to the location where they were collected. Additionally, three other ecotypes from England (England1 and England2) and Hungary were also included in the investigation.

Hairy Root Induction and MeJA Treatment

The rootlet explants of 14-day-old seedlings were prepared in a hormone-free Murashige and Skoog (MS) medium [28]. They were then inoculated with the 15834 strain of *Agrobacterium rhizogenes* using scalpel scraps. The inoculated explants were incubated in darkness at 25 °C on a hormone-free MS medium. After 48 h, they were moved to the selective MS medium containing 500 mL/L cefotaxime in the dark at 25 °C for 10 days. Following that, the explants were subcultured for 7 to 10 days on a hormone-free MS medium until the length of hairy roots reached approximately 5 cm.

Each hairy root explant was considered as a line and was excised into 1 cm pieces. Every 6 pieces were then transferred to a 50 mL MS liquid medium with 30 g/l sucrose and subcultured every 10 days. The cultures were shaken at 110 rpm and 25 °C in darkness. After one month, the line with the most vigorous growth from each ecotype is selected and divided into six samples by cutting them into the equal parts. Each sample is transferred to a 50 mL MS liquid medium in an Erlenmeyer flask, which serves as an experimental unit. After 15 days of active growth, three randomly selected flasks are treated with 100 mM MeJA, while the remaining three flasks serve as controls. This procedure is uniformly applied to all ecotypes.

Polymerase chain reaction (PCR) using rol C gene-specific primer (Forward: 5'-TAACATGGCTGAAGACGACC -3' and Reverse: 5'- AAACTTGCACTCGCCATGCC -3') was used to confirm the genetic transformation of the selected lines.

Extraction of SLM

Lyophilized hairy roots weighing up to 1 gram from each sample were defatted by using 10 mL of petroleum benzene at 40 °C for 8 h. SLM content of the samples was subsequently extracted using 10 mL of methanol at 55 °C for 15 h. The resulting extract was lyophilized, and the yellow dry residue was dissolved in 1 mL of methanol and stored in darkness at 4 °C [6].

HPLC Analysis

To quantify the components of SLM content, was applied high-performance liquid chromatography (HPLC, Knauer, Germany). The analysis utilized a 20 µl injector loop, a Nucleosil C18 5 µ (250 × 4.6 mm) column, and a PDA detector [29]. Solvent phase consisted of water, acetonitrile, and methanol was used according to the gradient program in Table 1. The elution time was 30 min with a flow rate of 0.2 ml/min, and peaks were detected at 288 nm.

To identify SLM components, the retention times of the sample SLM were compared to standards of SLM components. These standards included isosilybin B, isosilybin A, silybin B, silybin A, silydianin, silychristin and taxifolin that were provided by Sigma Chemicals. Various known concentrations (between 5 to 7 different amounts of various metabolites from 10 to 900 ppm) were used for each metabolite to plot the required calibration curve. Calibration curves were then used to calculate the amount of each metabolite in question based on peak area on each sample [30]. The SLM content was calculated by summing the quantities of its components. SLM measurements were conducted on two replicates for each treatment.

Experimental and Statistical Analysis

A 5×2 factorial experiment was conducted to assess the effects of five different ecotypes and two levels of MeJA treatment (0 and 100 mM) on SLM content. The experiment was designed using a completely randomized design (CRD) with three replications, although only two replications were measured for SLM content. The analysis of variance and Duncan's multiple range test (DMRT) were performed using SAS software (version 9.4, SAS Institute).

RESULTS

Hairy Root Induction

Out of the 20 ecotypes tested, only 5, namely P100, Manjil, Ardabil, Mashhad, and Isfahan, were able to produce a satisfactory amount of hairy roots after transformation. The transformation of these 5 ecotypes was confirmed through PCR, as mentioned in the method and materials section. In comparison to the common roots (Fig. 1), the hairy roots exhibited plagiotropic growth with extensive lateral branching. To ensure uniformity of repetitions, each hairy root line of the 5 ecotypes was divided into six parts (Fig. 2), which were then transferred to 100 mL Erlenmeyer flasks to create six similar experimental units.

Effect of Ecotype and MeJA on Fresh Weight of Hairy Roots

The analysis of variance showed that MeJA and its interaction with ecotype, did not significantly impact on root fresh weight. However, there was a significant difference among the ecotypes studied (Table 2).

Table 1 Solvents used in HPLC analysis

Time (min)	Methanol (ml/min)	Acetonitril (ml/min)	Water (Ph = 2.3 with 10% H3Po4) (ml/min)
0:00	22	15	63
7:30	22	15	63
15:00	40	20	40
30:00	22	15	63

Table 2 Analysis of variance of root fresh weight

Source of Variation	Degree of freedom	Means of square
Ecotype	4	7.06 **
MeJA	1	0.01 ns
Ecotype×MeJA	4	0.09 ns
Error	20	0.18
C.V%	18.44	

** and ns: significant at *P*=0.01 probability level and non-significant, respectively.

Table 3 Analysis of variance of SLM

Source of Variation	Degree of freedom	Means of square
Ecotype	4	68605.10 **
MeJA	1	8124.02 ns
Ecotype×MeJA	4	46375.86 *
Error	10	82005.91
C.V%	28.74	

**, * and ns: significant at *P=0.01*, *P=0.05* probability levels and non-significant, respectively.

The highest and the lowest root fresh weight were observed in Manjil (3.88 g) and Isfahan (1.18 g), respectively. Based on DMRT (*p=0.05*) the fresh weight of all ecotypes, except for P100 and Ardabil, exhibited significant differences from each other (Fig. 3).

Effect of Ecotypes and MeJA Treatment on the Biosynthesis of SLM

The effects of ecotype and the interaction between ecotype and MeJA were found to be significant on SLM content. However, treatment of hairy roots with 100 mM MeJA did not show a significant effect on SLM quantity compared to 0 Mm MeJA (Table 3). There were considerable variations in SLM content observed among different ecotypes, ranging from 129.43 to 489.84 ppm (Fig. 4). After conducting further analysis and comparing the mean

of the treatment combination of MeJA and ecotype (two-factor interaction) using DMRT, it was found that Mashhad and Ardabil ecotypes had the highest and lowest SLM amounts, 648.7 and 109.8 ppm, respectively, in the absence of MeJA treatment (Fig. 5).

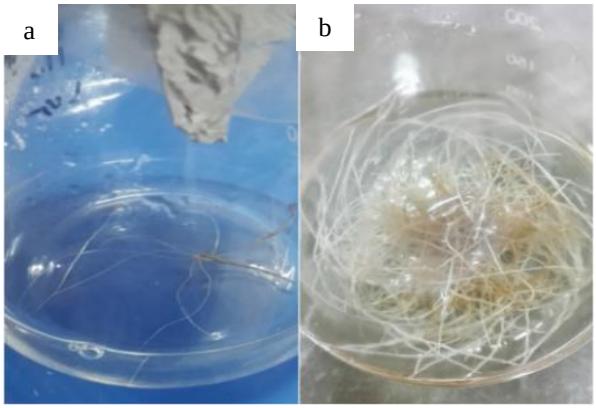

Fig. 1 Images of (a) common roots and (b) hairy roots.

Fig. 2 Dividing each hairy root line into 6 sections in order to create six identical experimental units.

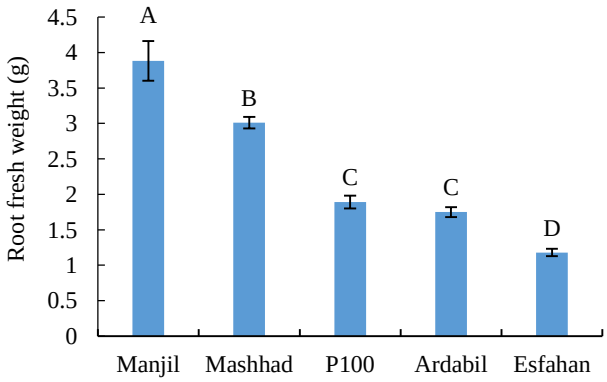

Fig. 3 Mean ± SE (n=6) and results of DMRT. Means with the same letter, do not have significant difference with each other at probability level of $P = 0.05$.

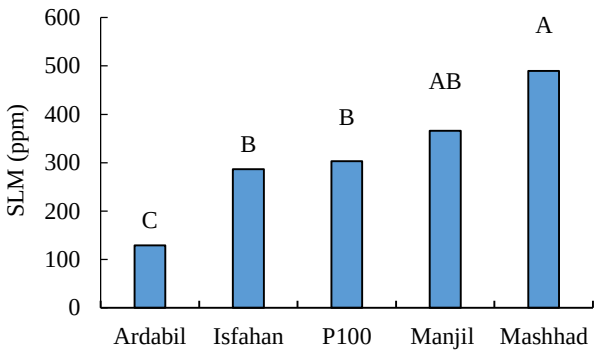

Fig. 4 SLM mean of genotypes ± SE (n=4) and results of DMRT. Means with the same letter, do not have a significant difference with each other at probability level of P=0.05.

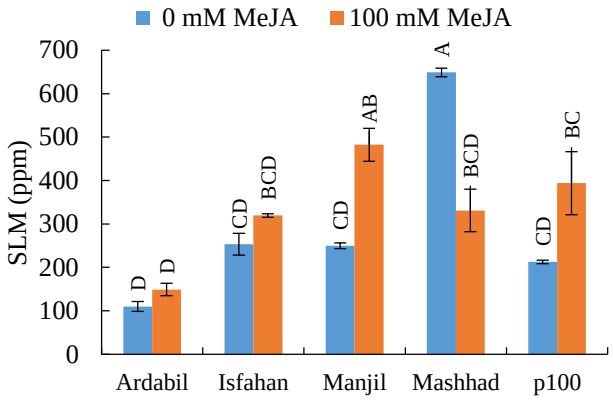

Fig. 5 Interactive effect of ecotype and MeJA on silymarin content ± SE (n = 2) and results of DMRT. Means with the same letter, do not have significant difference with each other at probability level of P=0.05.

DISCUSSION

The content of SLM without elicitation with MeJA ranged from 109.8 ppm in Ardabil to 647.8 ppm in Mashhad among different *S. marianum* ecotypes, indicating the presence of diversity in terms of this attribute (Fig. 5). Although the effect of MeJA on SLM amount was not significant (Table 3), there was a significant difference in SLM content between control and MeJA-treated in some of ecotypes (Fig. 5). MeJA significantly increased and decreased SLM content in Manjil and Mashhad ecotypes compared to their control (Fig. 5), respectively. This inconsistency is due to the significant interaction between ecotype and MeJA treatment (Table 3). In a study by Gharechahi *et al*., (2013), elicitation of *S. marianum* hairy roots with 100 mM MeJA for 24 h resulted in a decrease in SLM compared to the control [9]. However, with an increase in elicitation time to 48, 72, 96, and 120 h, SLM content increased compared to the control. The contrasting

responses of ecotypes to MeJA treatment, provide additional evidence of the considerable variation among different *S. marianum* ecotypes regarding this characteristic. Previous studies have also highlighted the wide molecular and morphological diversity among Iranian milk thistle ecotypes [20, 21].

Most of the studies conducted in this field have focused on investigating the effects of various stimulants on the amount of SLM. The effects of different elicitors such as various *Trichoderma* strains, chitosan, *Fusarium proliferatum*, *Aspergillus niger*, *Rhizoctonia solani*, *Piriformospora indica*, and yeast-extract on SLM production in *S. marianum* hairy roots culture have been examined. These elicitors increased the amount of SLM between 1.5 to 5.5 times compared to its control. In our study, we observed significant changes in SLM content, up to 5.۹ folds (Fig. 5), among different ecotypes. Evaluating the diversity of *S. marianum* genotypes in terms of SLM content in hairy root culture can greatly enhance *in vitro* SLM production.

CONCLUSION

The potential of genotype in the production of SLM in hairy root culture of *S. marianum* has not been fully utilized. Different biotic and abiotic elicitors, such as MejA, have been employed to boost the production of SLM in hairy root culture of this plant. The research findings indicate that as the existence of vast genetic diversity among milk thistle ecotypes, there is significant variation among various ecotypes of *S. marianum* in terms of SLM quantity in hairy root culture. This diversity is substantial enough to alter the impact of MejA on SLM content in different ecotypes. Many medicinal plant species also exhibit significant genetic diversity. It is recommended that researchers involved in this field besides the employing of various stimulants to enhance the production of active ingredients, also assess the germplasm of the target plant for diversity in secondary metabolites production in a tissue culture medium.

Conflict of Interests

The authors have not declared any conflict of interest.

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