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Reaserch Paper

The influence of *Serendipita indica* cell wall extract on silymarin production in cell suspension culture of *Silybum marianum* (L.) Gaertn

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Abstract

Milk thistle (*Silybum marianum* (L.) Gaertn) belonging to the Asteraceae family and known for its valuable secondary metabolite, silymarin. In order to get the cell suspension culture of *Silybum marianum*, the combination of 2,4-D and BAP hormones each with five different concentrations (0, 0.1, 1, 2 and 5 mg/L) and 3 different seedling explants (cotyledon, hypocotyl and root) was used to callus formation. High-quality callus was observed in eight different of hormone-explant combinations. Successful cell suspension culture was achieved only by using callus created from cotyledon explants treated with 0.1 mg/L 2,4-D and 5 mg/L BAP. Furthermore, the study examined the effects of 2% and 4% *Serendipita indica* cell wall extract as an elicitor on silymarin production in cell suspension culture at three different inoculation times (24, 48, and 72 h). After 48 h of inoculation with 2% fungal extract, the highest level of silymarin (199 ppm) was observed, which was significantly different from the control (46 ppm). The silymarin content of cells increased over time through elicitation with 4% fungal elicitor, while no similar pattern was observed with 2% fungal extract. Based on the results, the cell wall of *S. indica* can significantly enhance the amount of SLM in the cell suspension culture of *S. marianum*.

1. Introduction

Silymarin (SLM), the active ingredient found in *Silybum marianum* (L.) Gaertn., commonly known as milk thistle, possesses antioxidant and anti-inflammatory properties, which have convinced the pharmaceutical industry to utilize it. SLM is a mixture of silybin (A and B), silychristin, isosilybin (A and B), silydianin and taxifolin Producing SLM under *in vitro* condition is an attractive option due to the non-agricultural characteristics of milk thistle. Cell suspension cultures were utilized to produce SLM. However, according to the low amount of SLM in the cell suspension culture, different elicitors were employed in order to enhance the SLM content. Copper sulphate as an abiotic elicitor, increased 5-folds the SLM amount in cell suspension culture compared to the control (Elsharnouby and Hassan. 2018). Firouzi et al., (2013) using combination of phenylalanine, yeast extract and methyl jasmonate as an elicitor, boosted amount of SLM in the cell suspension culture to 8.6 folds than the control. Hassanen et al., (2021) also

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succeeded in increasing the SLM amount in cell suspension culture using L-Phenylalanine up to 66.33% compared to the control.

Serendipita indica (formerly known as *Piriformospora indica*) is an endophyte fungus that can be easily cultivated on a variety of growth media. *S. indica* is a soil born fungus and forms a symbiotic relationship with a wide range of plants and enhances the growth of the host plant (Varma et al., 2001). *S. indica* increased the tolerance of plants to biotic and abiotic stresses. *S. indica* also used as an elicitor to stimulate the production of secondary metabolites in tissue culture of medicinal plants. The addition of *S. indica* enhanced the production of pentacyclic triterpenoids in *Lantana camara* L. suspension cultures (Kumar et al., 2016). In another study, the co-culture of *S. indica* and *Sebacina vermifera* with cells suspension culture of *Linum album* led to an increase in podophyllotoxins production (Baldi et al., 2008). Moreover, *S. indica* was found to enhance withaferin A production in cell suspension cultures of *Withania somnifera*. Additionally, *S. indica* has been employed for the elicitation of hairy roots in various medicinal plants (Amani et al., 2021; Tashackori et al., 2018; Tashackori et al., 2016; Nouri and Farkhari. 2024).

In the current investigation studied the effect of different concentrations of *S. indica* cell wall extract as an elicitor at various inoculation times on the amount of silymarin in the cell suspension culture of *S. marianum*.

2. Method and material

Plant material

The Hungarian milk thistle seeds were washed under running water for twenty minutes. The seeds were sterilized by dipping them into ethanol (70% v/v) for 20 seconds, and then soaking them in a solution of sodium hypochlorite (2.5% v/v) for 15 minutes. Afterwards, the seeds were thoroughly rinsed with distilled water three times, each time for 10 minutes. Finally, the seeds were germinated in MS (Murashige and Skoog. 1962) medium, which contained 30 g/L of sucrose and 8 g/L of agar, under 16 h light/ 8 h dark photoperiod at 25°C.

Callus and cell suspension culture

Cotyledon, hypocotyl and roots of 10 days seedling were used for callus culture. The explants were placed on MS medium containing the plant growth regulators combinations of 2,4-D and BAP each with concentrations of 0, 0.1, 1, 2 and 5 mg/L. Subculture was carried out every 15 days to obtain calluses. Calluses were transferred into the erlenmeyer flask containing liquid MS medium and shacked in an orbital shaker with 100 rpm in darkness. At each time of subculture, 20 mL of the previous medium was added to 80 ml of the fresh medium. The subculture continued until individual cells were observed and the constant growth phase of the cells was reached.

Elicitor preparation

S. indica was cultured on CM medium (Hill-Käfer medium) and incubated at 26°C for 30 days. Afterwards, 3 to 6 pieces of 1 cm^2 agar discs were subcultured in 150 mL of liquid MYPG medium. The subcultures were incubated in a shaker incubator with a cycle of 16 h light/ 8 h dark, at a speed of 90 rpm, and a temperature of 26°C for 6 days. At the end of the log phase growth, the culture medium was filtered through a 0.22 mm filter. The fungal mat was washed with sterile double distilled water, dried at 40°C in an oven, and then crushed using a mortar. One gram of the dried cells was suspended in 10 mL of double distilled water and autoclaved at 121°C for 15 minutes. The suspension was then centrifuged at 5000 rpm for 15 minutes, and the collected supernatant was designated as 'cell extract' and used as an elicitor (Baldi et al., 2009).

Elicitor inoculation, experimental design and statistical analysis

The effect of two different concentrations of *S. indica* cell wall (2% and 4%) and 3 elicitation times (24, 48 and 72 h) were investigated on SLM content of *S. marianum* cell suspension culture. The experiment was done based on completely randomized design (CRD) with two replications. A 100 mL erlenmeyer flask with 50 mL cell suspension medium considered as an experimental unit. To apply ٢% and ٤% of fungal extract, 1 and 2 mL of fungal extract solution was added to the 49 and 48 mL culture medium, respectively. The erlenmeyer flasks were shaked with 100 rpm in an orbital shaker at 25°C in darkness. The SLM amount in control sample (0% elicitor) was measured only at 24 h.
Analysis of variance (ANOVA) was done by SAS software (version 9.4, SAS Institute) based on CRD. Duncan's multiple range mean test (DMRT) was applied for mean comparison as post hoc comparison method by SAS program.

Silymarin extraction and measurement

One gram cells fresh weight was homogenized with 15 mL of 80% (v/v) methanol. The mixture was then stored at −40°C for 48 h. Afterward, solution was filtered and its methanol was evaporated in a water bath at 40°C. The resulting dry residue was resuspended in 3 mL of distilled water and 6 mL of pure ethyl acetate, filtered and desiccated in a water bath at 40°C. The extract was dissolved in 1 mL of 1 mg/mL a-naphthol methanolic solution (Cacho et al., 1999). The components of SLM content were quantified via high-performance liquid chromatography (HPLC) with a 20 µL injector loop, a nucleosil C18 5 µ (250 × 4.6 mm) column, and S2500 UV detector (Knauer, Germany) (Cacho et al., 1999). All of the SLM component standards, isosilybin B, isosilybin A, silybin B, silybin A, silydianin, silychristin and taxifolin were provided by Sigma Chemicals (USA). SLM was calculated through the summation of its components. SLM measurement was done only for two replications for specific combinations of treatment as described in the Results section.

3. Results

Callus and cell suspension culture

Only eight out of the 75 different treatments, which comprise a combination of five different concentrations of 2,4-D and BAP, as well as three types of explants, showed desirable results in terms of callus appearance, color, and size up to the 6th subculture (Table 1).

Table1 Successful treatment combinations in callus formation

2,4-D	0.1 mg/L	0.1 mg/L	0.1 mg/L	0.1 mg/L	0.1 mg/L	0.1 mg/L	2 mg/L	5 mg/L
BAP	1 mg/L	2 mg/L	5 mg/L	1 mg/L	2 mg/L	2 mg/L	2 mg/L	1 mg/L
Explant	Cotyledon	Cotyledon	Cotyledon	Hypocotyl	Hypocotyl	Root	Root	Root

At the end of the 9th subculture of the callus suspension culture, among the calluses generated from eight different treatment combinations (as outlined in Table 1), the calluses derived from cotyledon explants treated with 0.1 mg/L of 2.4-D and 5 mg/L of BAP demonstrated the formation of single cells in suspension culture (Figure 1 and figure 2).

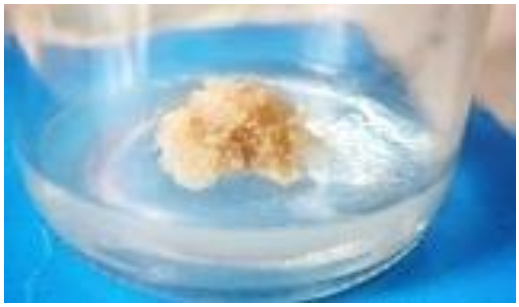

Fig. 1 derived callus of cotyledon explant, treated with 0.1 mg/L 2.4-D and 5 mg/L of BAP

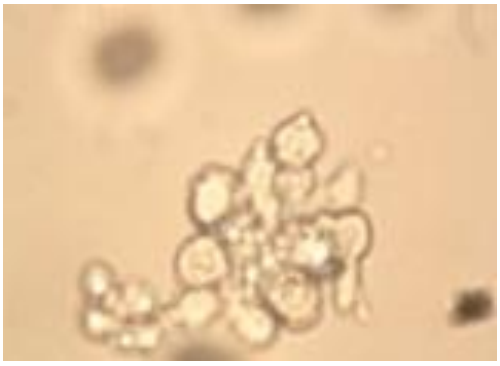

Fig. 2 Cells obtained from suspension culture of cotyledon calluses.

SLM measurement

The combination of elicitor concentrations and elicitation times significantly affected the amount of SLM in the cell suspension culture (Table 2). By 42 h of elicitation with 2% fungal elicitor, the maximum quantity of SLM (199 ppm) was achieved (as depicted in figure 3). The 2% *S. indica* cell wall elicitor at 24 h inoculation time also increased SLM content compared to the control.

Table 2 Analyze the variance of treatment combinations including elicitor concentrations and elicitation time for SLM content.

Source of variation	Degree of freedom	Means of square
treatment	6	2397.10*
Error	7	462.27
C.V%	40.18	

*, indicates significant at level of $P = 0.05$

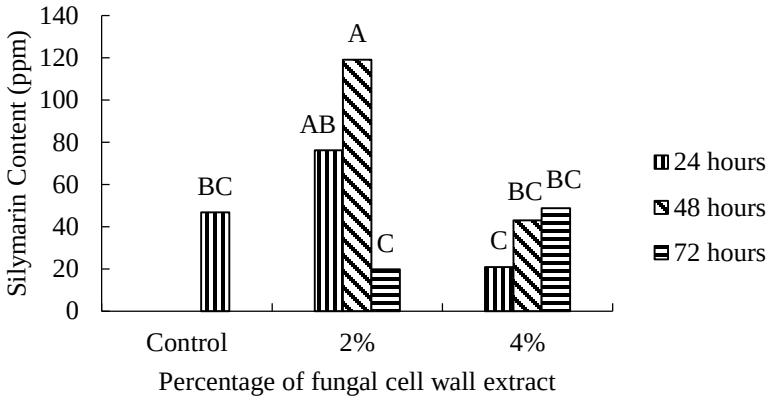

Fig. 3 The effect of varying concentrations (2% and 4%) of *S. indica* and different elicitation durations (24, 48, and 72 h) on silymarin production in the cell suspension culture of *S. marianum*.

4. Discussion

Lower ratios of auxin to cytokinin led to enhanced callus formation in cotyledon and hypocotyl explants. However, this trend was not observed in root explants (Table 1). Arekhi et al., (2012) reported that the combination of 1.5 mg/L kin and 1 mg/L 2,4-D resulted in the highest percentage (98%) of callus formation for root explants of *S. marianum*. Likewise, for hypocotyl explants, the greatest callus formation (77%) was achieved when using 1 mg/L kin in conjunction with 0.5 mg/L 2,4-D. In order to induce callus formation and produce cell suspension culture of *Silybum marianum*, Cacho et al., (1999) used cotyledon explants treated with a combination of 0.1 mg/L of 2,4-D and kin.

Based on results the production of SLM was significantly increased by treating cell suspension with 2% of *S. indica* cell wall for 24 h compared to the control. This treatment resulted in a four-fold increase in SLM production compared to the control. Other elicitation methods, such as using copper sulphate, phenylalanine+yeast extract+methyl jasmonate, and L-Phenylalanine on *S. marianum* cell suspension, resulted in increases of 5, 8.6, and less than 1 fold compared to the control, respectively (Elsharnouby and Hassan. 2018; Firouzi et al., 2013; Hassanen et al., 2021).

The SLM content increased over the time with the elicitation of cell suspension culture using 4% fungal elicitor. However, this trend was not observed at 2% fungal extract, suggesting the presence of an interaction effect between two factors. Factorial analysis revealed that the interaction between elicitor concentration and elicitation times was significant (Table 3). Hasanlo et al., (2013) reported that the elicitation of *Silybum marianum* hairy root culture with 10 mg *Phytophtora meloni* fungal extract also caused a reduction in SLM production over time (24, 48, and 72 h exposure). However, this trend was reversed when elicitation was done with 20 mg of fungal extract.

Table 3 Analysis of variance of elicitor concentrations and elicitation times based on factorial experiment for SLM content

Source of variation	Degree of freedom	Means of square
Elicitation time	2	2292.8^{nc}
Elicitor concentration	1	3496.0*
Two factor interaction	2	3096.8*
Error	6	3225.9
C.V%	40.18	

*, indicates significant at level of $P = 0.05$

This report is the first investigation into the impact of *S. indica* cell wall extract on SLM content of *Silybum marianum* cell suspension culture, based on the available information. Due to the complex nature of genetic control of SLM production, as well as the inclusion of different compounds in fungal cell wall extract, makes it necessary to have comprehensive information on the transcriptome and proteome of the samples in order to interpret the trend of SLM production affected by different concentrations of elicitor and elicitation times.

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