PROTEOME ANALYSIS OF MILK THISTLE (*SILYBUM MARIANUM* L.) CELL SUSPENSION CULTURES IN RESPONSE TO METHYL JASMONATE AND YEAST EXTRACT ELICITORS

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Abstract. Elicitors cause biosynthesis and accumulation of secondary metabolites by inducing defense responses. In this study, we treated the cell suspension cultures of *Silybum marianum* L. with MeJA (methyl jasmonate) (100 μM) and YE (yeast extract) (0.1 w/v) as elicitors and measured the content of Silymarin accumulation by HPLC (High Performance Liquid Chromatography). Accumulation of Silymarin significantly increased after 48 h of MeJA and YE application. In order to investigate the effect of abiotic (MeJA) and biotic (YE) stresses on expression of proteins in *S. marianum* cell suspension cultures, we employed high resolution two-dimensional gel electrophoresis coupled with MALDI-TOF-TOF (matrix assisted laser desorption ionization time of flightetime of flight) mass spectrometry. At least, 249 protein spots showed reproducible and significant changes in the gel. Spots were up or down regulated upon MeJA and YE treatments. Ten protein spots were identified using MALDI-TOF-TOF-MS. The identified proteins belong to different functional categories. The proteins were classified based on carbohydrate metabolism (spots 6 and 22), Nitrogen metabolism (spot 20), storage protein (spot 29), transport process (spot 12), protein modification and chaperones (spot 8), pathogenesis related (spot 1 and 2) and secondary metabolism (spot 3 and 53). The potential role of these proteins in the biosynthetic pathway of flavonoids and the finding of proteins in non-sequenced plants such as *S.marianum* requires further research.
Keywords: *elicitor, HPLC, silymarin, MALDI-TOF-TOF mass spectrometry, two-dimensional gel electrophoresis*

Abbreviations: MeJA, methyl jasmonate; JA, jasmonic acid; YE, yeast extract; MALDI-TOF/TOF, matrix assisted laser desorption ionization time of flightetime of flight; SLM, silymarin; 2-DE, two-dimensional gel electrophoresis; IEF, isoelectric focusing; IPG strip, immobilized pH gradient strip; SDS-PAGE, sodium dodecyl sulfate-poly acrylamide gel electrophoresis; IgG, immune globulin G; MS, mass spectrometry; CBB, coomassie brilliant blue

Introduction

Silybum marianum L. Gaertn. is an annual or biennial plant of the Asteraceae family (Karkanis et al., 2011). Silymarin (SLM) is a complex mixture of flavonolignans, including, silybin (SB), isosilybin (ISB), silychristin (SCN), silydianin (SDN) and taxifolin (TAX), which is isolated from the milk thistle plant.

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Silibinin is the main active component of silymarin (60-70%) (Kroll et al., 2007). Silymarin has been most widely utilized to treat liver, spleen and gall bladder disorders. Silymarin also has anti-cancer, chemopreventive, cardioprotective, neuroactive and neuroprotective activities (Fraschini et al., 2002; Murphy et al., 2000; Lorenz et al., 1984; Bahmani et al., 2015).

Plant cell culture technologies including Hairy roots, callus and cell suspension culture could be an alternative for the production of flavonolignans. Production of silymarin in cell cultures is low in comparison to the whole fruit (0.05–0.4% dry weight vs. 1–3% in fruits) (Cacho et al., 1999; Sánchez-Sampedro et al., 2005a; Sánchez-Sampedro et al., 2005b), in this regard, several approaches have been made to stimulate the productivity of flavonolignans in plant cell cultures. Elicitation is one of the most effective techniques for the large-scale production of secondary metabolites in *in vitro* cultures and for a better understanding of their biosynthesis (El-Garhy et al., 2016; Rahnama et al., 2008). Elicitors enhance the yield of secondary metabolites in cell cultures by regulating the rates of biosynthesis and accumulation (AbouZid, 2012; Barz et al., 1990; Hasanloo et al., 2014; Rahnama et al., 2008).

Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), have been reported to play an important role in signal transduction processes that regulate defense responses in plants, and enhance the production of secondary metabolites in cell cultures (Wang et al., 2015), i.e. rosmarinic acid in *Coleus blumei* (Szabo et al., 1999), or hypericin in *Hypericum perforatum* (Walker et al., 2002). Methyl jasmonate (MeJA), alone or in combination with yeast extract (YE), strongly promote the accumulation of silymarin (Elwekeel et al., 2012a; Elwekeel et al., 2012b; Sánchez-Sampedro et al., 2008). Treatment of cell suspension culture of *S.marianum* with YE elicitor improved production and release of silymarin into the culture medium to a level of about 3-fold higher than that of the control (Sánchez-Sampedro et al., 2005a).

Most proteomic studies regarding secondary matebolites production have been performed with elicited cell cultures. For instance, polyphenolic biosynthesis, stilbenoid in grapevin (Martinez-Esteso et al., 2011), flavonolignan in *S. marianum* (Corchete and Bru, 2013) have been analyzed at proteome level under the induction of elicitors, such as chitosan, cyclodextrins, methyl jasmonate or yeast extract. In the study by Corchete and Bru (2013), proteome alterations were analyzed in *S. marianum* cell cultures elicited with methyl jasmonate and methyl B cyclodextrin. They identified 19 differentially expressed proteins which belong to a few categories, including metabolism, stress and defense responses and transport processes. Gharechahi et al. (2013) studied proteins from *S. marianum* hairy roots exposed to MeJA and identified expressed proteins involved in various mechanisms like energy production, transpotation and secondary metabolism.

In the present study, we performed a proteomic analysis to assay the events occurring in *S. marianum* cell cultures elicited with MeJA and YE. Analysis of protein extracts by the high resolution 2-DE technique coupled with MALDI-TOF/TOF identified several proteins up or down regulated in response to elicitor treatments. Our aim was to investigate the changes of protein pattern and identify proteins in cell suspension cultures of *S. marianum* ellicited with MeJA and YE and discussed their putative role in the flavonolignan accumulation.

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Materials and Methods

Plant material and cell cultures

This research had been conducted in the Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran. The seeds of milk thistle (Budakalaszi cultivar) from Pakan bazr company, were immersed for 24 h in distilled water, sterilized by ethanol 70% (w/v) for 2-3 min then sterilized by dipping in sodium hypochlorite 2.5% (w/v), Tween 20, 0.1% (v/v), and rinsed exhaustively with sterile distilled water. The seeds were cultured in Murashige and Skoog (MS) medium without growth regulator and incubated in dark condition at 26±1 °C. After germination of seeds, plants were transferred to light. Cell cultures of *S. marianum* were developed from cotyledon and hypocotyl explants from 3-month old callus in MS liquid medium, supplemented with 30 g/L sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid and 2 mg/L kinitin at pH 5.8 (Pourjabar et al., 2012). Cultures were incubated in the dark at 25 °C and shaken at 90 rpm in darkness. Suspensions were subcultured every 2 weeks in the same medium and were maintained in 250 ml Erlenmeyer flasks with 50 ml of medium.

Culture treatments and elicitation

Suspensions were treated with 2 elicitors methyl jasmonate and YE: Methyl jasmonate (100 μM, final concentration) prepared as a filter-sterilized stock solution in ethanol and yeast extract (0.1% w/v) was dissolved in distilled water and then autoclaved (Sánchez-Sampedro et al., 2005a; Sánchez-Sampedro et al., 2005b). The control was the cell culture in same medium without adding any elicitor. Treatments were done 3 days after transfer, when cells had already started division. All culture experiments were performed in triplicate.

Flavonolignan analysis

One grams of cells were ground in liquid nitrogen and homogenized with 15 ml of 80% methanol. The homogenate was filtered and dried in vacuo below at 60 °C .The dry residue was resuspended in 3 ml distilled water, extracted twice with 6 ml pure ethyl acetate, the extracts were dried in vacuo at 60 °C and redissolved in 1 ml of methanol and kept at 4 °C in darkness (Hasanloo et al., 2008; Pourjabar et al., 2012). The content of flavonoeids was determined by HPLC system according to the method of (Hasanloo et al., 2008) on a Knauer liquid chromatography equipped with a Knauer injector with a 20 μl loop, a Nucleosil C18 5 μ (250 × 4.6 mm) column, Knauer K2600A UV detector and Chromgate software for peak integration. The elution time and flow rate were 30 min and 1 ml min−1, respectively and peaks were detected at 288 nm.

Protein extraction

After 48 h elicitation with MeJA (100 μM) and YE (0.1% w/v), control and treated cell suspension cultures were harvested separately. Proteins were extracted according to the method of (Yang et al., 2006) with some modifications. Five grams of cells were ground in liquid nitrogen. To remove DNA, 10 μl DNaseI (2 mg/ml) and 10 μl of 100X Reaction Buffer per ml (100X Reaction Buffer: 100 mM Tris-HCl (pH 7.5), 500 mM $MgCl_2$, 13 mM $CaCl_2$) was added and the mixture was incubated at 37 °C for 30 min. Then 2 ml pre-cooled homogenization buffer, 125 mM Tris-HCl (pH 7.5), 250 mM sucrose, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM

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dithiothreitol (DTT) and 1% Triton X-100 were added and incubated for 1 h. Thereafter, the homogenate was centrifuged at 13500 g for 20 min at 4 °C. The supernatant was mixed with 1/5 volume of cold 50% trichloroacetic acid (TCA) and kept in freezer -20 °C overnight. Then, the mixture was centrifuged at 13500 g for 20 min at 4 °C and the supernatant was discarded. The pellet was washed three times (each 20 min) with cooled acetone containing a 0.5% 2-Mercapto ethanol, centrifuged and completely dried. Pellet was resuspended in lysis buffer (8 M Urea, 2 M Thiourea, 4% CHAPS, 2% IPG buffer pH 4-7 (Bio-Rad), DTT 80 mM and protease inhibitor in 40 mM Tris-Base) and mix for 1 h in a rotator so as to enhance protein solubilization. Finally, the samples were centrifuged at 13500 × g at 4 °C for 10 min. Protein concentration was measured by Bradford assay using IgG as standard (Bradford, 1976).

Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-DE) was performed according to the method of Mostafaie et al. (2011). Briefly, the IPG strips [pH 4-7, 18 cm length, General electric (GE)] were rehydrated at room temperature overnight in 360 µl rehydration solution (8 M urea, 4% CHAPS, 80 mM DTT, 2% IPG buffers (pH 4-7), 40 mM Tris-Base and 0.002% bromophenol blue) in a reswelling tray (General electric). Isoelectric Focusing (IEF) was performed at 20 °C on an IPGphor Unit (GE Healthcare/Amersham Biosciences) using the following settings: 2 h 500 V, 2 h 3000 V and finally 7 h 8000 V until an accumulated voltage of 54000 V was achieved. For second dimension, focused strips were equilibrated twice, 15 min in 5 ml equilibration solution (50 mM Tris-HCl buffer, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT and 0.002% bromophenol blue) and then 15 min in the same solution containing 2.5% iodoacetamide instead of DTT. The second dimension was performed in 15% separation and 5% stacking gels. Protein spots in 2-DE gels were visualized by Coomassie Brilliant Blue (CBB) R- 350 according to the manufacturer's instructions (Görg et al., 2007; Mostafaie et al., 2011). Three gels for each sample were run and finally a total of eighteen gels were analyzed.

Image analysis

The gels were scanned at a resolution of 300 dots per inch using GS-800 densitometer (Bio-Rad) and were analyzed using the Melanie 6 software. Spot detection, protein quantification and spot pairing were carried out based on Melanie 6 default setting and spot pairs were investigated visually. The molecular masses of proteins on gels were determined by co electrophoresis of standard protein markers and pI of the proteins were determined by migration of the protein spots on 18 cm L (pH 4-7) IPG strips (Mostafaie et al., 2011). One 2-dimensional gel per sample was run for three biologically independent replicates, then percent volume (%vol) of each spot was extracted and analyzed by one way ANOVA test ($p < 0.05$). Only those spots that were present on all three replicate gels and had 1.5-fold changes were selected. Some differentially-expressed protein spots were selected as candidate proteins for MALDI-TOF-TOF analysis.

Protein identification and database search

Differentially-expressed spots were manually excised from preparative Coomassie blue-stained gels. Analysis was carried out by the Proteomics Laboratory; University of York, U.K., using MALDI-TOF-TOF mass spectrometry. Tryptic digestion was

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performed after reduction with DTE and S-carbamidomethylation with iodoacetamide. Gel pieces were washed two times with 50% (v:v) aqueous acetonitrile containing 25 mM ammonium bicarbonate, then once with acetonitrile and dried in a vacuum concentrator for 20 min. Sequencing-grade, modified porcine trypsin (Promega) was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5 times by adding 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.02 μg/μl. Gel pieces were rehydrated by adding 10 μl of trypsin solution, and after 5 min, adequate 25 mM ammonium bicarbonate solution was added to cover the gel pieces.

Digests were incubated overnight at 37 °C. A 1 μl aliquot of each peptide mixture was applied directly to the ground steel MALDI target plate, immediately followed by an equal volume of a freshly-prepared 5 mg/ml solution of 4-hydroxy- α -cyano-cinnamic acid (Sigma) in 50% aqueous (v:v) acetonitrile containing 0.1%, trifluoroacetic acid (v:v). Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with a Nd:YAG smart beam laser. MS spectra were acquired over a mass range of m/z 800-5000. Final mass spectra were externally calibrated against an adjacent spot containing 6 peptides (des-Arg1-Bradykinin, 904.681; Angiotensin I, 1296.685; Glu1-Fibrinopeptide B, 1750.677; ACTH (1-17 clip), 2093.086; ACTH (18-39 clip), 2465.198; ACTH (7-38 clip), 3657.929.). Monoisotopic masses were obtained using a SNAP averagine algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and a S/N threshold of 2.

For each spot, the ten strongest peaks of interest, with a S/N greater than 30, were selected for MS/MS fragmentation. Fragmentation was performed in LIFT mode without the introduction of a collision gas. The default calibration was used for MS/MS spectra, which were baseline-subtracted and smoothed (Savitsky-Golay, width 0.15 m/z, cycles 4); monoisotopic peak detection used a SNAP averagine algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) with a minimum S/N of 6. Bruker flexAnalysis software (version 3.3) was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra.

Tandem mass spectral data were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.6.1), through the Bruker BioTools interface (version 3.2). Search criteria included: Enzyme, Trypsin; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M); Peptide tolerance, 100 ppm; MS/MS tolerance, 0.5 Da; Instrument, MALDI-TOF-TOF (The version and size of the database can be obtained from the Mascot result page) (Hashemitabar et al., 2014).

Statistical analyses

Statistical analysis was carried out with SPSS (Version 16) software using one-way ANOVA test. All analytical values represent the means of three biological replications. Duncan's post hoc test was used for mean comparison ($p < 0.05$).

Results

Effect of elicitor on biosynthesis of SLM

To assay the effect of MeJA elicitation on SLM production, cell suspension cultures of *S. marianum* in the active growth phase were challenged with 100 μM MeJA and YE (0.1% w/v) separately. Result of this expriment showed that SLM content of the MeJA

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treated and YE treated cell cultures increased significantly after 48 h. Comparison between means showed that, there was no significant difference between the cell culture derived from cotyledon and hypocotyl in terms of silymarin content ($p < 0.05$). MeJA and YE treatment after 48 h increased the amount of silymarin production to 1.23 and 1.11 folds respectively in comparison to the control (*Fig. 1*). The highest amount of silymarin was obtained in cell suspension cultures treated with MeJA.

Proteome analysis

In order to investigate the changes in protein pattern in cell suspensions treated with elicitores compared to the control, we performed proteomics analysis of cell suspension cultures. We collected protein samples from control, MeJA treated and YE treated cell suspension cultures at 48 h after subculture, when they showed significant alteration in SLM content. A comparison between all the groups, control, MeJA and YE in both cell cultures derived from cotyledon and hypocotyl calli, leads to the detection of at least 249 protein spots (*Figs. 2 and 3*). One way ANOVA ($p < 0.05$) analysis showed that abundance of 40 protein spots were up or down regulated by at least 1.5 fold after MeJA and YE treatment. Out of the 40 candidate spots, 10 protein spots, which were seen on preparative CBB-stained gel, were selected and manually excised and analyzed by MALDI-TOF-TOF. *Table 1* shows the list of the identified proteins with their respective spot number, theoretical and experimental pI and molecular weight, percent of sequence coverage, protein name and accession number. The accession number was derived from NCBI protein database search and used to search the corresponding protein in uniprot database.

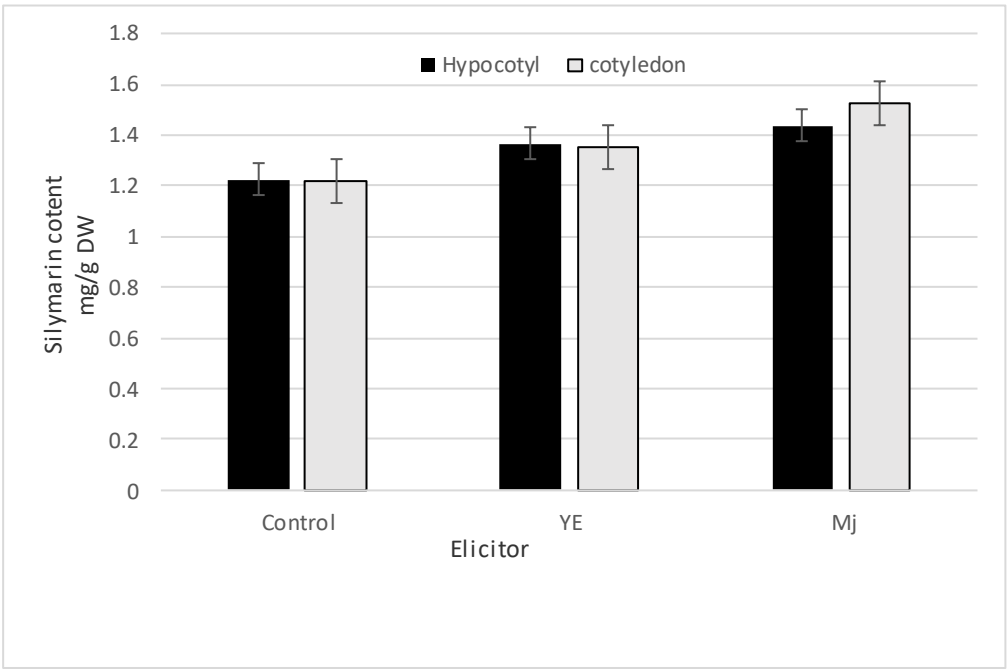

Figure 1. *SLM contents of S. marianum cell suspension cultures after MeJA and YE treatment at 48 hours after transfer of cell cultures. The values represent the average of three replicate experiments ±SD. (*p < 0.05)*

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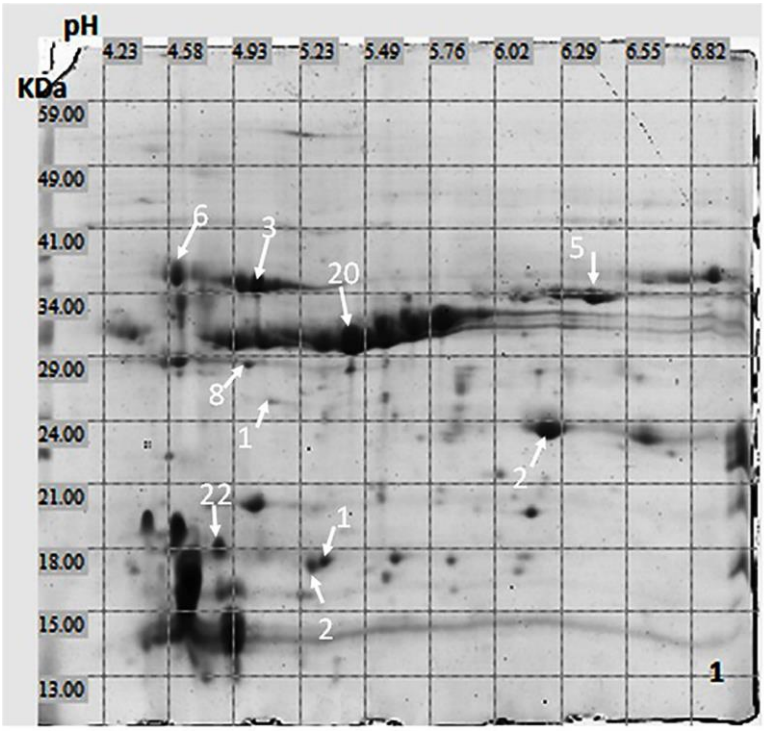

Figure 2. *Two-dimensional gel electrophoresis maps of cell suspension cultures. Total protein extract was separated in 18 cm IPG strip (pH 4-7) for the first dimension and 15% SDS-PAGE for the second dimension. The position of the identified proteins is shown in gel*

Table 1. *Proteins differentially expressed in elicited cell cultures of Sylibum marianum*

Spot No.[a]	Accession No.[b]	Protein name	Mw[c] (calc/theo) (Da)	Pi[d] (calc/theo)	Species	Coverage (%)
1	Q8LNX9	Pathogenesis-related protein	17/17	5.1/5.28	*Zinnia violacea* (Garden zinnia)	47
2	CAC43324	Pathogenesis-related protein	17/19	5.08/5.33	*Zinnia elegans*	30
3	AAM97498	O-methyltransferase	35/38	5.58/5.07	*Catharanthus roseus*	38
6	P48496	Triosephosphate isomerase,chloroplastic	36/34	6.45/4.65	*Spinacia oleracea* (Spinach)	60
8	AAN07899	20S Proteasome subunit alpha type	28/29	5.07/5.01	*Nicotiana benthamiana*	35
12	Q40520	Ras-related protein Rab11C	24/18	5.4/5.12	*Nicotiana tabacum* (Common tobacco)	51
20	O04999	Glutamine synthetase	39/30	5/5.7	*Medicago truncatu*	40
29	KVI12372.1	11-S seed storage protein	45/30	7.01/6.47	*Catharanthus roseus*	6
53	CAJ84723	Peroxidase	35/39	7.63/6.68	*Catharanthus roseus*	54

a Number corresponds to spot ID on gels
b accession number in www.uniprot
c Theoretical and calculated molecular weight
d Theoretical and calculated pI

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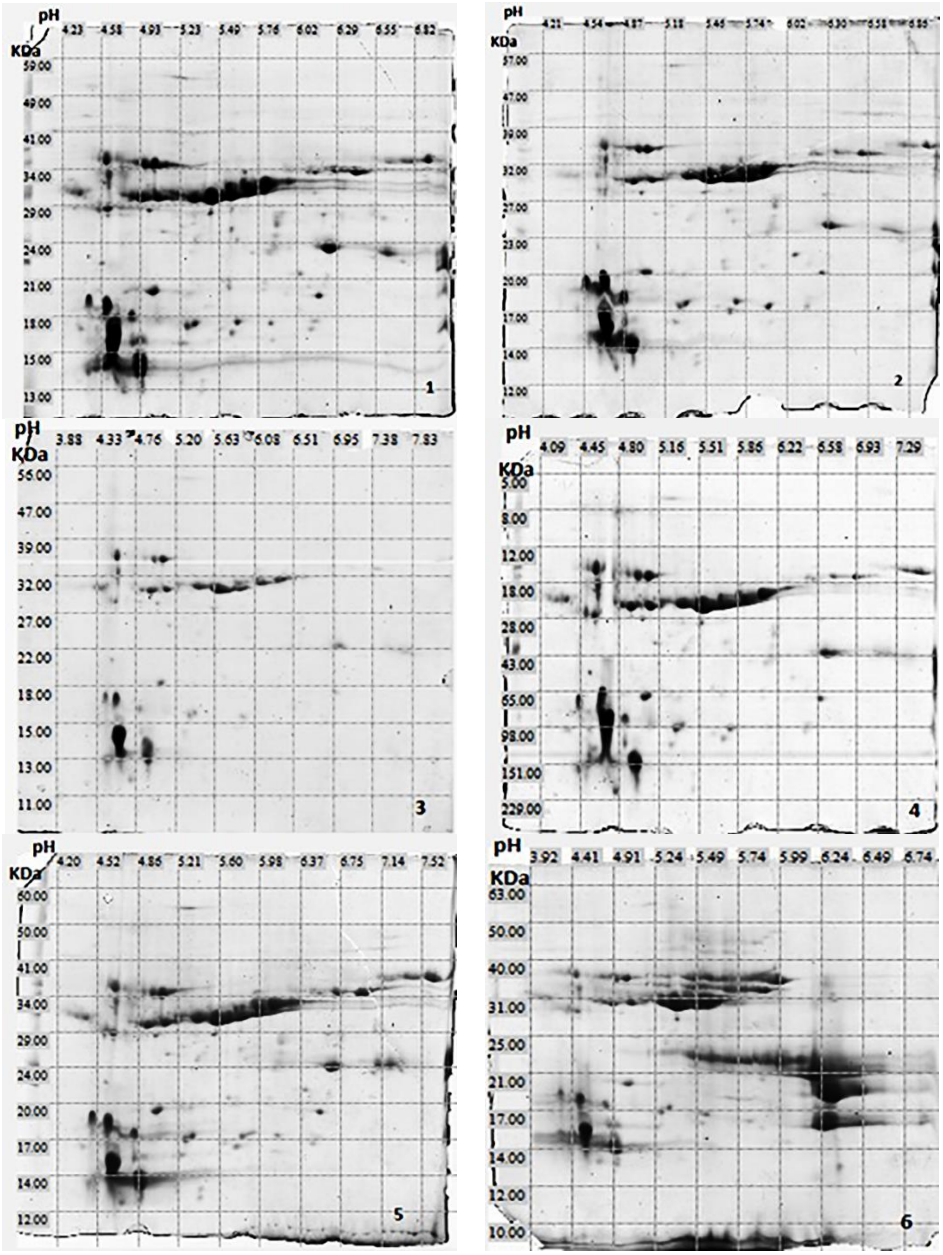

Figure 3. *Two-dimensional gel electrophoresis maps of cell suspension cultures 48 h after MeJA and Ye application and controls. 1- Cell suspension cultures Hypocotyl derived (control). 2- Cell suspension cultures Cotyledon derived (control). 3- Hypocotyl cell suspension cultures treated with MeJA. 4- Cotyledon cell suspension culture treated with MeJA. 5- Hypocotyl cell suspension cultures treated with YE. 6- Cotyledon cell suspension culture treated with YE*

These 10 identified spots were up or down regulated after elicitation with MeJA and YE compared with control and these proteins differentially accumulated in cell suspensions derived from cotyledons and hypocotyl explants (*Fig. 4*). *Fig. 4* shows the mean expression levels (mean percent volumes) of the differentially expressed spots on a 2-DE gel of proteins extracted from cell suspension cultures 48 h after MeJA and YE application. The expression levels of the identified spots which showed significant changes are presented in *Fig. 4*. Expression of proteins was even different in cell

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suspensions derived from cotyledons and hypocotyl explants. The identified proteins belong to different functional categories (*Table 1*). The proteins were classified based on carbohydrate metabolism (spots 6 and 22), Nitrogen metabolism (spot 20), storage protein (spot 29), transport process (spot 12), protein modification and chaperones (spot 8), pathogenesis related (spots 1 and 2) and secondary metabolism (spots 3 and 53).

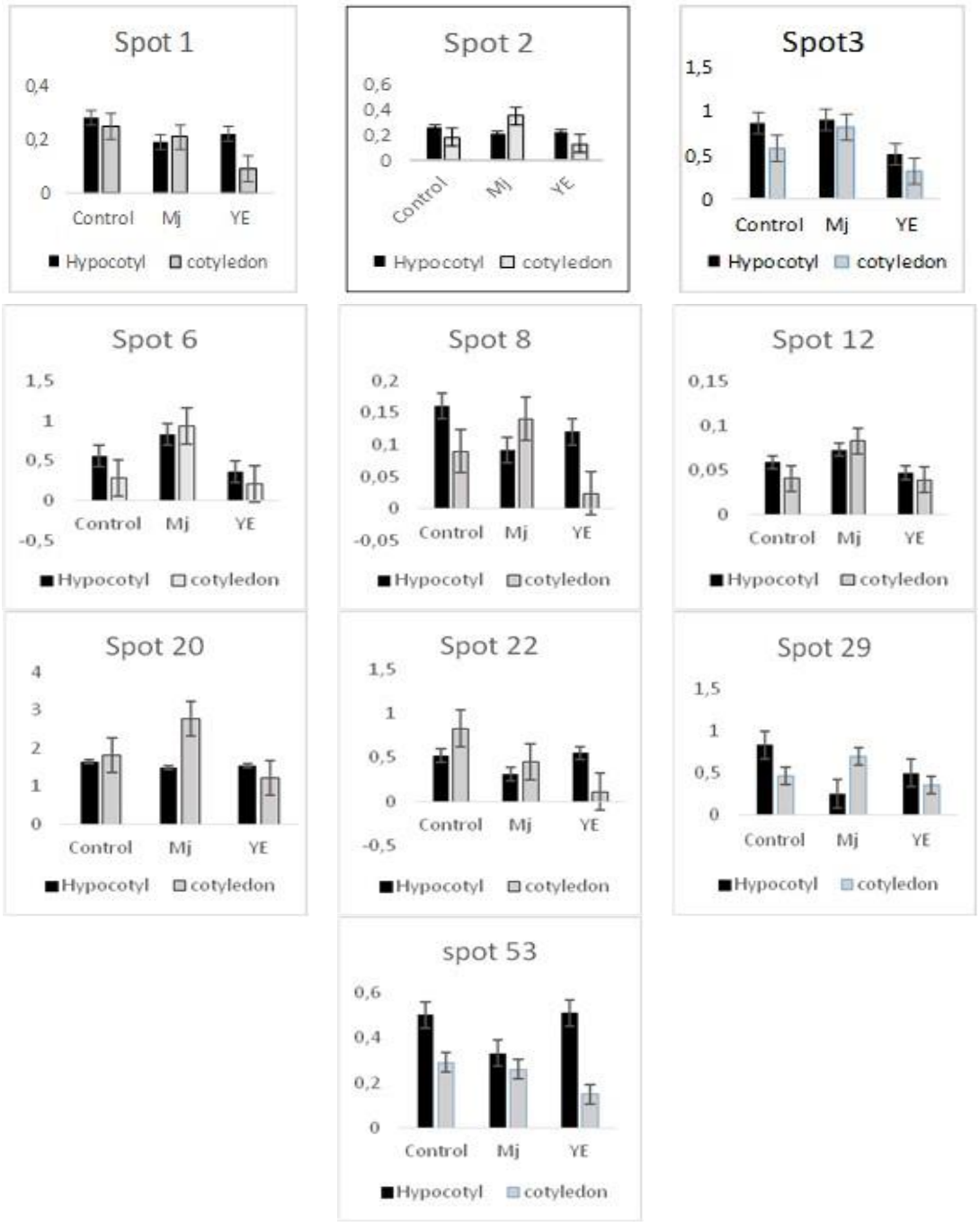

Figure 4. *The mean expression levels (mean percent volumes) of the differentially expressed proteins are shown ($p < 0.05$). The values represent the average of three replicate experiments ±SD. Y axes is the mean expression levels (mean percent volumes) of spots*

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Two identified proteins belong to carbohydrate metabolism (spots 6 and 22). Accumulation of these protein increased in the presence of MeJA. Alcohol dehydrogenase 2 (spot 22) and Triosephosphate isomerase, chloroplastic (spot 6) decreased in 48 h after treatment with YE (*Fig. 4*). Glutamine synthetase (GS, spot 20) was found to decrease. Cytosolic glutamine synthetase is encoded by a small family of genes that are well conserved across plant species. Members of the cytosolic glutamine synthetase gene family are regulated in response to plant nitrogen status, as well as to environmental cues, such as nitrogen availability and biotic/abiotic stresses.

Spot 12 (Ras-related protein Rab11C like protein of the Rab family), was upregulated by MeJA and YE elicitors (spot 12, Ras-related protein, up-regulated in all treatments). Rab proteins form the largest section of the Ras superfamily of small GTPases. Spots 1 and 2 were identified as Pathogenesis-related (PR) proteins. Those were up regulated after MeJA and YE treatment in cotyledon cell cultures. We observed the changes in the accumulation of this proteins, (Peroxidase (spot 53) and flavonoid O-methyltransferase (spot 3). It was possible to observe an increase in the accumulation of the protein peroxidase (spot 53) in the YE application in hypocotyl cell cultures and flavonoid O-methyltransferase (spot 3) in hypocotyl cell cultures after MeJA treatment. These two spots (3 and 53) were differentially accumulated in hypocotyl and cotyledon cell cultures after MeJA and YE treatments (*Fig. 4*).

Discussion

In this study, we investigated the effect of MeJA and YE elicitors on the accumulation of silymarin and protein profile in cell suspension cultures of milk thistle and we observed the changes in the protein levels in response to elicitation. We observed that, exposure of cell suspension cultures to MeJA and YE resulted in SLM accumulation after 48 h incubation. The results of our experiments showed that the use of MeJA and YE increased SLM accumulation in cell suspension cultures, but there was no significant difference between cell cultures derived from hypocotyl and cotyledon explants. This increase is related to the role of the elicitors in stimulating the biosynthesis of flavonoids in the cell suspension cultures. In other research studies, MeJA has been shown to enhance the production of SLM in cell cultures of *S. marianum* (Sánchez-Sampedro et al., 2005a), rosmarinic acid in *Lithospermum erythrorhizon* cell suspension cultures (Ogata et al., 2004), Resveratrol production in *Vitis vinifera* (Tassoni et al., 2005), Flavonoids in *Hypericum perforatum* (Wang et al., 2015), Phenylpropanoid and Isoflavonoid in *Medicago truncatula* cell cultures (Farag et al., 2008) and azadirachtin in hairy root cultures of *Azadirachta indica A.* (Satdive et al., 2007).

Some stresses, such as osmotic stress, wounding, drought, and exposure to elicitors, which include chitins, oligosaccharides, oligogalaturonides can induce JA signaling (Turner et al., 2002) and cause endogenous Jasmonic acid (JA) accumulation (Gharechahi et al., 2013). JA and its more active derivative, methyl jasmonate (MeJA) cause biosynthesis and accumulation of secondary metabolites by inducing defense responses (Gharechahi et al., 2013; Reymond and Farmer, 1998; Truman et al., 2007). Induction of secondary metabolite accumulation is an important stress response that depends on jasmonates as a regulatory signal (Blechert et al., 1995; Gundlach et al., 1992). Treatment of cell suspension cultures of *Rauvolfia canescens* and *Eschscholtzia californica* to a yeast cell wall elicitor leads to the rapid transient induction of

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endogenous jasmonic acid and methyl jasmonate (Gundlach et al., 1992). Jasmonate signaling pathway was supposed to be involved in the yeast extract induced production of silymarin. This arises by increase in lipoxygenase activity and linoleic acid content (Khalili et al., 2009).

These 10 identified spots showed different expression levels of the identified spots (*Fig. 2*). Proteins were found to be up and down regulated upon MeJA and YE treatment, even the expression of proteins in cell cultures derived from hypocotyl and cotyledon explants was different. Changes in expression of identified proteins in cell suspension cultures derived from Hypocotyl and cotyledon explants may be due to the differentiation and differently expressed proteins in the various plant tissues (cotyledons and hypocotyl), and this requires more research.

Some of the identified proteins in this study are involved in the general metabolism of the plants, including these spots (6, 8, 22, 29 and 20). The induction of stress and proteins related to defense, seen in this study, is associated with the role of the elicitors in plant defense. Change in expression levels of PR proteins was also demonstrated in this study. Spots 1 and 2 were identified as a Pathogenesis-related (PR) protein. It was up regulated after MeJA and YE treatment in cotyledon cell cultures. PR proteins are expressed in plants in response to many biotic and abiotic stresses (Reymond and Farmer, 1998).

A Ras-related protein Rab11C like protein of the Rab family, was upregulated by the two elicitor treatments (spot 12, Ras-related protein, up-regulated in all treatments). Rab proteins form the largest section of the Ras superfamily of small GTPases. The Rab family proteins involved in vesicular transport processes and secretion of secondary metabolites in plants (Nielsen et al., 2008). This is a good result to follow in future research.

We observed changes in the accumulation of the proteins flavonoid O-methyltransferase (spot 3) and peroxidase1 (spot 53). These proteins are involved in secondary metabolism. Regarding *S. marianum* as the non-model plant, an important challenge preventing the detection of specific proteins involved in secondary metabolism is the lack of fully sequenced genomes in non-model plants. Highly conserved proteins can be identified by sequence homology to *Arabidopsis thaliana* and other plant species (Bhattacharyya et al., 2012). Alternatively, specific EST databases have been made to identify proteins (Desgagné-Penix et al., 2010).

In this study, the proteomic approach has identified some of the events that have occurred in cell suspensions cultures of *S. marianum*. This is the basis for future research on *S. marianum* non-sequenced medicinal plant at molecular level. Much of the information obtained in comprehensive proteomic analysis can be achieved by mRNA sequencing at a lower cost and better range coverage. mRNA sequencing along with the proteomics method can be used for further research on secondary metabolism (Martínez-Esteso et al., 2015; Zubarev, 2013).

Conclusion

The results of this study showed that MeJA and YE elicitors caused significant increase in SLM content and activated defense-related proteins, proteins related to the transport mechanism with Extra cellular accumulation and secondary metabolism. The mechanism underlining the fact that metabolic pathways regulate defense response and accumulation of metabolites are largely unknown; however, investigation on analysis of

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proteins, and measurement of secondary metabolic will be essential for clear understanding of the pathway especially non-model species such as *S. marianum*. In summary, results shown in this study provide valuable basic information of the cell proteome of *S. marianum* that resulted due to enhanced accumulation of silymarin, and offers interesting possibilities for future research.

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