Comparison of Sample Preparation Methods for the Resolution of Metal-Regulated Proteins in *Helianthus Annuus* by 2-Dimensional Gel-Electrophoresis

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Abstract: The Dwarf Sunflower, *Helianthus annuus*, is a hyperaccumulator of the heavy metals cadmium, nickel, and chromium. The molecular mechanism of hyperaccumulation and adapting to the resulting abiotic stress is largely unknown. Metal-binding and other proteins induced in response to stress conditions may play indispensable roles in allowing the metals to accumulate and the plant to adapt itself. However, the presence of large amounts of the large subunit of ribulose1, 5 bisphosphate carboxylase/oxygenase (Rubisco) in leaves hampers the detection of many and, therefore also differentially expressed proteins, in gels. The aim of this study was to improve on methods of two-dimensional gel electrophoresis with high enough resolution for the purpose of identifying differentially expressed proteins for use in liquid chromatography and tandem mass spectroscopy and matrix-assisted laser desorption and ionization time-of-flight mass spectrometric analysis. The Teddy Bear cultivar of *H. annuus* was exposed to a mixture of cadmium, nickel, chromium, plus lead and lead only. Soluble proteins were extracted from leaf samples and several sample preparation procedures were tested. Use of a phenol extraction method enabled better resolution of proteins compared to either acetone or Trichloroacetic acid (TCA/acetone) precipitation techniques. TCA/acetone precipitation combined with a phenol treatment step (double precipitation) improved gel resolution with regard to reducing background staining and horizontal streaks, however, the heavy streaks associated with high concentrations of Rubisco were still present. Treatment of the samples with polyethylene glycol (PEG) was the most effective in reducing these streaks.

Keywords: *Helianthus annuus*, polyethylene glycol, proteomics, 2-DE.

INTRODUCTION

Contamination of soils and water with heavy metals has become a major environmental issue. Interest in the use of plants for environmental restoration (phytoremediation) has grown following the discovery that some plants are capable of accumulating toxic metals at levels many times greater (hyperaccumulators or hyperacumulator plants) than the 'non-accumulator' plants [1]. Evidence suggests that biochemical mechanisms for the hyperaccumulation of heavy metals are complex. We believe that the various proteins that are responding to the plant's exposure to heavy metals could be revealed by a proteomics approach, using two-dimensional gel electrophoresis (2-DE) and mass spectrometry. In this manner gene expression under different physiological conditions, such as environmental stress can be studied. Previously unknown proteins could be involved in metal binding or in general to the plant's response to toxins. Only identification of proteins expressed under defined experimental conditions could provide insight in stress response. These proteomics techniques have become the principal means to resolve and characterize proteins in such complex matrices as cellular extracts and serum [2]. While the analysis of proteins derived from animal and bacterial sources [3] has been the main focus, they have also been applied to a number of plant species including rice [4] and banana [5]. Compared to microorganisms and animal cells, however, the extraction and resolution of proteins from plant tissues presents a number of challenges due to a comparatively low concentration of protein per weight of tissue [6], high protease activities [7, 8], and the presence of various interfering substances, such as polyphenols and carbohydrates [5, 6, 9, 10]. Furthermore, it appears that the protein composition of each plant species is unique. Therefore, a well-defined set of conditions for each plant species is essential in obtaining reproducible and accurate results. In the 1980s, much effort has been directed towards the establishment of sample preparation methods for plant tissues [11]. Sample preparations generally use a one-step protocol, i.e., denaturing extraction in lysis buffer, which was shown to be suitable to obtain clean samples [5]. The majority of the protocols for sample preparation from plant tissues now involve two or more steps. These are precipitation-based [10] by the addition of high salt concentrations of divalent ions [5] followed by

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1875-0397/10

treatment of water miscible organic solvents such as acetone and acids [9], but a loss of proteins due to either incomplete precipitation or resolubilization of precipitants could present a serious problem. In addition, the electrophoretic separation of proteins from plant extracts is often complicated due to the presence of many non-protein contaminants indigenous to the plant, such as organic acids, lipids, polyphenols, pigments, and terpenes [12]. Moreover, a high abundance of certain proteins, e.g., the large subunit of ribulose1,5 bisphosphate carboxylase/oxygenase (Rubisco), can overshadow the identification of other, less abundant, proteins in 2-DE [13]. The main purpose of this study was to test and optimize sample preparation methods suitable for 2-DE analysis of proteins from *H. annuus*.

MATERIALS AND METHODS

Plant Materials and Metal Treatments

Seeds of the Teddy Bear cultivar of the Dwarf Sunflower *(H. annuus)* were purchased from the Jung Feed Company (Madison, WI). Seven plants were used in each experiment and were grown and treated with metal ions according to the procedures described previously [14]. Briefly, the plants were exposed to heavy metals in solutions containing a mixture of Cd, Cr, Ni (reflecting the contaminated soil conditions in Northeast Ohio) plus Pb or Pb alone. In some cases As was used instead of Pb. The metals were applied as Cd^{2+} ($CdSO_4 \cdot 8H_2O$), Cr^{3+} ($CrCl_3 \cdot 6H_2O$), Ni^{2+} ($NiSO_4 \cdot 6H_2O$), Pb^{2+} ($Pb(NO_3)_2$ and As as (Na_2HAsO_4) at 30 mg/l for each metal. Seeds of each cultivar were first grown hydroponically in Rockwool, a non-reactive, nonabsorbent support system and sustained with a standard hydroponic nutrient solution (Cropking Corp., Seville, OH). After a four-week growth period, plants were transferred to a PVC trough and subjected to the contaminant solution at a complete recycle rate of 1.59 l/h containing 30 mg/l of the toxins. After 17 days of exposure, the plants were harvested. Roots, leaves and stems were then sectioned and weighed. All the experiments were repeated twice and the tissues were used for analysis of metal levels and for protein isolation.

Extraction of Soluble Proteins from Leaves

The plant samples were ground in liquid N_2 using a pre-chilled mortar and pestle. The powdered tissue was stored in microcentrifuge tubes (1.5-2.0 ml) at –80°C until further use. A protein extraction kit was purchased from G-Biosciences (St. Louis, MO) and the protein extraction buffer was prepared by dissolving 1 g of dry protein extraction reagent in 1.1 ml of the Diluent which makes approximately 2 ml of the protein extraction buffer. Complete Plant Protease Inhibitor cocktail (G-Biosciences) was added (10 µl into1 ml protein extraction buffer) and vortexed. Ground tissue powder (approximately 0.2 g per tube) was placed in a microcentrifuge tube and mixed with 500 µl of extraction buffer. The resulting mixture was sonicated six times, ten s each, with one min breaks at 60 rpm in an ice bath and centrifuged at 14,000 x g for 30 min at 4°C. The pellet was resuspended in one-fourth of buffer used in the previous step and sonicated briefly. It was centrifuged again and the supernatants were pooled and stored at – 80°C until use.

Protein Assay

Protein concentration was determined using Bio-Rad Protein Assay reagent (Hercules, CA) with 1 mg/ml bovine serum albumin as a standard, according to the manufacturer's protocols. The buffers used in the determination of protein concentrations were different according to the sample preparation methods used. The protein solubilization buffer (G-Biosciences) was used only for the trichloroacetic acid and acetone precipitation. In the case of phenol extraction and PEG fractionation methods, the protein pellet was dissolved in a rehydration buffer (RB) (7 M urea, 2 M thiourea, 4 % NP-40, 50mM DTT, 1% Ampholytes and 0.002% Bromophenolblue). The assay involved addition of Coomassie Brilliant Blue G-250 to the protein extract and absorbance was measured at 595nm with a Beckman D-64 spectrophotometer.

Protein Extraction for 2-D Electrophoresis

In order to compare the protein extraction efficiency, resolution, and the presence of interfering substances in 2-DE, five protein extraction procedures were tested. In procedure 1, proteins were extracted using the method described by Jacobs *et al*. [6] and Damerval *et al*. [15]. It employs 10% trichloroacetic acid (TCA) containing 0.07% β-mercaptoethanol (2-ME). In procedure 2, proteins were precipitated using only ice-cold acetone/0.07 % 2-ME as the precipitant instead of using 10 % TCA/acetone/0.07% 2-ME mixture. A pellet was obtained using both procedures by 2 h incubation at –20° C followed by centrifugation (14,000 x g, 30 min, 4° C). In procedure 3, which was adapted from Wang *et al*. [12], with some modifications, SDS/phenol was used as the extraction agent. In the phenol extraction procedure, TCA/ Acetone and Acetone washed dry powder was suspended in 0.4 - 0.8 ml/0.1 g of SDS buffer (30% sucrose, 2 % SDS, 0.5 % 2-ME, 0.1 M Tris.HCl, pH 8.0) and Tris-buffered phenol (pH 8.0) in a 1:1 ratio. The mixture was vortexed and centrifuged (14,000 x g, 15 min, 4° C). The upper phenol phase was collected in a separate microcentrifuge tube and four volumes of 0.1 M ammonium acetate in 80% methanol was added and incubated overnight at –20° C. The mixture was centrifuged (14,000 x g, 15 min, 4° C) and the pellet was then washed twice with 0.1 M ammonium acetate in 80 % methanol followed by washing twice with 80% acetone. Finally the pellet was air dried and stored at 80° C until use. In procedure 4 (double precipitation), the TCA/acetone precipitation method was combined with the SDS/phenol extraction. The TCA/acetone and acetone washed powder was air-dried, mixed with 0.4 - 0.8 ml/0.1 g starting material of 1:1 SDS buffer/ Tris buffered phenol (v/v) and vortexed for 30 s. After centrifugation (16,000 x g, 5 min, 4° C), the upper phenol phase was mixed with four volumes of 0.1 M ammonium acetate in 80% methanol and incubated overnight at –20° C. The microcentrifuge tube was centrifuged (14,000 x g, 5 min, 4° C) and the pellet was then washed once with 0.1 M ammonium acetate in 80 % methanol followed by an acetone washing. The pellet was air-dried and dissolved in 500 µl of 1 % SDS buffer. The protein concentration was determined and 200 µg of protein was re-precipitated with 10 % TCA/acetone/0.07 % 2-ME. After the overnight incubation at –20°C, it was centrifuged (16,000 x g, 4°C, 10 min). The

pellet was washed twice with ice-cold acetone in 0.07 % 2-ME, air-dried and stored in –80° C until further use.

Finally, another procedure was tested, which employs polyethelene glycol (PEG) fractionation as described by Xi *et al*. [16]. Some modifications were made in order to minimize loss of proteins as described below. Dry tissue (0.2 g per tube) was homogenized in 1 ml of ice cold protein extraction buffer (0.5 M Tris.HCl, pH 7.8, 2% (v/v) NP-40, 20 mM $MgCl_2$, 2% 2-ME (v/v), and Complete Protease Inhibitor cocktail (G-Biosciences), 1 mM EDTA and 1 % (w/v) polyvinylpolypyrrolidone (PVPP)). The cell free slurry was prepared by sonication and mechanical grinding. It was centrifuged at 12,000 x g, 4° C for 15 min and the pellet was labeled as *F1*. The supernatant was then subjected to PEG fractionation. To the supernatant, 50 % (w/v) PEG (PEG 4000) stock solution was added to give a final PEG concentration of 16 %. The mixture was incubated in an ice bath for 30 min and centrifuged (12,000 x g, 4° C, 15 min) and the pellet was labeled as *F2*. The supernatant was mixed with four volumes of cold 10 % TCA/acetone with 0.07 % 2-ME and incubated overnight at –20° C. After the centrifugation (15,000x g, 4° C, 10 min) the resulting pellet gave rise to fraction 3 *(F3).* All pellets were washed with ice-cold acetone with 0.07 % 2-ME, vacuum dried and stored at –80° C until use.

Isoelectric Focusing

Proteins were first separated by isoelectric focusing (IEF) using the ZOOM IPGRunner system (Invitrogen, Carlsbad CA), pH 3-10 non-linear 7 cm IPG strips and rehydration buffer (RB; contained 7 M urea, 2 M thiourea, 4% NP-40, 50 mM DTT, 1 % ampholytes and 0.002% Bromophenol Blue) according to the manufacturer's instructions. Approximately 200 µg of the sample was diluted with RB to give a final volume of 160 µl. Samples were first applied in the mode of in-gel RB of the IPG strips and passive rehydration was carried out overnight at room temperature. Prior to rehydration, 50mM DTT and Bromophenol Blue (3.2µl /160µl RB) were added in to the buffer. IEF was performed at room temperature for 20 min at 200V, 15 min at 450V, 15 min at 750V and 1 h at 2000 V. The total volt-hours applied in the IEF step was 250. Prior to the second dimension analysis, the strips were equilibrated for 15 min in 10 ml equilibration solution (1 ml of 10 x sample reducing agent mixed with 9 ml of 1 x LDS sample buffer (Invitrogen) and subsequently for another 15 min in 10 ml of 125 mM alkylation solution (232 mg of iodoacetamide in 10 ml of 1x LDS sample buffer).

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The second dimension (SDS-PAGE) was performed in Nu-PAGE Novex 4-12 % Bis-Tris ZOOM gradient Gel (Invitrogen) with IPG wells following manufacturer's instructions. Protein molecular weight marker (Fermentas, Hanover, MD) was applied to the well provided on the gel for calibration of the molecular weight. The gels were run with an electrophoresis buffer (MES-SDS running buffer; Invitrogen) and electrophoresis was performed at 200 V for 35-40 min. Gels were stained with Simply Blue Safe Stain (Invitrogen) following the manufacturer's protocol.

RESULTS AND DISCUSSION

TCA-Acetone and Acetone Precipitation

Various conditions of protein extraction affect the number and kind of protein spots after two-dimensional gel electrophoresis, therefore, a good sample preparation is essential for obtaining reproducible 2-D gel results. The effectiveness of two commonly used protein precipitation methods was compared first. These employ trichloroacetic acid (TCA)-acetone [6] and acetone alone, respectively. The results of the 2-DE analysis of the TCA-acetone precipitation of proteins obtained from *H. annuus* (Teddy Bear) leaves are shown in Fig. (**1**). Overall, the number of spots detected in the TCA-acetone treated samples (Fig. **1A**) and acetone treated samples (Fig. **1B**) were similar. However, some of the protein spots were weaker in the TCA-acetone precipitated samples. This might be due to inability of certain proteins to dissolve, suggesting that some proteins in the samples were susceptible to irreversible denaturation by the acid treatment. Similar observations were previously reported when *Mycorrhiza* were treated in the same way [17]. Further, a number of protein spots present in the TCA precipitate were not detectable in the acetone-precipitated samples, but a number of new proteins appeared. This salting out effect of TCA was also observed in the study reported by Jiang *et al.* [18] who found that acetone precipitation resulted in an efficient sample concentration and desalting with good recovery of proteins in the 2-DE analysis for human plasma samples. It also is apparent that the leaf samples isolated from *H. annuus* contained materials that cause streaks throughout the gel. The streaks in the molecular mass region of 50-60 kDa were particularly heavy and were always present. The LC-MS-MS analysis indicated that they were due to the presence of Rubisco (data not shown). To minimize the effect of streaking, a number of methods were tried. The phenol extraction method was first described by Hurkman and Tanaka in 1986 [19]. Since then sample preparation based on phenol extraction has become the most commonly used protocol in plant proteomic studies [12, 20, 21], largely because phenol is effective in the removal of interfering compounds like polyphenols. To this end a combination of TCA/acetone precipitation with acetone washes followed by SDS/Phenol extraction [13, 21, 22] was tested. Fig. (**1C**) shows the 2-DE of the samples prepared by the phenol extraction procedure. The results depict a decrease in the number of protein spots, but also a lower amount of streaks as compared to the TCA-acetone or acetone protocols. Furthermore, a number of proteins not previously detected appeared. This difference could be the result of loss of nucleic acids that also could cause the streaking effect in 2-D gels [19, 21].

Double Precipitation

Another protocol tested was the double precipitation method recently described by Gomez-Vital *et al.* [23] to overcome the problem of high background staining. When the 2-D gel obtained using the double precipitation method (Fig. **2A**) was compared to either TCA/acetone (Fig. **1A**) or acetone precipitation (Fig. **1B**), the appearance of streaks or background staining did not change significantly. In addition, although the overall protein distribution did not change much, a number of proteins present in the acetone or TCA/

acetone protocols were not present when the double precipitation method was used indicating that this protocol resulted in a greater loss of proteins. On the other hand, when the protein pattern for the control samples (Cd, Cr, and Ni present, reflecting the condition of contaminated soils in Northeast Ohio) (Fig. **2A**) was compared to Cd, Cr, Ni, plus Pb treated plant sample (Fig. **2B**) four extra protein spots (labeled 1-4) were visible. In this regard, the double precipitation method improved over the TCA/acetone or acetone protocol, which only showed 2-3 extra proteins in the all metal-treated samples (data not shown). Analysis of these spots will be reported elsewhere.

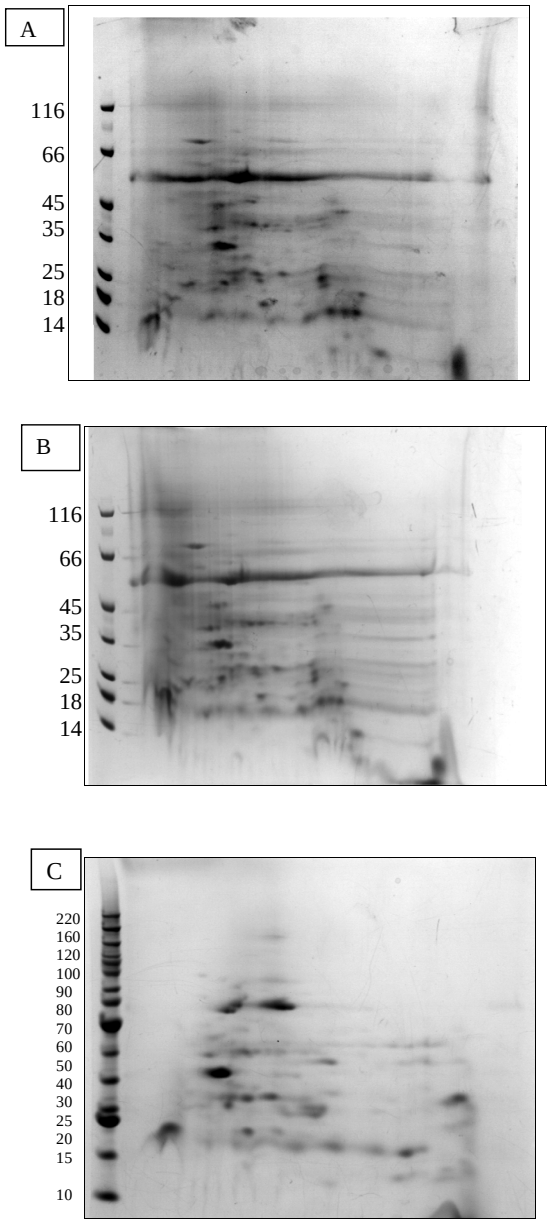

Fig. (1). 2-D gel of leaf proteins obtained from Teddy Bear cultivars. All proteins were obtained from 150 g of leaves from plants grown under hydroponic conditions. Three sample preparation methods were tested. **A**: 10% TCA/acetone precipitation. **B**: acetone precipitation. **C**: SDS/phenol extraction. Gels were stained with Coomassie Blue. The size of the marker proteins are given in kDa.

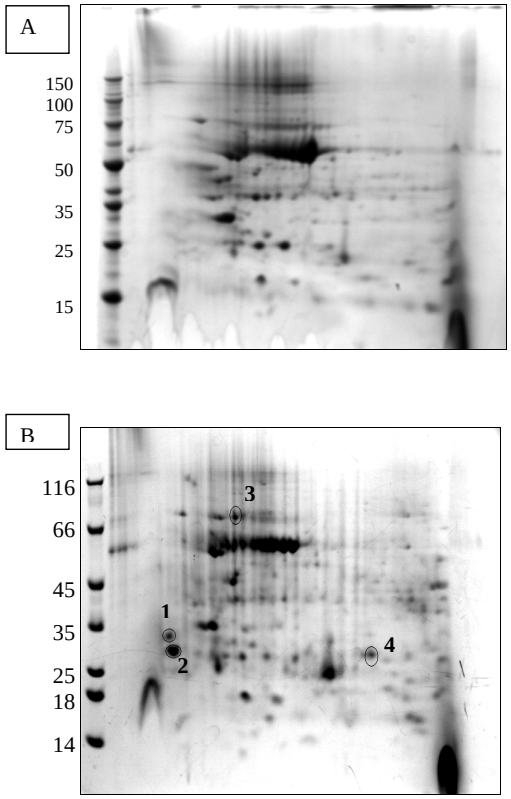

Fig. (2). 2-D gel obtained following double precipitation on leaf proteins (200 g) of Teddy Bear grown under hydroponic conditions. ***A***: Plants served as controls wherein they were exposed to Cd, Cr, Ni. ***B***: Plants were exposed to Cd, Cr, Ni and Pb.

PEG Fractionation

The polyethylene glycol fractionation (PEG) method described by Klose [24] for animal tissue was later adapted to plant tissues to overcome the problem of the low concentration of protein in plant tissues and minimizing the loss of proteins. In addition, the whole cell protein content made the protein gel more crowded, making identification and analysis of proteins difficult. The PEG-based method is thus intended to improve resolution over the precipitation methods. More importantly, the PEG fractionation might also reduce the amounts of the highly abundant Rubisco in the tissues of *H. annuus*. The PEG fractionation described recently by Xi *et al.* [16] indicated that Rubisco might be removed by PEG precipitation. This approach was tested on the Sunflower samples. Using the five-fractionation steps as described, with PEG concentrations up to 24%, it was found that three fractionations minimized the loss of proteins. The 1D PAGE (Fig. **3**) indicated that fraction 1 (F1) contained substantially higher levels of Rubisco than either fraction 2 (F2) or fraction 3 (F3), showing that Rubisco levels could be reduced by treatment of the sample simply with 16% PEG. The 2-DE profiles shown in Fig. (**4**) were from Teddy Bear leaf protein samples prepared according to this modified PEG method. Figure 4B shows the samples that were prepared from leaves exposed to the metals Cd, Cr, Ni, and As. Three proteins (labeled 1-3) (Fig. **4A**) were visible in the control samples and were absent in the metal-treated samples (Fig. **4B**) indicating that these proteins were absent when plants were exposed to all metals. Conversely, six proteins (labeled 4-9)

were new in samples from plants exposed to all these metals (Fig. **4B**), suggesting that these proteins were up-regulated due to metal-exposure.

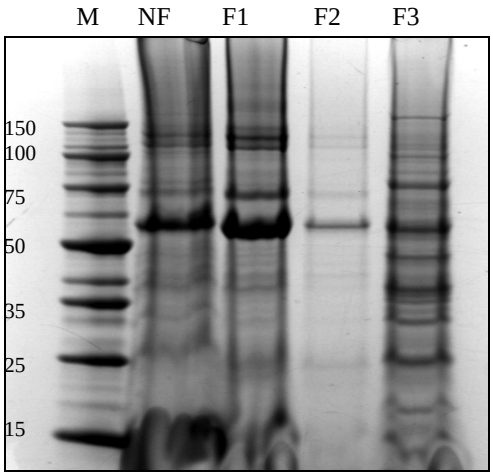

Fig. (3). SDS-PAGE obtained following the PEG fractionation on leaf proteins of Teddy Bear grown in soil. Plants were not exposed to the test metals. (M = Molecular Marker, NF = whole cell proteins prepared without fractionation, F1 = pellet obtained from cell free slurry after centrifugation (No PEG), F2 = Pellet obtained from 16% PEG, F3 =pellet obtained from the supernatant of F2 treated with 10% TCA/Acetone.

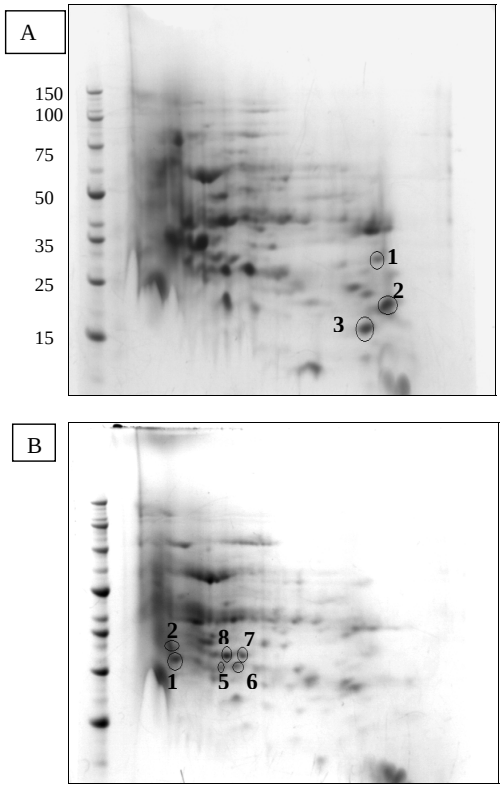

Fig. (4). 2-D gel obtained following the PEG fractionation (F3) on leaf proteins of Teddy Bear grown in soil. ***A***: Plants served as controls and spots labeled 1-3 were not observed in metal treated sample in Fig 4B. ***B***: Plants were exposed to testing metals Cd, Cr, Ni and As. Spots labeled as 1-6 were not observed in the control sample in Fig. (**4A**).

CONCLUSION

We have compared several sample preparation procedures for the Dwarf Sunflower (*Helianthus annuus*), to identify differentially expressed proteins for 2-DE analysis. Use of a phenol extraction method yielded better resolution of proteins compared to either acetone or trichloroacetic acid (TCA/acetone) precipitation techniques. TCA/acetone precipitation in combination with a phenol treatment (double precipitation) further improved gel resolution with regard to reducing background staining and horizontal streaks, however the heavy streaks associated with high concentrations of ribulose 1, 5-bisphosphate carboxylase still persisted. Treatment of the samples with polyethylene glycol (PEG) was simple and effective in reducing this masking protein.

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the Ohio Plant Biotechnology Consortium through The Ohio State University, Ohio Agricultural Research and Development Center. In addition funds were provided to HvK and RW from the Cleveland State University Research Council in the form of a Faculty Research Development Program award.

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Received: January 15, 2010 Revised: February 25, 2010 Accepted: March 02, 2010