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Selecting the optimum conditions for two-dimensional difference gel electrophoresis of proteins expressed in *Populus euphratica*

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ABSTRACT. We selected the optimum conditions for two-dimensional difference gel electrophoresis (2D-DIGE) of proteins expressed in the heteromorphic leaves of *Populus euphratica* Oliv. by adjusting the isoelectric focusing, the loading quantity, the concentration of the electrophoretic gel, and other parameters. The results of our study showed that protein separation was improved with many clear protein spots observed, and the differentiations were obvious. The findings of this study will be useful for future studies of protein expression in the heteromorphic leaves of *P. euphratica*.

Key words: *Populus euphratica* Oliv.; Heteromorphic leaf; Two-dimensional difference gel electrophoresis; Two-dimensional electrophoresis

INTRODUCTION

Two-dimensional electrophoresis (2D-E) is a highly sensitive and rapid protein identification technology that is an effective method of separating proteins in order to analyze gene function (Zhen et al., 2008), and was once the basis of proteomics. However, it does have some limitations: firstly, the silver stain is more sensitive than the Coomassie brilliant blue stain, but its limited dynamic range is not suitable for quantifying proteins; secondly, the silver stain is not compatible with mass spectra (MS) for analyzing and identifying proteins; thirdly, there are problems with the repeatability of the gel used in electrophoresis (Lilley and Friedman, 2004).

Two-dimensional difference gel electrophoresis (2D-DIGE) is based on using fluorescent dyes, including Cy2, Cy3, and Cy5, to label and mix protein samples and conduct electrophoresis on the same electrophoretic gel. Based on different fluorescent signals, each protein is separated and analyzed, which could overcome differences between electrophoretic gels (Song et al., 2012). Because of the sensitivity and linearity of the fluorescent dyes, 2D-DIGE is more accurate than other staining technologies. In addition, 2D-DIGE does not require fixation and decolorization after electrophoresis, which could reduce protein loss, particularly of proteins with low molecular weights (Van den Bergh and Arckens, 2004). Moreover, 2D-DIGE can be used to compare different protein samples on the same electrophoretic gel, without the confounding effect of gel differences. Differences in fluorescence intensity in each sample are caused by biological changes and not experimental error, which increases the reliability of the results (Marouga et al., 2005). Therefore, 2D-DIGE combined with MS, bioinformatics, and statistical analysis has improved the quantification and identification of plant proteins.

Warren et al. (2008) used 2D-DIGE to analyze the changes in phosphorylation in tropomyosin (Tm)-mutant mice (Glu54Lys) that were related to dilated cardiomyopathy. Tm phosphorylation decreased in the hearts of transgenic mice in comparison to that in non-transgenic mice, which indicated that the change in Tm phosphorylation is an important factor that is related to the E54K mutant in dilated cardiomyopathy. Park et al. (2007) used 2D-DIGE to identify the protein that is controlled by pX01 and plays a critical role in regulating its function in *Bacillus anthracis*. Holtappels et al. (2015) identified two proteins, FliC and CheY, in *Erwinia amylovora* using 2D-DIGE, which explained gradient differences in the toxicity of four bacterial strains.

2D-DIGE has been used in studies of plant tissues, organs, and physiological and biochemical characteristics under biotic and abiotic stress. Huang et al. (2006) used 2D-DIGE to analyze the proteins in the uppermost leaves of young wild-type Chinese fir trees. They found that the expression of 14 proteins increased and that of 15 decreased in the leaf tissue of a dwarf mutant. Song et al. (2012) used 2D-DIGE to analyze protein expression in the leaves of a wheat mutant. They reported that the chlorophyll-deficient mutant, Mt6172, exhibited the loss of a photosynthetic protein complex, a decrease in antioxidant capacity in the chloroplast, and an inhibition in editing after RNA transcription in the chloroplast. Amme et al. (2006) used 2D-DIGE to study *Arabidopsis thaliana* under low temperature stress, and detected proteins that were related to cellular responses to low temperature stress and monitored the changes in abundance of the proteins as the stress intensity gradually changed.

Populus euphratica Oliv. is a deciduous tree of the family Salicaceae that is mainly found in the arid deserts of Midwest Asia, North Africa, and southern Europe. As a typical

xerophile and mesophyte, the species can tolerate salinization and is an important species in desert riparian forests (Qiu et al., 2005). As an adaptation to living in a dry, continental climate, the leaf morphology of the species changes with the age of the plant. Young plants have lanceolate leaves, but as the plant grows, the number of broad, ovate leaves on the upper crown gradually increases. In addition, there are transitional leaf shapes between the upper and lower crowns such as acicular, diamond, oval, serrated, etc. (Zheng et al., 2007; Liu et al., 2015). There is an association between these heteromorphic leaves and ecological adaptation: initially, leaf genotypes on the same tree are the same, but in response to salt, water, or light stress, gene expression changes, resulting in changes to leaf shape (Bai et al., 2011).

The 2D-DIGE steps are similar to those of the classical 2D-E method, and mainly include sample preparation, first-dimension isoelectric focusing (IEF), second-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), gel dyeing, image acquisition, and image analysis. In order to obtain high-resolution images with a high repeatability, it is important to choose the appropriate gel, loading method and quantity, focusing conditions, and staining method. Therefore, in order to obtain satisfactory images for each sample, the correct conditions are required to construct a stable 2D-DIGE system with high resolution (Li et al., 2010).

In a previous study, Yue et al. (2009) investigated protein expression in *P. euphratica* leaves without optimizing the electrophoretic conditions, and found that the trichloroacetic acid (TCA)-acetone precipitation method with urea (7 M), thiourea (2 M), 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Deloitte Touche Tohmatsu (DTT, 80 mM), and 0.2% of the carrier ampholyte was suitable.

In our study, we selected two kinds of leaves, lanceolate and dentate broad-ovate. Based on previous study, we optimized the electrophoretic gel concentration, loading method, and focusing time, and obtained the optimum conditions for 2D-E that a subsequent 2D-DIGE was based upon.

MATERIAL AND METHODS

Material

The heteromorphic leaf samples were collected in July 2012 at the Beijing Forestry University, Beijing, China. All of the leaves were mature, healthy, and complete. After being frozen in liquid nitrogen, the leaves were stored at -80°C.

Protein extraction

The proteins were extracted by TCA-acetone precipitation and ground in liquid nitrogen. The extracted solution was stored at -20°C for 2 h and then centrifuged at 4°C with 15,000 *g* for 40 min. The precipitate was then washed with precooling acetone at 4°C for 1 h, and then centrifuged at 4°C with 15,000 *g* for 20 min. The precipitate was then freeze-dried to obtain a powder, which was resuspended with loading buffer at room temperature (RT). DTT was added to make a final concentration of 0.1%. The solution was then centrifuged at 20°C with 15,000 *g* for 40 min, and the pH was adjusted to 8.5. A Bradford protein assay was conducted to ensure that the protein concentration was suitable for fluorescent-dye labeling.

2D-E

Immobiline® DryStrip (IPG) pH3-10NL gels (GE Healthcare; pH 3-10, 17 cm long) were used for the IEF. The loading volume was 300 μL, which was calculated based on the loading quantity, loading buffer volume, and carrier ampholyte volume. The swelling and cup loading methods were used to load the samples. The IPG gels were taken from 4°C and placed at RT for 10 min, the covers were removed, and then they were placed upside-down and slowly covered with the mixed protein sample in the hydration disc, avoiding bubbles. Mineral oil (2 mL) was added to the gels, which were then hydrated for at least 12 h.

After hydration, the gels were washed with ddH_2O and superfluous items were removed using a filter, without touching the surface of the gels. Electrodes were then placed in the gels, which were covered with a wet filter and 4 mL of mineral oil. The IEF conditions were as follows: 50 V for 0.5 h, 200 V for 1 h, 2000 V for 1 h, 4000 V for 2 h, and 8000 V for 7 h. The voltage was gradually increased and the temperature maintained at 20°C with the maximum current (50 mA) in each gel.

After the IEF, the gels were washed with ddH_2O and balance liquid I was added. They were then placed on a rotator for balancing for 20 min, washed with ddH_2O, and balance liquid II was added. They were then placed on the rotator for balancing again for 15-20 min. The gels were then infiltrated with electrode buffer and placed on a second gelatinous plate at a concentration of 13% (Table 1). Agarose sealing liquid was added in order to remove bubbles, and the gels were placed on the gelatinous plate at 4°C for solidification. The electrophoresis conditions were as follows: 5 mA in each gel, electrophoresis for 1 h; the current was increased to 30 mA in each gel, and electrophoresis was run for 6-8 h until a bromophenol blue band was observed 1 cm from the button. The entire process was conducted under blackout conditions.

Table 1. Volume of each component in the second dimension gel.

Agents	Separating gel (15 mL) (T = 15%, C = 2.6%)	Separating gel (15 mL) (T = 13%, C = 2.6%)	Separating gel (15 mL) (T = 10%, C = 2.6%)	Stacking gel (10 mL) (T = 3%, C = 2.6%)
Secondary gel storage solution	7.5 mL	6.375 mL	5 mL	1.0 mL
Separating gel buffer	3.75 mL	3.75 mL	3.75 mL	-
Stacking gel buffer	-	-	-	2.5 mL
10% SDS	0.15 mL	0.15 mL	0.15 mL	0.1 mL
ddH_2O	3. 5 mL	4.575 mL	6 mL	6.3 mL
10% AP	0.1 mL	0.15 mL	0.1 mL	0.1 mL
TEMED	10 μL	10μL	10 μL	8 μL

SDS = sodium dodecyl sulfate.

After the 2D-E, the gels were washed with ddH_2O, a stationary liquid was added, and they were placed on a rotator for fixation for 1 h. They were then washed with ddH_2O four times (15 min each time), and stained with Coomassie brilliant blue overnight. They were decolorized using ddH_2O.

Image collection and analysis

The gels were imaged using a Typhoon Imager (GE Healthcare). Excitation wavelengths of 488, 532, and 633 nm were used to image the gels stained with Cy2, Cy3, and Cy5 separately. The PMT was adjusted in order to obtain protein spots. The images were analyzed using a PDQuest™ two-dimensional analysis system (Bio-Rad) for background

subtraction, protein spot resolving, internal standard matching, and differential expression analysis.

RESULTS AND DISCUSSION

Gel concentration selection in SDS-PAGE

Secondary gel concentration has a significant effect on separation, and different plant proteins require different separating concentrations. For example, in *Glycine max* and *Gossypium* spp, the separating concentration should be 12%, while in *Oryza sativa* L. it should be 10% (Ding et al., 2011).

At a high concentration of the separation gel (12%), most of the proteins were in the middle and upper parts of the image. At a lower concentration (10%), the spacing between the proteins significantly increased, and they were distributed more uniformly. After adjusting the loading quantity, more proteins were separated and obtained.

Because the proteins separated by SDS-PAGE only depend on the molecular size of the subunit, gel concentration selection is important. Usually, gel concentration selection is based on the molecular size of the protein sample. In our study, 5% stacking gel and 10-15% separating gel were suitable concentrations for analyzing proteins expressed in the heteromorphic leaves of *P. euphratica*. The different gel concentrations are shown in Table 1.

When comparing the images obtained from the three gel concentrations, the image obtained from the 15% gel concentration had a small aperture and the proteins had accumulated on the middle and upper parts of the gel. The samples were poorly separated and had a low resolution. The image obtained from the 10% gel concentration had a larger aperture but the proteins were pushed too far forwards, which resulted in the loss of low-molecular-weight proteins. The image obtained from the 13% gel concentration had better separation. The proteins were distributed uniformly on the gel with clear bands, and did not accumulate. Therefore, a 13% separating gel is the most suitable gel for analyzing proteins expressed in the heteromorphic leaves of *P. euphratica* (Figure 1).

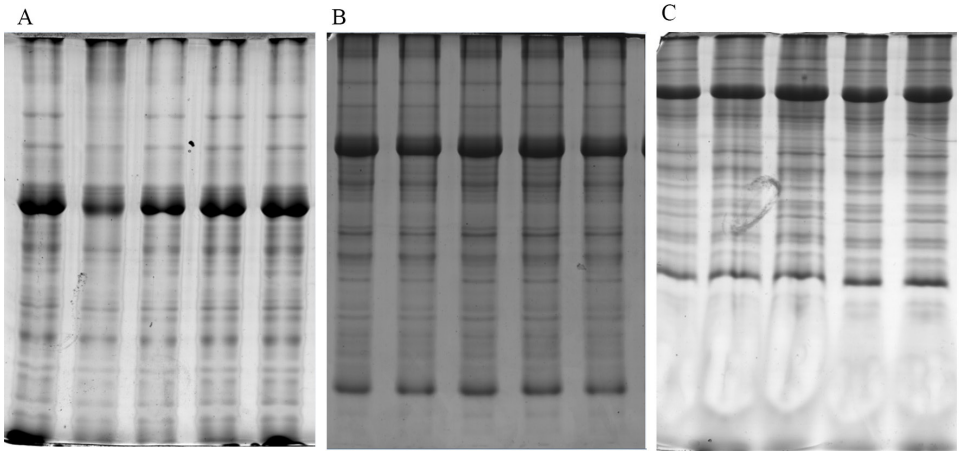

Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis images with different gel concentrations. **A.** 10% separating gel; **B.** 13% separating gel; **C.** 15% separating gel.

Selection of lysis and centrifugal times

In order to fully resolve the proteins in the lysis buffer, the period of time that the samples were resolved in the lysis buffer was a particularly important factor. In the secondary SDS-PAGE, if there were particles in the sample, vertical stripes appeared in the middle of the bands on the gel. Therefore, we used 30 and 60 min to select suitable lysis and centrifugal times. Separation with 60 min of lysis time was better than 30 min (Figure 2). The bands were clear and appeared on the middle and upper parts of the gel, without the vertical stripes that appeared on the gel with 30 min of lysis time, which indicated that 60 min of lysis time is optimal in obtaining a gel with a high protein concentration, uniform protein distribution, and few impurities.

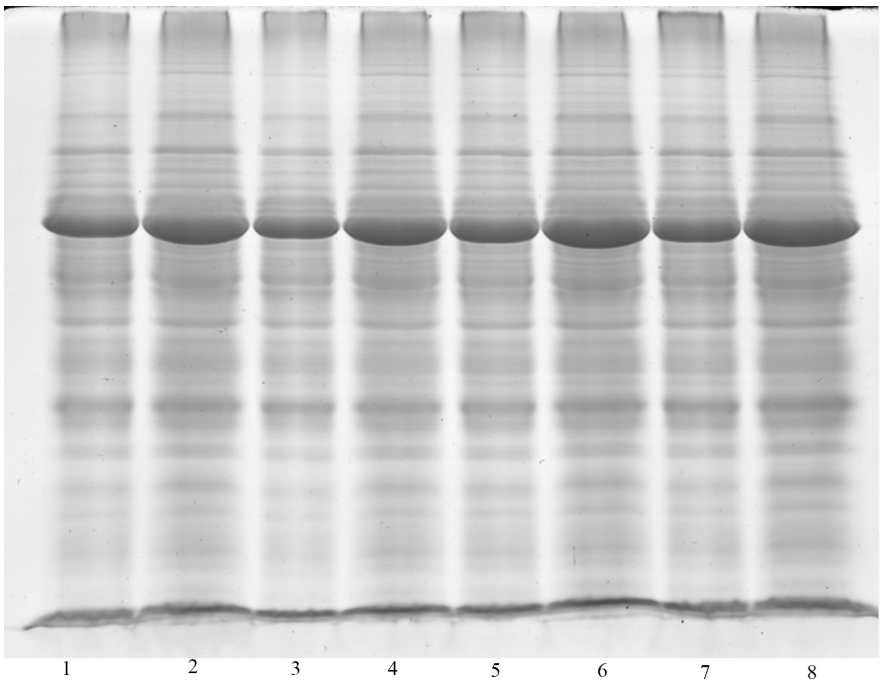

Figure 2. Effects of different lysis times on sample separation. *Lanes 1-4* = 30 min of lysis time; *lanes 5-8* = 60 min of lysis time; *lanes 1*, *3*, *5*, and *7* = 20-μg loading quantity; *lanes 2*, *4*, *6*, and *8* = 40-μg loading quantity.

Selection of IEF time and loading methods

IEF directly affects separation. Horizontal stripes or unregulated spots appear on gels with incomplete IEF, while electro-osmosis drift occurs and proteins appear on gels with excessive IEF (Li et al., 2010). Circular protein spots without lateral tailing appear on images with optimal IEF.

In our study, we firstly used the sample cup method to load the protein samples. The loading quantity was 500 μg, the voltage was increased to 8000 V with four steps, the IEF ran for 9.5 h, and the program was terminated when the voltage reached 60,000 V. After balancing, SDS-PAGE was conducted and Coomassie brilliant blue was used for staining.

Lateral diffusion occurred and the protein spots were unclear (Figure 3A), which indicated incomplete IEF with a poor image. Therefore, we reset the IEF program and extended the IEF time with 8000 V to 16 h, until the terminal voltage reached 900,000 V. Consequently, there was a great deal of diffusion around the protein spots, which indicated excessive IEF (Figure 3B). Therefore, we reduced the IEF period to 14.5 h with a voltage of 8000 V, and terminated the program when the voltage reached 80,000 V. The lateral diffusion was controlled. The protein spots were distributed on the gel with a higher resolution and better separation (Figure 3C).

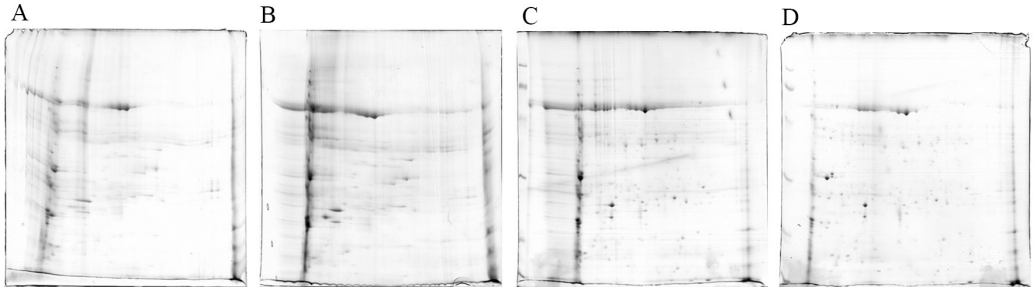

Figure 3. Two-dimensional electrophoresis separation of proteins expressed in the heteromorphic leaves of *Populus euphratica* Oliv. with different isoelectric focusing (IEF) times and loading methods. **A.** Sample cup method, IEF time 9.5 h, 60,000 V/h; **B.** Sample cup method, IEF time 16 h, 90,000 V/h; **C.** Sample cup method, IEF time 14.5 h, 8000 V/h; **D.** Swelling loading method, IEF time 14.5 h, 80,000 V/h.

Using the sample cup method, more proteins accumulated and there was poor separation (Figure 3C). Interference stripes affected the protein separation and the detection and analysis of the spots. Therefore, we used the swelling loading method and kept the other conditions as they were. Consequently, protein spot accumulation and the interference stripes decreased (Figure 3D). The protein samples were better separated, and the protein spots were clearly distributed on the gel with a higher resolution and a better image, which indicated that the swelling loading method was better than the sample cup method.

In conclusion, the swelling loading method was effective in separating proteins expressed in the heteromorphic leaves of *P. euphratica*, and the optimal IEF program conditions were as follows: 250 V, gradient, 30 min; 500 V, gradient, 60 min; 4000 V, gradient, 90 min; and 8000 V, step-n-hold, 11.5 h.

Selection of loading quantity

Gel concentration and loading quantity directly affect differentiating between protein spots in 2D-E (Song et al., 2013). According to Ding et al. (2011), loading quantity is important because of the detection of proteins that are in low abundance. However, with a large loading quantity, large salt ions in the sample would stop the voltage from increasing during the IEF, which would affect the IEF effect. In addition, a large loading quantity would induce the condensation and precipitation of the proteins during IEF, leading to lateral or vertical stripes appearing. The loading quantity should change according to the sample characteristics, gel length, and staining methods (Li et al., 2010).

In our study, we set the loading quantity at 500, 800, and 1000 μg. At the 500 μg loading quantity (Figure 4A), the proteins had separated well but there were few spots, and scarce proteins did not appear. At the 800 μg loading quantity (Figure 4B), the number of protein spots increased and scarce proteins appeared. The background was clear and the distribution of the spots was uniform, without any diffusion. At the 1000-μg loading quantity (Figure 4C), large-molecular-weight proteins were poorly separated with supersaturated protein spots. Therefore, the IPG gel (pH 3-10, 17 cm long) with an 800-μg loading quantity was optimal.

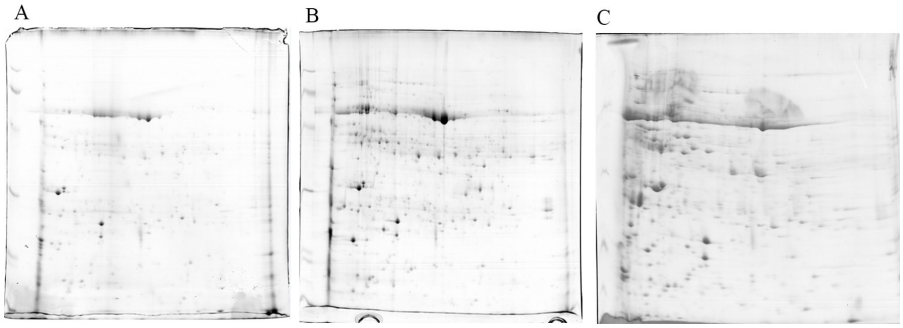

Figure 4. Two-dimensional electrophoresis separation of proteins expressed in the heteromorphic leaves of *Populus euphratica* Oliv. with different loading quantities. **A.** 500 μg; **B.** 800 μg; **C.** 1000 μg.

In conclusion, lysis buffer (Yue et al., 2009) containing urea (7 M), thiourea (2 M), 2% CHAPS, DTT (80 mM), and 0.2% carrier ampholyte; 60 min of lysis time; 13% separating gel; IEF with the following conditions: 250 V, gradient, 30 min; 500 V, gradient, 60 min; 4000 V, gradient, 90 min; 8000 V, step-n-hold, 11.5 h; and an 800-μg loading quantity were the optimal conditions for separating proteins expressed in the heteromorphic leaves of *P. euphratica*.

Separating proteins with 2D-DIGE

2D-DIGE involves additional fluorescent dye treatments to traditional 2D-E (Lv et al., 2015), the results of which are shown in Figures 5 and 6. Separation was better, with many clear protein spots visible. The differentiations were obvious.

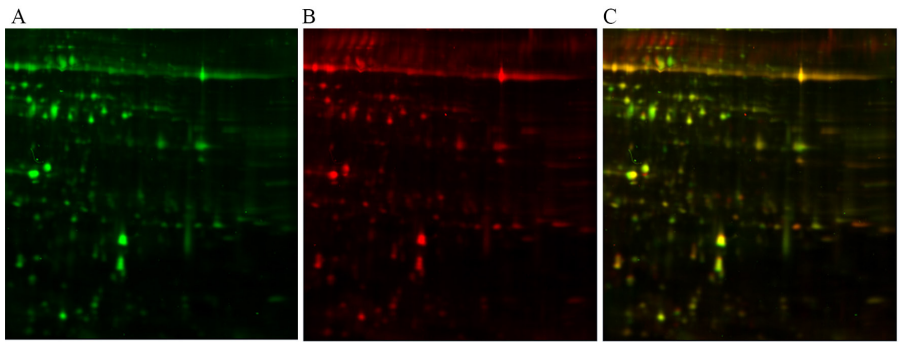

Figure 5. Two-dimensional difference gel electrophoresis analysis of lanceolate and dentate broad-ovate leaves of *Populus euphratica* Oliv. **A.** Green gel image of a lanceolate leaf labeled with Cy3; **B.** Red gel image of a dentate broad-ovate leaf labeled with Cy5; **C.** A superimposed image of A and B.

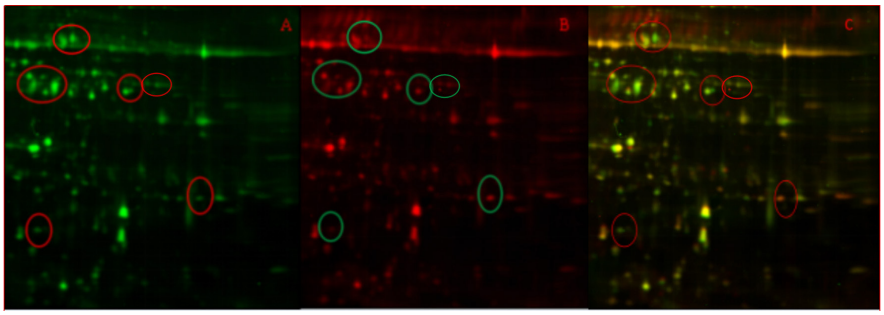

Figure 6. Two-dimensional difference gel electrophoresis images of lanceolate and dentate broad-ovate leaves of *Populus euphratica* Oliv. with protein spots circled. **A.** Green gel image of a lanceolate leaf labeled with Cy3; **B.** Red gel image of a dentate broad-ovate leaf labeled with Cy5; **C.** A superimposed image of A and B.

CONCLUSIONS

Our study has a few limitations. Firstly, *P. euphratica* Oliv. is a rare variety with limited representativeness. Secondly, there were individual and systematic differences in the 2D-DIGE images, and factors such as temperature and humidity affected the experiments. In addition, several gels were used that could not be made at the same time. Based on protein separation, the number of protein spots, image resolution, interference stripes, and the gel background we constructed a 2D-DIGE system that was suitable for separating proteins expressed in the heteromorphic leaves of *P. euphratica*, and obtained high-quality images. Based on these conditions, we analyzed the images and found differential protein spots, which may elucidate the mechanisms involved in the heteromorphic leaves of *P. euphratica* under stress.

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