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A general method for the extraction of citrus leaf proteins and separation by 2D electrophoresis: A follow up☆

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Abstract

With the aim of studying differentially expressed proteins as a function of abiotic and biotic stress in citrus plants, we optimized a protocol for the extraction of total leaf proteins and their 2-DE separation using commercially available immobilized pH gradient strips (IPGs) in the first dimension. Critical factors for good reproducibility of citrus leaf protein separation were identified: trichloroacetic acid (TCA)/acetone precipitation after extraction in lysis buffer, sample fractionation on narrow range overlapping IPGs and sample-cup loading at the anodic or cathodic end of the strip. The use of thiourea and a strong detergent (C7BzO) in the solubilization/rehydration buffer, coupled with the increase to 10% of SDS in the equilibration buffer before the second dimension seemed to affect positively the resolution of basic proteins. Using our protocol we resolved about 30 basic proteins on 6.3–8.3 pH range strips. Further, our protocol was successfully applied reproducibly on the analysis of control and salt exposed leaf samples of *Citrus reshni* Hort. Ex Tan.

Keywords: Citrus proteins; Narrow range immobilized pH gradient strips; Basic proteins; Low abundance proteins; C7BzO

1. Introduction

Citrus fruit production represents an important economic resource for most Mediterranean countries. Nevertheless, this area is largely affected by water shortage and soil salinity that are known to reduce growth and cause physiological disorders in citrus plants [1,2]. Citrus plants also suffer from different diseases that may considerably affect the fruit crop by reduction or suppression of tree development. For this reason, research focusing on the mechanisms by which these plants can overcome abiotic as well as biotic stresses is of noticeable importance to generate new resistant varieties. Proteomic approach, permitting simultaneous separation and identification of hundreds of proteins, might be a powerful tool for the comprehension of these processes. We are involved in an international project (INTERREG IIIA) dedicated to the understanding of response mechanisms to water shortage and salt salinity of different citrus rootstock varieties, using the proteomic approach, a strategy to find specifically expressed genes. Recently, a protocol for the extraction of proteins from citrus leaves followed by two-dimensional gel electrophoresis (2-DE) had been developed [3]. However, that paper showed two limitations: the use of a carrier ampholytes generated-pH gradient for the first dimension, instead of immobilized pH gradient strips (IPGs) and lack of information about the detection of low-copy-number proteins and the number of alkaline proteins recovered. Both low-abundant and basic proteins include regulatory proteins, receptors and most of all plasma membrane proteins that play key roles in cellular processes [4–6], and consequently, such kinds of proteins might be of noticeable importance in differential expression studies [7].

In this paper, we present an efficient protocol allowing the detection and the resolution by 2-DE of a maximum number of leaf citrus proteins including basic and low-copy-number ones. We used narrow (i.e. IPG 5–8; IPG 7–10) and ultra-narrow strips (i.e. IPG 6.3–8.3) to fractionate the sample, a new sulfobetaine [C7BzO, 3-(4-heptyl)phenyl-3-hydroxypropyl)

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Table 1
Recipes for the solutions used in CLP2 steps and 2-DE

Chemical components	Concentration	Source
Lysis buffer		
Urea	7 M	Fluka (51459)
Thiourea	2 M	Sigma–Aldrich
Tris	20 mM	Fluka (93304)
TCEP-HCl Tris (2-carboxy-ethyl)phosphine hydrochloride-	5 mM	Sigma–Aldrich
C7BzO 3-(4-heptyl)phenyl-3-hydroxypropyl)dimethylammoniopropanesulfonate	2%	Sigma–Aldrich
EDTA	5 mM	Fluka (ED2 SS)
PMFS phenylmethanesulfonyl fluoride	1%	Sigma–Aldrich
Glycerol anhydrous	10%	Fluka (49769)
Spermine base	25 mM	Fluka
Carborundum	50 mg	Geonatura
PVPP poly(vinylpolypyrrolidone)—(insoluble)	20 mg	Sigma–Aldrich
IPG buffer 3–10	2%	Bio-Rad
Solubilization/rehydration solution		
Urea	7 M	Sigma–Aldrich
Thiourea	2 M	Sigma–Aldrich
Tributylphosphine—TBP	5 mM	Sigma–Aldrich
C7BzO	1%	Sigma–Aldrich
IPG buffer	0.5%	Bio-Rad
Equilibration solution		
Urea	6 M	Sigma–Aldrich
Tris	50 mM	Sigma–Aldrich
Glycerol	30%	Fluka
SDS	10%	Fluka (71725)
TCEP-HCl Tris (2-carboxy-ethyl) phoshine hydrochloride-	5 mM	Sigma–Aldrich
IAA iodoacetamide	4%	Sigma–Aldrich

dimethylammonio propane sulfonate] and 10% SDS in the equilibration buffer before SDS-PAGE, to improve basic protein resolution. To test its efficiency, our protocol was applied to investigate the protein patterns of the leaves from control and salt stressed plants of *Citrus reshni* Hort. Ex Tan.

2. Experimental

2.1. Chemicals

All the commonly used reagents stated and not stated in Table 1 were purchased from Sigma–Aldrich S.r.l, Milano (Italy). "Carborundo" was purchased by Geonatura, Madrid, (Spain). (www.geonatura.com). Bio-Rad instrumentation and reagents were purchased by Bio-Rad Laboratories, S.r.l. Segrate-Milano (Italy).

All reagents were of analytical grade or better.

2.2. Plant materials and growth conditions

Citrus reshni Hort. Ex Tan. plants were grown for 1 year in 31 container filled with half sand/half soil and were watered once a week with a nutrient solution (Fertil 28-14-14, Boulogne Billancourt—France). Plants were grown in a climatic chamber at $28 \pm 1\,^{\circ}C$ with a light/dark cycle of 16/8 h. The room humidity was kept between 65% and 85%. Fluorescent tubes (General Electric F36W/54 and Osram Fluora L36W/77) were used to produce a PAR of 100 $\mu mol\,m^{-2}\,s^{-1}$.

A 50 mM NaCl salt treatment was applied by soaking pots in salt solution twice a week for 7 weeks.

Sampling of leaves was performed from control and salt treated plants. Two mature leaves from five of control or salt treated plants were pooled together and were frozen at $-80\,^{\circ}C$.

2.3. Citrus leaf protocol 2 (CLP2)

Leaves were ground in a pre-cooled mortar and pestle in liquid nitrogen. The powdered sample (0.5 g) was homogenized in 2.5 ml of lysis buffer (Table 1) for 5 min and then left solubilized for 1 h at room temperature under stirring.

The homogenate was centrifuged at $16{,}000 \times g$ (30 min, $20\,^{\circ}C$). The supernatant was mixed with three volumes of cold 10% trichloroacetic acid (TCA) in acetone. The proteins were allowed to precipitate overnight at $-20\,^{\circ}C$. The pellet was collected by centrifuging at $16{,}000 \times g$ (10 min, at $4\,^{\circ}C$) and washed two times with 1 ml of cold acetone and centrifuged again at $4\,^{\circ}C$. The pellet was air-dried at room temperature and then solubilized in 400 μl of solubilization/rehydration solution (Table 1) with stirring. During solubilization, the sample was reduced with 5 mM tributyl phosphine (TBP) for 1 h and then alkylated with 30 mM solution of iodoacetamide (IAA) for 3 h in the dark, as reported in the literature [8]. Finally, the sample was centrifuged at $16{,}000 \times g$ (5 min, at $20\,^{\circ}C$) to remove the insoluble material. Protein concentration was determined by RC-DC Protein Assay Kit (Bio-Rad) and then the samples were analyzed or stored at $-80\,^{\circ}C$, until use.

Table 2
Focusing conditions in CLP2 protocol

IPG strip type (Bio-Rad)		IPG buffer (Bio-Rad)	Protein loading (μg protein/strip)	Focusing duration (V/h)
pH 3–10 I	11 cm	pH 3–10 I	45/200	30000
pH 5–8	11 cm	pH 3–10 I	60	32000
pH 7–10 I	11 cm	pH 7–10 I	350	35000
pH 6.3–8.3	11 cm	pH 6.3–8.3	400	60000

Note that the focusing duration is calculated during the constant 8000 V stage.

The first dimension was performed on Protean IEF (Bio-Rad) on 11 cm IPG strips at 22 °C, limiting the current to 50 μA per strip. A 50 μl aliquot of suitably diluted proteins was applied through sample-cup loading at the cathodic or anodic end of a rehydrated strip. The voltage was set at 50 V for at least 4 h or overnight (depending on the strip pH range) and then 300 V for 2 h, 1000 V for 2 h, slow mode ramped to 8000 V over 2 h and then run at 8000 V until final volt-hours were reached (see Table 2).

Distilled water-wetted paper pads beneath the electrodes were always used during IEF. The paper pads act to remove salts and other interfering compounds so they do not interfere with the IEF. The focused strips were stored at least 2 h at −80 °C, and then were equilibrated for 15 min in 10 ml of equilibration solution (Table 1) to which TCEP-HCl [Tris (2-carboxyethyl) phosphine hydrochloride] (final concentration 5 mM) had been added. Strips were then equilibrated 15 min in 10 ml equilibration solution containing 4% IAA. Following equilibration, strips were sealed by 0.5% molten agarose in running buffer on a 12%, home-made gel and run on the Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare) at 25 mA/gel for about 4.5 h, in a buffer of 25 mM Tris, 192 mM glycine, 0.1% SDS, at 9 °C with cooling from a cooling device (Lauda ecoline RE 104, GMBH Co.Kg, Lauda—Konigshofen, Germany).

Staining was performed by placing gel into a fixative solution (40% ethanol, 10% acetic acid) for at least 2 h or overnight. Then an ammoniacal silver protocol was used [9]. The silver-stained gels were imaged by a back-illuminated scanner and the image analyzed on PD-Quest (Bio-Rad). Triplicates samples were done for both protein extraction and 2-DE analysis.

3. Results and discussion

The aim of our study was to develop a method for the 2-DE analysis of citrus leaf sample which allowed the visualization of a maximum of proteins especially low-copy-number and basic ones.

For good reproducibility of 2-DE, sample preparation is a critical step. Indeed, leaf tissues are not a good protein source, because of the presence of cell wall and vacuoles limiting protein yield and several interfering compounds such as phenols, proteolytic and oxidative enzymes, pigments and carbohydrates that may lead to irreproducible results in 2-DE protein separation [3,10].

Although a one-step procedure for protein extraction would be highly desirable with regard to simplicity and reproducibility [5], our first attempts to resolve citrus leaf proteins by applying the protocol used for the extraction of *Arabidopsis* leaf protein [11] gave irreproducible gels, streaks, and failure in reaching high voltage, as already observed for other plants [10].

Then, we compared the efficiency of protocols for the extraction of plant leaf protein including a precipitation step before 2-DE. We tested the classical TCA/acetone precipitation method [12]; the extraction method for citrus leaf proteins previously published [3] and the protocol for extraction of lemon fruit tissue proteins [13]. Among the three protocols we tested, first screenings showed that citrus leaf protocol [3] (namely CLP1) was the most suitable protocol giving reproducible 2-DE in all conditions applied. Thus, we decided to optimize CLP1 (extraction in Tris–HCl, pH 8.8; TCA/acetone precipitation; focussing in 9.5 M urea, 2% NP-40, 1% DTT, 2% 3–10 ampholytes) paying particular attention to the following steps:

(1) First dimension on immobilized pH gradient strips
(2) Protein extraction processes
(3) Equilibration step between first and second dimension electrophoresis.

(1) The first crucial modification was the use of commercially available IPGs, which overcomes the limitations of carrier ampholyte based 2-DE with respect to reproducibility, handling and resolution [5] and in this work we always used this focusing methodology, also when we tested citrus leaf protein samples processed according to CLP1.
(2) Tissue disintegration efficiency has a significant influence on total protein yield, especially when performing protein extraction from leaf tissues. This point had been stated in another paper [11]. We noted that the addition of an aliquot of "carborundo" (silica powder) and poly(vinylpolypyrrolidone) (PVPP) (insoluble) in the lysis buffer, resulted in a significant improvement of tissue disintegration due to mechanical abrasion and consequently in the increase of about 30% of the protein yield (about 14 mg/g fresh weight) in the crude extracts, before TCA/acetone precipitation.

We used a combination of two chaotropes, a strong detergent, and two reducing agents as components of our lysis and solubilization/rehydration buffers, as validated by other authors [14,15] as a method for optimal solubilization and 2-DE separation of plant proteins. Urea is a chaotrope effective in disrupting hydrogen bonds, while thiourea is better for breaking hydrophobic interactions and separation of membrane proteins [16]. Since the major problem associated with urea in aqueous solution might be the

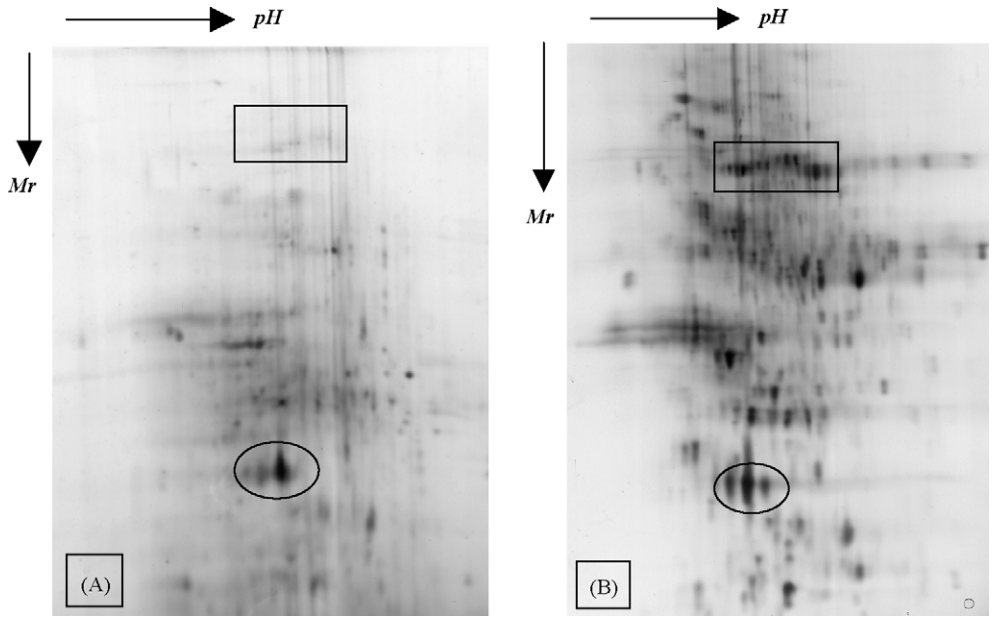

Fig. 1. Silver-stained 2-DE gels of 200 μg citrus leaf proteins. Panel (A) proteins extracted and focused according to CPL1 protocol, strip equilibration in 2% SDS buffer. Panel (B) protein extracted according to CPL1 protocol and focused in CPL2 solubilization buffer, strip equilibration in 10% SDS buffer. First dimension (IPG 3–10), SDS-PAGE 12% polyacrilamide gel, resolved Mr range 10–106 kDa.

presence of ammonium cyanate, which can introduce charge heterogeneities, we included carrier ampholyte (2%) as cyanate scavengers in our lysis buffer [5].

Detergents are utilized to avoid loss of proteins due to aggregation and precipitation, during solubilization and isoelectric focusing. Since the optimal choice of a detergent is largely empirical, we tested the extraction efficiency of the commonly used zwitterionic detergent CHAPS (with and without thiourea associated to urea), and other commercially available detergents, already used in 2-DE analysis, such as the sulfobetaine SB 3–10 [3-(Decyldimethyl-ammonio)propanesulfonate inner salt] (in 5 M urea and 2 M thiourea) [14,17], ASB14 (tetradecanoylamido propyl dimethyl ammonio propane sulfonate) [18], and C7BzO [6]. While CHAPS and SB 3–10 gave poor resolution in the basic pH range, ASB14 and C7BzO gave quite comparable results (data not shown). Thus, we decided to use C7BzO for extraction citrus leaf proteins because this detergent had been reported to be more efficient in resolving plant membrane proteins [6] and by our observations it did not give overestimation of protein concentration with respect to ASB14, at least when RC-DC Protein Assay Kit (Bio-Rad) was used.

At the same time, we replaced the reducing agent DTT, used in CPL1, with TBP and TCEP-HCl, because these reducing agents had been reported advantageous to obtain

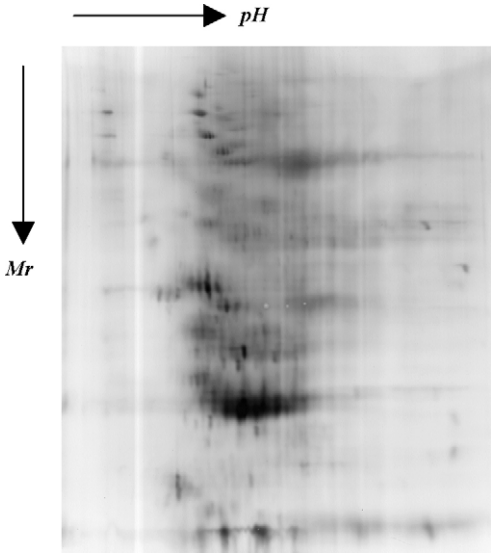

Fig. 2. Silver-stained 2-DE gels of 45 μg citrus leaf proteins processed according to CPL2 protocol. First dimension (IPG 3–10), SDS-PAGE 12% polyacrilamide gel, resolved Mr range 10–106 kDa.

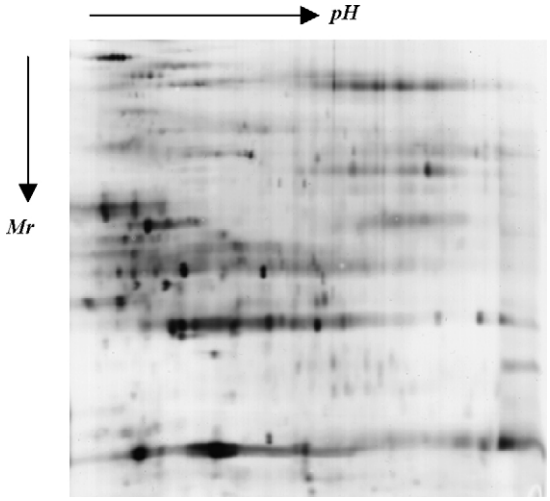

Fig. 3. Silver-stained 2-DE gels of 60 μg citrus leaf proteins processed according to CPL2 protocol. First dimension (IPG 5–8), SDS-PAGE 12% polyacrilamide gel, resolved Mr range 10–106 kDa.

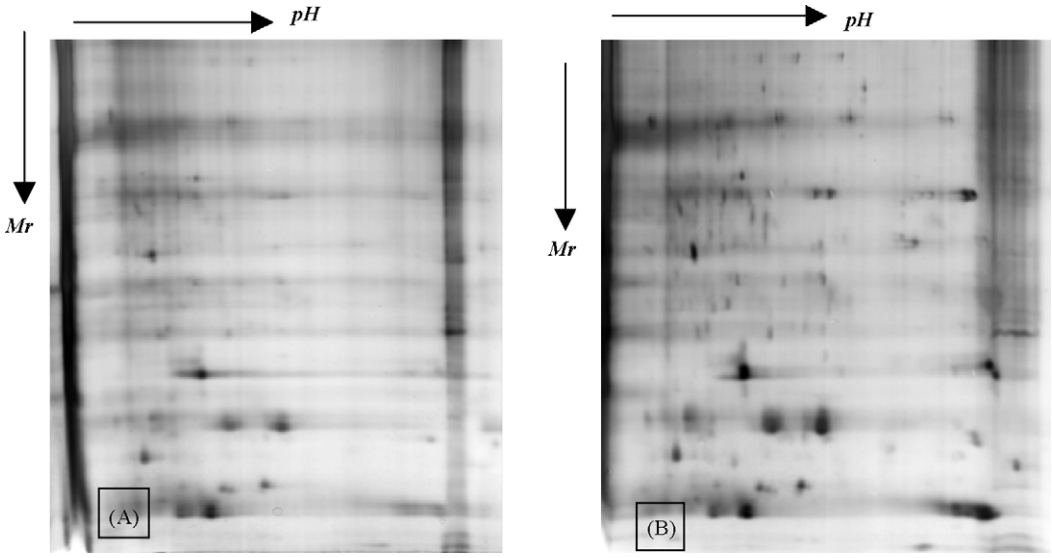

Fig. 4. Silver-stained 2-DE gels of 400 μg citrus leaf proteins extracted and focused with CPL2 buffers. Panel (A) strip equilibration in 2% SDS equilibration buffer. Panel (B) strip equilibration in 10% SDS equilibration buffer. First dimension (IPG 6.3–8.3), SDS-PAGE 12% polyacrilamide gel, resolved Mr range 10–106 kDa.

enhanced protein solubility during IEF as well as increased transfer to the second dimension [14,19].

(3) Taking into account that the equilibration step prior to SDS-PAGE is a critical point for visualization of a large amount of proteins, when using IPGs [5], we explored an optimization of this step. Recently it has been reported [20] that the basic membrane protein mABC1 was present in 2-DE gel only when SDS percentage in equilibration buffer had been increased to 10%. Thus, we adopted this modification in our protocol.

First, we tested the efficiency of our solubilization buffers on protein pellets extracted according to CLP1 and that of our equilibration buffer before SDS-PAGE.

Results are shown in Fig. 1.

In silver-stained gels of citrus leaf proteins extracted and solubilized according to CPL1, and focused on a IPG 3–10, about 450 spots were recovered (Fig. 1A). This spot number is in the same order to that reported in CLP1 paper [3]. About 500 well resolved spots were detected when proteins extracted according to the CPL1 protocol were focused in our rehydration buffer and the focused IPG strips were equilibrated in 10% SDS equilibration buffer, before second dimension (Fig. 1B). The increased number of proteins in the pattern shown in Fig. 1B seems a confirmation of the efficiency of thiourea and C7BzO to enhance the resolubilization of the TCA/acetone precipitated proteins. At the same time, the better resolution achieved especially in the basic range, could be due to the increase of SDS percentage in our equilibration buffer.

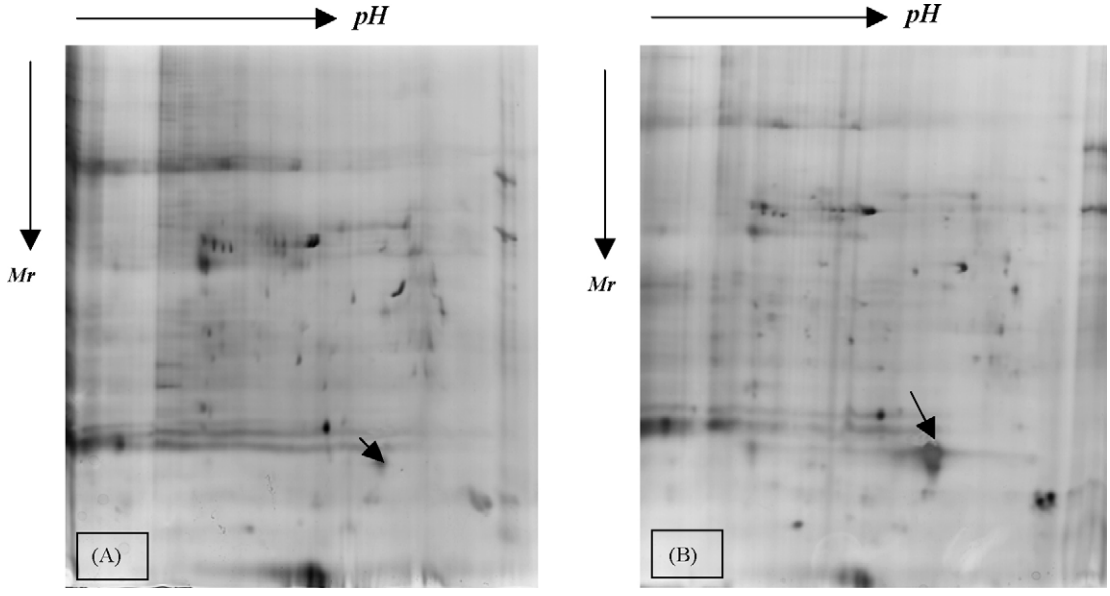

Fig. 5. Silver-stained 2-DE gels of 350 μg citrus leaf proteins from control (A) and 50 mM NaCl treated (B) plants. First dimension (IPG 7–10), SDS-PAGE 12% polyacrilamide gel, resolved Mr range 10–106 kDa.

About 700 spots were detected when CPL2 was applied to citrus leaf proteins 2-DE analysis (Fig. 2), but at the expense of the amount of proteins loadable, gel background staining and resolution of single protein spot.

To overcome these drawbacks, we found useful the fractionation of the sample on narrow overlapping range strips (IPG 3–6, 5–8, 7–10), associated to sample-cup loading at anodic or cathodic end of the strip, as previously reported [13].

In Fig. 3, the pattern of citrus leaf protein loaded on a IPG 5–8 is shown.

Further, we tried the fractionation on ultra-narrow range strips (e.g. IPG 3.9–5.1, or 6.3–8.3) that allow the application of higher proteins amounts for the detection of low-copy-number proteins. An example of the pattern in the basic range (IPG 6.3–8.3) is reported in Fig. 4A and B. To avoid protein precipitation and to achieve a good protein separation, we found compulsory to apply low voltage (50 V) at least for 6–7 h during the initial stage of the IEF.

From the comparison of the patterns shown in Fig. 4A and B, the efficiency of 10% SDS equilibration buffer for detecting more and well resolved basic proteins seems to be confirmed.

After standardizing the protocol on control samples to optimize experimental conditions, we applied the same procedure on 50 mM NaCl treated samples to investigate whether CPL2 was suitable to detect differentially expressed proteins as a function of environmental conditions.

For example, the different protein patterns from control (Fig. 5A) and salt treated (Fig. 5B) citrus leaves focused on IPG 7–10 were reported. Salt-responsive proteins (may up or down regulated) are circled. The arrows point out a protein spot that seemed to be modified as a function of salt treatment. Further investigations are still in progress to confirm these preliminary results.

4. Concluding remarks

During all extraction and separation steps for 2-DE, protein loss is unavoidable [5]. However, our results clearly show that the use of two chaotropes (urea and thiourea), a strong detergent (C7BzO) and more efficient reducing agents (TBP or TCEP) in the 2-DE buffers increased noticeably the number of spots detected.

The increase of the SDS percentage from 2% to 10% in the equilibration buffer between first and second dimension electrophoresis seems to positively affect the resolution and the number of basic protein spots. As the mechanism is still unknown, further investigation should be made before this modification can be routinely used.

As far as we know, this is the first time that a number of citrus leaf basic proteins (about 30 spots) are well resolved in the basic pH range (IPG 7–10 or 6.3–8.3). These preliminary results could be the first step for providing a basic proteome reference map of citrus leaf. Protein identification by MS analysis is still in progress.

Proteomic methodologies are continuously implemented and further technical modifications could be carried out to improve 2-DE analysis of citrus leaf proteins. Moreover the protocol which is presented in this paper appears reproducible and efficient to detect a large number of total citrus leaf proteins as well as to detect specifically expressed proteins as a function of environmental parameters by using zooming-in gels with ultra-narrow range pH strips.

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