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## Research Article

# Protein extraction from *Phoenix dactylifera* L. leaves, a recalcitrant material, for two-dimensional electrophoresis

This work was aimed at optimizing a protein extraction procedure for date palm (*Phoenix dactylifera* L.) leaves, a highly recalcitrant plant tissue for 2-DE. Five protein extraction protocols based on different protein precipitation agents (TCA/acetone vs. phenol (Ph) methods) and protein resolubilization methods (physical treatments, e.g., sonication, shaking and/or heating) were tested. Ph/SDS extraction with methanol/ammonium acetate precipitation, followed by DOC preincubation and TCA/acetone precipitation and, finally, solubilization by shaking in rehydration solution was found to be the best protein extraction method. We conclude that DOC with TCA/acetone precipitation step eliminates interfering compounds, thus allowing efficient resolubilization of date palm leaf proteins. This method could be appropriate for proteomic studies such as date palm colonization by entomopathogenic fungi.

### Keywords:

2-DE / Leaf protein extraction / *Phoenix dactylifera* DOI 10.1002/elps.200700380

## 1 Introduction

Preparation of adequate protein samples for 2-DE analysis is the most critical step for any proteomics study. Proteins should be denatured, reduced and solubilized or rehydrated in order to obtain a complete disruption of their intra- and intermolecular secondary bonds and, hence, to assure that each spot in the gel represents an individual polypeptide [1, 2]. Moreover, proteins extracted from plant tissues are typically more difficult to resolve by 2-DE than those from other organisms. This is due to the abundance of interfering compounds, particularly when working with recalcitrant tissues from woody plant species. Removal of those compounds that can interfere in electrophoretic separation becomes a crucial matter for sample preparation. Salts, polysaccharides, pigments, nucleic acids, polyphenols and other secondary metabolites can cause vertical and horizontal streaking, smearing and reduced number of distinctly resolved protein spots in 2-D gels. There are many studies addressing the development of optimal sample preparation protocols for proteomics analysis of different recalcitrant plant tissues [3–

6], although the utility of some of these protocols as standard or universal protocols valid for any type of sample has been seldom realized [7]. However, standard protocols can be more valuable for comparative proteomics analyses of different plant species tissues than for the analysis of a given plant tissue, for which an optimized specific protocol can be raised. Indeed, as most available protein extraction protocols are based on the utilization of a very limited and common repertoire of physical treatments, solvents and buffers, the best choice when facing to analyze a recalcitrant plant tissue not previously subjected to proteomics studies, is developing a specific protocol optimized rather than apply a given universal protocol. This is the case of date palm leaf, a recalcitrant tissue for which there are no proteome reports. Consequently, contrasted protocols for obtaining good protein preparations are lacking.

Palms are among the most important ornamental crops in Spain. Eastern Spain produces ca. two million palms per year for exporting. Our group is exploring the use of entomopathogenic fungi for biological control of date palm pests [8, 9] which have been introduced as a consequence of the increase of palm imports in Spain. We have recently described the endophytic behaviour of different entomopathogenic fungi inoculated in date palm leaf petioles. This endophytic colonization might modulate date palm defences [9]. We are currently studying the induction of proteins (related with plant defence) in date palm leaf petioles colonized by entomopathogenic fungi. In this paper, we have evaluated five methods for extracting and solubilizing palm leaf proteins suitable for 2-DE studies.

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**Abbreviations:** **FW**, fresh tissue weight; **-H**, heat; **Ph**, phenol; **-S**, shaking; **-U**, ultrasounds

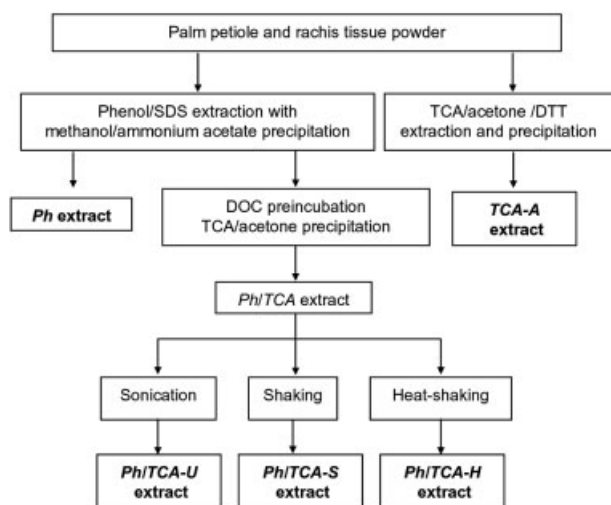
## 2 Materials and methods

### 2.1 Plant material

We sampled *Phoenix dactylifera* leaves from the Alicante University campus (Sant Vicent del Raspeig, Alicante, SE Spain). Leaves from pest- and pathogen-free young palms (1.0–1.5 m high, <2 years old) were cut and carried to the laboratory in plastic bags. After limb and spines removal, the petioles and rachis were washed once with tap water containing a few drops of commercial detergent. The studies were carried out in the petiole and rachis since they are the most likely areas for endophytic fungus spread. Besides, spines are extremely fibrous and even more difficult to process than petioles. Petioles and rachis were rinsed once in distilled water and finally in 70% v/v ethanol (5 min/rinse). They were cut into small pieces, were frozen in liquid nitrogen and were ground to a fine powder using a prechilled grinder (Taurus MS50) with liquid nitrogen for 2–3 min. Ground tissue was stored at  $-20^{\circ}\text{C}$  until protein extraction (24–48 h).

### 2.2 Protein extraction

Protein extraction was performed using several protocols as summarized in Fig. 1.



**Figure 1.** Flow-chart of the protocols for protein extraction from date palm petiole and rachis used in this paper.

#### 2.2.1 Phenol method (*Ph* extract)

*Ph* extraction procedure was based on a method modified from Wang *et al.* [3] for olive leaves. For preparing dry tissue powder, 4 g of ground tissue (three replicates) was resuspended in 4–5 mL ice-cold acetone, vortexed thoroughly for 1 min and centrifuged at 8000 rpm for 5 min at  $4^{\circ}\text{C}$  (Eppendorf Centrifuge 5804R). The remaining pellet was washed

twice with ice-cold acetone and then transferred into a mortar and allowed to dry at room temperature (*ca.* 20 min). The dried pellet was further ground with pestle to a finer powder and then sequentially rinsed with ice-cold 10% w/v TCA in acetone (five times), with cold aqueous 10% w/v TCA (three times), and finally with cold 80% v/v acetone (three times). Each time the pellet was completely resuspended in the different washing media by vortexing for 1 min and then recovered by centrifugation at 8000 rpm for 3–5 min ( $4^{\circ}\text{C}$ ). The final pellet was dried at room temperature (1 h).

For protein extraction, 2 g *ca.* of the above dry pellet was resuspended in 10 mL *Ph* (Tris-buffered, pH 8.0; Amresco) and then an equal volume of dense SDS buffer (30% w/v sucrose, 2% w/v SDS, 0.1 M Tris-HCl, pH 8.0, 5% v/v  $\beta$ -mercaptoethanol) was added. The mixture was vortexed thoroughly for 1 min and after separation from the aqueous phase by centrifugation at 8000 rpm for 5 min (twice). The upper *Ph* phase was collected and precipitated with five volumes of cold methanol plus 0.1 M ammonium acetate at  $-20^{\circ}\text{C}$  for 30 min. Precipitated proteins were recovered by centrifugation at 8000 rpm for 5 min ( $4^{\circ}\text{C}$ ), and then washed three times with cold methanolic ammonium acetate and cold 80% v/v acetone. The final pellet (*Ph* extract, see Fig. 1) was air-dried (30 min) and redissolved in SDS-PAGE sample buffer (0.5 M Tris-HCl pH 6.8; 10% w/v SDS, 10% v/v glycerol, 0.5% w/v bromophenol blue and 5% v/v  $\beta$ -mercaptoethanol), or in 2-DE rehydration solution (8 M urea, 2% w/v CHAPS, 20 mM DTT, 0.5% v/v Bio-lytes pH 3–10 (BioRad) and 0.001% w/v bromophenol blue) by shaking at 160 rpm for 1 h at  $25^{\circ}\text{C}$ . The supernatant was recovered by centrifugation (12 000 rpm for 15 min) and stored at  $-20^{\circ}\text{C}$  (24–48 h) for later analysis.

#### 2.2.2 *Ph*/TCA and sonication method (*Ph*/TCA-U extract)

Four grams of ground tissue (three replicates) was processed as described above to obtain the *Ph* extract. This extract was then air-dried and subjected to DOC incubation and a TCA precipitation step according to Granier [10] and Bensadoun and Weinstein [11] as described by Casado-Vela *et al.* [12]. Briefly, the dried pellet was resuspended in 750  $\mu\text{L}$  deionized water and, after adding 8.5  $\mu\text{L}$  2% w/v DOC, the mixture was incubated for 10 min on ice. Then, 250  $\mu\text{L}$  cold 24% w/v TCA in water were added and the sample was vortexed thoroughly, left to precipitate on ice for 30 min and centrifuged at 11 000 rpm for 10 min ( $4^{\circ}\text{C}$ ). The remaining pellet was washed with 1 mL ice-cold acetone, incubated 10 min on ice and centrifuged at 11 000 rpm for 10 min ( $4^{\circ}\text{C}$ ) (three times). The recovered pellet (*Ph*/TCA extract, see Fig. 1) was finally air-dried at room temperature (30 min) and redissolved either in SDS-PAGE sample buffer or in 2-DE rehydration solution by sonication in an ultrasonic bath (Ultrasons, Selecta, Barcelona, Spain) for 1 h on ice. Supernatant was recovered by

centrifugation at 12 000 rpm for 15 min and stored at  $-20^{\circ}\text{C}$  (24–48 h) for later analysis (*Ph/TCA-U* extract, see Fig. 1).

### 2.2.3 Ph/TCA and shaking method (*Ph/TCA-S* extract)

Four grams of ground tissue (three replicates) was processed as described above to obtain the *Ph/TCA* extract. This extract was then air dried at room temperature (30 min) and redissolved either in SDS-PAGE sample buffer or in 2-DE rehydration solution by shaking at 160 rpm for 1 h at  $25^{\circ}\text{C}$ . The supernatant from 2-DE rehydration solution was recovered by centrifugation and the pellet was redissolved once more as above (shaking in rehydration solution at 160 rpm for 1 h at  $25^{\circ}\text{C}$ ). The remaining insoluble residue was removed by centrifugation (12 000 rpm, 15 min) and the protein-containing supernatants were pooled and stored at  $-20^{\circ}\text{C}$  (24–48 h) for later analysis (*Ph/TCA-S* extract, see Fig. 1).

### 2.2.4 Ph/TCA and heating method (*Ph/TCA-H* extract)

Four grams of ground tissue (three replicates) was processed as described above to obtain the *Ph/TCA* extract. This extract was then air dried at room temperature (30 min) and redissolved either in SDS-PAGE sample buffer or in 2-DE rehydration solution without urea by heating for 1 h at  $90^{\circ}\text{C}$ . The supernatant from 2-DE rehydration solution without urea was recovered by centrifugation and the pellet was redissolved twice by shaking in complete 2-DE rehydration solution at 160 rpm for 1 h at  $25^{\circ}\text{C}$ . The ratio of the rehydration solution without urea over the complete rehydration solution used for each sample was 1:2.7. Supernatants were recovered by centrifugation (12 000 rpm, 15 min), pooled and stored at  $-20^{\circ}\text{C}$  (24–48 h) for later analysis (*Ph/TCA-H* extract, see Fig. 1).

### 2.2.5 TCA-acetone method (*TCA-A* extract)

In addition to the above four Ph-based extraction protocols, protein extraction was also performed by TCA/acetone precipitation according to the classical method by Damerval *et al.* [13] with minor modifications. Four grams of ground tissue (with three replicates) obtained as in Section 2.1 was homogenized in a mortar with pestle in the presence of washed sea sand and four volumes of ice-cold 10% w/v TCA in acetone containing 0.07% w/v DTT. Proteins were allowed to precipitate for 1 h at  $-20^{\circ}\text{C}$  and were recovered, after gauze filtration (to eliminate sea sand) and centrifugation (20 000 rpm for 30 min at  $4^{\circ}\text{C}$ ). They were washed twice with ice-cold acetone containing 0.07% DTT for 30 min, air-dried at room temperature (30 min) and solubilized by shaking at 160 rpm for 1 h in SDS-PAGE sample buffer or in 2-DE rehydration solution. The remaining insoluble residue was

removed by centrifugation (12 000 rpm, 15 min) and the protein-containing supernatant was stored at  $-20^{\circ}\text{C}$  (24–48 h) for later analysis (*TCA-A* extract, see Fig. 1).

## 2.3 Protein quantitation

Protein concentration in the various extracts was measured using three different methods: (i) RC-DC™ Protein Assay kit (BioRad Laboratories, Hercules, CA, USA), which was used according to the manufacturer's protocol. Absorbance at 700 nm was measured in microtiter plates against a reagent blank (rehydration solution treated in the same manner as for extracts but containing no protein) and serum ovalbumin as a standard, using a TECAN Model GENios spectrophotometer. (ii) BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA), based on bicinchoninic acid, which was used according to the manufacturer's protocol. Absorbance was measured in microtiter plates as described above at 595 nm. (iii) A variation of the original Bradford method [14] as described by Ramagli and Rodríguez [15]. Briefly, replicates of 10  $\mu\text{L}$  of each sample were mixed with 10  $\mu\text{L}$  of 0.1 M HCl and 80  $\mu\text{L}$  of distilled water. Afterwards, 3.5 mL of 1:3 diluted dye reagent mix (BioRad Protein Assay; BioRad Laboratories, Munich, Germany) was added and vortexed. Absorbance at 595 nm was measured after 5 min in microtiter plates as described above. The protein concentration was expressed as microgram *per* gram fresh palm tissue weight. Three replicates were used to evaluate the protein extraction protocols and protein yield was presented as mean  $\pm$  SD.

## 2.4 Electrophoresis and gel image analysis

1-D SDS-PAGE analysis was carried out as described by Laemmli [16] with protein pellets directly redissolved in SDS-PAGE sample buffer as we described previously. 1-D SDS-PAGE was also performed with 20  $\mu\text{g}$  solubilized protein (in rehydration solution) *per* gel lane, which were applied with sample buffer for SDS-PAGE (volume up to 25  $\mu\text{L}$ ). We used 3.9% stacking and 12% resolving polyacrylamide home-cast gels which were run at 200 V constantly for 1 h in a Mini-Protein 3 cell (BioRad).

For 2-DE analysis of protein extracts, the first IEF dimension was carried out on IPG strips (7 cm, 3–10 and 5–8 linear pH gradient; BioRad) which were rehydrated for 12 h with 150  $\mu\text{g}$  of protein in 125  $\mu\text{L}$  rehydration buffer. Focusing was carried out in a Ettan IPGPhor (Amersham Biosciences) at 50  $\mu\text{A}$  constantly *per* strip at  $20^{\circ}\text{C}$ , applying the following program: a linear increase from 0 to 250 V over 20 min, 250 to 4000 V over 2 h, and then a rapid gradient of 4000 V until 10 000 V·h were reached. After IEF, the IPG strips were immediately equilibrated for 15 min with an equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% w/v glycerol, 2% w/v SDS) containing 2% w/v DTT, followed for 15 min with the same buffer but containing 135 mM iodoacetamide. The second dimension (SDS-PAGE) was

performed as described above but on 13% polyacrylamide home-cast gels. Protein quantities quoted in the text were estimated using the RC-DC method.

Gels were stained with CBB G-250 (BioRad). Gel images were digitalized with an EPSON Expression 1680 Pro Scanner and analyzed with the Phoretix Software (Nonlinear Dynamics). Experimental  $M_r$  values were calculated by mobility comparisons with protein standard markers (SDS-PAGE Standards Broad Range, BioRad) run in a separate marker lane on the SDS-gel, while  $pI$  was determined by using a 5–8 linear scale over the total dimension of the IPG strip. The analysis was re-evaluated by visual inspection. For statistical analyses, we used the Kruskal–Wallis nonparametric test (H), and the  $U$  Mann–Whitney test when statistical differences were found.

### 3 Results and discussion

To the best of our knowledge no reports on date palm 2-DE studies are currently available. We have therefore tested and adapted several procedures to extract and solubilize date palm petiole and rachis proteins for 2-DE. Date palm leaf is a recalcitrant material which requires special treatments for obtaining extracts with adequate amounts of protein free from electrophoresis interfering compounds. As examples of such compounds, date palm tissue contains high levels of tannins and various other phenolic compounds (*e.g.*, ferulic, *p*-coumaric, *p*-hydroxybenzoic and caffeoylshikimic acids, quercetin and isorhamnetin heterosides, and (+)-catechin and (–)-epicatechin flavanols [17–19]), carbohydrates (*e.g.*, abundant starch grains in parenchymatic tissue [9]), and certain oxidative enzymes (*e.g.*, polyphenol oxidase [20]). We have consequently tested two main procedures for optimizing protein extraction. One is based on Ph extraction while the other one uses TCA/acetone precipitation (Fig. 1). Ph has been used to extract proteins from recalcitrant plant materials such as leaves (*e.g.*, olive and pine) or fruit layers (exocarp and mesocarp) of several plant species (kiwi, banana, avocado, orange and grape berry) [3–5, 7, 21] with good results. Methods based on Ph extraction are usually time consuming, so we compared them with TCA/acetone extraction, a general method which has broadly been used for plant protein extraction [5, 22–25].

#### 3.1 Protein yields

Protein contents of the different extracts obtained from date palm leaf tissue, as quantified by three different protein assay protocols are shown in Table 1. Although results were highly influenced by the assay protocol, the highest protein content was consistently obtained for the *Ph/TCA-U* extract irrespectively of the quantification protocol used. For the remaining extracts, a similar classification on the basis of their protein concentrations (namely, *Ph/TCA-S* > *Ph/TCA-H* > *Ph* > *TCA-A*) was obtained with the RC-DC and modified

**Table 1.** Protein yields of date palm petiole and rachis tissue extracted by five methods: (*Ph*) Ph/SDS extraction methanol ammonium acetate precipitation, previous extract plus DOC with TCA/acetone precipitation and solubilization in 2-DE rehydration solution by either sonication (*Ph/TCA-U*), shaking (*Ph/TCA-S*) or heat-shaking (*Ph/TCA-H*), and finally, TCA/acetone extraction (*TCA-A*)

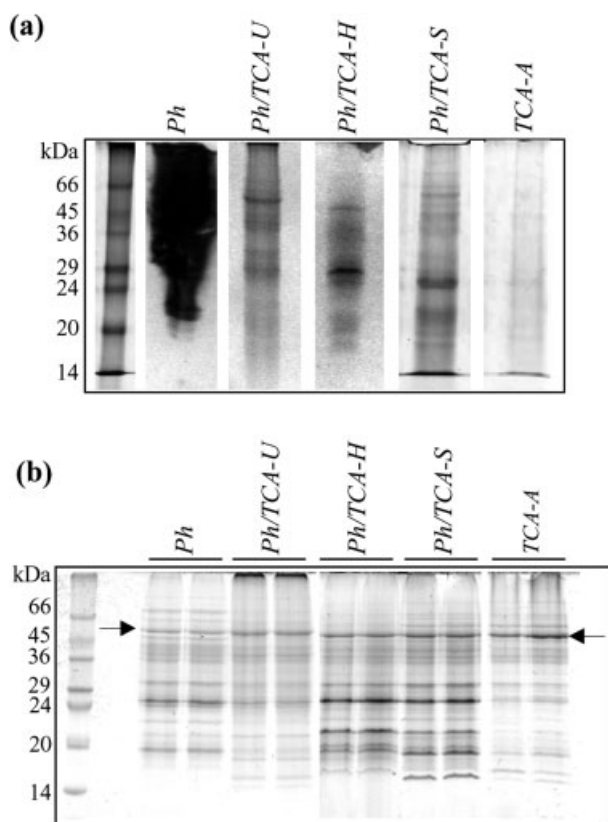
Extraction and solubilization methods	Protein yield ( $\mu\text{g}$ protein/g fresh tissue)		
	RC-DC Protein Assay	BCA Protein Assay	Modified Bradford
Ph	41 $\pm$ 15	113 $\pm$ 28	79 $\pm$ 21
<i>Ph/TCA-U</i>	457 $\pm$ 57	622 $\pm$ 89	255 $\pm$ 15
<i>Ph/TCA-S</i>	101 $\pm$ 12	251 $\pm$ 77	98 $\pm$ 6
<i>Ph/TCA-H</i>	72 $\pm$ 18	326 $\pm$ 14	92 $\pm$ 20
<i>TCA-A</i>	12 $\pm$ 4	275 $\pm$ 32	42 $\pm$ 9

Protein content in extracts was quantified by three methods based on different principles. Data show average values (each one from three independent experiments) with their SDs.

Bradford methods. With the BCA method a different result (namely, *Ph/TCA-H* > *TCA-A* > *Ph/TCA-S* > *Ph*) was obtained. These variations between protein assays, the BCA method, yielding different results from the other methods, thought rather surprisingly might be explained by differences between methods at the level of chemical principle and tolerance to interfering substances. With the assay protocol that gave highest values (BCA Protein Assay), the average yield of proteins for all extraction methods was between 0.6 and 0.1 mg/g fresh tissue weight (FW). The higher value of this range is about a half of that obtained for olive young leaf extracts [7]. However, protein yields for extracts of most plant tissues are usually comprised between 1 and 10 mg/g FW. Poorer protein extracts, with concentration levels within the 0.1–1.0 mg/g FW range, are typically obtained from plant tissues with low protein content and large amounts of interfering compounds [4, 5, 7, 26, 27]. Therefore, our extracts protein yields clearly indicate that date palm leaves fall within this last group of highly recalcitrant plant tissues.

#### 3.2 SDS-PAGE analysis

Protein extracts from date palm petiole and rachis were first analyzed by SDS-PAGE (Fig. 2). SDS-PAGE of samples in standard sample buffer revealed interfering compounds, as smearing highly stained with colloidal Coomassie blue, for *Ph* extract (Fig. 2a, lane 2) or few and poorly resolved bands for *Ph/TCA-U*, *Ph/TCA-S* and *Ph/TCA-H* extracts (Fig. 2a, lanes 3–5). The latter showed however a much clearer background than the *Ph* extract. This indicated that DOC with TCA/acetone precipitation efficiently eliminated interfering compounds. Cholate and DOC are anionic detergents, just



**Figure 2.** SDS-PAGE separation of proteins from date palm petiole and rachis protein extracts. (a) Proteins obtained by Ph-based and TCA/acetone protocols and solubilized in SDS-PAGE sample buffer (0.5 M Tris-HCl pH 6.8; 10% w/v SDS, 10% v/v glycerol, 0.5% w/v bromophenol blue and 5% v/v  $\beta$ -mercaptoethanol). (b) Proteins (20  $\mu$ g) obtained by Ph-based protocols and TCA/acetone protocol and resuspended in 2-DE rehydration buffer (8 M urea, 2% w/v CHAPS, 20 mM DTT, 0.5% v/v Bio-lytes pH 3–10 (BioRad) and 0.001% w/v bromophenol blue). Gels were stained with colloidal CBB (G-250). Arrows in (b) probably indicate the protein band corresponding to Rubisco. Each protein extraction protocol is represented by two independent extracts (two adjacent lanes) (b).

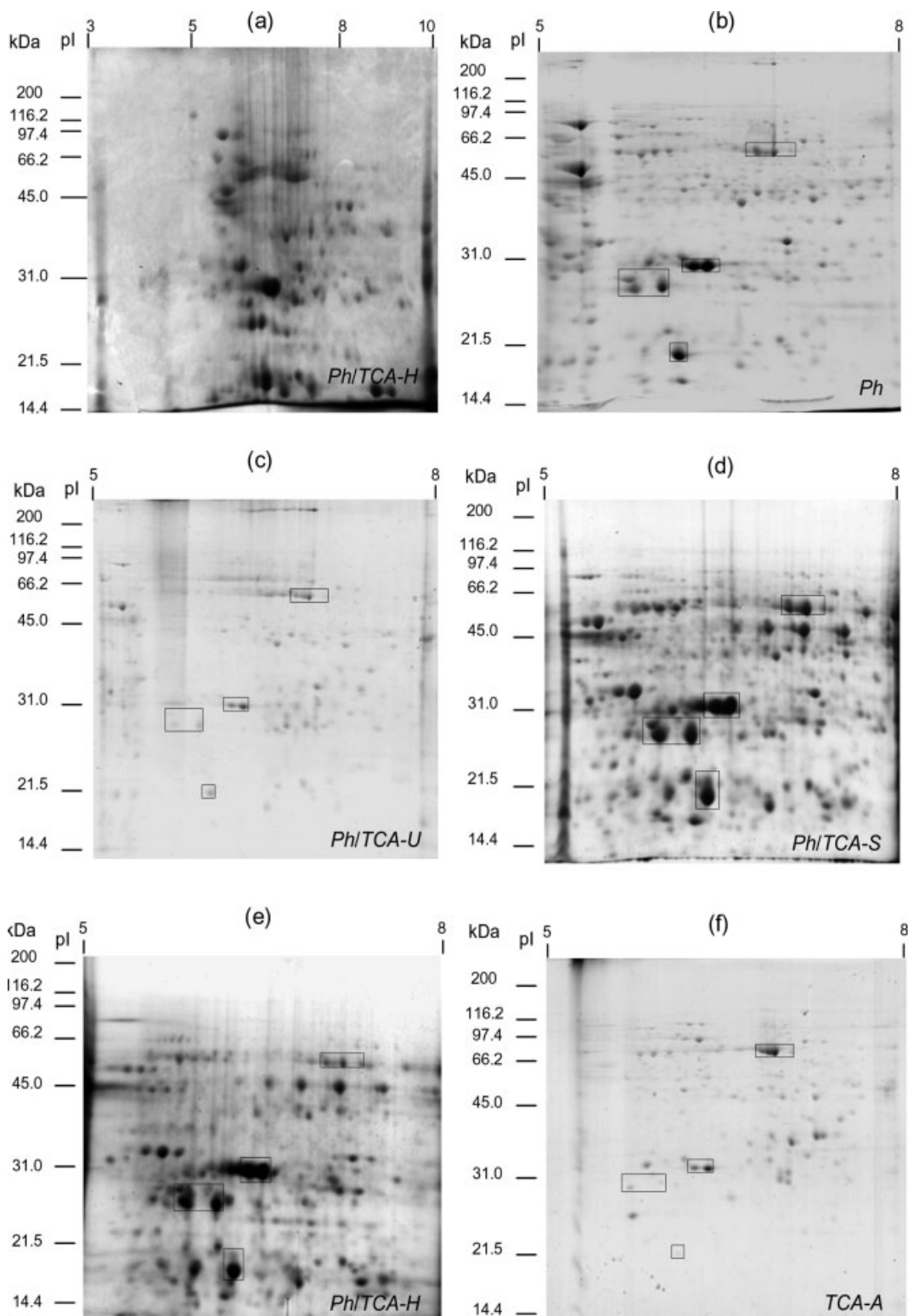
like SDS, very effective at solubilizing integral membrane proteins and dissociating protein complexes [28–30]. Nevertheless, SDS is very denaturing and more difficult to eliminate for avoiding interference with IEF [31]. Both DOC and TCA probably helped protein solubilization and precipitation, and eliminated interfering compounds (possibly lipids, salts, Phs) allowing a better protein rehydration. TCA-A extract showed very few resolved bands (Fig. 2a, lane 6). Probably the main problem associated with TCA precipitation is the resolubilization of the protein pellet, as indicated by the fact that TCA-A extract gave the lowest protein yields in two out of the three methods assayed (Table 1).

SDS-PAGE was greatly improved when samples were redissolved in rehydration solution instead of in sample buffer. The composition of rehydration solution favours proteins

solubilization. Urea is quite efficient in disrupting hydrogen bonds whereas zwitterionic detergents as CHAPS are able to solubilize hydrophobic membrane proteins. DTT prevents oxidation of the proteins, while ampholytes also improve protein solubility [2, 32, 33]. Twenty to thirty well-resolved bands (between 14 and 66 kDa) without background were obtained (Fig. 2b). All extracts displayed a similar prominent band of *ca.* 51 kDa which could correspond to the ribulose biphosphate carboxylase/oxygenase (Rubisco) large subunit (Fig. 2b, arrows). This protein of about 55 kDa is typically a major component of leaf protein extracts [3, 4, 34, 35]. However, extracts varied in the set of protein bands present in gels (Fig. 2b), with Ph/TCA-S and Ph/TCA-H giving the best results and Ph/TCA-U the worst one in terms of intensity and number of bands (Fig. 2b). The differences found between extracts point out also to a lack of correlation, clearly evidenced with Ph/TCA-U, between the protein yields estimated either from protein assays (Table 1) or SDS-PAGE band number and intensity data (Fig. 2b). However, apart from the poor 1-DE profile for Ph/TCA-U, gel profiles of others extracts were fairly consistent with their protein contents estimated by either RC-DC or modified Bradford methods. Such conflicting results probably indicate a differential coextraction by the various methods of palm leaf constituents together with proteins which may interfere in electrophoresis. Palm tissues contain few proteins and high levels of starch or phenolics which are compounds able to interfere with both protein assays [36] and protein electrophoresis [3–4, 5, 26]. Shaking and heating would extract protein with less contaminating compounds therefore increasing protein resuspension efficiency whereas the coextraction of such compounds could have been facilitated by the sonication step included in the Ph/TCA-U method. Furthermore, among the various physical treatments (sonication, shaking and heating) used to improve protein extraction and resuspension, sonication did not produce the same positive effect on protein solubilization as the others. Thus, in the conditions used in this work, sonication seems to have been little efficient in separating proteins from cell membranes or to dissociate protein aggregates from date palm petiole and rachis tissues. This was indicated by the band of highest molecular weight which was stronger for Ph/TCA-U than for the remaining extracts (Fig. 2).

### 3.3 2-DE analysis

Protein extracts were then evaluated by 2-DE (Fig. 3). Ph/TCA-H extract was preliminary analyzed by using 3–10 linear IPG strips for the first-dimensional IEF step and the resulting 2-DE gel showed that the majority of protein spots were focused in the 5–8 pH range (Fig. 3a). We selected this range as the standard condition for resolving the majority of proteins. Otherwise two gels or more should have been performed *per* each sample to include narrower pI ranges. We envisage such detailed electrophoresis analyses in future studies. Therefore, all extracts were run in a 5–8 linear pH



**Figure 3.** 2-DE separation of proteins from date palm petiole and rachis using the five extraction methods (*Ph*, *Ph/TCA-U*, *Ph/TCA-S*, *Ph/TCA-H* and *TCA-A* extracts) described in Fig. 2b. Proteins (150  $\mu$ g estimated using RC-DC Protein Assay) solubilized in 2-DE rehydration buffer (8 M urea, 2% w/v CHAPS, 20 mM DTT, 0.5% v/v Bio-lytes pH 3–10 (BioRad) and 0.001% w/v bromophenol blue) were separated on a 3–10 (a) and 5–8 (b–f) linear pH gradient in the first dimension (IEF) and visualized using CBB G-250 in 7  $\times$  10 cm gels. Squares represent spots present on all gels with 5–8 linear pH gradient (b–f).

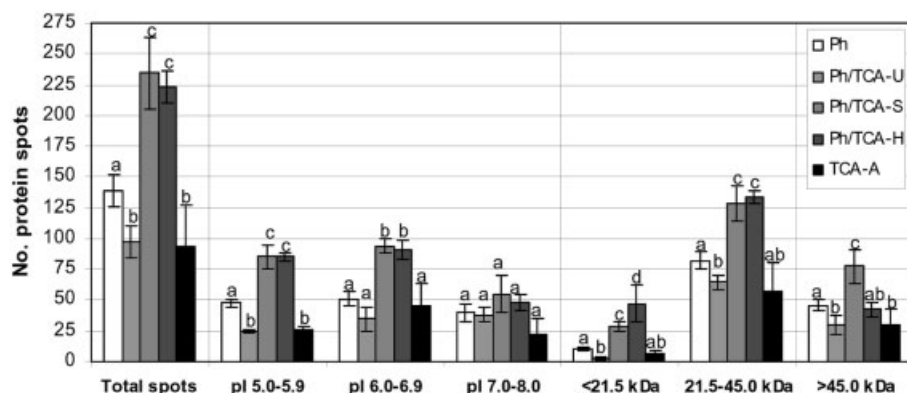
gradient, a range that was selected as representing for our samples the best compromise between number and resolution of separated spots (Figs. 3b–f). The resulting 2-DE gels presented well-resolved protein maps of date palm petiole and rachis tissue relatively free of horizontal streaking. This was especially true for *Ph/TCA-S* and *Ph/TCA-H* extracts. The average number of spots was significantly higher ( $p \leq 0.05$ ) for *Ph/TCA-S* and *Ph/TCA-H* ( $234 \pm 29$  and  $223 \pm 13$ , respectively) than for the rest of the extracts (Fig. 4). Among these, *Ph* extract showed a greater number of spots ( $139 \pm 13$ ) than either *Ph/TCA-U* or *TCA-A* ( $97 \pm 12$  and  $93 \pm 33$ , respectively; Fig. 4). These 2-DE profiles were in agreement with SDS-PAGE results and hence pointed to a degree of discrepancy or consistency with the protein concentration data obtained with the different assay methods similar to that previously discussed for 1-DE. For further analyses of differences between the protein extracts, we compared *pI* and  $M_r$  ranges of the resolved spots. Regarding *pI*, the number of spots for *Ph/TCA-S* and *Ph/TCA-H* at both 5.0–5.9 and 6.0–6.9 *pI* ranges, was found significantly higher than that for the remaining extracts. Number of spots for *Ph* extracts was higher at the 5.0–5.9 *pI* range than for *Ph/TCA-U* or *TCA-A* extracts (Fig. 4). Regarding  $M_r$ , *Ph/TCA-H* extracts contained higher number of spots of  $M_r < 21.5$  kDa than the rest. Spots of  $M_r$  between 21.5 and 45 kDa were more abundant in *Ph/TCA-S* and *Ph/TCA-H* extracts. Proteins larger than 45 kDa were more abundant in *Ph/TCA-S* extracts (Fig. 4). Regarding number of spots and *pI* and  $M_r$  ranges, both *Ph/TCA-S* and *Ph/TCA-H* extracts showed equivalent high quality extraction. However, 2-D patterns showed that *Ph/TCA-H* extract (Fig. 3e) had a poorer resolution than *Ph/TCA-S* (Fig. 3d).

To further explore differences between extracts, we calculated the matching ratios (number of matched spots with reference gel/total spots of reference gel) of the resolved spots in their 2-DE gels (Table 2). Even though protein spots and patterns were different, nine spots were clearly present in all gels (see Fig. 3 and Table 2). Many of the spots present on one gel were matched with spots of other gels. For example, from the 151 spots of *Ph* gel, 73 (48%) and 79 (52%) spots were also detected in *Ph/TCA-U* and *Ph/TCA-S* gels,

respectively. From the 106 spots of *Ph/TCA-U* gel, 73 spots (69%) were also found in *Ph* gel. Between *Ph/TCA-S* and *Ph/TCA-H* gels, 139 spots (52 and 58%, respectively, with total spots of the reference gel) were matched. And from 122 spots of *TCA-A* gel, 68 spots (56%) were also in *Ph* gel. Regarding exclusive spots of each extract, the percentage varied between 5 and 20%. The highest percentages were obtained from the gels with larger numbers of spots (*Ph*, *Ph/TCA-S* and *Ph/TCA-H*) (Table 2).

## 4 Concluding remarks

Date palm leaf is a highly recalcitrant plant material which seems to produce only very diluted protein extracts with high levels of interfering (nonprotein) compounds. In order to minimize such constraints we have tested five different extraction protocols, which as a whole encompass the main extraction techniques currently available for plant tissues. They differed either at the protein precipitation level (TCA/acetone vs. Ph methods) or at the protein resolubilization level (physical treatments as sonication, shaking and/or heating). According to our results, Ph/SDS extraction with methanol/ammonium acetate precipitation, followed by DOC and TCA/acetone precipitation and, finally, heat and/or shaking solubilization in rehydration solution (*Ph/TCA-H* and *Ph/TCA-S* extracts, respectively) seemed to be the best protocols to perform 2-DE analysis of date palm leaf proteins. The same protocol but using sonication instead of shaking and/or heating in the solubilization step gave an extract (*Ph/TCA-U*) which produced, taking together the four Ph-based protocols, the poorest results in terms of number and intensity of spots after 2-DE analysis. Since this extract provided the highest protein yield as quantified by the three protein quantitation assays tested which are based on different principles, one might conclude that sonication, under the conditions used in our work, extracted nonprotein substances which interfere with proteins during electrophoresis and not to disaggregate protein from homogeneous or heterogeneous complexes. The nonprotein compounds might have caused either poor solubilization or precipitation of palm



**Figure 4.** Protein spots in date palm petiole and rachis tissue detected by 2-DE gels using five extraction methods (*Ph*, *Ph/TCA-U*, *Ph/TCA-S*, *Ph/TCA-H* and *TCA-A* extracts). Spots were classified by total values  $M_r$  and *pI* ranges. Values on bars correspond to the mean of three replicates. Vertical lines represent the SD. Significant differences were calculated for extraction methods within each *pI* and  $M_r$  ranges ( $\alpha = 0.05$ ).

**Table 2.** Matching ratios (number of matched spots with reference gel/total spots of reference gel) between gels prepared from date palm samples obtained with methods shown in Table 1

Reference gel	Total spots of reference gel	Gels matched with reference gel										Exclusive spots		Spots present on all gels	
		<i>Ph</i>		<i>Ph/TCA-U</i>		<i>Ph/TCA-S</i>		<i>Ph/TCA-H</i>		<i>TCA-A</i>		N°	%	N°	%
		MS	%	MS	%	MS	%	MS	%	MS	%				
<i>Ph</i>	151	151	<b>100</b>	73	48	79	52	48	32	68	45	28	18	9	6
<i>Ph/TCA-U</i>	106	73	69	106	<b>100</b>	41	39	34	32	52	55	11	10	9	8
<i>Ph/TCA-S</i>	268	79	29	41	15	268	<b>100</b>	139	52	59	22	53	20	9	3
<i>Ph/TCA-H</i>	238	48	20	34	14	139	58	238	<b>100</b>	48	20	46	19	9	4
<i>TCA-A</i>	122	68	56	52	43	59	48	48	39	122	<b>100</b>	6	5	9	7

Gels analyzed (*Ph*, *Ph/TCA-U*, *Ph/TCA-S*, *Ph/TCA-H* and *TCA-A*) are those shown in Fig. 3. Each row presents an analysis set (Phoretix) with numbers of matched spots (MS) and percentage corresponding to total spots of reference gel.

petiole and rachis proteins. These artifacts could be the possible causes for the discrepancies for in protein yield estimations of the protein extraction methods tested. The simplest protocol based on Ph/SDS extraction with methanol/ammonium acetate precipitation and solubilization in rehydration solution gave an extract (*Ph*) that produced intermediate results in terms of both number of spots in 2-D gels and protein content. As an alternative to Ph extraction, we tested one protocol based on TCA/acetone precipitation [13] followed by solubilization shaking in rehydration solution. This resulted in an extract (*TCA-A*) which produced 2-DE spot patterns as poor as those for *Ph/TCA-U* although with protein yield significantly lower than that for this latter extract. TCA/acetone precipitation is a very useful method for minimizing protein degradation and removing interfering compounds. However, protein losses due to incomplete precipitation and/or resolubilization of proteins can occur [2, 5]. The main differences among the 2-DE patterns of the various extracts were quantitative (in number and intensity of spots). However, minor differences regarding *pI* and *M<sub>r</sub>* ranges were also found between extracts, which would indicate that extracts varied in the *pI* and *M<sub>r</sub>* of proteins they contained. Regarding the patterns of 2-DE gels, matching ratios of spots were calculated and the results showed that the different gels showed significant percentages of exclusive spots and ample variations in spot concordance degree. This raises a question as to whether the combined 2-DE analysis of various extracts rather than that of a unique, optimal one must be required for obtaining information on as much palm protein spots as possible. For a precise answer to above question, a maximal number of spots, specific or not, should be excised from the gels and MS analysed for protein identification. Such approach could also help in defining which types of proteins are specifically or preferentially extracted by the various methods and hence in better explaining the relative efficiency of each protocol. However, this strategy might not prove very relevant due to the lack of sufficient genomic or proteomic information in data bases on date palm.

By looking at the 2-DE patterns, the *Ph/TCA-S* method seems the most suitable in terms of protein quantity and number of spots of all five methods for protein extraction from palm leaf tissues tested. Horizontal striking is usually present in protein extracts from tissue with high amounts of Ph compounds, as date palm leaf. In gels from *Ph/TCA-S* horizontal striking was not present. The protocol described seems to eliminate these interfering compounds. Plant cells are rich in these and others interfering compounds (salts, phenolics, organic acids, pigments, polysaccharides) but in general protein content and concentration of plant material are lower than those obtained from microorganisms and animal cells.

TCA/acetone precipitation and Ph extraction methanol/ammonium acetate precipitation are the methods of choice for extracting proteins from recalcitrant plant tissues [5]. These methods increase protein concentration in extracts and help to remove contaminants. However, some polymeric contaminants are often coextracted [4] and precipitated proteins, especially those of high *M<sub>r</sub>*, are difficult to resolubilize [2, 5]. Ph extraction methods seemed to minimize proteolysis and optimize the extraction of membrane proteins [35]. They also reduce polysaccharide content [4]. Although comparative studies are scarce, Ph-based protocols seem to be superior to TCA/acetone methods for protein extraction from recalcitrant plant tissues [4, 21]. Nevertheless, both methods should be tested for a given recalcitrant tissue [5]. Accordingly, several recently proposed protocols for extracting proteins from various recalcitrant plant tissues are either based on TCA/acetone precipitation [26], Ph extraction [3, 27] or even a combination of both [7]. Our conclusion with date palm petiole and rachis tissue is that the method based on Ph extraction with methanol/ammonium acetate precipitation works better than that based on TCA/acetone precipitation. However, subsequent steps of DOC with TCA/acetone precipitation and physical aided resolubilization by shaking must be included to obtain an optimum Ph-based protocol. Both the anionic detergent DOC and TCA allowed a better pro-



tein rehydration possibly by the elimination of interfering compounds (probably lipids, salts, Phs).

In summary, we present a protein extraction protocol (*Ph/TCA-S*) adequate for date palm leaves, a recalcitrant material, which can be applied to proteomics studies. We are currently using this protocol to analyse palm defensive responses elicited upon colonization by nonhost fungal pathogens (entomopathogenic fungi). These important biocontrol agents have been recently found to be endophytic colonizers of date palm leaves [9]. They could, as has been found for other antagonists, modulate plant host defences [37] as part of their mode of action. Our preliminary experiments using this extraction method in palm tissues inoculated with several entomopathogenic fungi (Gómez-Vidal, 2007, unpublished results) indicate that the method is a useful tool for undertaking 2-DE studies of palm fungus interactions. Identification of up and down-regulated proteins in palm tissue inoculated with entomopathogenic *versus* uninoculated controls is in progress in our laboratory.

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