Efficient method of protein extraction from *Theobroma cacao* L. roots for two-dimensional gel electrophoresis and mass spectrometry analyses

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ABSTRACT. *Theobroma cacao* is a woody and recalcitrant plant with a very high level of interfering compounds. Standard protocols for protein extraction were proposed for various types of samples, but the presence of interfering compounds in many samples prevented the isolation of proteins suitable for two-dimensional gel electrophoresis (2-DE). An efficient method to extract root proteins for 2-DE was established to overcome these problems. The main features of this protocol are: i) precipitation with trichloroacetic acid/acetone overnight to prepare the acetone dry powder (ADP), ii) several additional steps of sonication in the ADP preparation and extractions with dense sodium dodecyl sulfate and phenol, and iii) adding two stages of phenol extractions.

Proteins were extracted from roots using this new protocol (Method B) and a protocol described in the literature for *T. cacao* leaves and meristems (Method A). Using these methods, we obtained a protein yield of about 0.7 and 2.5 mg per 1.0 g lyophilized root, and a total of 60 and 400 spots could be separated, respectively. Through Method B, it was possible to isolate high-quality protein and a high yield of roots from *T. cacao* for high-quality 2-DE gels. To demonstrate the quality of the extracted proteins from roots of *T. cacao* using Method B, several protein spots were cut from the 2-DE gels, analyzed by tandem mass spectrometry, and identified. Method B was further tested on *Citrus* roots, with a protein yield of about 2.7 mg per 1.0 g lyophilized root and 800 detected spots.

Key words: Cocoa; Plant protein extraction; Root;
Two-dimensional gel electrophoresis

INTRODUCTION

Theobroma cacao L. (Malvaceae) is a preferentially allogamous perennial woody species with geographical origin in South America and several series in the Amazon and Guyana regions (Almeida and Valle, 2007). The species is cultivated in the American, African, and Asian continents, and many countries worldwide are involved in cocoa production, marketing, and consumption (Almeida and Valle, 2007, 2009). The cacao tree is commercially explored for seed production because seeds are fermented, dried, and ground to produce liquor and fat. These two products are mixed with sugar, milk, and other ingredients to produce the most popular derivative of cacao: chocolate. Cacao is an important commodity: more than 20 million people depend directly on cocoa for their livelihood, and approximately 90% of the production is exported in the form of beans or semi-manufactured cocoa products to Europe and the USA (Food and Agriculture Organization, http://www.fao.org).

Biotic and abiotic stresses are a major problem for cocoa production, causing heavy crop losses. Diseases caused by pathogens, such *Phytophthora* spp and *Moniliophthora* spp, destroy cacao tree plantations and cause great economic losses and social changes in affected areas (Purdy and Schmidt, 1996; Andebrhan et al., 1999). Furthermore, water stress, flooding of the soil, and excessive solar radiation can affect *T. cacao* in the different areas that have been cultivated; these events can also cause plant death and reduced production (Sena Gomes and Kozlowski, 1986; Almeida and Valle, 2007). The economic losses and social impacts of different stresses that affect cocoa pushed forward the advancement of genomic studies to understand the genomic program of cacao during infection by pathogens (Jones et al., 2002; Verica et al., 2004; Gesteira et al., 2007; Leal et al., 2007; Argout et al., 2008, 2011), providing information in databases that can be used to identify proteins by mass spectrometry (MS). However, cacao has received little attention with respect to proteomic research. Until now, few studies have been carried out to analyze the protein pattern of cacao during its response to different stresses (Pirovani et al., 2008, 2010; Rehem et al., 2011). In particular, proteomics offers the possibility of identifying post-translational modifications of proteins, which are largely not predictable from genome sequences. Proteomics can also test whether proteins are

translated and accumulated after the expression of their encoding genes (Gallardo et al., 2001; Hoa le et al., 2004).

Sample preparation is one of the most crucial steps in obtaining high-quality resolution of proteins in proteomic analysis, yet it can be problematic (Görg et al., 2004). Proteins isolated from plant tissues are often difficult to resolve by two-dimensional gel electrophoresis (2-DE) because of the abundance of secondary metabolites. In particular, plant tissues such as roots contain lower concentrations of proteins and often contain high levels of materials that strongly interfere with 2-DE, resulting in horizontal and vertical streaking, smearing, and reduced numbers of distinctly resolved protein spots (Wang et al., 2003; Saravanan and Rose, 2004). These interfering compounds, which are present in plant materials such as *T. cacao*, disturb protein separation and proteome analysis by 2-DE (Görg et al., 2004; Saravanan and Rose, 2004). To our knowledge, there are no reports on successful protein extraction from the roots of *T. cacao* for 2-DE.

To overcome these problems, we developed a new protocol for *T. cacao* root protein extraction that is based on a protocol for protein extraction from *T. cacao* leaves and meristems (Pirovani et al., 2008). This protocol attempts to minimize the presence of interfering compounds and has a higher yield of obtained protein. In our study, we also compared the protocol that we developed with the protocol of protein extraction from *T. cacao* leaves and meristems, and we evaluated their suitability for MS analysis. Our protocol was tested further on *Citrus* roots, which are known for their difficult protein extraction.

MATERIAL AND METHODS

Plant materials

The roots were obtained from plants of *T. cacao* and *Citrus*. The *T. cacao* plants were grown in a greenhouse at the Universidade Estadual de Santa Cruz (UESC), Ilhéus, BA, Brazil (14° 47' S, 39° 16' W, 55 m above sea level). The plants were grown in plastic pots with a 25-L capacity that were filled with organic substrate (peat and shredded *Pinus* cortex + shredded coconut fiber in a 1:1 ratio) and enriched with mineral macronutrients and micronutrients according to the nutritional needs of the species (Souza, 2007). The *Citrus* plants were grown in a greenhouse at the Embrapa Mandioca e Fruticultura, Cruz das Almas, Ba, Brazil (12° 40' 39" S, 39° 6' 23" W, 226 m above sea level). The plants were grown in plastic pots with a 15-L capacity that were filled with the substrate Plantmax (Eucatex Agro, Brazil), washed sand, and clay in a 2:1:1 ratio and fertilized via the shoot. After being washed with water, the root samples were stored at -80°C after fixation in liquid nitrogen and subsequently lyophilized and stored at -20°C.

Protein extraction

The preparation of the acetone dry powder (ADP) from the plant total extract was based on the protocol described by Pirovani et al. (2008) (Method A) with modifications (Method B) that are described below (Figure 1). Initially, 1.0 g plant tissue (lyophilized roots) was finely pounded into a powder in liquid nitrogen using a pestle and mortar and intermixed with 7% polyvinylpolypyrrolidone (w/w). The fine powder was resuspended in 10 mL 10%

trichloroacetic acid (TCA) in acetone (cold) and 0.07% 2-mercaptoethanol and sonicated on ice (3 pulses of 5 s each, 70% output, 10-s intervals) on an Ultrasonic processor (Gex 130, 130 W, USA). This process was repeated four times at intervals of 10 min. The mixture was incubated overnight at -20°C for complete precipitation of proteins. The mixture was then centrifuged at 10,000 g and 4°C for 10 min. The supernatant was discarded, and the pellet was washed three times with the same volume of cold acetone and 0.07% 2-mercaptoethanol. The pellet was completely resuspended by sonication on ice (3 pulses of 5 s each, 70% output, 10-s intervals) and centrifuged at 10,000 g for 10 min at 4°C. The final pellet was dried at room temperature and used for protein extraction or stored at -20°C for future use.

Roots harvesting
Lyophilization

Grinding roots under N$_2$ liquid

Method A

Acetone dry powder preparation
TCA in water, sonication

Method B

TCA/acetone dry powder preparation/
Sonication (3X, 10-min intervals)

Incubated overnight –20°C

Acetone, sonication

PROTEIN EXTRACTION
Extraction phenol/SDS dense,
Sonication

PROTEIN EXTRACTION
Three extractions phenol/SDS dense,
Sonication

PRECIPITATION
0.1 M Ammonium acetate/Methanol,
-20°C, overnigth

PRECIPITATION
0.1 M Ammonium acetate/Methanol,
-20°C, overnigth

PROTEIN ANALYSIS
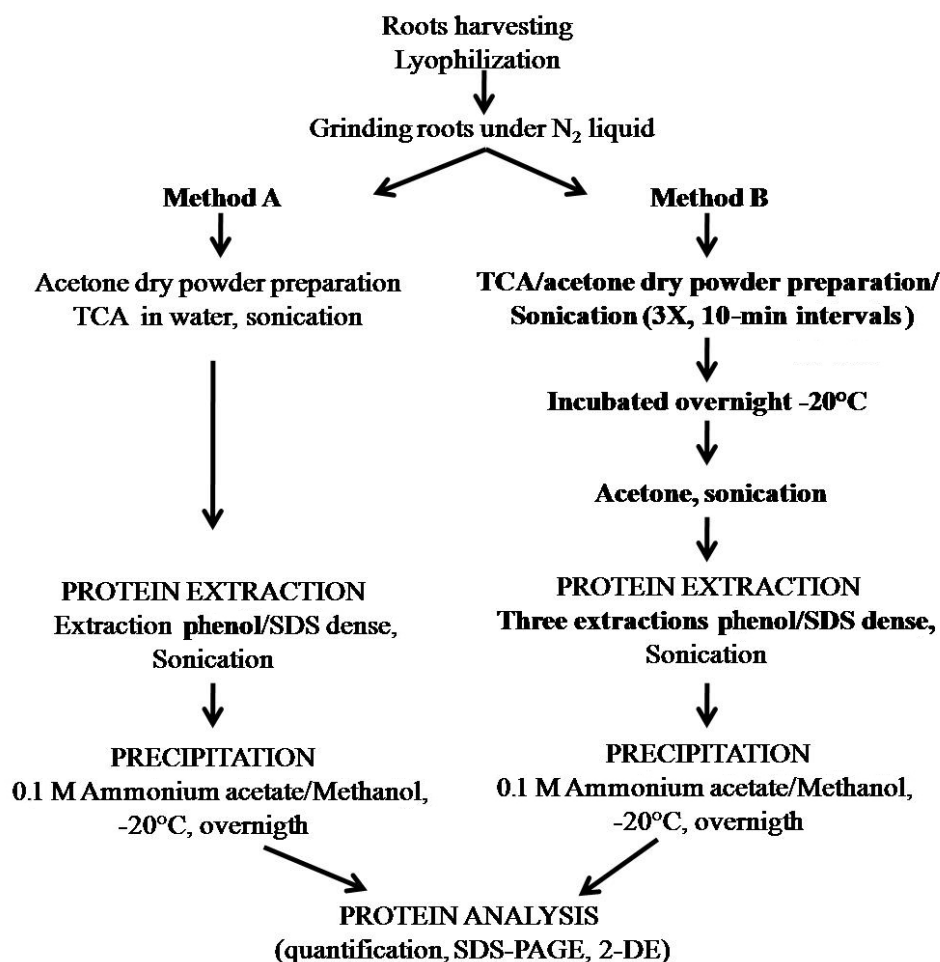**(quantification, SDS-PAGE, 2-DE)**

Figure 1. Scheme showing the Methods A and B of protein extraction of the roots.

Protein extraction from ADP was based on the protocol described by Pirovani et al. (2008) (Method A) with modifications (Method B). The ADP was resuspended in 10 mL dense sodium dodecyl sulfate (SDS) extracting buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH

8.0, 5% 2-mercaptoethanol). Samples were sonicated on ice (4 pulses of 10 s each, 70% output, 10-s intervals), and then 5 mL saturated phenol, pH 8.0, was added. The sample was then centrifuged at 10,000 g and 4°C for 15 min. The supernatant was then transferred to a new tube and stored on ice. The proteins from the lower phase were extracted two times with one volume of dense SDS buffer and phenol. Phenol phases from the three extractions were grouped and incubated overnight at -20°C with five volumes of 0.1 M ammonium acetate in methanol. The pellet was generated through centrifugation at 10,000 g and 4°C for 20 min. Proteins were washed twice with 1 M ammonium acetate in methanol, twice with acetone, and once with 80% ethanol, and then the pellets were finally dried at room temperature. In each wash, the sample was centrifuged at 10,000 g and 4°C for 5 min.

The pellets were treated with the 2-D Clean-Up kit according to manufacturer recommendations (GE Healthcare, UK) and resuspended in the appropriate rehydration buffer. Proteins were quantified using the 2-D Quant Kit according to manufacturer instructions (GE Healthcare).

2-DE

For 2-DE, the first dimension of separation was performed on an Ettan IPGphor system (GE Healthcare). Protein samples were applied in 250 µL 2-DE rehydration solution by reswelling 13 cm Immobiline DryStrip (pH 4-7, GE Healthcare) for 12 h. Afterwards, focusing was performed on the same apparatus under the following conditions: step and hold at 500 V for 1 h, gradient 1000 V for 1 h, gradient 8000 V for 2.5 h, and step and hold 8000 V for 55 min. After isoelectric focusing, the strips were stored at -80°C until the second-dimension analysis. Before the SDS-polyacrylamide gel electrophoresis (PAGE), the strips were incubated for 15 min in equilibration buffer (6 M urea, 7.5 mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue) with 1% dithiothreitol (w/v) and for another 15 min in equilibration buffer with 2.5% iodoacetamide (w/v). The strips were transferred to vertical 12.5% SDS-PAGE gels. The second dimension (SDS-PAGE) was performed on a Ruby SE600 system (GE Healthcare) under the following conditions: 15 mA for 45 min, 40 mA for 30 min, and 50 mA per gel for 3 h for every strip at a constant temperature of 11°C. The High-Range Rainbow Molecular Weight Marker was used (GE Healthcare). All 2-D gel separations were repeated three times using Methods A and B. After electrophoresis, proteins were visualized with 0.08% colloidal Coomassie G-250 (w/v) (Neuhoff et al., 1988). Gels were scanned using ImageScanner II (Amersham, GE Healthcare) and analyzed using ImageMaster 2D Platinum (GE Healthcare).

Protein identification

Selected protein spots were excised from the 2-DE gel, equilibrated with 50% acetonitrile containing 25 mM ammonium bicarbonate to remove Coomassie blue stain, and rinsed with distilled water. The gel plugs were dehydrated with 100% acetonitrile, vacuum dried, digested with 4 µL 25 ng/µL Trypsin Gold, Mass Spectrometry Grade (Promega, USA) in 25 mM ammonium bicarbonate, and incubated overnight at 37°C. The tryptic fragments were eluted from the gel with 50% acetonitrile and 5% formic acid (Yin et al., 2005). The extracts were dried under vacuum until a volume of 15 µL was reached.

The solution containing the proteolytic digests was then fractionated in an ionic exchange column. Subsequently, the digests were fractionated on a reversed-phase C18 column using two mobile phases, phase A, containing H_2O and 0.1% formic acid, and phase B, containing acetonitrile and 0.1% formic acid. For peptide separation, a linear gradient of 5-95% acetonitrile was used. The eluted peptides were directly introduced to the quadrupole mass spectrometer Q-TOFmicro (Waters, Manchester, UK) by its electrospray probe. The most abundant ions that were observed in the MS spectrum were automatically selected for collision-induced dissociation using the Masslynx software, generating their MS/MS spectra. Argon gas was used for peptide collision.

The resulting spectra were processed by the algorithm MaxEnt3 of the Masslynx ProteinLynx software to generate a list of masses, which correspond to peaks obtained in the spectra analyzed. The list of peaks generated by Proteinlynx 2.4 was searched against the *T. cacao* genome and National Center for Biotechnology Information databases. For this search, we used MASCOT version 2.1.0 (Matrix Science). The identification was made by the peptide mass fingerprint and sequenced by MS/MS.

RESULTS

The quantitative comparisons of protein extracted using these two protocols and the number of spots are listed in Table 1. Method B gave significant yields that were greater than those of Method A. With Method B, 1.0 g lyophilized roots of *T. cacao* typically yielded approximately 2.5 mg protein, which was more than the 0.7 mg yield that was obtained by Method A.

Table 1. Yield proteins of *Theobroma cacao* root (mg/g dry weight) and total number of spots using two extraction methods (A and B).

Method	Protein yield (mg/g dry weight)	Spot number
A	0.65	60
B	2.45	400

The quality and amount of protein extracted using the two methods (A and B) were monitored by one-dimensional SDS-PAGE and 2-DE. Both experiments were repeated at least three times to confirm the reproducibility. Our main modifications in Method B compared with the original (Method A) were increasing the number of extractions with phenol and sonication of the samples at the end of the process to maximize the solubilization of the extracted proteins.

Method B proved to be more efficient for the extraction of root proteins than Method A (Figure 2, columns 1 and 2). Method A did not eliminate interferents that may affect the protein quantification of the samples. This demonstrates that our new method of protein extraction improved the quality and the quantity of proteins extracted from *T. cacao* roots.

In the 2-DE analysis, protein extracts that were obtained by the two methods were tested despite the low quality of the protein extract from Method A. Lower resolution and fewer spots were observed on 2-DE gels with protein obtained by Method A than with protein obtained by Method B (Figure 3). Approximately 60 spots were observed on protein gels obtained by Method A, while 400 spots were observed from Method B as estimated by the ImageMaster 2D Platinum software (GE Healthcare). Thirteen spots were excised from the gel

and identified by MS (Table 2). From these 13 spots, 14 proteins were identified and listed in Table 2 with their properties. It was possible to identify proteins from different cellular compartments by Method B, such as mitochondrion, nucleus, plasma membrane, cytosol, apoplast, ribosome, endoplasmic reticulum lumen, vacuole, and peroxisome.
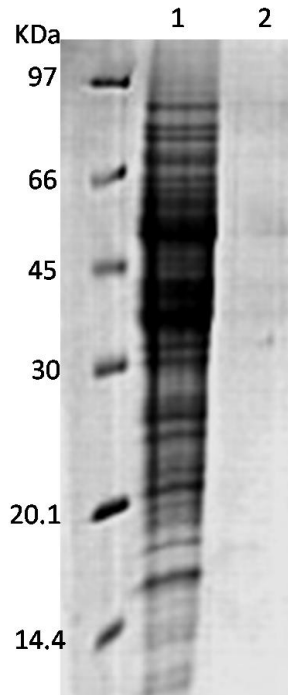

Figure 2. SDS-PAGE root proteins. Method B (column 1) and Method A (column 2) of protein extraction. Twenty micrograms of proteins was loaded on 12.5% acrylamide gel.
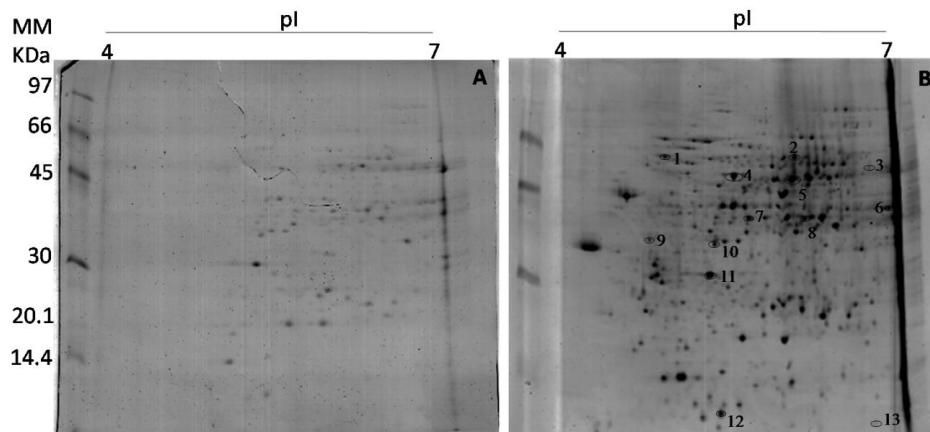

Figure 3. 2-DE proteins from *Theobroma cacao* root. **A.** Method A of protein extraction. **B.** Method B of protein extraction. Each gel was loaded with 350 mg of total protein and stained with 0.08% colloidal Coomassie G-250 (w/v).

Table 2. Proteins identified by mass spectrometry.

Spot No.[a]	Protein name/accession number	E-value	Theoretical MW (kDa)/pI	Biological process	Subcellular location
1	Rubisco subunit binding-protein alpha subunit/BAE71311.1	0.0	63.8/4.9	Protein refolding Auxin biosynthetic process Unfolded protein binding	Mitochondrion Cytosolic ribosome Membrane/Apoplast
2	Bisphosphoglycerate-independent phosphoglycerate mutase/XP_002519975.1	0.0	61.1/5.5	Response to cold Response to cadmium ion	Plasma membrane Mitochondrial envelope Cytosol/Apoplast
3	Methylmalonate-semialdehyde dehydrogenase/XP_002518343.1	1,80E-139	57.8/8.0	Glycolysis Response to oxidative stress Oxidation reduction Valine metabolic process	Mitochondrion
4	ATP synthase beta chain/ACS83602.1	0.0	59.7/6.0	Plasma membrane ATP synthesis coupled proton transport	Mitochondrial proton-transporting ATP synthase, catalytic core
5	Enolase/ABW21688.1	0.0	47.9/5.8	Response to salt stress Response to light stimulus Response to cold	Cell surface Phosphopyruvate hydratase complex
6	Alcohol dehydrogenase a/ABO41830.1	0.0	41.6/6.6	Oxidation reduction Alcohol dehydrogenase (NAD) activity	Cytosol
	Pyruvate dehydrogenase e1 alpha subunit/ACJ11743.1	0.0	43.5/7.2	Oxidation reduction Glycolysis Response to salt stress	Nucleus Cytosol
7	Caffeic acid o-methyltransferase/ACT32029.1	0.0	40.0/5.2	Lignin biosynthetic process	Cytosol
8	Protein disulfide/AEK80406.1	0.0	39.5/5.5	Pollen tube development Electron transport chain Response to endoplasmic reticulum stress Cell redox homeostasis	Plant-type cell wall Cytoplasmic membrane-bounded vesicle Endoplasmic reticulum lumen
9	Late embryogenesis abundant group 2/XP_002533345.1	9,86E-136	30.3/4.5	Embryo sac development Response to cadmium ion Response to desiccation Embryonic development ending in seed dormancy	Plasma membrane
10	Cysteine synthase/XP_002512253.1	0.0	34.3/5.0	Cysteine biosynthetic process from serine	Cytoplasm
11	Class I chitinase/Q41596.1	0.0	34.8/5.1	Chitin catabolic process Defense response Cell wall macromolecule catabolic process	Vacuole
12	Mlp-like protein 28/gi|21592592|AAM64541.1	4,66E-73	17.6/5.2	Response to biotic stimulus Defense response	Cytosol
13	Nucleoside diphosphate kinase 1/ADB85102.1	1,53E-93	16.4/6.4	Auxin biosynthetic process Response to salt stress Response to cadmium ion CTP biosynthetic process	Peroxisome Vacuole Plasma membrane Apoplast

[a]Spot numbers correspond to the numbers on the 2-D gel images in Figure 3B.

Protein extraction by Method B was also tested in *Citrus* roots. With this method, 1.0 g lyophilized *Citrus* roots yielded about 2.7 mg protein. The proteins isolated from *Citrus* roots by Method B gave high-resolution separation and minimal contamination on 2-D gels (Figure 4). Approximately 800 spots were estimated by the ImageMaster 2D Platinum software (GE Healthcare).
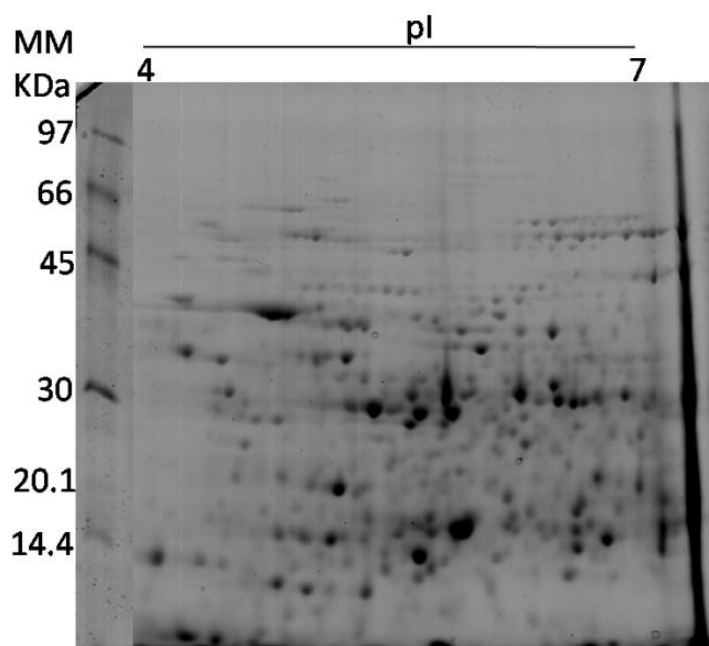

Figure 4. 2-DE proteins from *Citrus* root using Method B of protein extraction. The gel was loaded with 350 mg of total protein and stained with 0.08% colloidal Coomassie G-250 (w/v).

DISCUSSION

Standard protocols have been proposed for various types of samples, but the particularities of many samples require the use of specific protocols that are optimized according to the objective of the study, the specific type of tissue, and the age of the organ (Görg et al., 2004; Islam et al., 2004). *T. cacao*, in particular, has a very high level of interfering compounds, such as polysaccharides and phenolic compounds (Gesteira et al., 2003), that possibly explain the absence of data in the literature for obtaining quality proteins and studies of successful 2-DE proteome analysis of *T. cacao* roots, making it necessary to develop an efficient protocol for protein extraction from roots of this species.

Protein extraction from plant samples is often challenging, especially in woody plants, which have higher lignin contents than other plant species, making the disruption of the cell wall (especially in roots and stems) problematic. Roots, especially of recalcitrant species such as *T. cacao* (Figueira et al., 1994; Gesteira et al., 2003), besides being an organ containing

tissues that are difficult of macerate, have low protein content (Isaacson et al., 2006; Xie et al., 2007). Roots have many non-protein contaminants that affect 2-DE, including polysaccharides, polyphenols, nucleic acids, terpenes, and organic acids; these contaminants accumulate mainly in the vacuole in various soluble forms (Tsugita and Kamo, 1999; Pan, 2000). The contaminants can be coextracted with protein and affect protein migration in 2-DE, resulting in streaking (Görg et al., 2000).

According to Vâlcu and Schlink (2006), the grinding of the roots in liquid nitrogen, followed by precipitation by TCA/acetone and sonication, is one of the most effective approaches for plant samples that are rich in lignin. In this study, we found that compared to leaf samples (Pirovani et al., 2008), preparing the ADP of the *T. cacao* roots required precipitation with TCA/acetone overnight with several stages of sonication to obtain a high yield of protein (Table 1).

The extraction of proteins from ADP was performed using a mixture of phenol and dense SDS buffer (Wang et al., 2003; Pirovani et al., 2008). SDS is a good solubilizing agent, and phenol minimizes the protein degradation that often occurs during sample preparation because of the action of proteolytic enzymes that are found in the sample itself (Schuster and Davies, 1983); phenol has been reported to remove interfering compounds, such as polyphenols, efficiently prior to electrophoresis (Wang et al., 2003; Saravanan and Rose, 2004). The difference between the two methods that were compared in this study was that two extractions with phenol were added in Method B. Additional extractions with phenol, which were associated with changes that were made in the preparation of ADP, allowed the acquisition of proteins that were free of degradation and high resolution (Figures 2 and 4), indicating the efficiency of Method B in obtaining high-quality protein in a high quantity.

When separated on 2-DE gels, the protein extract that was obtained by Method B showed high resolution and clear separation of more spots than were described in the literature for other methodologies. Caetano et al. (2007), using the methodology of De Mot and Vanderleyden (1989) for extraction of proteins from roots of *Vigna unguiculata*, obtained 2-DE gels with approximately 250 spots at pI 4-7, while Bona et al. (2007), using the method Bestel-Corre et al. (2002) for the extraction of proteins from roots of *Cannabis sativa*, obtained 300 spots on 2-DE gels at pI 4-7.

The clear separation of a large number of protein spots with minimal streaking on 2-DE gels and the successful identification of protein spots by mass-spectrum comparisons to protein mass databases for *T. cacao* demonstrates that extraction Method B was suitable to obtain the quantity and quality of proteins that are required for proteomic studies of roots.

The efficiency of Method B for protein extraction from recalcitrant roots of other species was demonstrated by its use in *Citrus*. *Citrus*, similar to *T. cacao*, is a genus with recalcitrant species (Moore et al., 1992); its roots are a difficult material to extract proteins because they have low protein content and a large amount of interfering contaminants. To our knowledge, no reports of protein extraction from roots of *Citrus* have been successful for the 2-DE method. The proteins isolated by Method B showed high yield and quality and 2-DE gels with well-resolved spots that were free of stains and streaks.

In conclusion, through Method B, we succeeded in isolating a high yield of high-quality proteins from *T. cacao* roots. The 2-DE gels that were obtained were of high quality, free of smearing and streaking, and could be used for protein identification by MS. It is expected that our protocol could also be applied for other recalcitrant plant roots, despite plants varying considerably in the amounts and types of interfering compounds they produce.

ACKNOWLEDGMENTS

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