EXTRACTION METHODS FOR ANALYSIS OF OIL PALM LEAF AND ROOT PROTEINS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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ABSTRACT

*Oil palm (*Elaeis guineensis *Jacq.) is an important crop in Malaysia, and as such, it has become the second largest export earner for the country. This study ventured into developing suitable gel-based proteomics methods for specific oil palm tissues with the aim of understanding its biological systems. An optimal procedure for extraction of proteins from oil palm root and leaf tissues was developed through evaluations and modifications of three existing methods. The TCA/acetone, phenol/ammonium acetate and chloroform/acetone protein extraction approaches were tested on the root and leaf tissues of oil palm. The total protein yields and resulting 2-DGE protein spot profiles revealed that TCA/acetone method is the most effective approach for both tissues. Thus, TCA/acetone method will be used to produce total proteins for downstream analyses by mass spectrometry.*

Keywords: protein extraction, two-dimensional gel electrophoresis, oil palm.

Date received: 23 July 2013; **Sent for revision:** 27 August 2013; **Received in final form:** 18 November 2013; **Accepted:** 16 January 2014.

INTRODUCTION

A proteomics approach using a combination of two-dimensional gel electrophoresis (2-DGE) with mass spectrometry has the potential to be a powerful tool in the selection and evaluation of new varieties, since it permits simultaneous separation and identification of hundreds of proteins (Zukas and Breksa III, 2005). Sample preparation is the most important step of gel-based proteomics studies employing 2-DGE because the quality of the gel pattern largely depends on it. In this regard, proteome analysis of plant tissues involves a number of practical challenges that are typically more problematic than other organisms. In addition to having relatively low protein concentrations, plant tissues are often rich in proteases and interfering compounds such as polysaccharides, phenolic compounds, lipids, organic acids, pigments, proteases as well as other secondary metabolites (Görg *et al*., 2004; Shaw and Riederer, 2003). These interfering compounds are the reasons for horizontal and vertical streaking, smearing and a reduction in the distinctly resolved protein spots (Saravanan and Rose, 2004). These difficulties must be overcome to increase the efficiency of gel-based proteomics technique for a successful protein identification utilising mass spectrometry approach.

Various protein extraction methods have been developed in previous studies due to the fact that protein extraction efficiency varies with the type of tissues and plant species (Carpentier *et al*., 2005; Gorg *et al*., 2004; Shaw and Riederer, 2003). The common protocol for protein extraction from plant tissue is the trichloroacetic acid (TCA)/acetone extraction method (Xu *et al*., 2008; Li *et al*., 2007;

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Askari *et al*., 2006). The phenol-based extraction had been reported as an efficient method for recalcitrant tissues (Agrawal and Thelen, 2006; Mooney *et al*., 2006; Sheffield *et al*., 2006). Another method employed for perennial *Bupleurum* root tissues involves protein solubilisation in chloroform followed by precipitation of the proteins with acetone (Xie *et al*., 2007).

In this study, three extraction methods were evaluated for oil palm root and leaf tissues using 2-DGE and the results were further examined for protein identification by mass spectrometry. To our knowledge, this is the first published protein extraction protocol from perennial oil palm root and leaf tissues that has given reproducible results.

MATERIALS AND METHODS

Chemicals and Equipment

Mini-Protean III equipment, PROTEAN IEF Cell, PDQuest 2-D software, VersaDoc imaging system, immobilised pH gradient (IPG) strip of 7 cm length and linear pH of 3-10, ReadyPrep 2-D Cleanup kit, 30% acrylamide/bis solution, Coomassie Brilliant Blue (CBB) G-250, urea, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), Bio-Lyte® 3/10 buffer, tetramethylethylenediamine (TEMED), dithiothreitol (DTT), iodoacetamide, glycine and sodium dodecyl sulphate (SDS) were purchased from Bio-Rad (Hercules, CA). The 2-Mercaptoethanol, thiourea, tris base, trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), chloroform:isoamyl alcohol (24:1), sucrose, phenylmethanesulfonyl fluoride (PMSF), phenol, ammonium acetate and potassium chloride (KCl) were from Sigma-Aldrich (Taufkirchen, Germany). Methanol, acetone and ethanol were from Merck (Darmstadt, Germany). Protein assay 2-D Quant Kit and bovine serum albumin (BSA) as a standard were from GE Healthcare (Piscataway, NJ). Sodium chloride (NaCl) was from Fisher Scientific (Leicestershire, UK). All chemicals used were analytical grade and MiliQ water was used for all buffers and solutions.

Plant Materials

The root and leaf tissues of *dura* x *pisifera* (DxP) variety of commercial oil palm (*Elaeis guineensis* Jacq.) were obtained from the Malaysian Palm Oil Board (MPOB) Research Station in Kluang, Johor, Malaysia. Leaf and root tissues were collected and thoroughly washed with water, cut into small pieces, snap-frozen in liquid nitrogen and stored at -80°C prior to protein extraction.

Protein Extraction

TCA/acetone extraction. TCA/acetone precipitation was carried out according to Damerval *et al.* (1986) and Görg *et al*. (1997) with slight modifications. One gramme of root or leaf material was ground to fine powder in a pre-cooled mortar in the presence of liquid nitrogen. Four millilitres of extraction buffer containing 0.1 M Tris-HCl, pH 8.3, 0.5 M NaCl, 5 mM DTT and 1 mM PMSF was added to the ground tissues. The suspension was sonicated in cold water-bath sonicator for 15 min and centrifugation was performed at 3200 x *g* for 15 min at 4°C. Subsequently, 3 ml of cold acetone containing 10% TCA (w/v) and 1 mM DTT was added to the suspension to promote protein precipitation prior to overnight incubation at -20°C. The protein pellet was recovered by centrifugation at 3200 x *g* for 15 min at 4°C. The protein pellet was washed in 2 ml of cold acetone containing 1 mM DTT followed by centrifugation at 3200 x *g* for about 5 min at 4°C. The protein pellet was then air-dried and resuspended in 1 ml of rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 60 mM DTT and 1% (v/v) ampholytes). The supernatant was used for further analysis.

Phenol/ammonium acetate extraction. The method of Hurkman and Tanaka (1986) was adapted for our samples. Ground root or leaf materials (1 g) were homogenised on ice in 4 ml extraction buffer (50% phenol, 0.45 M sucrose, 25 mM EDTA, 1% [v/v] 2-mercaptoethanol, 250 mM Tris-base at pH 8.8) for 10 min. The extracts were transferred to new tubes and incubated in an incubator shaker for 30 min at 4°C. Following centrifugation at 5000 x *g* for 10 min, the upper phenol-phase was collected and precipitated for 2 hr at -20°C in five volumes of ice-cold 0.1 M ammonium acetate and 1% (v/v) 2-mercaptoethanol in methanol. The precipitate obtained by centrifugation at 12 000 x *g* for 10 min at 4°C was then washed twice with 20 ml of cold 0.1 M ammonium acetate in methanol followed by cold 80% acetone. Cold 70% ethanol was used for the final wash. The resulting protein pellet was air-dried and resuspended in 1 ml rehydration buffer and stored at -80°C for analysis.

Chloroform/acetone extraction. Ground root or leaf samples (1 g) were homogenised in 4 ml of extraction buffer (0.1 M KCl, 0.5 M Tris-base, pH 7.5, 0.05 M EDTA, and 2% 2-mercaptoethanol) for 10 min according to Xie *et al.* (2007). The mixture was vortexed for 2 hr at 4°C and centrifuged at 12 000 x *g* for 15 min at 4°C. The supernatant was transferred to a new tube, and an equal volume of water-saturated

chloroform and isoamyl alcohol (24/1, v/v) was added. The tube was shaken at 4°C for 30 min and then kept on ice for 10 min before centrifugation at 10 000 x *g* for 10 min at 4°C. The upper and lower phases were carefully removed from the tube. After that, 4 ml of water was added to the interphase together with an equal volume of water-saturated chloroform and isoamyl alcohol (24/1, v/v). The mixture was mixed well and allowed to stand on ice for 10 min before centrifugation at 10 000 x *g* for 10 min at 4°C. This step was repeated twice. The interphase was washed with ice-cold acetone for a total of three times. The resulting protein pellet was air-dried and resuspended in 1 ml of rehydration buffer and stored at -80°C for analysis.

Protein Quantification

Protein contents were determined using the 2-D Quant Kit (GE Healthcare, Piscataway, NJ) with bovine serum albumin (BSA) as the protein standard.

Sample Clean-up

Protein samples were cleaned-up prior to isoelectric focusing (IEF) separation using the ReadyPrep 2-D Cleanup kit (Bio-Rad, Hercules, CA) to reduce streaking, background staining and other gel artifacts associated with substances contaminating 2-DGE samples.

Protein Separation by SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970) in 12% polyacrylamide gel using a Bio-Rad Mini-Protean III equipment (Bio-Rad, Hercules, CA) at 50 V for 15 min followed by a 50 V increase to 200 V for another hour. Twenty-five μg of solubilised protein was loaded in each lane. The gels were stained overnight with Blue Silver (Candiano *et al*., 2004).

Two-dimensional Gel Electrophoresis

IPG strip of 7 cm length and a linear pH gradient of 3-10 (Bio-Rad, Hercules, CA) was passively rehydrated overnight with 100 μg of proteins in 100 μl rehydration buffer. IEF was carried out using a PROTEAN IEF Cell (Bio-Rad, Hercules, CA) at constant current of 50 μA per strip until 20 kVh was reached for 7 cm strip. Following IEF, the IPG strip was stored at -20°C until the second dimension separation. Subsequently, the strip was equilibrated twice for 10 min in 5 ml of equilibration buffer (6 M urea, 2% SDS, 1.5 M Tris/HCl, pH 8.8 and 50% glycerol) containing 2% DTT and 2.5% iodoacetamide, for reduction and alkylation of the proteins respectively. Second dimension SDS-PAGE was performed as described previously and visualised using Blue Silver stain.

Gel Analysis

The 2-DGE gel images were analysed using PDQuest 2-D software (Bio-Rad, Hercules, CA). After spot detection, 2-DGE gels were aligned, matched and a quantitative determination of the spot volumes was performed. The total numbers of protein spots were determined in leaf and root samples for the respective extraction methods.

Statistical Analysis

The experiments were performed in completely randomised design and repeated three times. For each treatment, three replications (n=3) were maintained. Statistical analysis was performed using SPSS Version 16.0 for Windows (John Wiley and Sons Australia Ltd., Milton, Queensland). One-way ANOVA was used to compare among different extraction of leaf and root for parameters used in the study, *i.e.* total protein content and total number of protein spots. Post hoc analysis for individual comparison was then done using Tukey test. The p-value of less than 0.05 (<0.05) indicated that there was a significant difference between extraction means.

RESULTS

Evaluation of Protein Yields and Number of Proteins Spots

The objective of this study was to generate suitable methods for protein extraction from oil palm leaf and root and subsequently separate using the 2-DGE. Oil palm is a woody plant that possesses high levels of phenolic compounds and lipids. The challenge is not just to get the most proteins but also to eliminate as much interfering compounds as possible from the protein extracts. All the protocols utilised have the ability to reduce phenolic and carbohydrate contaminants but with varying efficiencies. For this work, the three different methods of protein extraction, namely TCA/acetone, phenol/ammonium acetate and chloroform/acetone methods were adapted to suit the different oil palm tissues and evaluated based on their total protein yields and 2-DGE profiles.

Precipitation with 10% of TCA in acetone significantly (<0.05) increased the protein yield, as well as the number of protein spots in leaf and root tissues (*Table 1*). The TCA/acetone method produced the highest protein yield, *i.e.* 0.291 ± 0.015 mg g^{-1} fresh weight (FW) for leaf and 0.352 ± 0.051 mg g^{-1}

TABLE 1. PROTEIN YIELD AND TOTAL NUMBER OF SPOTS USING DIFFERENT PROTEIN EXTRACTION METHODS

Methods	Protein yield (mg g^{-1} FW)		No. of protein spots	
	Leaf	Root	Leaf	Root
TCA/acetone	0.291 ± 0.015	0.352 ± 0.051	158 ± 3	268 ± 7
Phenol/ammonium acetate	0.242 ± 0.011	0.080 ± 0.027	145 ± 6	75 ± 5
Chloroform/ acetone	0.082 ± 0.017	0.057 ± 0.016	114 ± 8	21 ± 5

(FW) for root compared to the other two methods. In leaf, TCA/acetone and phenol/ammonium acetate methods resulted in 70% more protein spots than chloroform/acetone approach. The TCA/acetone extraction of root samples produced 3.71 times more protein spots than the phenol extraction and 10.68 times more protein spots than the chloroform extraction. Thus, the TCA/acetone precipitation and washing steps were deemed sufficient to purify and concentrate proteins from the leaf and root tissues.

The result is supported by a similar finding in turfgrass leaves where the TCA/acetone produced higher protein yield compared to chloroform/acetone, tris-base/acetone and phenol/ammonium acetate protocols. In the same study, both the TCA/acetone and phenol/ammonium acetate protocols generated relatively higher protein yields in the root tissues (Xu *et al.*, 2008). However, a comparative study on the three protein extraction protocols on tomato tissues showed that the phenol-based method produced a higher protein yield compared to TCA/acetone (Saravanan and Rose, 2004). In another study using a woody plant (citrus), the optimal protein extraction protocol was phenol-based (Tris–HCl, KCl and phenol) coupled with a solvent precipitation to purify and concentrate the proteins (Zukas and Breksa III, 2005).

Efficiency of the Three Protein Extraction Methods Based on SDS-PAGE Profiles

Apart from the total amount of soluble proteins extracted, another criteria used to select an optimal extraction protocol is the number of protein bands, ideally covering a wide range of molecular sizes. The quantitative characteristics of protein extracts from oil palm leaf and root tissues obtained using the three protocols were evaluated using the SDS-PAGE. The proteins were resolved into distinct bands that spanned from less than 25 kDa to more than 250 kDa. SDS-PAGE revealed different protein band patterns for the three methods for root protein (*Figure 1*). Root proteins extracted using TCA/acetone exhibited the best protein band resolution over a wide range of M_r compared to the other methods. Furthermore, many protein bands were diffused or absent in chloroform/acetone and phenol/ammonium acetate extracted samples.

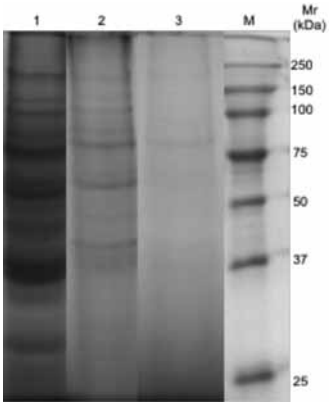

Figure. 1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of proteins extracted from oil palm root using different protein extraction methods: (1) trichloroacetic acid (TCA)/ acetone, (2) chloroform/acetone and (3) phenol/ammonium acetate and (M_r) protein standards (Bio-Rad, Hercules, CA). Twenty-five µg of proteins were loaded in each lane and the gel was stained with Blue Silver.

Comparison of the Three Protein Extraction Methods using 2-DGE Approach

The bands of the leaf and root proteins generated by SDS-PAGE represent a physical separation of the proteins present in the whole proteomes. The numbers of electrophoretic bands produced by SDS-PAGE do not reflect the total protein entities due to the lack of resolving power. The 2-DGE offers a better analysis for the proteomes combining protein separations based on molecular weight and isoelectric point. The 2-DGE profiles for the leaf proteins (*Figure 2*) and root proteins (*Figure 3*) extracted using three different methods showed that the quality of separated protein spot intensities varied as indicated with the marked regions.

In leaf, TCA/acetone method produced clear background in the high-M_r region (region I in *Figure 2a*) compared to the other methods (region I in *Figures 2b* and *2c*). The chloroform/acetone method generated the least number of protein spots in the low-M_r region (region II in *Figure 2c*) than the other two methods (region II in *Figures 2a* and *2b*). In conclusion, leaf proteins for both high-M_r and low-M_r regions extracted with TCA/acetone method were relatively well-separated and this method has

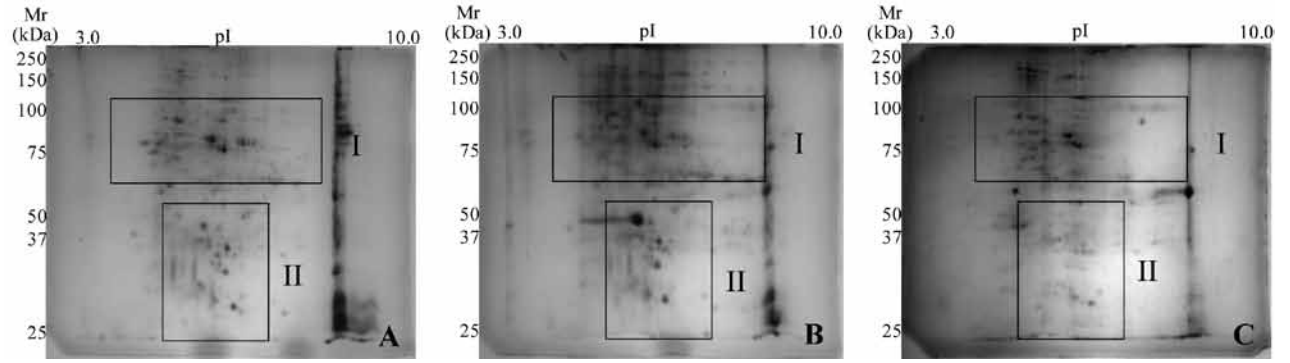

Figure 2. Comparison of two-dimensional gel electrophoresis protein patterns in leaf using different protein extraction methods: (a) trichloroacetic acid (TCA)/acetone, (b) phenol/ammonium acetate and (c) chloroform/acetone. Proteins (100 µg) were separated in the first dimension using immobilised pH gradient strips (pH 3-10) followed by the second dimension using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Blue Silver.

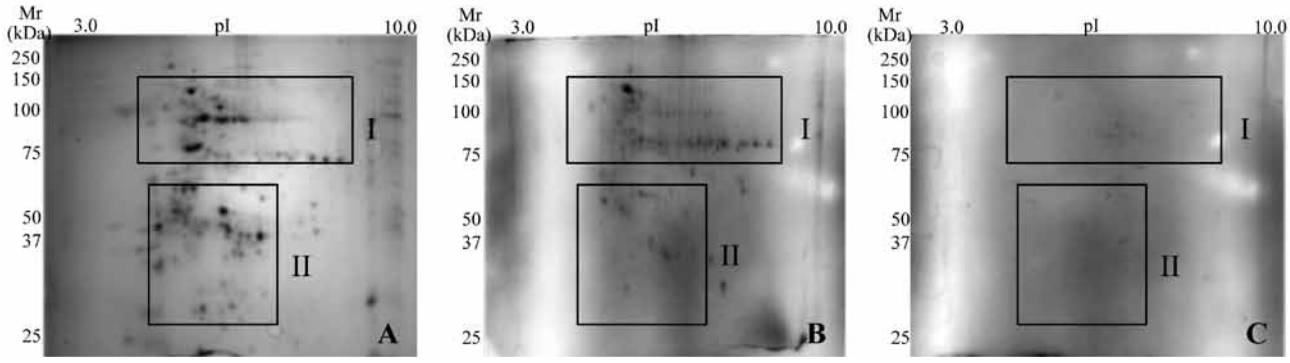

Figure 3. Comparison of two-dimensional gel electrophoresis protein patterns in root using different protein extraction methods: (a) trichloroacetic acid (TCA)/acetone, (b) phenol/ammonium acetate and (c) chloroform/acetone. Proteins (100 µg) were separated in the first dimension using immobilised pH gradient strips (pH 3-10) followed by the second dimension using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Blue Silver.

recovered more protein spots (158 ± 3 protein spots) compared to the other two methods.

The protein in the roots extracted using TCA/acetone (*Figure 3a*) resulted in good quality well-resolved protein profiles compared to other methods. Root proteins extracted using the chloroform/acetone method produced deficient protein separation, and low number and intensity of protein spots. The TCA/acetone method yielded more protein spots (268 ± 7 protein spots) with a higher protein spot intensity in the high-M_r and low-M_r regions (regions I and II in *Figure 3a*) than the phenol/ammonium acetate extraction method (75 ± 5 protein spots) (*Figure 3b*).

DISCUSSION

The critical step required in any proteome profiling study is the evaluation of protein extraction protocols to identify the most efficient approach for the type of plant tissues studied. Depending on the types of tissues, different extraction protocols may produce different levels of efficiency which are normally evaluated based on the amount of total proteins extracted, the numbers of different proteins obtained, the molecular mass range of the proteins and the levels of contaminants present. Ideally, the best extraction method should generate the highest number of different protein types covering a wide range of sizes from a few kDa to hundreds of kDa. In addition, the extraction method should also be able to extract low abundance proteins which are most likely to be key regulatory proteins of cellular events (Sebastiana *et al*., 2013). Previous studies have suggested that the optimisation of the protein extraction protocol for different plant samples is important because different plant species and tissues vary in the amounts and types of non-protein interfering compounds (Shaw and Riederer, 2003; Görg *et al*., 2004; Carpentier *et al*., 2005).

The TCA/acetone protocol was initially developed by Damerval *et al*. (1986) and is based on protein denaturation and precipitation under acidic and hydrophobic conditions, which help to concentrate and isolate the proteins from lipids, phenols, polysaccharides and nucleic acids as well as inactivate proteases that would cause artifact spots and loss of high mass proteins (Görg *et al*., 2004;

Wang *et al.*, 2008). The sample preparation should be as simple as possible to reduce gel-to-gel variations since reproducibility is one of the obstacles in 2-DGE technique. Thus, TCA/acetone approach is the most potential candidate and simplest among protein extraction methods. Our results were consistent with a few other studies conducted in different species of woody plants such as spruce (*Picea abies*), needles and beech (*Fagus sylvatica* L.) leaves and roots resulting in very high protein yields extracted from the TCA/acetone method (Valcu and Schlink, 2006). Xu *et al.* (2008) suggested that the TCA/acetone extraction method was the most effective method in cool-season turfgrass for the leaf tissue while a combination of the TCA/acetone and phenol methods was suitable for the root tissue. While the TCA/acetone method is very useful in minimising protein degradation and removing interfering compounds, the protein losses cannot always be avoided due to incomplete protein precipitation and insufficient protein solubilisation (Xu *et al*., 2008). This approach was also found to be unsuitable for more complex plant tissues such as tomato, banana, avocado, orange, apple and tomato tissues due to the co-extraction of polymeric contaminants (Saravanan and Rose, 2004; Carpentier *et al.*, 2005).

Phenol extraction of protein has been reported as the most suitable protein extraction method for recalcitrant tissues containing relatively lower amount of protein and high content of interfering compounds that affect electrophoresis (Saravanan and Rose, 2004; Wang *et al.*, 2008). The phenol extraction is capable of generating samples with higher purity than TCA/acetone. Compounds such as polysaccharides and other water-soluble contaminants are separated from the proteins since these compounds are not soluble in the phenolic layer (Hurkman and Tanaka, 1986). This method is based on selectivity as organic solvents like phenol with the presence of sucrose, are able to separate proteins from contaminating substances like nucleic acids, carbohydrates and cellular debris by eliminating it in the aqueous phase through phase separation. Then, solubilised and mostly purified proteins can be precipitated with methanol and ammonium acetate (Faurobert *et al.*, 2007). This method was reported to be highly effective when applied to tomato *Lycopersicon esculentum* L., avocado *Persea americana* L., orange *Citrus sinensis* L., banana *Musa sapientum* L., potato *Solanum tuberosum* L., apple *Malus domestica* L. and cotton *Gossypium hirsutum* L. (Wang *et al*., 2008; Saravanan and Rose, 2004; Carpentier *et al*., 2005). Recently, Sebastiana *et al.* (2013) reported that the phenol extraction protocol allowed an efficient proteome isolation and 2-DGE separation of different woody recalcitrant plant tissues namely grapevine leaves, pine needles and cork oak ectomycorrhizal roots. However, the disadvantage of the phenol extraction method is that it is time-consuming and phenol is toxic. Moreover, this method is less efficient than the TCA/acetone method for proteins, producing fewer spots and lower spot intensity in high M_r.

The chloroform-acetone method has been reported to be efficient method for protein extraction from thick and woody plants containing high levels of interfering compounds such as lipids and phenolics (Xie *et al.*, 2007). This method is able to detect more protein spots in the low-M_r region but fewer protein spots in the high-M_r region. Nevertheless, the disadvantage of this method is the low efficiency of protein extraction and detection, due to incomplete protein extraction; incomplete removal of non-protein contaminants in the extracts; or loss of proteins during the intricate extraction procedure.

Three methods of protein extraction examined in this study varied in their efficiency in protein quantity and profiles using 2-DGE for leaf and root tissues of oil palm. This study showed that different protein extraction methods are required to obtain high protein yield and high quality 2-DGE protein detection and separation in both oil palm tissues. In this study, the TCA/acetone method was modified according to Görg *et al.* (1997) by replacing 2-mercaptoethanol with DTT in order to extract more pH basic proteins instead of acidic proteins and to remove the TCA and salt from the sample, which can be observed by horizontal streaking in the 2-DGE gels (Westermeier *et al.*, 2008). The TCA/acetone method gave very high protein yield and good gel resolutions for leaf and root proteins. Clearly this indicates that the use of TCA and acetone to remove non-protein contaminants improves the protein extraction from these tissues (Xie *et al*., 2007). Thus, the TCA/acetone method will be used for downstream analyses by mass spectrometry for protein identification from oil palm root and leaf tissues in the future.

CONCLUSION

Three most common plant protein extraction methods for oil palm leaf and root were evaluated. Among these methods, the TCA-acetone method is the most suitable protein extraction approach for the oil palm root and leaf tissues based on the protein yields, SDS-PAGE and 2-DGE gel analyses.

ACKNOWLEDGEMENT

We thank the Director-General of MPOB for permission to publish this article, Dr Mohd Din Amiruddin and Mr Marhalil Marjuni of Breeding

& Quantitative Genetics Group, Advanced Biotechnology and Breeding Centre, MPOB for providing oil palm sample. The technical assistance of Mr Abdul Rahim Mohamad and Mr Abdul Fadzil Suhaimi of Proteomics and Metabolomics Group, Advanced Biotechnology and Breeding Centre, MPOB is gratefully acknowledged. This study was funded by the MPOB Board Approved Programme (R005606000).

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