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Evaluation of different protein extraction methods for banana (*Musa* spp.) root proteome analysis by two-dimensional electrophoresis

M Mayil Vaganan*, S Sarumathi, A Nandakumar, I Ravi and M M Mustaffa

Physiology and Biochemistry Laboratory, ICAR National Research Centre for Banana, Thayanur Post, Thogamalai Road, Tiruchirapalli, Tamil Nadu, India 620 102

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Four protocols *viz*., the trichloroacetic acid-acetone (TCA), phenol-ammonium acetate (PAA), phenol/SDS-ammonium acetate (PSA) and trisbase-acetone (TBA) were evaluated with modifications for protein extraction from banana (Grand Naine) roots, considered as recalcitrant tissues for proteomic analysis. The two-dimensional electrophoresis (2-DE) separated proteins were compared based on protein yield, number of resolved proteins, sum of spot quantity, average spot intensity and proteins resolved in 4-7 p*I* range. The PAA protocol yielded more proteins (0.89 mg/g of tissues) and protein spots (584) in 2-DE gel than TCA and other protocols. Also, the PAA protocol was superior in terms of sum of total spot quantity and average spot intensity than TCA and other protocols, suggesting phenol as extractant and ammonium acetate as precipitant of proteins were the most suitable for banana rooteomics analysis by 2-DE. In addition, 1:3 ratios of root tissue to extraction buffer and overnight protein precipitation were most efficient to obtain maximum protein yield.

Keywords: Plant protein extraction, Phenol-ammonium acetate, Trichloroacetic acid-acetone, Two-dimensional electrophoresis, Banana rooteomics

Proteomic analysis of plant tissues poses many practical challenges as having relatively low protein concentrations, high activity of proteases and oxidative enzymes and coextraction of interfering non-protein materials[1]. The non-protein contaminants are problem for 2-DE in relation to proteins migrations, for example reversible phenolic-protein complex formation and irreversible oxidation by covalent condensations, leading to proteins degradation and charge heterogeneity and resulting in horizontal and vertical streaking, smearing, distortion and a reduction in the number of distinctly resolved protein spots[2,3]. Tissues of banana (*Musa* spp.) contain high levels of oxidative enzymes (polyphenol oxidase) activity[4,5], phenol compounds and high levels of latex and soluble carbohydrates, which interfere with electrophoresis by blocking gel pores causing precipitation and extended focusing times[6] and considered as recalcitrant from the point of protein sample preparation and proteomics analysis[6,7].

Many methods have been employed for protein extraction from banana tissues. Carpentier *et al*[3] used the classical TCA-acetone to profile banana meristem proteome and could resolve 1137 spots on silver nitrate-stained 2-D gels. Song *et al*[8] isolated proteins using hot-SDS buffer and TCA-acetone from banana peel and pulp and detected 510 and 394 peptide spots, respectively on silver-stained 2-D gels. Recently, more than 550 protein spots could be observed on 2-D gels stained with CBB from banana peel from the total proteins isolated by phenol-based method[9]. However, a suitable and efficient protocol for protein extraction from root tissue has not been identified. Roots as critical primary organs are vulnerable to different stresses, such as pathogens, salts, drought, heavy metals and anoxia in the soil, with consequent effects on plants growth and production in general and on development and quality of fruits in particular[10].

In this study, four protocols *viz*., the trichloroacetic acid-acetone (TCA), phenol-ammonium acetate (PAA), phenol/SDS-ammonium acetate (PSA) and trisbase-acetone (TBA) were evaluated with modifications for protein extraction from banana (Grand Naine) roots, considered as recalcitrant tissues for proteomic

*Corresponding author
E-mail: mmayilv@gmail.com
Tel: +91 94425 53117

Abbreviations: CBB, Coommassie brilliant blue; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); 2-DE, two-dimensional electrophoresis; DTT, dithiothreitol; IEF, isoelectric focusing; IPG, immobilised pH gradient; PAA, phenol-ammonium acetate; PAGE, polyacrylamide gel electrophoresis; PSA, phenol/SDS-ammonium acetate; PVPP, polyvinylpolypyrollidone; SDS, sodium dodecyl sulphate; TBA, tris base-acetone; TCA, trichloroacetic acid-acetone.

analysis. The two-dimensional electrophoresis (2-DE) separated proteins were compared based on protein yield, number of resolved proteins, sum of spot quantity, average spot intensity and proteins resolved in 4-7 p*I* range.

Materials and Methods

Plant materials and protein extraction

Tissue culture plants of *cv*. Grand Naine of banana were grown in sterilised soil in green house maintained with 25/20°C day/night temperature and 70% RH with watering on alternate days with nutrient solution containing macro and microelements[11]. Root tissue was excised and immediately finely powdered using liquid nitrogen. TCA extraction and acetone precipitation (TCA), phenol extraction and ammonium acetate precipitation (PAA), phenol-SDS extraction and ammonium acetate precipitation (PSA) and Tris-base extraction and acetone precipitation (TBA) protocols were evaluated for protein extraction from banana root tissues with modifications with respect to quantity of tissue (1, 2 and 3 g), volume of extraction buffer (1:1, 1:3 and 1:5) and duration of incubation time (2, 6 and 12 h). Four replications were performed for all the protocols.

TCA extraction and acetone precipitation (TCA)

Root tissue powder was mixed thoroughly with ice-cold acetone containing 10% (v/v) TCA, 1.5% (w/v) polyvinylpolypyrollidone (PVPP) and 0.07% (w/v) DTT[12] by vortexing for 15 s and incubated at -20°C for proteins precipitation. The proteins along with tissue debris were pelleted by centrifugation at 16,000 x *g* for 20 min at 4°C. Supernatant was removed and the pellet was washed twice or thrice in ice-cold acetone containing 0.07% DTT until the supernatant was colourless. The protein pellets were incubated at -20°C for 1 h between the washes. Final pellet was lyophilized and suspended in resolubilization buffer containing 7 M urea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylam onio]-1-propanesulfonate (CHAPS), 1% (v/v) ampholytes (3/10), 1% (w/v) DTT and 35 mM Tris base and vortexed for 1 h at room temperature. Insoluble root tissue debris was removed by centrifugation at 16,000 x *g* for 30 min.

Phenol extraction and ammonium acetate precipitation (PAA)

Tissue powder with ice-cold extraction buffer (50 mM Tris-HCl (pH 8.5), 5 mM EDTA, 100 mM KCl, 1% DTT, 30% sucrose, 1.5% PVPP and 100 µl protease inhibitor cocktail (Sigma) was mixed by vortexing for 30 s and an equal volume of ice-cold Tris-buffered phenol (pH 8.0)[3,13] was added and again vortexed for another 15 min at 4°C. Following centrifugation at 16,000 x *g* for 30 min, the phenolic (upper) phase was reextracted with an equal volume of extraction buffer. The phenolic phase was thoroughly mixed with five volumes of ice-cold 100 mM ammonium acetate in methanol and stored at -20°C for proteins precipitation. The protein was pelleted by centrifuging at 6,000 x *g* for 3 min and washed twice in ice-cold acetone containing 0.2% DTT and protein pellets were incubated at -20°C for 1 h between the two washes. The pellets were dried under nitrogen gas and resuspended in solubilization buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.8% (v/v) ampholytes and 1% (w/v) DTT.

Phenol-SDS extraction and ammonium acetate precipitation (PSA)

The protein extraction buffer used was Tris-buffered phenol (pH 8.0) and SDS buffer containing 30% (w/v) sucrose, 2% (w/v) SDS, 0.1 M Tris-HCl (pH 8.0) and 0.5% (w/v) DTT in ratio of $1:1^2$ and the remaining steps followed were similar to phenol extraction and ammonium acetate precipitation method.

Tris-base extraction and acetone precipitation (TBA)

Root tissue powder with extraction buffer containing 40 mM Tris base, 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1.5% (w/v) PVPP and 2% (w/v) DTT[14,15] was thoroughly mixed by vortexing for 15 s on ice. Following centrifugation at 12,000 x *g* for 20 min at 4°C, the supernatant containing the protein was carefully collected and protein was precipitated by thoroughly mixing with four volume of ice-cold acetone containing 0.07% DTT and incubating at -20°C. The precipitated protein was pelleted by centrifuging at 12000 x *g* for 15 min at 4°C and the protein pellets were washed twice with ice-cold acetone containing 0.07% (w/v) 2-mercaptoethanol and the protein pellets were incubated at -20°C for 1 h between the washes. Finally, protein pellets were lyophilised and suspended in resolubilization urea buffer as explained above. Protein content from the preparations was determined using Bradford method[16] with BSA as standard.

2-Dimensional (2-D) gel electrophoresis

The isoelectric focusing was carried out in 18 cm IPG bluestrips (pH 3–10, linear gradient, Serva) by using an IEF100 (Hoefer, CA, USA). IPG strips were

passively rehydrated with 350 µl rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 1% ampholytes and 0.002% bromophenol blue) containing 250 µg of root proteins for 12 h. The voltage settings for IEF were: 500 V for 1 h, 1000 V for 1 h and 3000 V for 11 h equalling to 58.5 kV h at working temperature of 20°C. The proteins in the strips were denatured with equilibration solution containing 10 mg DTT and 2.5 mg iodoacetamide per ml for 15 min separately.

The second dimension separation of proteins was carried out with SDS gels containing 12% (v/v) polyacrylamide in PROTEAN II XL (Bio-Rad) vertical gel electrophoresis apparatus at 17°C and at a constant voltage of 150 V. Proteins resolved in 2-DE gels were fixed, developed and detected by silver staining protocol[17] and imaged using Epson Perfection V750-M Pro scanner (Syngene) and the images were analysed using Melanie7 software. Statistical analysis of data was done as suggested by Panse and Sukhatme[18].

Results

Of the four protocols, TCA and PAA had significantly higher protein yield of 0.86 ± 0.043 and 0.89 ± 0.054 mg/g root tissue respectively, compared to PSA and TBA protocols. The PAA protocol yielded highest protein of 0.42 ± 0.037 mg/0.1 ml lysis buffer, which was significantly higher than the TCA protocol (0.34 ± 0.028 mg/0.1 ml) (Table 1). Clear qualitative and quantitative differences were observed in the protein resolving pattern between the methods tested for banana rooteomics study. Proteins extracted from roots using TCA and PAA protocols (Fig. 1a & b) exhibited clear protein profiles, whereas the proteins in the 2-D gels of PSA and TBA protocols (Fig. 1c & d) did not show clear separation with prominent streaking and distortion of spots, thus reducing the distinctly resolved spots.

Higher yield of proteins by the PAA and TCA protocols was reflected in the more number of protein spots detected in the gels, compared to other two methods. PAA protocol produced highest number of proteins in 2-DE gels among the four methods tested with a total of 584 spots, followed by the TCA protocol, which showed 546 protein spots, approximately 100% more protein spots yield than the PSA and TBA protocols, with only 261 and 258 spots, respectively. The PAA and TCA protocols produced better spot resolution, being free from any streaking and distortion of proteins. Comparison for common protein spots between the TCA and PAA methods showed that a total of 433 spots and 32 protein spots were disappeared in TCA method compared to PAA method.

In addition, the results showed that 1 g of tissue in 1:3 volumes of extraction buffer and overnight protein precipitation at -20°C produced highest protein yield. Extraction of protein from more amount of root tissue yielded proportionately higher amount of protein, but the protein yield from 1 g of tissue was found sufficient to perform the 2-D proteomic analysis, even using 24 cm IPG strips and for silver or CBB staining. The low and high tissue to extraction buffer ratios resulted in lower protein yield from root tissue, particularly by PAA protocol. Incubation for 2 or 6 h was insufficient for complete protein precipitation, as overnight incubation yielded significantly higher protein quantity.

Consequent on detection of highest number of protein spots, the sum of protein spots quantity was highest by the PAA protocol, followed by the TCA protocol and the other two protocols had very low sum of proteins intensity. Also, PAA protocol showed highest average protein spot intensity, followed by TCA protocol (Fig. 2). Comparison of protocols for number of proteins resolved in 4-7 p*I* range showed distribution of more than two-third of the total spots in PAA and TCA protocols (Fig. 3). Distribution of resolved proteins along the different M_r regions exhibited that one-third of total protein spots

Table 1—Protein yield, concentration and number of spots from banana roots by four different extraction protocols
[Values represent mean ± SD of four experiments]

Extraction method	Protein yield (mg/g root tissue)	Protein conc. (mg/100 µl lysis buffer)[a]	No. of protein spots
TCA-acetone (TCA)	0.86 ± 0.043	0.34 ± 0.028	546 ± 25.3
Phenol-ammonium acetate (PAA)	0.89 ± 0.054	0.42 ± 0.037	584 ± 20.6
Phenol/SDS-ammonium acetate (PSA)	0.63 ± 0.036	0.28 ± 0.025	261 ± 32.7
Tris buffer-acetone (TBA)	0.56 ± 0.032	0.20 ± 0.022	258 ± 14.1

[a]Quantity of protein solubilised when total protein extracted from 1 g of root tissue was solubilised in 0.1 ml lysis buffer

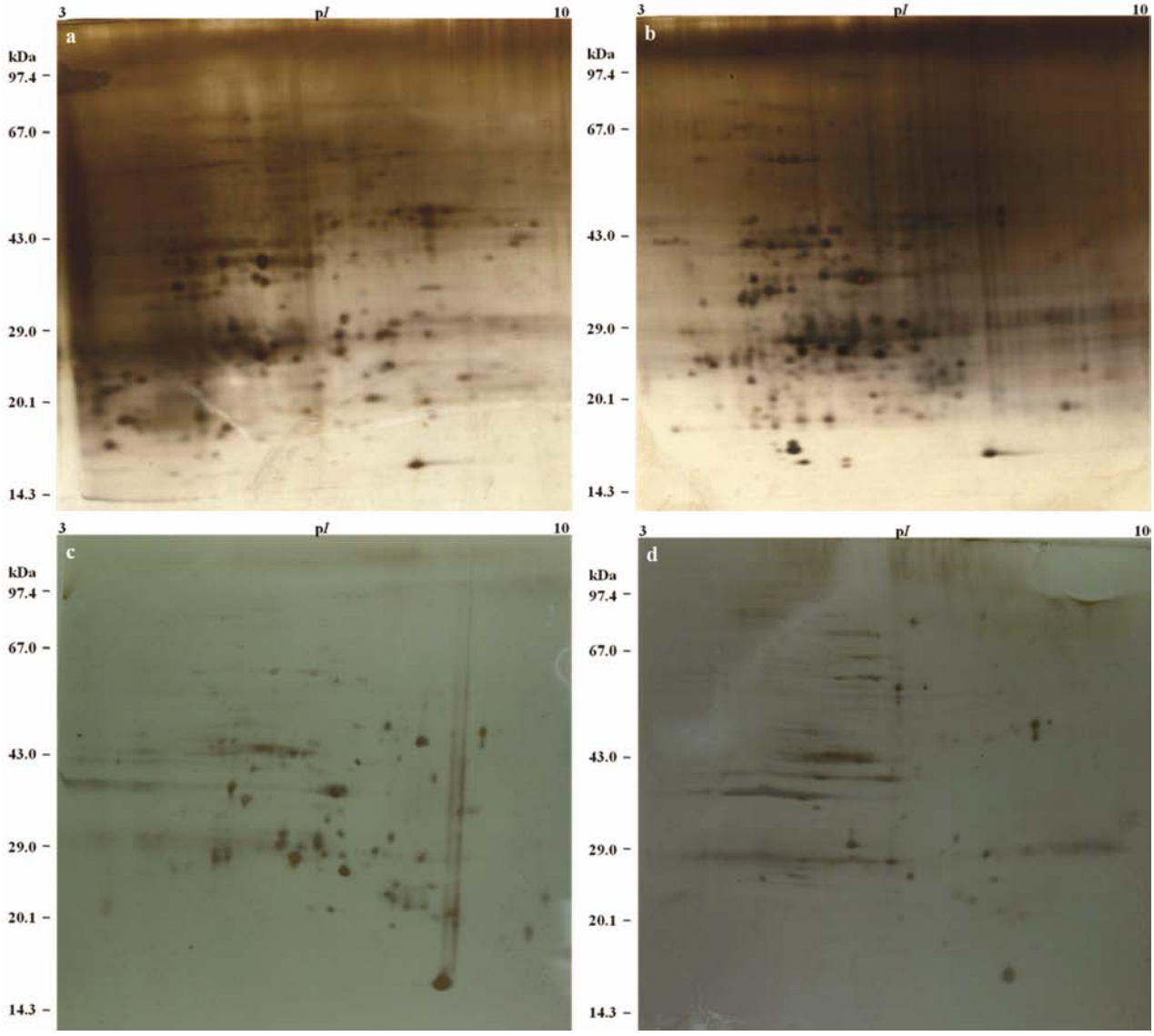

Fig. 1—2-DE patterns of banana root proteins using four extraction protocols: (a) Trichloroacetic acid-acetone protocol; (b) Phenol-ammonium acetate protocol; (c) Phenol/SDS-ammonium acetate protocol; and (d) Tris base-acetone protocol [Proteins (250 µg) were separated in the first dimension using IPG strips of pH 3-10, followed by the second dimension using12% SDS-PAGE and stained with silver nitrate]

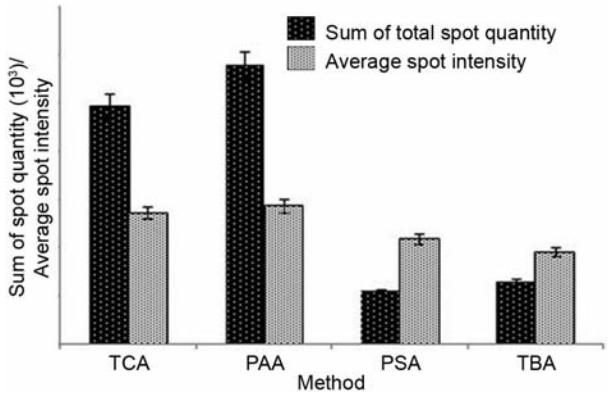

Fig. 2—Comparison of extraction protocols for sum of total spot quantity (10^3) and average intensity of total spots [Values are mean ± SE (n = 4)]

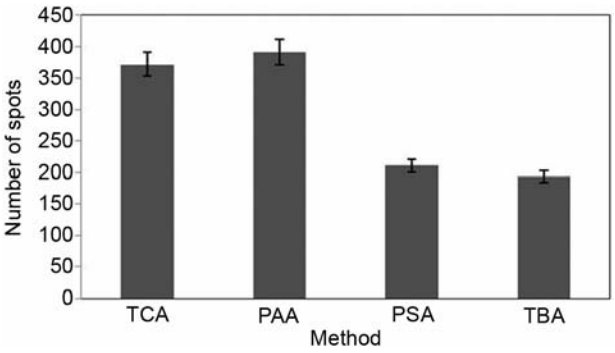

Fig. 3—Distribution of proteins in 4-7 p*I* range [Values are mean ± SE (n = 4)]

in low-M_r region of <29 kDa and around 60% of proteins in middle-M_r region of 29-67 kDa in two main protocols. In the high-M_r region of >67 kDa, only less than 10% of protein spots were found to be resolved.

Discussion

Banana tissue particularly roots are very recalcitrant to biochemical and molecular analyses. In terms of protein yield, the classical TCA and PAA protocols were equally efficient in extraction of proteins from banana root tissue. However, the TCA protocol yielded significantly low protein concentration, implying lower solubilization of proteins in lysis buffer, although TCA is a very efficient protein precipitant and known to instantly eliminate proteolytic and other protein modifying enzymes[12]. TCA protocol yielded a protein pellet that was more difficult to resuspend in lysis buffer compared with PAA protocol[19] and that might be the reason that 32 proteins disappeared in TCA 2-DE gel. On protein yield and concentrations, the results were in agreement with the findings for tomato roots, banana leaves and peels and grapevine leaves and roots[3,7,9,20]. Use of phenol and SDS as extraction agents yielded low protein quantity in this study, whereas Wang *et al*[2] have reported SDS-phenol combination yielding 2.49 mg protein from 1 g of mature olive leaf.

Maximum number of proteins detected in 2-DE gel of PAA protocol is attributed to minimum protein degradation due to endogenous proteolytic activity and more efficient resolubilization of proteins in lysis buffer[2,20]. Although TCA is a very effective protein precipitant and an instant arrestor of proteolytic activity as a strong acid[12,21], the incomplete resolubilization of the protein in TCA protocol might have resulted in less number of spots and under-representation of proteins in 2-DE gel. Moreover, PAA protocol produced better spot focusing and resolution, compared to TCA protocol. Results of many previous comparative evaluations of methods supported the findings of the present study. PAA protocol has produced higher number of proteins than TCA protocol from tomato, *Agrostis* and *Vitis* roots[7,15,20]. Also, the same protocol has exhibited more number of spots from banana meristem[3] and fruit peel[9], rice leaves[22] and *Arabidopsis* cell suspension[23].

Earlier studies have reported a few hundred to 3000 resolved proteins using 2-DE from different biological samples[2,24]. Detection of around 550 spots obtained from *Musa* spp. roots from this study was comparable with results of around 600 spots from grapevine roots[20], though another study has reported more than 1000 proteins from *Agrostis* grass roots[15].

Greatest sum of total spot quantity and average spot intensity matched with total number of spots obtained with PAA protocol. In addition, PAA protocol invariably gave greatest sum of total spot quantity and average spot intensity along different p*I* range and M_r regions in comparison to TCA protocol, which was in agreement with the findings of Jellouli *et al*[20] for grapevine roots. These results negated the hypothesis that TCA protocol leads to resolubilization of greater quantity of specific group of proteins along particular p*I* and M_r[3,25].

The data on number of proteins resolved in 4-7 p*I* range are culled and presented separately, as many studies[24,26] have been carried out 1-D IEF using IPG strips of non-linear pH 4-7. The results of such experiments have demonstrated that distribution of root proteins is not homogenous and uniform with most of the proteins focusing and resolving between 4 and 7. The present results also demonstrated that majority of the proteins were found resolved in 4-7 pH gradient and this behaviour of proteins was associated with basic biochemical characteristics of *Musa* spp. proteins on charge formation. The narrow separation of proteins indicated again that the non-linear pH gradient IPG strips are an apt choice for study of plant rooteomics[15,20,26].

In conclusion, the phenol as extractant and ammonium acetate as precipitant of proteins appeared to be efficient for the extraction of proteins from banana roots. Consistently, the PAA protocol produced higher protein yield, efficient resolubilization of proteins and resolving of significantly higher number of protein spots in 2-DE. Also, the sum of proteins spot quantity and average spot intensity were higher by the PAA protocol and the phenol-based method provided superior protein spots resolution and focusing. This study is the first report on the evaluation of methods for protein extraction for 2-DE separation of *Musa* spp. roots and the information may prove useful in banana rooteomics analysis in response to biotic and abiotic stresses.

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References

1 Gorg A, Weiss W& Dunn M J (2004) *Proteomics* 4, 3665-3685

2 Wang W, Scali M, Vignani R, Spadafora A, Sensi E, Mazzuca S & Cresti M (2003) *Electrophoresis* 24, 2369-2375

3 Carpentier S C, Witters E, Laukens K,Deckers P, Swennen R & Panis B (2005) *Proteomics* 5, 2497-2507

4 Gooding P S, Bird C & Robinson S P (2001) *Planta* 213, 748-757

5 Wuyts N, Waele D & Swennen R (2006) *Plant Physiol Biochem* 44, 308-314

6 Carpentier S C, Panis B, Vertommen A, Swennen R,Sergeant K, Renaut J, Laukens K, Witters E, Samyn B & Devreese B (2008) *Mass Spectrometry Rev* 27, 354-37

7 Saravanan R S & Rose J K (2004) *Proteomics* 4, 2522-2532

8 Song J, Braun G, Bevis E & Doncaster K (2006) *Electrophoresis* 15, 3144-315

9 Zhang L L, Feng R J & Zhang Y D (2012) *J Sci Food Agric* 10, 2106-2115

10 Marsoni M, Vannini C, Campa M, Cucchi U, Espen L & Bracale M (2005) *Vitis* 44, 181-186

11 Henriet C, Draye X, Oppitz I, Swennen R & Delvaux B (2006) *Plant Soil* 287, 359-374

12 Damerval C, Vienne D, Zivy M & Thiellement H (1986) *Electrophoresis* 7, 52-54

13 Hurkman W J & Tanaka C K (1986) *Plant Physiol* 81, 802-806

14 Rabilloud T (1998) *Electrophoresis*19, 758-760

15 Xu C, Xu Y & Huang B (2008) *Phytochemistry* 69, 38-48

16 Bradford M M(1976) *Anal Biochem* 72, 248-254

17 Blum H, Beier H& Gross H J(1987) *Electrophoresis* 8, 93-99

18 Panse V G & Sukhatme P V (1985) In: *Statistical methods for agricultural workers*, pp. 34-47, Indian Council of Agricultural Research, New Delhi

19 Nandakumar M P, Shen J, Raman B & Marten M R (2003) *J Proteome Res* 2, 89-93

20 Jellouli N, Salem A B, Ghorbel A & Jouira H B (2010) *J Integr Plant Biol* 52, 933-94

21 Wu F S, Wang M Y, Wu F S & Wang M Y (1984) *Anal Biochem*1 39, 100-103

22 Islam N, Lonsdale M, Upadhyaya N M, Higgins T J, Hirano H & Akhurst R (2004) *Proteomics* 4, 1903-1908

23 Sarry J E, Kuhn L, Ducruix C, Lafaye A, Junot C, Hugouvieux V, Jourdain A, Bastien O, Fieve J B, Vailhen D, Amekraz B, Moulin C, Ezan E, Garin J & Bourguignon J (2006) *Proteomics* 7, 2180-2198

24 Chevalier F, Martin O, Rofidal V, Devauchelle A D, Barteau S, Ommerer N & Rossignol M (2004) *Proteomics* 5, 1372-1381

25 Vincent D, Wheatley M D & Cramer G R (2006) *Electrophoresis* 27, 1853-1865

26 Zorb C, Schmitt S, Neeb A, Karl S, Linder M & Schubert S (2004) *Plant Sci* 167, 91-100