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OPTIMIZATION OF PROTEIN ISOLATION FROM DATE PALM PLANTS AND ITS UTILIZATION IN DIFFERENTIAL PROTEOMICS ASSOCIATED WITH RED PALM WEEVIL INFESTATION

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Differential proteomics is considered as one of the most powerful tools for evaluating relative expression of molecular moieties either in plants or animals. The present study focuses first on optimizing a rapid and sensitive protocol for the isolation of high quality protein to be used in two dimensional gel electrophoresis (2DE) from date palm samples, and then comparing differentially expressed peptides associated with red palm weevil (RPW) infestation of this plant using uninfested plants as control. Among the several methodologies we used for optimization, it was revealed that Phenol/ SDS extraction followed by methanolic ammonium acetate precipitation (designated as protocol 3 in this study) yielded high quality protein. Moreover, 2DE protein analysis demonstrated both qualitative and quantitative differences between control and infested date palm samples. Our differential proteomic methodologies showed 22 differential spots having modulation level ≥1.5 fold. Subsequently, these differentially expressed peptides were subjected to MALDI-TOF peptide mass fingerprinting analysis for their characterization. The 11 peptides identified through these methodologies fall into three major functional groups including stress/defense (5), photosynthesis (2), ion transport (1) related proteins and three with other functions. Our data revealed that proteins related to date palm defense or stress response were up-regulated in infested samples while the proteins involved in photosynthetic activities were down regulated. The present results indicated that RPW infestation of date palm plants induced molecular changes manifested through differential expression of proteins. Differentially expressed peptides besides increasing our understanding relevant to RPW infestation will help us in developing methodologies for early detection of RPW infestation beneficial for curbing this problem in economically important date palm trees.
Keywords: Red palm weevil, date palm, proteomics, infestation

INTRODUCTION

The date palm, *Phoenix dactylifera* L. (Arecaceae, Arecales) is cultivated in tropical and subtropical regions of the world mainly in West Asia and North Africa between 10°N and 39°N in North hemisphere and between 5°S to 33° 51°S in the Southern hemisphere (Al-Khalifah *et al.*, 2013). About 3000 (Zaid, 1999) to 5000 (Bashah, 1996) date palm cultivars are planted in various parts of the world and serving people nutritional needs since times immemorial (Chandrasekaran and Bahkali, 2013).

The total world date palm production is 7.4 million tons and the Arab World is contributing 5.4 million tons annually (FAO, 2009). The Kingdom of Saudi Arabia is the third largest producer of fine quality dates (FAO, 2012) worldwide having 23 million date palm trees yielding about 970,488 tons of dates annually (Alhudaib *et al.*, 2007). Unfortunately, this valuable fruit crop is under severe attack by RPW (*Rhynchophorus ferrugineus*), the most destructive pest of the date palm tree (*Phoenix dactylifera*) (Aldawood and Rasool, 2011). According to an estimate, economic loss on the management and eradication of this deadly pest is up to $130 million annually in the Middle East at only 5% infestation in date palm plantation (El-Sabea *et al.*, 2009). Moreover, millions of dollars losses have been reported on coconut and other palm species (Faleiro, 2006).

In the past, several detection techniques, including visual inspections, acoustic sensors, sniffer dogs, and pheromone traps have been tested for the early detection of RPW infestations followed by removal of infested plants to curb further spread of this insect however, a quick and earliest detection procedure is still awaited. Recently, scientists are trying to identify the plant responses for detection of pathogenic infection or herbivore attack using proteomic approaches. For example, *Plutella xylostella* feeding on *Arabidopsis thaliana* leaves left proteins footprints on 2DE gel where 38 additional protein spots (out of 1100 spots) have been detected after infestation (Liu *et al.*, 2010).

Also, it has been reported that plants like humans have innate and adaptive defense responses when attacked by herbivore that induce direct and indirect damages (Kessler and Baldwin, 2002). Even oral secretions released into plant tissues by plant feeding insects elicit special acquired defensive responses in the plants (Felton and Tumlinson, 2008; Halitschke *et al*., 2001). Furthermore, insect regurgitates and other oral secretions also modulate plants defense proteins or stimulate release of volatile compounds (Korth and Dixon, 1997; Turlings *et al*., 1990). These volatile compounds help to protect infested plant against herbivores attacks through direct and indirect defense and tolerance reaction such as secretion of secondary metabolites (Kessler and Baldwin, 2002). Sometimes herbivore feeding induces proteinase inhibitors (PI) in plants that inactivate insect digestive enzymes thus starving insects to death (Tamayo *et al*., 2000).

Recently, proteomics approaches have been successfully used to investigate plant responses against pathogenic infection and herbivores feeding on them. Proteomic analysis of healthy and brittle leaf diseased date palm leaflets showed quantitative differences in many proteins. In differentially expressed proteins, Mn-binding PSBO and PSBP proteins were decreased, whereas, other proteins were increased in diseased samples (Marqués *et al*., 2011). Proteomic analysis of date palm responses to entomopathogenic fungi: *Beauveria bassiana, Lecanicillium dimorphum* and *L.* cf. *psalliotae,* was studied using 2D proteomic techniques. Results revealed that plant defense/ stress, photosynthesis and energy metabolism associated proteins were differentially expressed in entomopathogenic fungi affected date palm leaves as compared to healthy samples (Gómez-Vidal *et al*., 2009). Pea (*Pisum sativum*) responses to powdery mildew (*Erysiphe pisi*) were examined using 2DE and leaf proteins from control non-inoculated and inoculated susceptible (Messire) and resistant (JI2480) plants exhibited some quantitative and qualitative differences (Curto *et al*., 2006).

Major objective of the present study was to optimize protein isolation methodology from date palm barely used for proteomic studies in the past and observe molecular changes in the plant subsequent to infestation with one of the highly damaging insect of this plant, the RPW. We firmly believe that protein isolation methodology developed in our laboratory will be beneficial for several molecular studies of this plant to be ensued in future. Optimized protein expression methodology developed in this study was used for differential proteomics of plants infested with RPW. We observed a highly intriguing modulation of proteins associated with RPW infestation in date palm that could be utilized for early detection of infestation.

MATERIALS AND METHODS

***Date palm plants and infestation with RPW*:** Tissue cultured date palm plants of Khudry cultivar were obtained from Al Rajhi Tissue Culture Laboratory, Riyadh, Saudi Arabia and divided into 3 groups (each group having three replicates). Mechanical wounding and infestation with RPW to date palm was carried out as described previously (Lippert *et al*., 2007). Briefly, nine plants were divided into three groups each having 3 replicates. Group one was artificially infested with RPW larvae, second was artificially wounded whereas third was kept as control without any treatment. Artificial infestation of date palm was carried out by 5 second instar RPW healthy larvae introduced into the plant through making holes in the stem using drill machine with 6-mm size bit. Subsequently, the stem part of the plants was wrapped up with fine steel mesh.

***Protein extraction and SDS-PAGE*:** Leave samples of above treated (infested, non-infested) and control (without treated) date palm plants were taken after 3-days for protein extraction. The leaves were cut from the plants and rinsed with distilled water. After getting dried with blotting paper, the leaves were cut into small pieces using clean scissor. Samples were weighed 6 gm each and grinded to fine powder in liquid nitrogen using pestle and mortar. The leave samples were also chopped in moulinex blender (LM 209) prior to grinding in liquid nitrogen. Five existing protocols with some modifications were tried in order to identify a high protein yielding protocol with reduced number of steps. The protocols/ methods used in this study are provided in Table 1.

***Protocol 1. TCA-Acetone precipitation extraction*:** This procedure was modified from a published TCA-acetone precipitation protocol (Damerval *et al*., 1988). Two hundred mg ground tissue powder from date palm samples (leaves) was dissolved in 1 ml of TCA solution (10% w/v TCA in

Table 1. Comparative efficiency of different protocols for protein extraction from the date palm samples

	Protocols used for date palm protein extraction	Protein yield (µg/200mg)
1	TCA/ acetone/ DTT extraction and precipitation	220
2	Simple extraction buffer/ DTT and acetone precipitation	30
3	Phenol/ SDS extraction with methanolic ammonium acetate precipitation	810
4	Phenol/ buffer with methanolic ammonium acetate precipitation	792
5	Acetone/ TCA washing/ Phenol/ SDS extraction with methanolic ammonium acetate precipitation	756

acetone with 0.07% 2-mercaptoethanol) and incubated at -20°C for 1 hour. Pellet was recovered by centrifuging at 10,000 x g for 20 min at 4°C. The supernatant was removed, and proteins were washed by adding one ml of ice-cold acetone containing 0.07%, 2-mercaptoethanol (twice). Samples were stored at -20°C for at least 30 min. Pellet was recovered by centrifuging at 10,000 x g for 20 min between washes. Supernatants were discarded, and pellets were dried at room temperature. Dried pellet was solubilized in SDS buffer for SDS PAGE analysis.

***Protocol 2. Simple buffer extraction*:** In this method, a total of 200 mg ground tissue powder was resuspended in 2 ml extraction buffer (50 mM Tris-Cl pH 8.8, 5 mM EDTA, 20 mM DTT, 100 mM KCl). Each sample was grinded for 30 min to enhance the extraction of protein. Cell debris removed by centrifuging at 10,000 x g for 20 min at 4°C. The supernatant was transferred to new 15 ml falcon tube, and proteins precipitatedby adding 5 volume of 100% ice-cold acetone. Samples were stored at -20°C for at least 2 hours and then centrifuged at -20°C for 20 min. Pellets were washed twice with 5 volume of 80% acetone. Each time, sample was kept at -20°C for 30 min and recovered by centrifuging at 10,000 x g for 20 min. After discarding supernatant, pellet was dried at room temperature and solubilized in SDS buffer.

***Protocol 3. Phenol-SDS extraction*:** In this protocol, proteins were extracted using phenol/SDS extraction followed by methanolic ammonium acetate precipitation (Wang *et al*., 2003). For this, 200 mg powder was re-suspended in 1 ml phenol (Tris-buffered, pH 8.0) and 1 ml dense SDS buffer [30% w/v sucrose, 2% w/v SDS (Sigma), 0.1 M Tris-HCl, pH 8.0, 5% v/v 2-mercaptoethanol]. The blend was mixed thoroughly by vortexing and then centrifuged for 5 minutes at 10000 xg at 4°C. The upper phenol phase was collected carefully without disturbing interphase and precipitated with five volumes of cold 0.1 M ammonium acetate in methanol. The mixture was incubated at -20°C for 30 min. Precipitated proteins were recovered by centrifugation at 1000 xg for 5 min at 4°C and then washed two times with cold methanol solution containing 0.1 M ammonium acetate and two times cold 80% v/v acetone. Each time, protein pellet was recovered by centrifugation at 8000 xg for 5 minutes. Protein pellet was air-dried at room temperature for 1 hour.

***Protocol 4. Phenol-simple buffer extraction*:** In this method proteins from date palm leave samples were extracted using phenol-simple buffer extraction followed by methanolic ammonium acetate precipitation. This method was described previously for proteomic studies by Hurkman and Tanaka (1986). For protein extraction, the 200 mg powder was resuspended in 1 ml phenol (Tris-buffered, pH 8.0) and 1 ml extraction buffer (50 mM Tris-Cl pH 8.8, 5 mM EDTA, 20 mM DTT, 100 mM KCl). The mixture was mixed thoroughly by vortexing and then centrifuged for 5 min at 10000 xg at 4°C. The upper phenol phase was transferred to new falcon tube and lower phase was again extracted with 1 ml phenol and 1 ml extraction buffer. The upper phase was again collected after centrifugation and mixed with above collected phenolic phase. Protein was precipitated with five volumes of cold 0.1 M ammonium acetate in 100% methanol. The mixture was incubated at -20°C for 1 hour. Precipitated proteins were recovered by centrifugation at 1000 xg for 20 min at 4°C and then washed two times with cold methanol solution containing 0.1 M ammonium acetate and two times cold 80% v/v acetone. Each time, protein pellet was kept at 20°C for 30 min and recovered by centrifugation at 8000 xg for 5 minutes. Protein pellet was air-dried at room temperature for 1 hour.

***Protocol 5. TCA/ acetone/ phenol/ SDS buffer extraction*:** Proteins from date palm leaves were extracted using TCA/acetone/ phenol/ SDS extraction with methanol/ ammonium acetate precipitation as previously described by Gomez *et al.* (2008). Before protein extraction, 200 mg ground tissue (three replicates) of each sample was re-suspended in 5 ml ice cold acetone and insoluble materials were recovered by centrifugation at 5000 xg at 4°C. The pellet thus obtained was sequentially rinsed with ice-cold 10% w/v TCA in acetone (five times), cold aqueous 10% w/v TCA (three times) and finally cold 80% v/v acetone (three times). Each time pellet was recovered by centrifugation at 5000 xg at 4°C for 5 min. The pellet was dried for at least 1 hour at room temperature and then used for protein extraction. For protein extraction, dried pellet was re-suspended in 1 ml phenol (Tris-buffered, pH 8.0; and 1 ml dense SDS buffer (30% w/v sucrose, 2% w/v SDS (Sigma), 0.1 M Tris-HCl, pH 8.0, 5% v/v 2-mercaptoethanol. The blend was mixed thoroughly by vortexing and then centrifuged for 5 min at 10000 xg at 4°C. The phenol phase was collected and precipitated with five volumes of cold methanol plus 0.1 M ammonium acetate at -20°C for 30 min. Precipitated proteins were recovered by centrifugation at 1000 xg for 5 min at 4°C and then washed three times with cold methanol solution containing 0.1 M ammonium acetate and cold 80% v/v acetone. Each time, protein pellet was recovered by centrifugation at 8000 xg for 5 min. Protein pellet was air-dried at room temperature for 1 hour.

***Sample preparation and SDS-PAGE analysis*:** For total protein analysis on SDS-PAGE, aliquot of each sample was suspended on 100 mM Tris buffer (pH 8.0) and then mixed with equal volume 2X SDS-reducing buffer (100 mM Tris-Cl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol and 200 mM mercaptoethanol). One dimension PAGE (12%) as described previously by Tufail *et al.* (2006) was employed to analyze the sample for total protein analysis. Mini VE (GE healthcare) apparatus was used for SDS-PAGE.

***2DE analysis*:** Two-dimensional gel electrophoresis (2DE) was carried out as previously described by Gómez-Vidal *et al.* (2008). Dried protein samples were solubilized in rehydration buffer containing chaotropic agent urea, alongside surfactants CHAPS and thiourea (7 M urea, 2 M thiourea, 2% CHAPS w/v, 2% DTT, 0.5% IPG buffer pH 3-11, 0.002% bromophenol blue) by shaking at 150 rpm for 1 h at 25°C. Protein concentration was measured using 2-D Quant kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocol using bovine serum albumin (BSA) as a reference standard. The samples were further cleaned for 2D using the 2D Clean-Up Kit (GE Healthcare, Little Chalfont, UK). The 450 µg extracted protein was loaded on 24 cm, pH 3-11, immobilized pH gradient strips. These strips were rehydrated for 16 hours at 20°C and then isoelectric focusing (IEF)was performed using Ettan IPGphor3 IEF unit (GE Healthcare, Bucks UK) at 50 µA per strip at 20°C according to following program: 1) step 400V for 1 hour, 2) Gradient 1000V for 1 hour, 3) Gradient 3500V for 1 hour, 4) Step 3500 V for 3 hour. These strips were then equilibrated for 15 min at room temperature under gentle agitation in an equilibration buffer (0.05 M Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS,) containing 20 mM DTT, followed by another 15 min equilibration in the same buffer containing 125 mM iodoacetamide. After equilibration, strips were then loaded on 12.5% SDS-polyacrylamide gels and separated using Ettan DALT six electrophoresis Unit (GE Healthcare, Little Chalfont, UK). After electrophoresis, gels were removed and stained with Colloidal Coomassie Brilliant Blue G-250 (CCB), scanned, and analyzed using Progenesis SameSpots software version 3.3 (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK.). One way ANOVA was used to calculate the fold difference values and P-values. A threshold level was set of 1.5 fold up- or down-regulation, at $p < 0.05$ level.

***Protein identification by mass spectrometry*:** Differentially expressed twenty two spots were cut, digested, analyzed by MALDI TOF-MS and identified by PMF, as previously described by Alfadda *et al.* (2013). In brief, excised protein spots were destained and digested with trypsin with 10 µl trypsin at a concentration of 2 ng/µl (Promega, USA) according to the recommended procedure by the manufacturer. The resulting tryptic digests were extracted by adding 50% acetonitrile/0.1% Triflouroacetic acid followed by drying to 10 µl using vacuum centrifugation. The 0.5 µl peptides was mixed with matrix (10 mg α-Cyano-4-hydroxycinnamic acid in 1 ml of 30% acetonitrile containing 0.1% TFA) and applied on MALDI- target and dried before MS analysis and after that subjected to MALDI-TOF-MS (UltraFlexTrem, Bruker Daltonics, Germany). Peptide mass fingerprints were processed using flex analysis software (version 2.4, Bruker Daltonics, Germany). MS data were interpreted by BioTools3.2 (Bruker Daltonics, Germany) in combination with the Mascot search algorithm (version 2.0. 04) against Swiss-Prot database for green plants. Protein spots were also counted manually and false background spots detected by software excluded from the analysis.

RESULTS AND DISCUSSION

Among the molecular techniques currently being used, proteomics has proved to be one of the most powerful and reliable for evaluating relative expression of molecular moieties in normal and diseased plants or animal tissues. Although proteomics and genomics in animal studies led to translational benefits, however, their usage in plant disease assessment have been relatively low. In the current study, we optimized protein isolation methodology from date palm tree followed by utilizing the highly quality isolated protein in evaluating differential proteomics responses in this fruit tree upon exposure with one of its highly injurious insect, the red palm weevil. The RPW is a very serious and rapidly spreading pest of the date palm trees in Gulf region and particularly in the Kingdom of Saudi Arabia that produces majority of export quality fruit from this tree. A bottleneck in controlling RPW infestation has been the early detection methodology. The infestation symptoms in the tree appear at later stages when it is too late to save the infested plant. Our data provides differential proteomics information from the RPW infested plants that could be utilized for developing highly sensitive molecular techniques to identify infested plants at their early stage of infestation.

***Protein extraction optimization and protein yield*:** Though an initial methodology for protein isolation from date palm has been described previously by Gómez-Vidal *et al.* (2008); however, differential proteomics need procedures that can provide high quality protein. Thus, firstly we optimized a rapid and high yielding protein extraction protocol from the date palm. To achieve the purpose, five previously existing protein extraction methods with some modification were used to extract and solubilize the date palm proteins for 2DE analysis. The extracted proteins were quantified using 2D quant kit (Table 1).

Relative quality and quantity of proteins isolated through various extraction protocols was confirmed by SDS-PAGE analysis. The protein profiling on SDS-PAGE revealed an interesting pattern of isolated proteins. Total protein contents were either much low (designated as protocol 2 in this study) or yield of high molecular weight proteins was low (designated as protocol 1) and also protein profile was not promising comparing other protocols (Fig. 1). Protein quantification revealed that the other three methods (protocols 3-5) yielded almost equal amount of protein from a plant sample of 200 mg. Relative amounts of protein from protocol 1 based on TCA acetone extraction and precipitation yielded almost 220 µg proteins/ 200 mg sample while protocol 2 based on simple buffer extraction gave low yield (only 30 µg). Other three protocols yielded almost

equal amount of proteins, ~800 µg/200 mg sample (Table 1). However, the protocols 3 and 4 yielded optimal protein and also had relatively reduced number of procedural steps and time. The main reason for low protein yield in protocol 1 (TCA-acetone method) compared to other phenol based methods (protocols 3, 4 and 5) could be due to the low solubility of protein pellet in SDS buffer as compared to phenol-based methods (Chen and Harmon, 2006). Furthermore, TCA-acetone protocol was suggested more effective with tissues from young plants and suitable for complex tissues (Saravanan and Rose, 2004; Carpentier *et al*., 2005; Wang*et al*., 2003). These quantitative results revealed that phenol-based methods gave higher protein yield as compared to TCA-acetone and simple buffer method. Of phenol-based methods, protocol 3 was finally chosen for further analysis because of its reduced number of steps and higher yield compared to others methods.

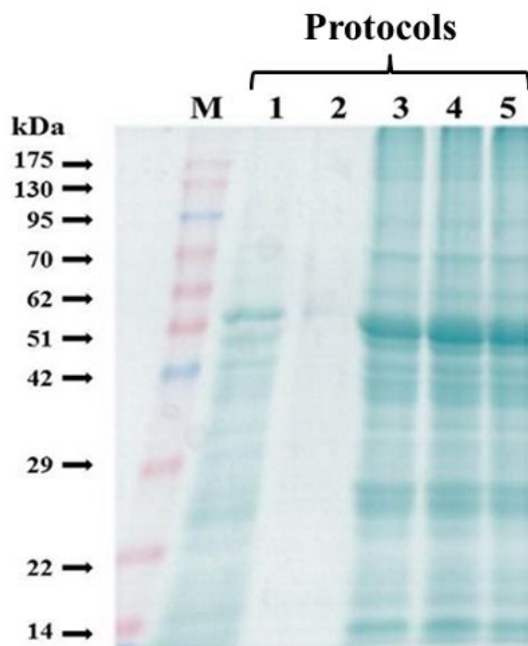

Figure 1. Comparative efficiency of five protocols (indicated in Table 1) for protein extraction from the date palm samples through SDS-PAGE. M stands for the protein molecular marker while lanes 1-5 indicate respective protocols used for the optimization of protein extraction.

***Evaluation of protein profiling by SDS-PAGE and 2DE*:** After optimization of protein extraction procedure, the best selected method (phenol-SDS extraction method, protocol 3) was used to isolate proteins from control, infested and wounded date palm samples for differential expression profiling analysis using SDS-PAGE. Approximately, 10 µg aliquots of each sample was solubilized in SDS loading buffer and separated on 12.5 % SDS-PAGE before staining. Protein profile after staining with Coomassie brilliant blue G250 showed good reproducibility among replicates, consistent solubilization and reproducible extraction (Fig. 2). Also, the SDS-PAGE data confirmed that protein profile isolated from different samples was consistent among the samples and replicates, however, SDS-PAGE failed to reveal the differential diagnostic bands (Fig. 2).

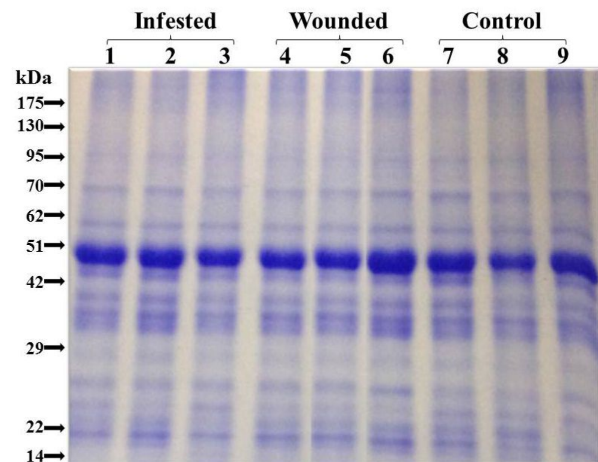

Figure 2. Comparative protein expression profiling of the control, infested and wounded date palm samples using SDS-PAGE. Lanes 1-3 represent total cell proteins from 3-infested replicates, while lanes 4-6 represent proteins from wounded date palm samples, and lanes 7-9, represent proteins from control date palm samples.

***Differential proteomics analysis*:** Protein expression profiles were compared in RPW infested date palm samples with uninfested controls (artificially wounded plants) and control. The extracted proteins were initially quantified using 2D quant kit after solubilizing in 2D-rehydration buffer. Each sample was evaluated by 2DE to compare differences among control, infested and wounded samples. The 2DE gels were scanned using Biometra Gel Documentation System (Biometra, Goettingen, Germany) and the protein spots were detected and analyzed using Progenesis Samespots software. On average, 227 proteins spots were detected in each gel using 24 cm IPG strip, pH 3-11 by image analysis. The statistical analysis of the gels was carried out between control vs infested, control vs the wounded and wounded vs infested. When proteins spots were compared in these combinations, majority of protein spots expression was unchanged as per threshold defined in our study. There were 22 spots showing statistically significant differences ($p \leq 0.05$) and showing more than1.5-fold modulation. Data generated is depicted in the Venn diagram (Fig. 3); 11 spots appeared

to be increased in abundance in infested in relation to the control and wounded. Five protein spots appeared to be increased in wounded samples compared to control and infested, 6 appeared to be increased in control compared to wounded and infested. According to our knowledge this report is highly unique in nature as far as date palm proteomics is concerned.

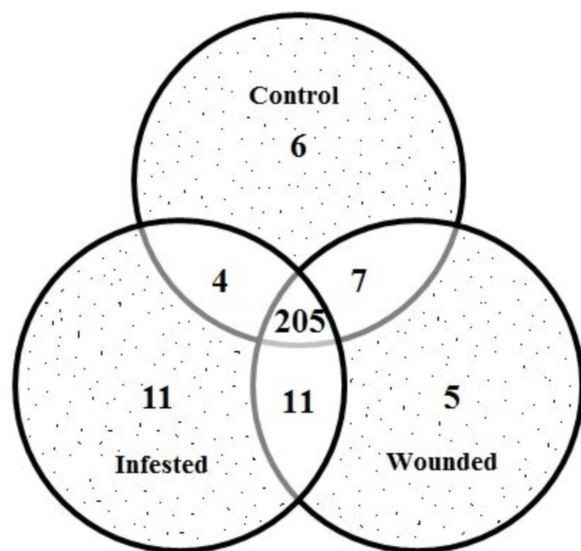

Figure 3. Venn diagram for the relative distribution of proteins spots in control, mechanically wounded and RPW infested date palm samples. The non-overlapping segment of diagram represent the number of proteins which were significantly up-regulated (>1.5-fold) in the corresponding group when compared with the other two groups. The overlapping region between any two groups represents the number of proteins spots significantly up-regulated (>1.5-fold) compared to the third one. While the central overlapping region depicts the protein spots where no any statistically significant change in up or down regulation was observed.

***Identification of differentially expressed peptides by mass spectrometry*:** Basic proteomics coupled with mass spectrometry has helped to pinpoint exactly molecular moieties modulated subsequent to artificial intervention or infestation in plants. To proceed further with the identification of differentially expressed peptides according to our predefined threshold criterion preparative gel was run with equal quantity of each protein. The gel was subsequently stained with colloidal Coomassie blue G-250 and imaged. Differentially modulated 22-protein spots (with 1.5 fold change in intensity) were selected, manually excised very carefully from preparative gel followed by trypsin digestion before subjecting them to MS analysis (Fig.4).

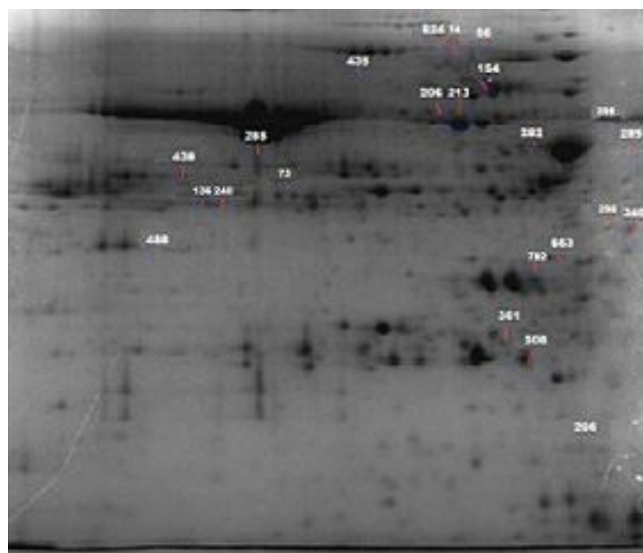

Figure 4. Reference gel showing differentially expressed spots used for Mas Spectrometric analysis.

Data generated from MS of differentially expressed peptides was processed by BioTools 3.2 (Bruker Daltonics, Germany) in combination with the Mascot search algorithm (version 2.0.04) against green plants database. Of the 22-differentially expressed spots analyzed by MS, we were able to match only 11-proteins (50%) in the existing protein dataset whereas for the remaining spots either low score or no hits were observed. This observation is quite intriguing and also expected as the date palm proteomics is in quite infancy and several new proteins will be added to the plant proteins database. Table 2 provides complete information about the potentially identified protein spots including spot number, Uniprot accession number, protein description, function, theoretical pI, molecular weight, protein coverage (%), score, and matching organism for the differentially expressed proteins. All identified proteins have shown homology to other species mainly *Zea mays* (Maize), *Oryza sativa* (Rice), *Solanum demissum, Solanum tuberosum L*., Palm tree, *Mesembryanthemum crystallinum, heterophylla* (White cedar), and *Arabidopsis thaliana*. The percentages of sequence coverage of the identified proteins were 20-47%. Among 11-proteins matched in the plants proteins database 10-proteins increased in infested compared to control. One heat shock protein, 2-Cys peroxiredoxin BAS1, chloroplastic, Oligopeptide transporter 3, and Ferredoxin-NADP reductase, chloroplastic were specifically increased in infested compared to control. Identified proteins were classified into three functional groups based on their main biological process: Stress and defense related protein (46%), proteins involved in Photosynthetic activities (18%), and ion transport proteins (9%), and others (27%) and have been shown in Fig. 5.

Table 2. Differentially expressed proteins identified by Mass Spectrometry in date palm associated with RPW infestation

S. No.	FC (I)	FC (W)	Accession (Uniprot)	Protein description	Function	pI	MW	Coverage %	Score	Organism
782	1.63↑	0.92↓	Q6ER94	2-Cys peroxiredoxin BAS1, chloroplastic	Stress response detoxification	5.67	28307	37	65	*Oryza sativa* (Rice)
488	3.45↑	2.45↑	P41343	Ferredoxin--NADP reductase, chloroplastic	Stress response	8.54	41322	32	93	*Mesembryanthemum crystallinum*
508	2.33↑	1.62↑	O23482	Oligopeptide transporter 3	Stress response	6.31	140853	20	62	*Solanum demissum* (wild potato)
361	1.93↑	1.55↑	Q60CZ8	Putative late blight resistance protein homolog R1A-10	Hyper sensitive response Defense	5.78	15312	20	57	*Solanum demissum* (wild potato)
435	1.89↑	1.53↑	P49087	V-type proton ATPase catalytic subunit A	Ion transport	5.89	62198	45	166	*Zea mays* (Maize)
74	2.15↑	1.38↑	P11143	Heat shock 70 kDa protein	Stress response	5.22	70871	32	130	*Zea mays* (Maize)
542	3.14↑	1.32↑	Q42572	DNA ligase 1	DNA repair	8.20	88427	21	62	*Arabidopsis thaliana*
206	1.69↑	2.17↑	P31542	ATP-dependent Clp protease ATP-binding subunit clpA homolog CD4B, chloroplastic	Protease Protein metabolic process	5.86	10246	34	128	*Solanum lycopersicum* (Tomato)
136	0.44↓	0.41↓	Q37282	Ribulose bisphosphate carboxylase large chain	Photosynthesis Calvin cycle	6.04	52482	27	72	*Tabebuia Heterophylla* (White cedar)
240	1.69↑	0.74↓	P28259	Ribulose bisphosphate carboxylase large chain	Photosynthesis Calvin cycle	6.33	52710	47	109	*Drymophloeus subdistichus* (Palm tree)
346	2.57↑	1.49↑	P54260	Aminomethyltransferase, mitochondrial	Plant metabolism Glycine cleavage	8.77	44648	40	60	*Solanum tuberosum* L.

Arrows indicate the proteins up (↑) and down (↓) regulations, FC = Fold change, I = RPW infested samples, W = Mechanically wounded samples, pI = Isoelectric point, MW = Molecular weight.

***Stress and defense associated proteins*:** Among the eleven characterized proteins, 5 (46 %) were recognized as defense and stress response proteins based on gene ontology classification.

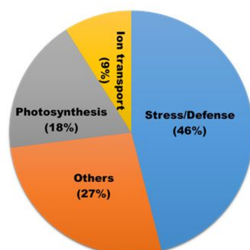

Figure 5. A Pie chart depicting the physiological classification of potentially identified proteins through Mass Spectrometry analysis.

The expression levels of these proteins in infested sample appeared to be increased compared to control. Relatively higher expression of proteins involved in defense and stress responses might be induced by the stresses associated with infestation. One of the stress-related proteins was heat-shock protein (Hsp) and coded by spot no.74, significantly accumulated in infested and wounded samples (2.15 and 1.38 folds in infested and wounded samples respectively). During stress the Hsps are usually up-regulated and their main function is to fold protein properly, and to stabilize proteins against heat or other stresses.

The other stress responsive proteins were identified from four spots (spots No: 782, 488 508 and 361) (Table 2). Protein identified from spot 782 was 2-Cys peroxiredoxin BAS1, chloroplastic, associated with stress responses and cells detoxification, and had shown high expression both in infested as well as in wounded date palm samples. Hydrogen peroxide produced in chloroplast serves as a signaling molecule that takes part in cellular communication (Apel and Hirt, 2004; Foyer and Noctor, 2000), especially for long distance (Karpinski *et al*., 1999). When level of H_2O_2 enhanced in response to different abiotic and biotic stresses, it may pose an oxidation threat to plant cells (Mittler *et al*., 2004). In order to balance the toxic and signaling activities of hydrogen peroxide the chloroplasts are equipped with 2-Cys peroxiredoxins. The peroxiredoxin is thiol-based peroxidases which reduce hydrogen and organic peroxides.

Moreover, when biotic agents elicit overproduction of reactive oxygen species, a corresponding overexpression of 2-Cys peroxiredoxins involved in the detoxification process anticipated. The second spot (no. 488) was identified as ferredoxin-NADP reductase, chloroplastic protein and related to stress response showed high expression in infested as well as wounded date palm samples. These proteins are located in the thylakoid membrane and their expression increases in response to oxidative stress. Upregulation of Ferredoxin in tobacco produces resistance to *P. syringae* and *Erwinia carotovora* (Huang *et al*., 2007). Ferredoxin-NADP reductase over-expression after biotic stress may predict their role in defense (Bilgin *et al*., 2010).

The other important protein identified (spot no. 508) was oligopeptide transporter 3 protein and upregulated both in infested and wounded date palm samples. In spruce *Picea sitchensis* genes associated with transportation processes oligopeptide transporter were up-regulated after weevil feeding (Ralph *et al*., 2006). Oligopeptide transporters are involved in the translocation of small peptides across cellular membranes including glutathione, glutamyl peptides, hormone-amino acid conjugates, peptide phytotoxins, and systemin inducing systemic signaling against herbivores attack (Stacey *et al*., 2002). Oligopeptide transporters were up regulated in grapes infested with leaf-galling *phylloxera* (Nabity *et al*., 2013). Moreover, differential expression pattern of oligopeptide transporters in rice seedlings exposed to abiotic and biotic stresses was also reported (Liu *et al*., 2012).

Another pathogen resistance protein (spot 361) identified as putative late blight resistance protein homolog R1B-10 and was also found to be up-regulated both in infested and wounded samples as we expected and is again in agreement to the previously published reports (Poupard *et al*., 2003; Tarchevsky *et al*., 2010). This protein is involved in providing some safeguards to the plant against pathogen and eventually stops the pathogen growth. The overexpression of this protein indicated that this protein may activate the specific downstream genes, thus preparing the plant for upcoming encounters.

Differentially expressed protein spots related to photosynthesis (spot No: 136 and 240) were identified as ribulose bis phosphate carboxylase large chain. These proteins have very close Mr and pI values or differ only very slightly and belong to the same functional family. The existence of such isoforms with slight difference in Mr and pI has been reported previously in date palm (Marqués *et al*., 2011; Sghaier-Hammami *et al*., 2009) and also in other species like *Arabidopsis* (Sghaier-Hammami *et al*., 2012). However, expression of these proteins is down-regulated in infested sample, as we expected, and this should not be surprising as many photosynthetic genes are reported to be down-regulated following insect or pathogen attacks and abiotic stresses (Bazargani *et al*., 2011; Bilgin *et al*., 2010; Nabity *et al*., 2009). The reduction of photosynthetic activity probably leads to trade off from growth to defense (Bilgin *et al*., 2008; Li *et al*., 2011; Zou *et al*., 2005).

Spots number 346 and 435 were identified as aminomethyltransferase, and mitochondrial and V-type proton ATPase catalytic subunit A proteins, respectively. Aminomethyltransferase, mitochondrial protein is also known as glycine cleavage system responsible for catalyzing glycine degradation (Walker and Oliver, 1986). V-type proton ATPase catalytic subunit A protein is responsible for acidifying a variety of intracellular compartments in eukaryotic cells (Persike *et al*., 2012). V-type ATPases are the large membrane protein complexes present in eukaryotic cells and acidify various intracellular compartments with the transport of protons across the membrane (Du *et al*., 2010). These ATPases generate a proton electrochemical gradient across vacuolar membrane Na+/H+antiporter, to compartmentalize Na+ into the vacuole (Chinnusamy *et al*., 2005), thus playing a key role in biological energy metabolism.

Spot 206 was identified as ATP-dependent Clp protease ATP-binding subunit ClpA homolog CD4B, chloroplastic. These proteases involve in chloroplast biogenesis (Adam *et al*., 2006) and up regulation of this protein in infested samples lead to enhance the activity of this protease for the formation and maintenance of a functional thylakoid electron transport. Our results are in agreement with those previously described (Olinares *et al*., 2011).

Date palm tree is mainly woody in nature and manifestation of stress responses associated with RPW infestation is opening new avenues of scientific research relevant to this historical plants mainly growing in Arabian Peninsula and several other parts of the world. Proteomics/genomics strategies will help in future selective cultivation of date palms besides saving them from insects and pests.

Overall, our study provides information regarding an optimized protocol for the isolation of high quality proteins from date palm tree to be used for proteomic studies and also set a paradigm for differential proteomics associated with infestation of this plant with highly injurious insect, the RPW. Among the proteins identified majority are stress related or involved in photosynthetic machinery. Limited proteomics data available from other plants also suggest similar changes in expression levels. It is quite possible that plant kingdom might have similar acquired defense response like in humans and observation still to be supported from future studies.

***Conclusions*:** Our report is highly unique as being the first on optimization of protein isolation from the date palm trees. The results of the present study demonstrate that Phenol/SDS extraction with methanolic ammonium acetate precipitation is the best technique for rapid and better protein harvest from the date palm samples. Moreover, twenty two

differentially expressed protein spots were recognized having intensity fold difference ≥1.5. The mass spectrometry analysis identified proteins related to stress/defense response, photosynthetic activity and some miscellaneous functions. Our results conclusively reveal that RPW infestation induce responses that regulates differential expression of proteins associated with defense, stress and photosynthetic systems of the palm tree. These differentially expressed proteins can be utilized for developing biomarkers for detection of RPW at an early stage of infestation.

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