Differential Proteomic Analysis of Date Palm Leaves Infested with the Red Palm Weevil (Coleoptera: Curculionidae)

Authors: Rasool, Khawaja Ghulam, Khan, Muhammad Altaf, Tufail, Muhammad, Husain, Mureed, Mehmood, Khalid, et al.

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Differential proteomic analysis of date palm leaves infested with the red palm weevil (Coleoptera: Curculionidae)

Khawaja Ghulam Rasool[1,2,], Muhammad Altaf Khan[3], Muhammad Tufail[1,2], Mureed Husain[1], Khalid Mehmood[1], Muhammad Mukhtar[4], Makio Takeda[2], and Abdulrahman Saad Aldawood[1]*

Abstract

The red palm weevil, Rhynchophorus ferrugineus (Oliv.) (Coleoptera: Curculionidae), is a highly damaging pest of palm trees worldwide. The infestation is highly concealed in nature. Hence, a highly sensitive and reliable early detection technique needs to be applied in the field for identification and treatment of the infested date palms to curtail further infestation. We have recently reported the differential proteomic analysis of the date palm stem tissues associated with the red palm weevil infestation. In this study, we examine the response of date palm infested with red palm weevil based on the leaf proteome expression changes detected using two-dimensional differential gel electrophoresis (2D-DIGE) followed by Matrix-Assisted Laser Desorption/Ionization–Time-of-Flight (MALDI-TOF). We observed qualitative and quantitative proteome differences between the control and weevil-infested date palm samples. The red palm weevil infestation induced specific responses attributable to weevil feeding, relative to artificially wounded trees (which were used as a control). Differential proteomics led to the identification of 32 red palm weevil infestation-specific protein spots ($P \leq 0.05$ having ≥ 1.5-fold modulation) that were further subjected to mass spectrometer analysis for identification and characterization. Proteins involved in plant stress and plant defense, photosynthesis, carbohydrate utilization, and protein degradation were affected in infested plants. The differentially expressed red palm weevil infestation-specific peptides can be used as biomarkers for the identification of early infestation with this insect in date palm trees. Moreover, our study demonstrates the potential use of proteomic strategies in diagnosing phyto-infestation caused by insect pests, diseases, and perhaps even for variety selection.

Key Words: Rhynchophorus ferrugineus infestation; proteins; differential expression; 2D-DIGE; MALDI-TOF

Resumen

El picudo rojo de la palma, Rhynchophorus ferrugineus (Oliv.) (Coleoptera: Curculionidae), es una plaga muy dañina de las palmeras en todo el mundo. La infestación está muy oculta en la naturaleza. Por lo tanto, se debe aplicar una técnica de detección temprana altamente sensible y confiable en el campo para la identificación y el tratamiento de las palmeras datileras infestadas para reducir infestaciones en el futuro. Recientemente hemos informado sobre el análisis proteómico diferencial de los tejidos del tallo de palmera datilera asociados con la infestación del picudo rojo de la palmera. En este estudio, examinamos la respuesta de la palmera datilera infestada con picudo rojo en base a los cambios en la expresión del proteoma foliar mediante electroforesis en gel diferencial bidimensional (2D-DIGE) seguida de Desorción/Ionización de Láser Asistida por Matriz-Tiempo-de-Vuelo (MALDI-TOF). Observamos diferencias proteómicas cualitativas y cuantitativas entre las muestras de control y las datileras infestadas de picudos. La infestación del picudo rojo indujo respuestas específicas atribuibles a la alimentación del picudo, en relación con los árboles heridos artificialmente (que se usaron como control). La proteómica diferencial resultó en la identificación de 32 sitios de proteínas específicas de la infestación de picudos rojos ($P \leq 0.05$ con modulación ≥ 1.5 veces mayor) que fueron sometidas adicionalmente a análisis de espectrómetro de masas para identificación y caracterización. Las proteínas involucradas en el estrés de la planta y la defensa de la planta, la fotosíntesis, la utilización de carbohidratos y la degradación de las proteínas se vieron afectadas en las plantas infestadas. Los péptidos específicos de la infestación del picudo de la palmera roja expresados diferencialmente se pueden utilizar como biomarcadores para la identificación de la infestación temprana con este insecto en palmeras datileras. Por otra parte, nuestro estudio demuestra el uso potencial de las estrategias proteómicas en el diagnóstico de la fito-infestación causada por plagas de insectos, enfermedades, y tal vez incluso para la selección de variedades.

Palabras Clave: infestación de Rhynchophorus ferrugineus; proteínas; expresión diferencial; 2D-DIGE; MALDI-TOF

[1]Economic Entomology Research Unit, Department of Plant Protection, College of Food and Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia; E-mails: gkhawaja@ksu.edu.sa (K. G. R.); mtufail@ksu.edu.sa (M. T.); mbukhsh@ksu.edu.sa (M. H.); kmehmood@ksu.edu.sa (K. M.); aldawood@ksu.edu.sa (A. S. A.)
[2]Organization of Advanced Science and Technology, Kobe University, Kobe 657-8501, Japan; E-mail: mtakeda@kobe-u.ac.jp (M. T.)
[3]Department of Plant Production, College of Food and Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia; E-mail: altafksu@gmail.com (M. A. K.)
[4]Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, Lebuhraya Tun Razak 26300 Gambang Kuantan, Pahang Darul Makmur, Malaysia; E-mail: mukhtar.muhammad@gmail.com (M. M.)
*Corresponding author; E-mail: gkhawaja@ksu.edu.sa

Red palm weevil, Rhynchophorus ferrugineus (Oliv.) (Coleoptera: Curculionidae), infestation has been reported in several parts of the world. The Kingdom of Saudi Arabia, the second largest producer of fine-quality dates (FAO 2011) is suffering major losses. Area under cultivation with date palm is 150,744 ha of land, with over 23 million trees, producing 970,488 tons of dates annually (Alhudaib et al. 2007). Unfortunately, this important fruit crop is threatened by this highly invasive pest.

Rhynchophorus ferrugineus was first recorded in the Persian Gulf region early in the 1980s (Abraham et al. 2001). An estimated 80,000 palm trees in Saudi Arabia are infested with red palm weevil, which poses a threat to surrounding plants (Mukhtar et al. 2011). Furthermore, infestation with red palm weevil has been reported in over 50% of the date palm-growing countries, including all those in the Middle East (Faleiro 2006). Damage to date palm trees by red palm weevil is caused principally by the insect larvae feeding within the trunk. The concealed feeding habit of the larvae makes it extremely difficult to detect early infestations. Eventually, severe damage to the internal tissues will result in death of the tree (Abraham et al. 1998). The weevil completes several generations per year within the same host; ultimately, the tree collapses (Rajamanickam et al. 1995; Avand 1996). Yield loss due to infestation can range from mild to severe (Gush 1997).

Pesticides application to infested date palm trees have been partially successful in controlling red palm weevil; however, there is still an urgent need for early detection and more effective and environmentally safer control measures. Several detection methodologies, including visual inspections, acoustic sensors (Potamitis et al. 2009), sniffer dogs (Nakash et al. 2000), and pheromone traps (Faleiro & Kumar 2008) have been tested to assist quarantine efforts and identify infestations at early stages; however, all methods developed thus far suffer from logistic and implementation issues. Efforts to identify environmentally safe biological management of weevils (Abdullah 2009; Guerri-Agullo et al. 2010), or early removal of infested plants thus curtailing further spread of this insect, are the safest options to be applied in the field.

In the post-genomic era, proteomics methodologies provide highly reliable assessment of change in living organisms. Proteome analysis of healthy palms, and those infected with brittle leaf disease, demonstrated quantitative variations in various proteins expressions (Marqués et al. 2011; Sghaier-Hammami et al. 2012). A number of proteins have been identified in Vitis vinifera L. (Vitaceae), Acca sellowiana (O. Berg.) (Myrtaceae), and Cyclamen persicum Mill. (Primulaceae), using differential proteomics, which differentiate responses to biotic and abiotic stresses (Winkelmann et al. 2006; Marsoni et al. 2008; Cangahuala-Inocente et al. 2009; Zhang et al. 2009). Moreover, date palm leaves infected with entomopathogenic fungi also revealed differential expression in plant defense-related proteins in comparison to healthy date palm leaf tissues (Gómez-Vidal et al. 2008).

Recently, we have reported optimization of protein isolation from date palm plants, and its use in differential proteomics associated with red palm weevil infestation (Rasool et al. 2014). Also, we have identified 32 proteins modulated in the date palm stem infested with red palm weevil using two-dimensional differential gel electrophoresis and mass spectrometry (2D-DIGE-MS). The identified proteins belonged to ion transport proteins, lipid biosynthesis, protein folding, plant defense, carbohydrate metabolism, lignin biosynthesis, cytoskeletal proteins, and a few others (Rasool et al. 2015).

In studies presented herein, we report differential proteome analysis of healthy and weevil-infested date palm leaves using a highly sensitive two-dimensional differential gel electrophoresis followed by Matrix-Assisted Laser Desorption/Ionization–Time of Flight (MALDI-TOF). Our results revealed significant and reproducible differences in date palm leaf peptides upon infestation with weevils that could be used to devise an early detection method for removing infested plants to control further spread of this insect.

Materials and Methods

MECHANICAL WOUNDING AND INFESTATION WITH RED PALM WEEVIL

Tissue-cultured date palm plants of the cultivar 'Khudry' used in this study were purchased from Alrajhi tissue culture laboratory, Riyadh, Saudi Arabia. Mechanical wounding and artificial infestation of date palm cultivars were carried out as described previously (Lippert et al. 2007). Briefly, plants were separated into 3 groups. Group 1 was artificially infested with red palm weevil larvae, group 2 was artificially wounded, and group 3 was without any treatment and served as a control. Artificial infestation (group 1) was made with 5 second-instar red palm weevil larvae by piercing holes in the stem using a drill with a 6 mm diam bit. The stem part of the treated plants was wrapped with fine steel mesh to prevent escape of larvae. The artificial wound (group 2) also was created with the same drill bit. These plants then were harvested after 3 d. Leaf tissues for differential proteomic study were taken from each plant and stored at –80 °C.

TOTAL PROTEIN EXTRACTION AND SDS-PAGE

Leaf samples from infested (weevils plus drilling), uninfested (no drilling or weevils), and artificially wounded (drilling but no weevils) date palm plants were taken 3 d post-infestation for protein extraction. Leaves were removed from the plant with sterile scissors and rinsed in distilled water. Proteins were extracted from control, infested, and wounded date palm leaves samples in triplicate (each treatment containing 3 plants) using the phenol/SDS extraction method as described previously (Gomez-Vidal et al. 2008) with slight variations (Rasool et al. 2014). In short, leaves were ground into fine powder in liquid nitrogen using mortar and pestle, and the 1.0 gram powder was subjected to protein extraction. The powder was suspended in 5 mL phenol and 5 mL dense SDS buffer (30% w/v sucrose, 2% w/v SDS (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), 0.1 M Tris-HCl, pH 8.0, 5% v/v 2-mercaptoethanol). The sample was mixed thoroughly by vortexing and then centrifuged for 5 min at 10,000 rpm at 4 °C. The upper phenolic phase was collected carefully without disturbing interphase and precipitated with 5 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 1,000 rpm for 5 min at 4 °C and then washed 2 times with cold methanol solution containing 0.1 M ammonium acetate and then 2 times with cold 80% v/v acetone. Each time, the protein pellet was recovered by centrifugation at 8,000 rpm for 5 min. The protein pellet was air-dried at room temperature for 1 h and an aliquot of each sample was suspended in 100 mM Tris buffer, pH 8.0, and then mixed with equal volume of 2× SDS-reducing buffer (100 mM Tris–Cl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 200 mM mercaptoethanol for SDS-PAGE analysis as described previously (Tufail et al. 2000; Laemmli 1970). Afterward, electrophoresis gel was stained with Coommassie brilliant blue G-250 with constant and gentle agitation overnight. Upon destaining, resolved protein fractions were visible in the form of light and dark bands.

TWO DIMENSIONAL (2D) DIFFERENTIAL GEL ELECTROPHORESIS

Protein samples were quantified by 2D quant kit (GE Healthcare, Bio-Sciences Corp., Piscataway, New Jersey, USA) and labeled with Cy-

Dye DIGE Fluor minimal dyes according to the manufacturer's recommendation (GE Healthcare) before electrophoresis. In short, 50 µg protein of each sample was labeled with 400 pmol CyDye Fluor minimal dyes. The control, artificially wounded, and infested samples were labeled alternatively with Cy3 or Cy5 (Table 1). The pooled internal standard, containing equal amount of protein amounts from all samples, was labeled with Cy2. After labeling, proteins samples were combined for electrophoresis according to the experimental design shown in Table 1. Five IPG Immobiline DryStrips 24 cm, pH 3 to 10 (GE Healthcare, Bucks, United Kingdom) were rehydrated overnight in 450 µl 2DE rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 0.5% pH 3 to 11 ampholytes (GE Healthcare), 1% DTT, trace bromophenol blue). After rehydration, samples were focused using an Ettan IPGphor IEF unit (GE healthcare) according to the manufacturer's conditions. Further DryStrip cover oil then was pipetted across the surface to cover the IPG strip. Isoelectric focusing was carried out using Ettan IPGphor IEF unit (GE Healthcare, Bio-Sciences Corp.) at 50 µA per strip at 20 °C. After IEF, strips were equilibrated in equilibration buffers (2% SDS, 75 mM Tris-HCl pH 8.8, 6M urea, 30% (v/v) glycerol, 0.002% bromophenol blue) containing DTT (100 mg per 10 mL buffer) or 2-iodoacetamide (250 mg per 10 mL buffer), respectively, before second dimension separation of proteins on 5 to 20% SDS polyacrylamide gels in low fluorescent glass plates. The equilibrated strip was placed on the 5 to 20% polyacrylamide gradient gel surface and sealed in place with molten agarose (1% (w/v) agarose, 0.002% (w/v) bromophenol blue in Tris-glycine SDS electrophoresis buffer). Double-distilled (dd) H_2O was pipetted onto the gel up to the top of the glass cassette. Gels were run in a Hoefer DALT tank using the Ettan DALT 6 vertical units (GE Healthcare, Little Chalfont, and United Kingdom) at 15 °C for 1W per gel for 1 h and then 2W per gel until the bromophenol blue dye reached the end of gel.

IMAGE ACQUISITION AND ANALYSIS

The gels were scanned with fluorescence gel scanner, Typoon imager (Trio) (GE Healthcare), using suitable wavelengths and filters for Cy2, Cy3, and Cy5 dyes as per the manufacturer's recommendations The gels images were examined using Progenesis SameSpot software version 3.3 (Nonlinear Dynamics Ltd, Newcastle Upon Tyne, United Kingdom). Differentially expressed peptides were assessed using normalized protein spots in the Cy5 and Cy3 channels compared to the internal standard (Cy2). Spots of red palm weevil infested and artificially wounded date palm samples also were compared to control samples. One-way analysis of variance (ANOVA) was used to analyze the fold difference values. Threshold level was fixed at 1.5 fold up- or down-regulation at $P < 0.05$ level.

PROTEIN IDENTIFICATION BY MASS SPECTROMETRY

Differentially expressed peptides ascertained through 2D gel electrophoresis were identified and characterized by running a preparative gel using a 700 µg total protein sample obtained by pooling all the samples present in the experimental design. The gel was stained with colloidal Coomassie blue for 5 d followed by rinsing in Milli Q water and stored until spots were picked and identified by mass spectrometry. The differential protein spots, after matching with reference gel, were excised manually from Coommassie-stained preparative gels and digested with trypsin for Matrix-Assisted Laser Desorption/Ionization–Time of Flight analysis according to previously described methods (Alfadda et al. 2013). Following trypsin digestion, peptides were extracted by adding 50% acetonitrile per 0.1% Triflouroacetic acid per 49.9% water (v/v) followed by drying. The 0.5 µl peptides were mixed with matrix (10 mg α-Cyano-4-hydroxycinnamic acid in 1 mL of 30% acetonitrile containing 0.1% Trifluoroacetic acid (TFA) and applied on the Matrix-Assisted Laser Desorption/Ionization-target and dried before being subjected to Matrix-Assisted Laser Desorption/Ionization–Time of Flight-mass spectrometry (UltraFlexTrem, Bruker Daltonics, Bremen, Germany) as described previously (Alfadda et al. 2013). Mass spectrometry data were interpreted by BioTools 3.2 (Bruker Daltonics) in combination with the Mascot search algorithm (version 2.0.04) for Swiss-Prot database for green plants. The identified protein was not accepted as correct until the Mascot score was above 60.

Table 1. Experimental design for two-dimentional differential gel electrophoresis. Three replicates from control, infested, and wounded protein samples were labeled and combined for two-dimentional differential gel electrophoresis.

Gel No.	Cy2	Cy3	Cy5
1	Pooled sample	Control 1	Wounded 1
2	Pooled sample	Control 2	Wounded 2
3	Pooled sample	Control 3	Infested 3
4	Pooled sample	Infested 1	Wounded 3
5	Pooled sample	Infested 2	

Results and Discussion

EVALUATION OF PROTEIN PROFILING BY TWO-DIMENSIONAL DIFFERENTIAL GEL ELECTROPHORESIS AND MASS SPECTROMETRY

Plants, like humans, are susceptible to pathogens like bacteria, mycoplasma, viruses, fungi, nematodes, and protozoa. Phytopathogens elicit defense responses at molecular levels in addition to physiological and anatomical changes in plants (Jones & Dangl 2006). This study was designed to investigate the date palm plant molecular responses subsequent to infestation with red palm weevil. This pest severely damages the date palm trees, ultimately leading to the death of the plant if not managed properly (Abraham et al. 2001).

Briefly, two-dimensional differential gel electrophoresis and mass spectrometry were used to identify differential proteome changes among control, weevil-infested, and mechanically wounded date palm samples. This powerful technique bypasses the limitation associated with conventional two-dimensional gel electrophoresis by introducing fluorescent reagents for protein labeling (difference gel electrophoresis or differential gel electrophoresis) as they provide higher sensitivity compared to normal staining; furthermore, spot volume quantification is greatly improved by the addition of an internal standard and running multiple samples in the same gel (Alfadda et al. 2013). We also compared protein expression profile of red palm weevil-infested date palm with uninfested controls to identify specificity of molecular changes associated with pest infestation.

Date palm plant leaves from control, infested, and artificially wounded plants were alternatively labeled with either Cy3 or Cy5 dyes, while the internal standard was consistently labeled with Cy2 dye, and the internal standard contained equal amounts of each sample present in the experimental design (Table 1). Five gels were run by multiplexing the 2 samples in each gel along with the internal standard, except that 1 gel contained a single sample with internal standard. This multiplexing makes the sample comparison simpler by eliminating the variability of 2DE profile, which was introduced by gel-to-gel rather than biological variation. Furthermore, incorporation of the Cy2 labeled internal standard helps inter-gel matching and improves accuracy of quantitation, thus minimizing the impact of gel-to-gel variation on quantification. The two-dimensional gel electrophoresis gels were scanned using fluorescence gel scanner, Typoon imager (Trio) (GE Healthcare). The representative gels of 2D-differential gel electrophoresis are shown in Figure 1, revealing date palm control sample labeled with Cy3 dye, date palm artificially wounded sample labeled with Cy5 dye, date palm sample infested with red palm weevil labeled with Cy3 dye, date palm sample pooled from all and labeled with Cy2 dye and overlay gel of control, infested, and wounded along with internal standard. Relative protein expression levels were compared among control, infested, and wounded samples. Differentially expressed protein spots were detected and analyzed using Progenesis SameSpot software. Our analyses revealed, on average, 745 proteins spots in each gel using 24 cm immobilized pH gradient (IPG) strip, pH to 11 by image analysis.

Statistical analysis of the gels was computed between control versus infested, control versus wounded, and wounded versus infested. Figure 2 shows the distribution of protein spots in a Venn diagram. In total, 745 spots were detected in each gel of control, wounded, and infested samples. Among the 745 peptides, expression levels of 713 were unchanged at a predetermined threshold level (≥ 1.5-fold modulation) that we adopted in our study. Thirty-two spots appeared to be differentially expressed in either control, infested, or wounded. Non-overlapping segments of the Venn diagram revealed that the number of significantly up-regulated spots in the infested sample compared to both wounded and control samples are 13 (10 were identified: 461; 483; 437; 976; 438; 833; 1,299; 558; 621; and 392), whereas number of significantly up-regulated spots in the wounded date palm sample compared to both control and infested were 8. Similarly, the number of up-regulated spots in the control sample compared to both wounded and infested were 11. The overlapping segment in both wounded and infested represented that the number of up-regulated spots in these samples compared to control are 12, whereas the number of up-regulated spots in both control and wounded are 15 compared with infested. We observed that significantly up-regulated spots in both control and infested compared to wounded are 5.

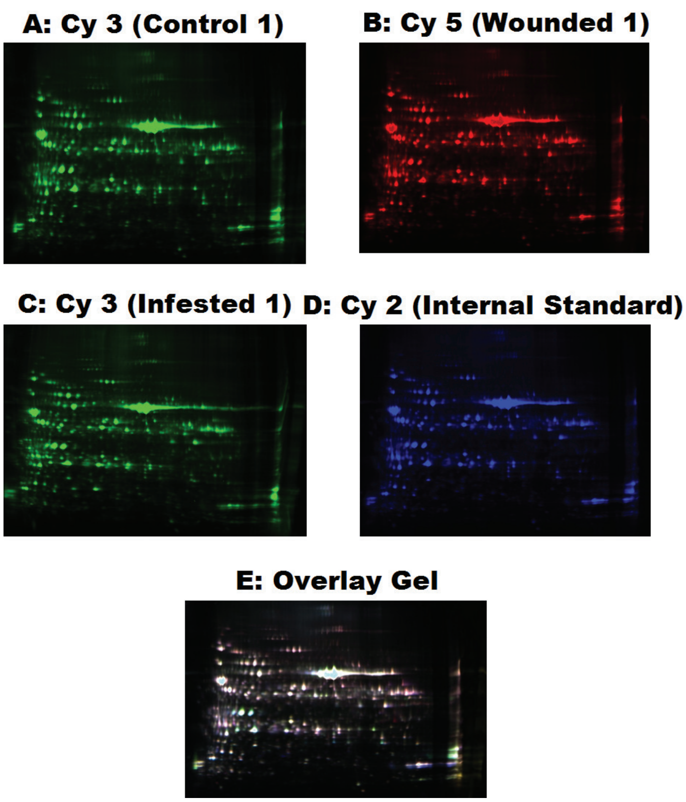

Fig. 1. Two-dimensional differential gel electrophoresis representative images of date palm proteins. The protein sample of control, wounded, infested, and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by two-dimensional differential gel electrophoresis followed by image scanning. (A) image of date palm control sample and labeled with cy3 dye; (B) image of date palm artificially wounded sample labeled with cy5 dye; (C) image of date palm sample infested with red palm weevil and labeled with cy3 dye; (D) image of date palm sample pooled from all and labeled with cy2 dye; (E) overlay gel of control, infested, and wounded along with internal standard.

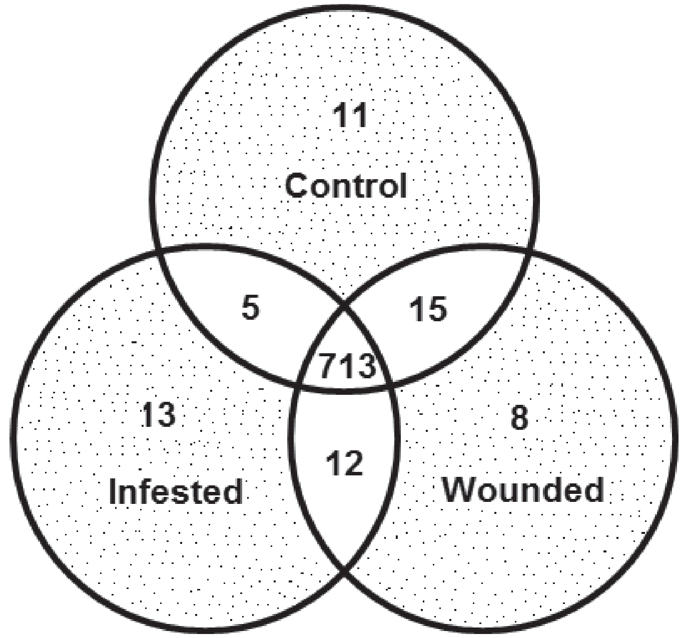

Fig. 2. Venn diagram for the relative distribution of proteins spots in control, mechanically wounded, and red palm weevil infested date palm samples. The non-overlapping segment of diagram represent the number of proteins which were significantly up-regulated (> 1.5-fold) in the corresponding group when compared with the other two groups. The overlapping region between any two groups represents the number of protein spots significantly up-regulated (> 1.5-fold) compared to the third one. The central overlapping region depicts the protein spots where no statistically significant change in up- or down-regulation was observed.

PROTEIN IDENTIFICATION BY MASS SPECTROMETRY

For the identification and characterization of differentially expressed peptides, a preparative gel was run using equal amounts of each sample, stained by Colloidal Coommassie blue G-250, and imaged. The 32 differential spots were excised from preparative gels, digested enzymatically with trypsin, and identified by mass spectrometry. The collected mass spectrometric data were processed by BioTools 3.2 (Bruker Daltonics) in combination with the Mascot search algorithm (version 2.0.04) against the green plants database, but only 20 differential spots were identified. Table 2 showed the spot number, Swiss-Prot accession number, protein description, function, theoretical pI, molecular weight, protein coverage (%), score, and matching organism for the differentially expressed proteins. All these differentially expressed peptides are of great interest in finding a highly reliable biomarker associated with early infestation of red palm weevil. To the best of our knowledge, this is the first report of its type reporting differential protein in date palm leaf tissues after red palm weevil infestation.

Differentially expressed proteins were matched with specific proteins of Arabidopsis thaliana Heynh. (Brassicaceae) (20%); Zea mays L. (Poaceae) (maize) (15%); Solanum lycopersicum L. (Solanaceae)

Table 2. Differentially expressed proteins between red palm weevil-infested, artificially wounded, and control date palm leaves identified by Matrix-Assisted Laser Desorption/Ionization–Time of Flight peptide mass fingerprinting after two-dimensional differential gel electrophoresis.

Spot No.	FC (I/C)	FC (W/C)	Accession (uniprot)	Protein description	Function	pI	M_w	Cover%	Score	Organism
Stress and defense related proteins 35%										
461	1.63↑	1.53↑	Q01899	Heat shock 70 kDa protein, mitochondrial	Stress response	5.95	72721	24	71	Phaseolus vulgaris (kidney bean)
483	2.22↑	2.06↑	P42755	Em protein H5	Stress response	5.14	10054	68	60	Triticum aestivum (wheat)
437	1.68↑	1.31↑	P11143	Heat shock 70 kDa protein	Stress response	5.22	70871	35	133	Zea mays (maize)
558	1.71↑	1.53↑	Q6L3X3	Putative late blight resistance protein homolog R1B-8	Hyper sensitive responseDefense	6.31	140853	20	62	Solanum demissum (wild potato)
621	1.93↑	1.65↑	Q60CZ8	Putative late blight resistance protein homolog R1A-10	Hyper sensitive responseDefense	5.78	153116	20	60	Solanum demissum (wild potato)
392	1.62↑	1.47↑	Q69QQ6	Heat shock protein 81-2	Stress response	4.98	80435	27	92	Oryza sativa subsp. Japonica (rice)
433	1.85↑	1.5↑	P11143	Heat shock 70 kDa protein	Stress response	5.22	70871	32	130	Zea mays (maize)
Photosynthesis and Calvin cycle 30%										
702	2.19↓	1.66↓	Q37282	Ribulose bisphosphate carboxylase large chain	PhotosynthesisCalvin cycle	6.04	52482	27	72	Tabebuia heterophylla (Pink trumpet tree)
528	1.36↓	1.85↓	P25829	Ribulose bisphosphate carboxylase large chain	Photosynthesis	6.44	52236	55	202	Calamus usitatus (palm tree)
661	1.21↓	1.89↓	Q7X999	Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic	Photosynthesis	6.62	51811	60	242	Larrea tridentate (creosote bush)
1299	1.71↓	1.54↓	Q0INY7	Ribulose bisphosphate carboxylase small chain, chloroplastic	Photosynthesis	9.04	19862	13	74	Oryza sativa subsp. Japonica (Rice)
635	1.97↓	1.62↓	Q33406	Ribulose bisphosphate carboxylase large chain	Photosynthesis	6.33	52746	29	87	Deppea grandiflora
438	1.68↑	1.53↑	P49087	V-type proton ATPase catalytic subunit A	Ion transport	5.89	62198	45	166	Zea mays (maize)
Carbohydrate biosynthesis 10%										
627	1.66↑	1.52↑	P55232	Glucose-1-phosphate adenylyltransferase small subunit, chloroplastic/ amyloplastic	Starch biosynthesis ATP binding	5.59	54105	28	65	Beta vulgaris (sugar beet)
955	1.70↓	1.55↑	Q9SZL9	Cellulose synthase-like protein D4	Cellulose synthesis	6.19	125713	14	60	Arabidopsis thaliana
Protein turnover 10 %										
347	1.77↑	1.61↑	P31542	ATP-dependent Clp protease ATP-binding subunit clpA homolog CD4B, chloroplastic	Protease Protein metabolic process	5.86	102463	34	128	Solanum lycopersicum (tomato)
395	2.07↑	1.65↑	Q9LZW3	U-box domain-containing protein 16	Protein turnover	6.82	74181	27	72	Arabidopsis thaliana
Proteins related with other function 15%										
1200	1.42↓	2.53↓	Q9ZS62	Phytochrome B1	Transcription regulation	5.78	126698	22	64	Solanum lycopersicum (tomato)
833	1.75↑	1.58↓	Q42572	DNA ligase 1	DNA repair	8.20	88427	21	62	Arabidopsis thaliana
976	1.89↑	1.59↑	Q9LZU9	Putative respiratory burst oxidase homolog protein J	Oxidase	9.48	103498	21	65	Arabidopsis thaliana

Arrows indicate the proteins up (↑) and down (↓) regulations, FC = Fold change, I = red palm weevil-infested samples, W = mechanically wounded samples, pI = Isoelectric point, MW = Molecular Weight.

(tomato) (10%); Solanum demissum Lindl. (Solanaceae) (nightshade) (10%); Oryza sativa subsp. Japonica Shig.Kato (Poaceae) (rice) (10%); Phaseolus vulgaris L. (Fabaceae) (bean) (5%); Triticum aestivum Host (Poaceae) (wheat) (5%); Tabebuia heterophylla (DC.) Britt. (Bignoniaceae) (pink trumpet tree) (5%); Calamus usitatus Blanco (Arecaceae) (palm tree) (5%); Larrea tridentate (DC.) Coville (Zygophyllaceae) (Creosote bush) (5%); Deppea grandiflora Schltdl. (Rubiaceae) (5%); and Beta vulgaris L. (Amaranthaceae) (sugar beet) (5%). The identified proteins were classified into the following groups (showing number of proteins in parenthesis): stress and defense (7), photosynthesis (6), carbohydrate biosynthesis (2), protein turnover (2), and peptides of unknown function (3), as depicted in Figure 3. The majority of differentially expressed proteins subsequent to infestation with red palm weevil were stress related, and several others potentially could be involved in the response to this damaging insect infestation (Table 2). Proteins significantly up-regulated in infested samples, as well as in wounded date palm samples, whereas some others (especially belonging to photosynthesis) were down-regulated.

The 7 differentially expressed peptides associated with red palm weevil infestation (spots 461, 483, 437, 558, 621, 392, and 433) fall in the category of stress related protein or related to plant defense (Table 2). A group of heat shock proteins was identified, and these proteins protect plants against various stresses (e.g., biotic and abiotic) by folding and unfolding of other proteins. These heat shock proteins were recognized according to their molecular weight. In our case, 2 differentially expressed peptides upon weevil infestation are heat shock peptides, Hsp 70KDa and Hsp 81-2. The Hsp81-2 also is named Hsp90. The heat shock protein family modulated in infested date palm was Hsp70 (spots 433,437, and 461) and Hsp 81-2 (392). The Hsp70 is an ATP-dependent molecular chaperone mainly induced by heat or other abiotic stresses, whereas others are not heat inducible, and are present under normal growth conditions in some tissues (Su & Li 2008). They facilitate the folding process of newly synthesized proteins and minimize aggregation (Lee & Schöffl 1996). A high level of heat shock protein spots 461, 437, and 433 were observed in infested samples. Similar findings have been reported in other plants as well (Fink 1999; Lee & Schöffl 1996). The heat shock proteins of 90 kDa (Hsp90) also are molecular chaperones that promote folding, structural maintenance, and regulation of a subset of proteins involved in transduction of signals, cell cycle control, etc. The Hsp90 also triggers growth and development of organisms involving conformational regulation of many regulatory proteins and protecting cells under stress (DeRocher & Vierling 1994). The Hsp90 may have some role in disease resistance (Kozeko 2010). Some recent proteomics works also identified several Hsp modulation in response to different stress conditions in pea (Lu et al. 2003; Curto et al. 2006) and triticale under a low nitrogen fertilization level (Castillejo et al. 2010a).

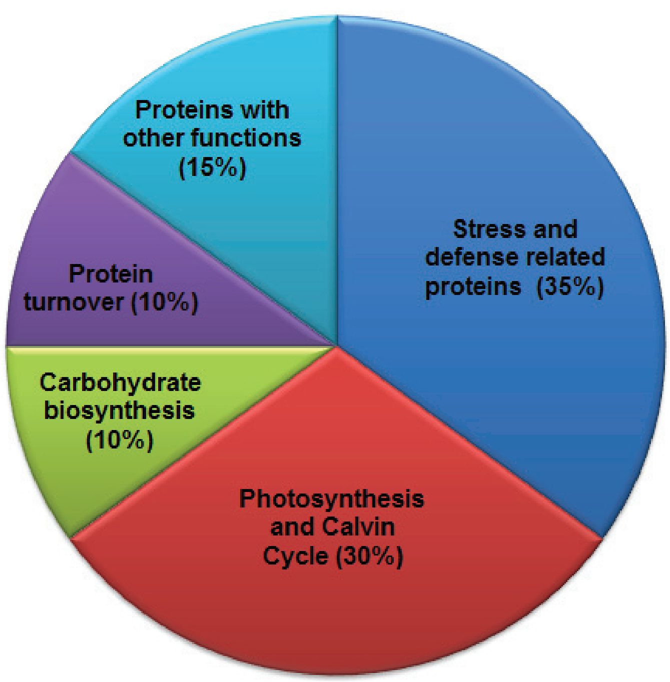

Fig. 3. A pie chart presenting the classification of identified proteins according to their biological functions, expressed in percentage.

Red palm weevil infestation led to up-regulation of 2 pathogen resistance proteins (spots 558 and 621) identified as putative late blight resistance protein homolog R1B-8 and putative late blight resistance protein homolog R1B-10. Previous reports have shown similar elevation of these proteins upon infestation or injury (Castillejo et al. 2010b; Poupard et al. 2003). These are the resistance proteins that guard the plant against pathogens, and ultimately stop the pathogens from inflicting damage. The up-regulation of defense-related disease resistance protein indicated that this protein may activate the specific downstream genes, thus preparing the date palm plant for upcoming deleterious challenges associated with weevil infestation.

The protein spots 702, 528, 635, 661, and 1,299 modulated upon infestation with red palm weevil are related to photosynthetic machinery and identified as ribulose bisphosphate carboxylase large chain. These proteins have very close Mr and pI values. These slight variations could be attributed to post-transcriptional modification, suggesting that these different members may belong to same functional family. The existence of these isoforms with slight difference in molecular weight and pI has been reported previously in date palm (Tarchevsky et al. 2010; Sghaier-Hammami et al. 2009) and in other species like Arabidopsis (Marqués et al. 2011). However, the expression level of these proteins is reduced in infested date palm samples, as was expected. There are many photosynthetic peptides showing reduced expression following attack by insects or pathogens and abiotic stresses (Nabity et al. 2009; Bilgin et al. 2010; Bazargani et al. 2011), because reduction of photosynthetic activity leads to change in resources from growth to defense (Bilgin 2008). Another reason for this reduction is the hyper-response, which leads to activation of numerous defense reactions as repression of photosynthesis-related genes during hyper-response, which also has been reported previously (Zou et al. 2005; Li et al. 2011).

A unique differentially expressed peptide spot (438) associated with red palm weevil infestation corresponds to vacuolar-type ATPases (V-type ATPases). These are the large membrane protein complexes in eukaryotic cells that acidify various intracellular compartments with the transport of protons through the membrane (Du et al. 2010). The ATPases generate a proton electrochemical gradient across the vacuolar membrane Na+/H+ antiporter, to compartmentalize Na+ into the vacuole (Chinnusamy et al. 2005), thus playing a key role in biological energy metabolism.

The differentially expressed peptide spot 347 was characterized as ATP-dependent Clp protease ATP-binding subunit clpA homolog CD4B, chloroplastic. These proteases contribute in chloroplast biogenesis through the degradation of certain proteins during environmental changes (Adam et al. 2006). Up-regulation of this protein in infested samples suggested the important role for the photosystem complexes, as increased activity of proteases is required for the formation and maintenance of a functional thylakoid electron transport. Our results are in agreement with those previously described (Olinares et al. 2011).

One of the differentially expressed peptides, i.e. spot 395, matched with U-box domain-containing protein 16 found in Arabidopsis thaliana. This protein is the component of ubiquitin ligase that is involved in regulatory mechanism of controlling various responses. Actually, ubiquitination is not only associated with proteasome-mediated protein degradation, but it also regulates protein function in a proteasome independent way. It changes protein localization, activity, and interactions (Schnell & Hicke 2003). U-box domains look like the RING finger domain (Aravind & Koonin 2000). The U-box domain is essential for the ubiquitin activity, and the significance of this has been shown in different ways. This domain interacts with E2 proteins (Pringa et al. 2001), and lack of ubiquitination activity after the deletion of the U-box domain also has been reported (Ohi et al. 2003; Stone et al. 2003; Zeng et al. 2004). In plants, ubiquitination plays an important role to control environmental and endogenous signals, including responses to pathogen attack (Hare et al. 2003). Moreover, the involvement of E3 ligase in plant pathogen response has been previously identified in Arabidopsis RING finger proteins RPM1-interacting protein2 (RIN2) and RIN3 (Kawasaki et al. 2005), and in rice (Oryza sativa) U-box spotted leaf11 (SPL11) (Zeng et al. 2004). The up-regulation of this protein in response to infestation suggests the activation of the date palm defensive role. This ubiquitin protein ligase also plays a vital role in the regulation of a variety of cellular functions including cell cycle, transcription development, signal transduction, and nutrient sensing (Jonkers & Rep 2009). In addition to this, it recently has been reported that the proteolytic function of the ubiquitin-proteasome system regulates the virulence of pathogenic fungi (Liu & Xue 2011). Therefore, appearance or up- or down-regulation of ubiquitin ligase in infested and wounded proteins suggests its strong role in infestation, and has potential to serve as a biomarker in early detection.

Spot 627 exhibited homology to glucose-1-phosphate adenylyl transferase small subunit, and has a regulatory role for the biosynthesis of starch. Spot 955 showed homology to cellulose synthase-like protein D4 of Arabidopsis thaliana (Q9SZL9) and is supposed to be a Golgi-localized beta-glycan synthase that polymerizes the backbones of noncellulosic polysaccharides (hemicelluloses) of plant cell walls. Spot 833 belongs to DNA ligase 1 and is matched with Arabidopsis thaliana, involved in sealing nicks in double-stranded DNA during DNA replication, DNA recombination, and DNA repair. Our proteomic study revealed that 1 protein matching with putative respiratory burst oxidase homolog protein J, a calcium-dependent NADPH oxidase responsible for superoxide generation. We believe that upon pathogen attack, the earliest cellular response in plants is an increase in reactive oxygen species, known as oxidative burst, and this in turn leads to the activation of local and systemic resistance responses (Mendoza 2011), resulting in cell wall reinforcement, programmed cell death, and expression of defense genes. The superoxide radicals produced are converted into H_2O_2 that triggers the hyper-response in plants to kill the pathogen, and moreover it induces the transcription of various resistance genes (Mellersh et al. 2002).

Our present study on differential proteomic analysis of date palm leaves is an extension of our previous studies where we have reported the proteins modulated in the date palm stem associated with red palm weevil infestation (Rasool et al. 2015). Both studies have displayed a very clear modulated response of various unlike proteins except 2 proteins, the heat shock 70kDa and V-type proton ATPase catalytic subunit A, which were found up-regulated in both stem and leaf tissues. The up-regulation of V-type proton ATPase catalytic subunit A was 1.68- and 5.31-fold in leaf and stem tissues, respectively, when compared with the control treatments. It has been reported that V-type proton ATPase catalytic subunit A is principally responsible for acidifying various intracellular compartments in eukaryotic cells (Sun-Wada et al. 2003). We assume that an over-regulation of this protein (in both leaf and stem tissues) is attributed to the red palm weevil infestation that leads toward acidic pH in date palm (KGR, unpublished data), and has a possibility to be used as a molecular marker for early detection of this weevil in date palms; however, it needs further characterization and validation of these results. Moreover, heat shock 70kDa proteins are commonly present in both plant and animal cells. They were originally reported as heat shock-associated proteins (Ritossa 1962) but later described to be induced by various kinds of stresses such as biotic and abiotic stresses (Boston et al. 1996; Vierling 1991). The heat shock 70kDa protein could be another possible option, because of its up-regulation in both leaf and stem tissues, for developing molecular markers but needs further investigation.

Although our objective was to determine red palm weevil specific molecular responses, it was interesting to note that similar molecular moieties are up-regulated in artificial wounding, as well as in red palm weevil infestation. However, relative modulation (down-regulation or up-regulation) was quite different, although we could not retrieve any unique differential protein related to weevil infestation. As such, we have to further characterize these responses, especially different spatial and temporal responses of weevil infestation. We need to establish certain baselines of proteomic changes for characterizing weevil-specific molecular changes. We believe that the protein molecules displaying modulated response, especially those with up-regulated expression patterns in infested date palm samples, will be helpful in developing diagnostic molecular markers for early detection of red palm weevil infestation beneficial for manipulating this crucial problem of date palm wreckage. But this needs further comprehensive research work, including spatial and temporal protein expression that was not possible within our limited budget. Proteomic work involves advanced equipment, as well as very expensive diagnostic procedures and chemical reagents.

Infestation by this highly damaging insect is a major threat in date palm-growing countries including the Kingdom of Saudi Arabia, and removal of infested trees has been recognized as the most effective tactic to prevent further spread of this insect. We have identified several molecular moieties to be used in the future for developing a highly sensitive, early infestation-detection molecular test to be used in screening for red palm weevil-infested date palm trees. Furthermore, our study has opened avenues for using proteomic strategies in diagnosing phyto-infestations caused by insect pests, diseases, and for plant varietal selection, including several desirable traits identified in plants.

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Disclosure

We declare that no author has any commercial or associative interest that represents a conflict of interest in connection with the subject of this manuscript.

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