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

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

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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 ≤ 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 electrotérfosis en gel 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 ≤ 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

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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.

Rynchophorus 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 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-Aguillo 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 inflicted 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; Canga et al. 2009). 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.

**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% w/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% w/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 Coomassie 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 recommenda-
tion (GE Healthcare) before electrophoresis. In short, 50 µg pro-
tein of each sample was labeled with 400 pmol CyDye Fluor minimal
dyes. The control, artificially wounded, and infested samples were la-
beled alternatively with Cy3 or Cy5 (Table 1). The pooled internal stan-
dard, 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 Health-
care, 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 Dry-
Strip 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% polyacryl-
amide 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) H₂O 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 im-
ager (Trios) (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 nor-
malized protein spots in the Cy5 and Cy3 channels compared to the in-
ternal 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 elec-
trophoresis 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 spectrom-
etry. The differential protein spots, after matching with reference gel,
were excised manually from Coomassie-stained preparative gels and
digested with trypsin for Matrix-Assisted Laser Desorption/Ioniza-
tion—Time of Flight analysis according to previously described meth-
ods (Alfadda et al. 2013). Following trypsin digestion, peptides were
extracted by adding 50% acetonitrile per 0.1% Trifluoroacetic acid per
49.9% water (v/v) followed by drying. The 0.5 µL peptides were mixed
with matrix (10 mg α-Cyano-4-hydroxycomnic 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 be-
fore 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-Prote database for green plants. The identified protein was not
accepted as correct until the Mascot score was above 60.

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, my-
coplasm, 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 sub-
sequent to infestation with red palm weevil. This pest severely dam-
ages 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 electrophore-
sis 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 com-
pared 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 multiplex-
ing makes the sample comparison simpler by eliminating the variability
of 2DE profile, which was introduced by gel-to-gel rather than biologi-
cal variation. Furthermore, incorporation of the Cy2 labeled internal
standard helps inter-gel matching and improves accuracy of quantita-
tion, thus minimizing the impact of gel-to-gel variation on quantifica-
tion. The two-dimensional gel electrophoresis gels were scanned using

Table 1. Experimental design for two-dimentional differential gel electrophore-
sis. 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     | Control 1  | Wounded 1  |
| 2       | Pooled     | Control 2  | Wounded 2  |
| 3       | Pooled     | Control 3  | Infested 3 |
| 4       | Pooled     | Infested 1 | Wounded 3  |
| 5       | Pooled     | Infested 2 |            |
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; 383; 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.

**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)
| Spot No. | FC (I/C) | FC (W/C) | Accession (uniprot) | Protein description | Function | pI | MW | 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↑ | 1.3↑ | 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 R18-8 | Hyper sensitive response | 6.31 | 140853 | 20 | 62 | Solanum demissum (wild potato) |
| 621 | 1.93↑ | 1.65↑ | Q6GC28 | Putative late blight resistance protein homolog R1A-10 | Hyper sensitive response | 5.78 | 153116 | 20 | 60 | Solanum demissum (wild potato) |
| 392 | 1.62↑ | 1.47↑ | Q69QG6 | 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 | Photosynthesis | 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 utilis (palm tree) |
| 661 | 1.21↓ | 1.89↑ | Q7X999 | Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic | Photosynthesis | 6.62 | 51811 | 60 | 242 | Larrea tridentata (creosote bush) |
| 1299 | 1.71↓ | 1.54↓ | Q0NY7 | 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 adenyltransferase small subunit, chloroplastic/ amyloplastic | Starch biosynthesis | 5.59 | 54105 | 28 | 65 | Beta vulgaris (sugar beet) |
| 955 | 1.70↓ | 1.55↓ | Q9S2L9 | Cellulose synthase-like protein D4 | ATP binding | 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 CD48, chloroplastic | Protease Protein metabolic process | 5.86 | 102463 | 34 | 128 | Solanum lycopersicum (tomato) |
| 395 | 2.07↑ | 1.65↑ | Q9LW3 | 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↓ | Q5Z562 | 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↑ | Q9LZJ9 | 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.
spots 461, 437, and 433 were observed in infested samples. Similar aggregation (Lee & Schöffl 1996). A high level of heat shock protein (Hsp) plays a role in the folding process of newly synthesized proteins and minimizes protein misfolding and unfolding of other proteins. These heat shock proteins were recognized according to their molecular weight. In our case, 2 differentially expressed peptides were identified: Hsp 70KDa and Hsp 81-2. The Hsp81-2 also is named Hsp90. The Hsp90 may have some role in disease resistance (Kozeko 2010). 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 the 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 (Oliñares et al. 2011).

One of the differentially expressed peptides, i.e. spot 395, matched with U-box domain-containing protein 16 found in Arabi-
dopsis 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 (Oh 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 (Mishra 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 of 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 adenyllyl 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. This protein is the component of ubiquitin ligase that is attributed to the red palm weevil infestation (Rasool et al. 2015). Both studies have revealed that 1 protein matching with putative respiratory burst oxidase homolog protein 1, 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$_2$O$_2$, 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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