Molecular Profiling of Date Palm: Phoenix dactylifera L. Infested with Red Palm Weevil: Rhynchophorus ferrugineus (Oliv.) (Curculionidae : Coleoptera)

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［ヤシオサゾウムシ***Rhynchophorus ferrugineus*** **(Oliv.) (Curculionidae : Coleoptera)** によるナツメヤシ　***Phoenix dactylifera*** L. 食害の分子マーカーを用いた早期検出法］

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Dedications

I dedicate this dissertation to my loving and dearest wife: Khalida Rasool Khawaja, my adorable son: Adil Rasool Khawaja and my three lovely daughters: Mahnoor Rasool Khawaja, Fatima Rasool Khawaja and Rania Rasool Khawaja for their constant support and unconditional love.

I love you all dearly

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Chapter ONE

1 General introduction and objectives

1.1 Introduction

The date palm (*Phoenix dactylifera* L.) is considered as one of the oldest cultivated fruit trees on earth. An earliest evidence of date palm cultivation goes back to 4000 BCE in Iraq (Wrigley, 1995) and 3000 BCE in the Nile Valley (Dowson, 1982). Date palm has significant agricultural and socio-economic importance for centuries in Middle East and North Africa (Morton, 1987) where date palm cultivation has been the main livelihood source for farmers. Beside a staple food for millions of people (Vayalil, 2012) dates have potential health benefits as dates are rich in nutrients and bioactive compounds (Vayalil, 2012; Maier and Metzler, 1965; Al-Farsi *et al*., 2005). Date fruits are a high energy food containing carbohydrates, salts, minerals, dietary fiber, vitamins, fatty acids and amino acids (Al-Shahib and Marshall, 2003). The composition of date fruit comprises of 73-79% carbohydrates, 14-18% total dietary fibers, 2.0-3.2% fat, 2.5% ash, and 2.1-3.0 % protein (Elleuch *et al*., 2008; Al-Farsi *et al*., 2007) subjected to the variety/ cultivar. Also, several studies have reported that dates have antioxidant, anti-mutagenic and antimicrobial properties (Saddiq and Bawazir, 2010; Vayalil, 2002). Several other medicinal uses of dates have also been reported such as curing intestinal ailments, colds, sore throat,

toothaches, fever, liver and abdominal pain, cystitis, gonorrhea, and cough (El-Hadrami and Al-Kjhayri, 2012; Vayalil, 2002; Vayalil, 2012).

In addition, date palm is used as a wind breaker and inhibits soil degradation and desertification (EI-Mously, 1998). Date fruits, date palm trunk and leaves are the commonly used parts of the date palm that have several commercial and medicinal values.

According to the recent FAO report, Egypt, Iran, Saudi Arabia, Algeria, Pakistan and Iraq are the five leading date producers and constitute about 71% of global dates production (FAO, 2012). There are about 3000 date palm varieties worldwide (Zaid, 2002) of which over 400 have been reported from Saudi Arabia (Anonymous, 2006). The annual production of dates in Saudi Arabia is 1050000 metric tons from 160000 hectares (FAO, 2012). Dates have great importance in Judaism, Christianity and Islam, and are being used as a major breakfast food item during the fasting of Ramadan. Moreover, dates have high nutritional value, and long shelf life therefore, it was referred to as 'tree of life' in the Bible (UN, 2003).

Unfortunately, a highly invasive pest, the red palm weevil (RPW) *Rhynchophorus ferrugineus*, is posing a severe threat to this important fruit crop. RPW has also been classified as a category-1 pest in the Middle-East by Food and Agriculture Organization of the United Nation (El-Sabea *et al*., 2009). Originally, RPW was described as a deadly pest of coconut palm in northern India (Lefroy, 1906) but later it was also reported as pest of date palms (Madan, 1917). The pest has spreaded rapidly and its invasion has been reported from 15% of the coconut growing and 50% in palm growing countries (Ju and Ajlan, 2011). RPW is attacking almost 26 palm species belonging to 16 different genera

worldwide (Dembilio and Jacas, 2012). Generally, RPW infests young palms below 20 years (Faleiro, 2006).

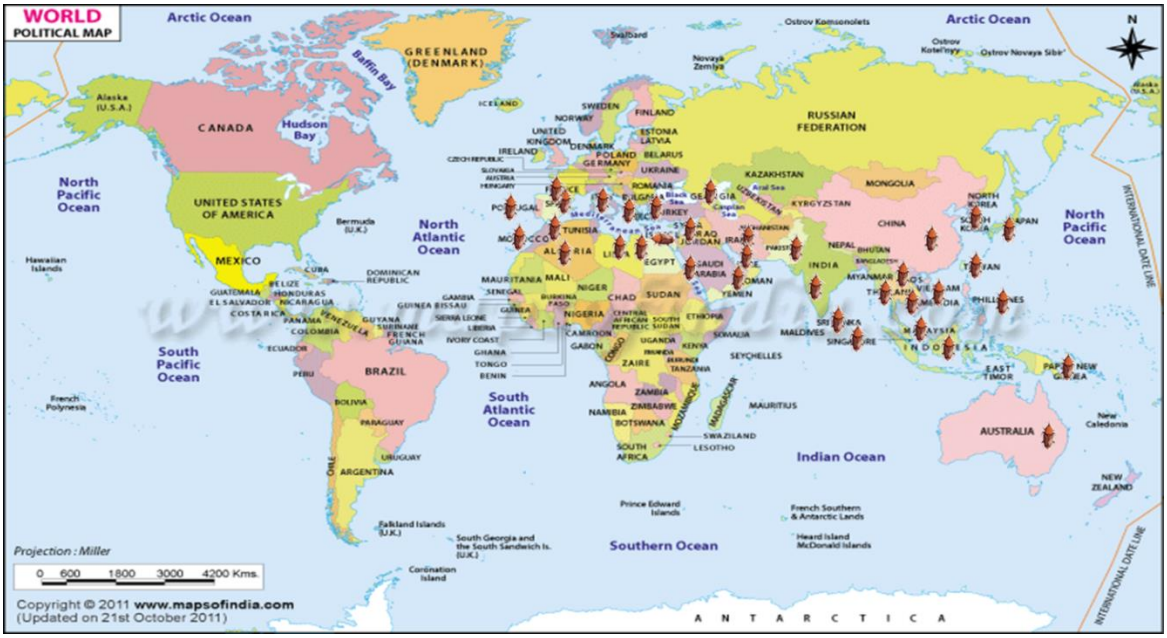
List of red palm weevil primary host plants

S. No.	Host Plants	Technical Name
1.	Date palm	*Phoenix dactylifera*
2.	Canary island palm	*Phoenix canariensis*
3.	East Indian wine palm	*Phoenix sylvestris*
4.	Sago palm	*Metroxylon sagu*
5.	Thorny palm	*Oncosperma horrida*
6.	Queen palm	*Arecastrum romanzoffianum*
7.	Toddy palm	*Borassus flabellifer*
8.	Palasan	*Calamus merrillii*
9.	Betelnut palm	*Areca catechu*
10.	Sugar palm	*Arenga pinnata*
11.	Fishtail palm	*Caryota cumingii*
12.	Mountain fish tail palm	*Caryota maxima*
13.	Chinese fan palm	*Livistona chinensis* var. *Subglobosa*
14.	Nibung palm	*Oncosperma tigillarium*
15.	Cuban royal palm	*Roystonea regia*
16.	Coconut	*Cocos nucifera*
17.	Gebang palm	*Corypha utan* (= *C. Gebanga* and *C. Elata*)
18.	African oil palm	*Elaeis guineensis*
19.	Ribbon fan palm	*Livistona decipiens*
20.	Chinese fan palm	*Livistona chinensis*
21.	Chinese windmill palm	*Trachycarpus fortunei*
22.	Washington palms	*Washingtonia* sp.
23.	Regal palm	*Roystonea regia*
24.	Hispaniola palm	*Sabal blackburniana* (= *umbraculifera*)

(EPPO, 2008; Murphy and Briscoe, 1999)

RPW is a strong flier that can cover up to one kilometer in a single attempt and up to 7 km in 3-5 days (Abbas *et al.*, 2006). This characteristic enhances the RPW ability to disperse, colonize and breed at new host (Murphy and Briscoe, 1999). RPW very rapidly extended its westwards geographical distribution since its discovery in the Gulf in 1980s. It was reported from Saudi Arabia and the United Arab Emirates in 1985, and has rapidly spreaded throughout the Middle East and into the Egypt. In 1994, RPW was reported from

Spain, Israel, and Jordan. It reached to Palestine in 1999, Italy in 2004, Canary Islands in 2005, whereas, RPW infestation was reported from Balearic Islands, France, and Greece in 2006, and Turkey in 2007 (Malumphy and Moran, 2007).



Red palm weevil worldwide distribution map

The neonate larvae of RPW feed on palm tissues inside the trunk and larval span completes in two months while pupal stage takes three weeks to adult emergence. Adult stage lives for 2 to 3 months and having multiple mattings and laying 250 eggs on average that take about 3-days to hatch (Murphy and Briscoe, 1999).

It is difficult to detect early RPW infestation symptoms because neither RPW larva nor damage can be observed. Generally, symptoms appear at later stage of infestation including: presence of tunnels in the trunk, oozing out of a brownish viscous liquid having distinctive fermented odor, protruding of chewed fibers from the holes around the trunk, gnawing sound caused by feeding grubs can be heard while placing the ear near the palm

trunk, empty pupal cocoons and dead adult weevils can be seen near the infested trees, and collapsing of the tree/ crown (Hussain *et al*., 2013).

In Japan, RPW was recorded for the first time in 1975 in Okinawa damaging palm trees especially Canary Island date palm (*P*. *canariensis*) and then invaded southern Japan in 1998 (Usui *et al*., 2006). Later, RPW has also been reported from other areas of Japan including Daito Islands, Okinawajima Islands, Kagoshima (mainland), Miyazaki, Kumamoto, Nagasaki (mainland), Fukuoka (mainland and Nokonoshima Islands), Hiroshima, Okayama, Awajishima Islands, Wakayama Prefectures and now extending to the northern part of Nagasaki (Yoshimoto, 2011). In southern Japan, RPW emerged from the host trees in March and revealed some population peaks in summer and autumn and ceased their activities in winter. RPW continues to multiply even in severe winter because of its concealed nature living inside the trunk protecting from life-threatening climatic conditions. In RPW, about 3-4 generations per year have been reported (Abe *et al*., 2009). Abe *et al*. (2010) reported that the temperature of RPW damaged palm tissues is much increased (30-40^{o}C) even in cold winter and this raise in temperature is attributed to the microbial fermentation. He further pointed out that this increase in temperature favors RPW overwintering under extremely cold winter without any interruption in Japan.

Larval stage of RPW is mainly responsible for causing damage to the date palm while feeding which makes tunnels and large cavities within date palm trunk. The concealed feeding behavior of the RPW larvae makes early detection very difficult. Often cutting and decaying of the vascular system leads to the death of the date palm tree (Abraham *et al.*, 1998). RPW completes various generations per year within the same host until the tree collapses (Rajamanickam *et al.,* 1995; Avand Faghih, 1996).

In Saudi Arabia, about 80,000 date palm trees have been infested with RPW, and pose a threat to other surrounding trees (Mukhtar *et al.,* 2011). Although, it is difficult to estimate the actual loss caused by RPW to date palm growing community, yet, in Saudi Arabia, the annual loss solely for extermination of heavily infested date palms by this pest has been reported US$1.74 to 8.69 million at 1-5% infestation, respectively (El-Sabea *et al.,* 2009).

Although, applications of systemic pesticides through injection and spray to infested date palm tree have been partially effective in controlling RPW; however, an effective and efficient early detection methodology is urgently needed for the successful management of RPW. Scientists have tried to find and implement nondestructive methods for the early detection of RPW like insects having concealed feeding behavior (Mankin and Fisher, 2002; Mankin *et al*., 2002; Lemaster *et al*., 1997; Hagstrum *et al*., 1996; Shuman *et al*., 1993). Unfortunately, RPW infestation symptoms appear at later stages when it is too late to recover the palm tree. Therefore, failure in early detection of RPW infestation is the major obstacle in its successful management. Currently, some detection techniques, including visual inspections, acoustic sensors (Potamitis *et al.,* 2009), sniffer dogs (Nakash *et al*., 2000), and pheromone traps (Faleiro and Kumar, 2008) have been tried out to detect RPW infestations at early stages; however, each technique has suffered certain logistic and implementation issues. Efforts to identify environmentally safe biological management of the RPW (Abdullah, 2009; Guerri-Agullo *et a.*, 2009) are only in their early stages.

Recently, proteomics techniques have been successfully used to diagnose infections/ diseases in humans. But their use in investigating plant responses against pathogenic infection and herbivores feeding is relatively minor. Some potential investigations on

plant proteomics encouraged us to explore proteomics methodologies to identify the differentially expressed protein moieties to be used for the early detection of RPW infestation in date palm. For example, comparison of proteome analysis of date palm leaves infected with brittle leaf disease with their healthy counterparts showed quantitative differences in several proteins (Marqués *et al*., 2011). In another attempt, proteome analysis of date palm leaves infected with brittle leaf disease revealed differential expression at early disease stage and MSP-33 kDa subunit protein associated with Mn deficiency was taken as brittle leaf disease biomarker (Sghaier-Hammami, 2012). Moreover, Gómez-Vidal *et al.* (2009) examined the plant defense/stress, photosynthesis and energy metabolism related proteins showed differential expression in the date palm (*Phoenix dactylifera*) leaves in response to the attack by entomopathogenic fungi (*Beauveria bassiana*, *Lecanicillium dimorphum* and *L. cf. psalliotae*) as compared to control samples. In another study, proteome analysis of date palm sap identified some proteins associated with *Saccharomyces cerevisiae* (Ben Thabet, 2010).

Plants have evolved various direct and indirect defense mechanism against insect pests (Kessler and Baldwin, 2002). In direct defenses mechanism plants have developed special characteristics such as thorns, trichomes, and primary and secondary metabolites (Kessler and Baldwin, 2002). Herbivores induce proteinase inhibitors (PI) in plants which prevent digestive enzymes required for digestion in insects (Tamayo *et al.*, 2000). Indirect defenses mechanisms facilitate the release of volatile organic compounds which attract herbivores predators and parasitoids to regulate their population (Dicke and Van Loon, 2000). Herbivores regurgitates and other oral secretions released into the plant tissues

trigger the plant defense mechanism to release volatile organic compounds (Korth and Dixon, 1997; Turlings *et al.*, 1990; Felton and Tumlinson, 2008; Halitschke *et al.*, 2001). Therefore, we plan to identify the differentially expressed peptides of date palm possibly associated with RPW infestation. This study may be helpful in developing some molecular marker(s) for the early detection of RPW. The objectives of our study are the molecular profiling of the date palm infested with RPW, and identification and characterization of differentially expressed peptides using MALDI-TOF.

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Chapter TWO

2 Optimization of protein isolation from date palm plants and its utilization in differential proteomics associated with red palm weevil infestation

2.1 ABSTRACT

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 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.

2.2 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 to 5000 date palm cultivars are planted in various parts of the world and serving people nutritional needs since times immemorial (Zaid, 1999; Bashah, 1996; 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.

2.3 MATERIALS AND METHODS

2.3.1 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.

2.3.2 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.

2.3.3 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 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.

2.3.4 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 was 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 precipitated by 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.

2.3.5 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.

2.3.6 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.

2.3.7 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.

2.3.8 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.

2.3.9 Two-dimensional gel electrophoresis 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.

2.3.10 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.

2.4 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.

2.4.1 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.

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**

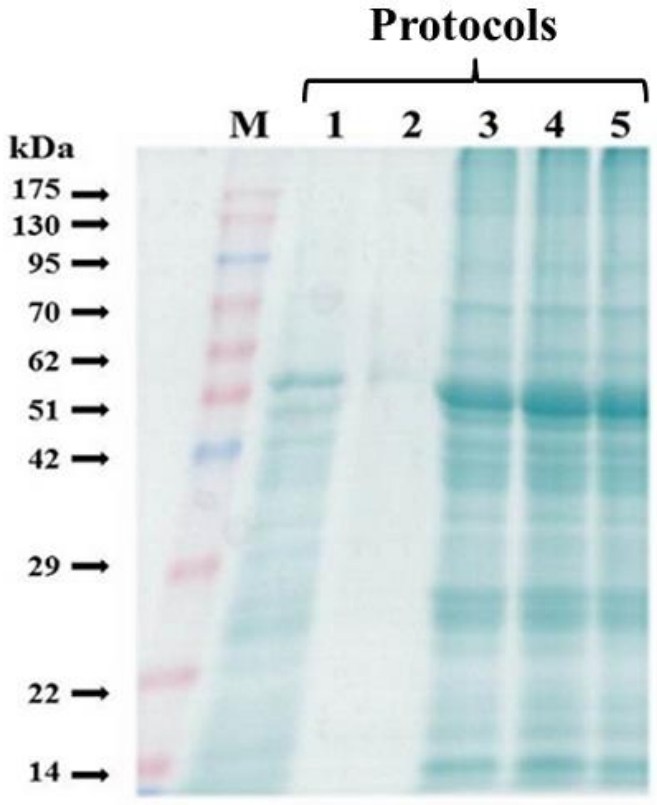


Fig. 1. Comparative efficiency of five protocols (indicated in Table 1) for protein extraction from the date palm samples through SDS-PAGE. M stands for the protein molecular marker while lanes 1-5 indicate respective protocols used for the optimization of protein extraction.

2.4.2 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).

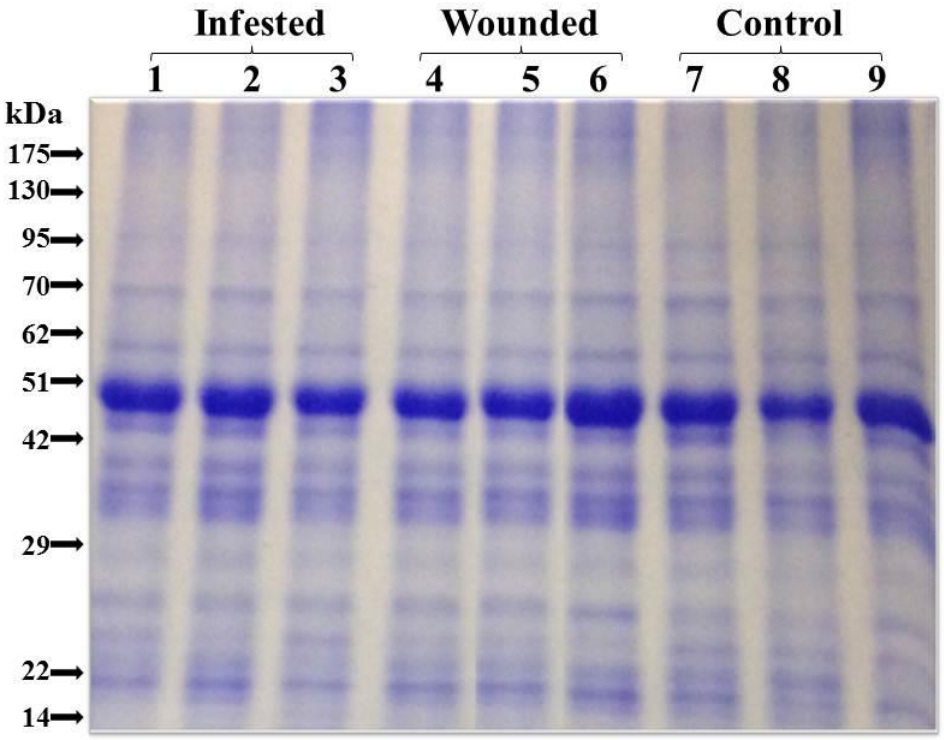
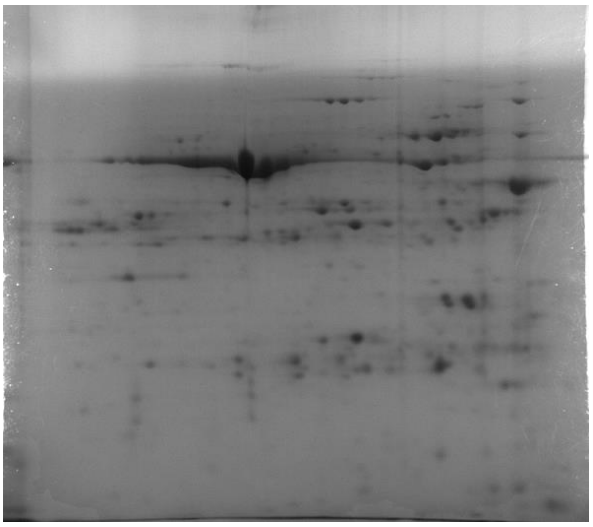


Fig. 2. Comparative protein expression profiling of the control, infested and wounded date palm samples using SDS-PAGE. Lanes 1-3 represent total cell proteins from 3-infested replicates, while lanes 4-6 represent proteins from wounded date palm samples, and lanes 7-9, represent proteins from control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

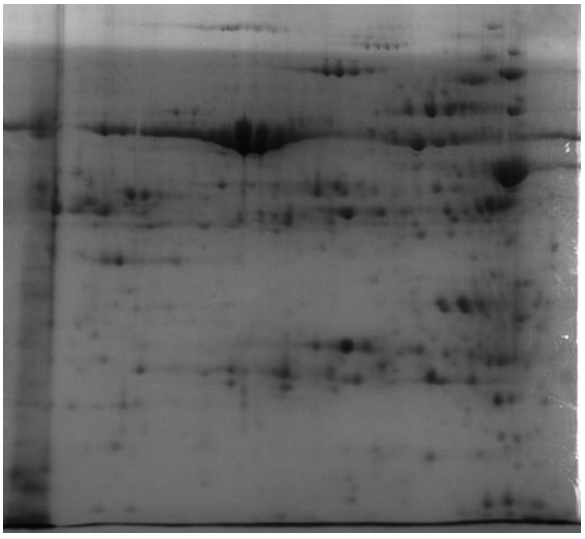
2.4.3 Differential proteomics analysis

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.

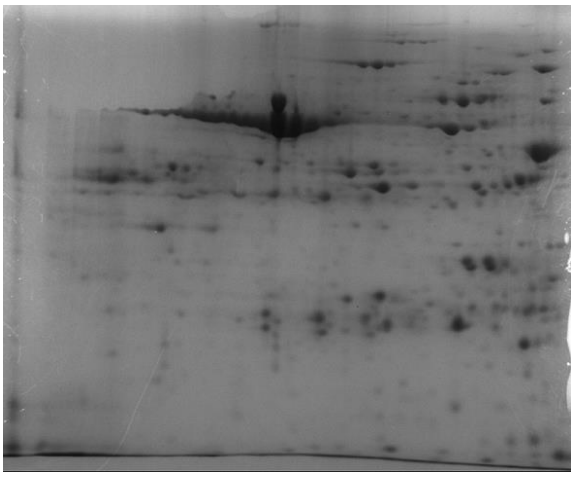
Gel (Control-1)



Gel (wounded-1)



Gel (Infested-1)



Representative Gels: Two Dimensional Electrophoresis (2DE)

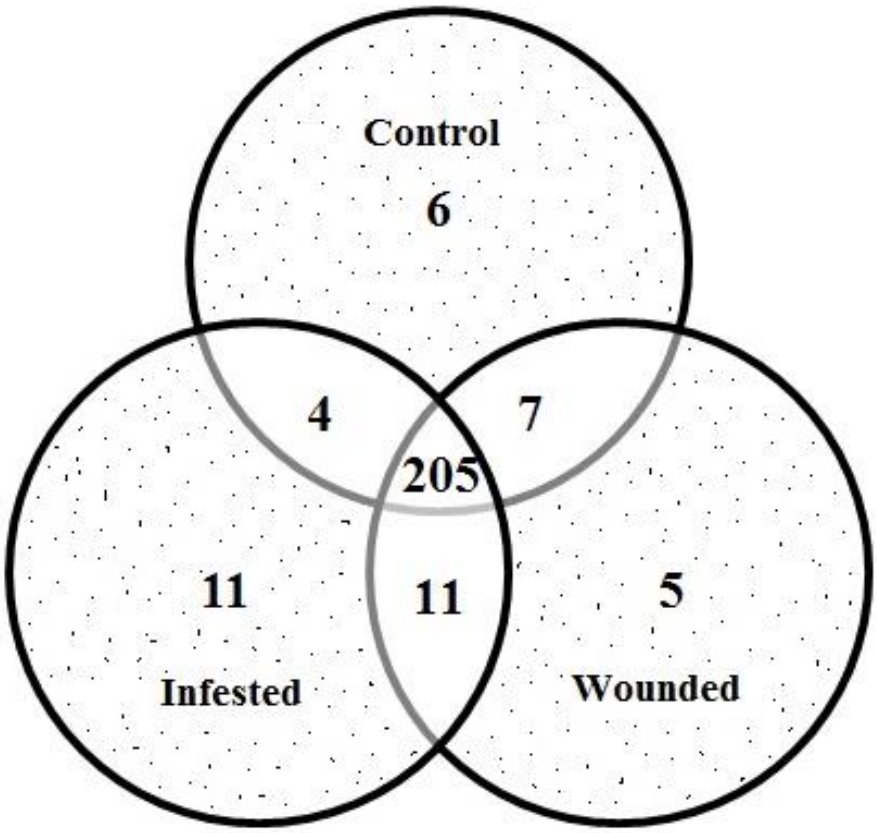


Fig. 3. Venn diagram for the relative distribution of proteins spots in control, mechanically wounded and RPW infested date palm samples. The non-overlapping segment of diagram represent the number of proteins which were significantly up-regulated (>1.5-fold) in the corresponding group when compared 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.

2.4.4 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).

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 *A. thaliana*.

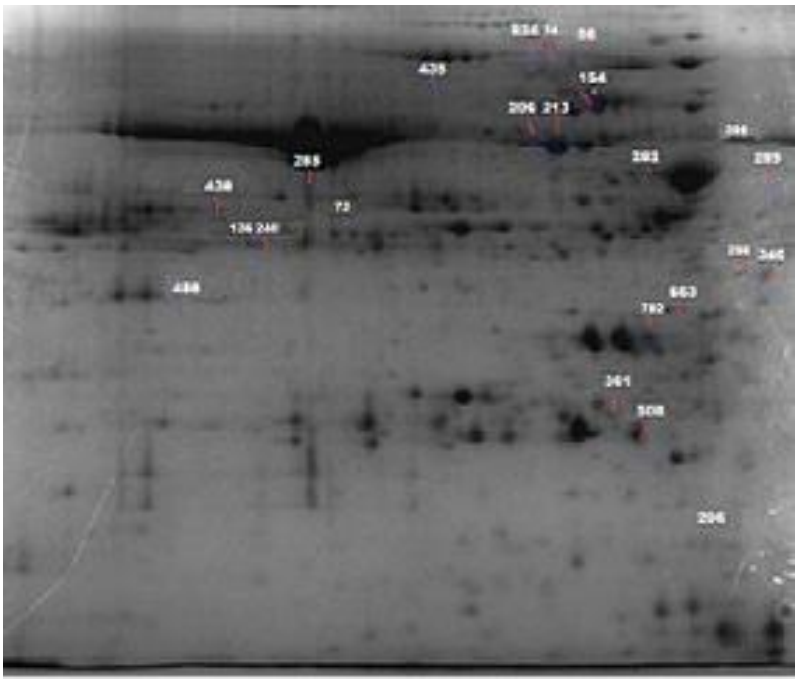


Fig. 4. Reference gel showing differentially expressed spots used for Mas Spectrometric analysis.

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.

2.4.5 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. 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.

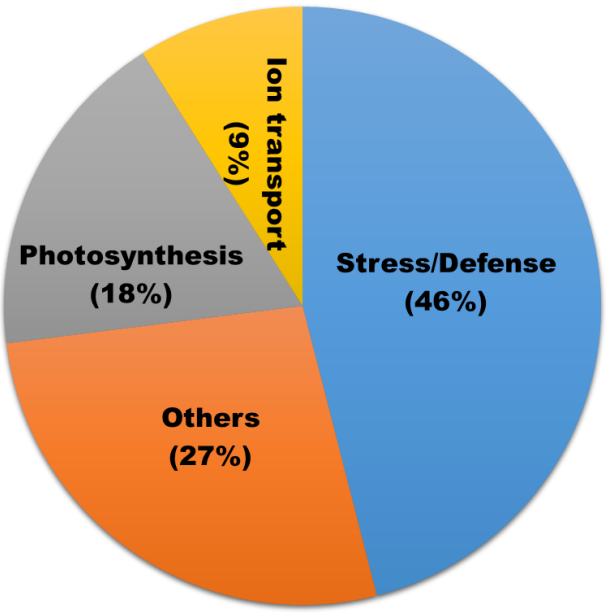


Fig. 5. A Pie chart depicting the physiological classification of potentially identified proteins through Mass Spectrometry analysis.

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

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.

2.5 CONCLUSION

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 regulate 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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Chapter THREE

3 Molecular profiling of the date palm, *Phoenix dactylifera* L. infested with RPW, *Rhynchophorus ferrugineus* (oliv.) (Curculionidae: Coleoptera) leave samples using 2D-DIGE analysis and MALDI-TOF

3.1 ABSTRACT

The RPW (*Rhynchophorus ferrugineus*) is damaging date palm trees in several regions of the World including Saudi Arabia. This insect infestation cycle is highly concealed in nature and early detection followed by destruction of the affected trees is the only viable strategy to control further spread. A highly sensitive and reliable early detection method of the infested plants is awaited to be applied in the field for removing infested plants thus curtailing further spread of this insect. Post-genomic era techniques have made possible to identify earlier responses of livings including plants to any injury/ infections. As such, we compared date palm protein expression differences of infested plants leaves with the healthy one by a highly sensitive two-dimensional differential gel electrophoresis (2D-DIGE) followed by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF-TOF). The 2D-DIGE results revealed qualitative and quantitative proteome differences between control and RPW infested date palm samples. The RPW infestation induces injury to the plants and as such to identify infestation

specific responses artificially wounded trees were used as a control. Differential proteomics led to the identification of thirty two RPW infestation specific protein spots ($p \leq 0.05$ having ≥ 1.5 fold modulation) further subjected to mass spectrometric analysis for their identification and characterization. Protein involved in stress/defense related, photosynthetic, carbohydrate utilization system and protein degradation were mainly modulated in infested plants. These differentially expressed RPW infestation specific peptides can be used as biomarker for the identification of early infestation with this insect in date palm trees.

3.2 INTRODUCTION

Red Palm Weevil infestation have been reported in several parts of the World, however, 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 having over 23 million trees producing 970,488 tons of dates annually (Alhudaib *et al.* 2007). Unfortunately, this important fruit crop is under threat due to highly invasive pest, the red palm weevil (RPW), *Rhynchophorus ferrugineus*, first recorded in early 1980's(Abraham et al. 2001) in the Gulf.

It has been estimated that 80,000 palm trees in Saudi Arabia are infested with RPW posing a danger to surrounding plants (Mukhtar *et al*. 2011). Furthermore, infestation with RPW have been reported in over 50% of the date palm growing countries, sparing none in the Middle East (Faleiro, 2006), however, Saudi dates have special value as the pilgrimage on their return brings dates as gift, a Muslims tradition spanning over centuries. Damage to date palm trees is mainly caused by the insect larvae feeding within

the trunk of palms. This concealed feeding habit of the larvae, makes it extremely difficult to detect infestation at early stages. Often severe damage to the internal tissues leads to the death of the date palm tree (Abraham *et al.* 1998). The weevil completes several generations per year within the same host until the tree collapses (Rajamanickam *et al.* 1995; Avand Faghih, 1996). Yield loss due to infestation could be mild to severe (Gush, 1997).

Pesticides application to infested date palm tree have been partially successful in controlling RPW; however, there is still an urgent need for early detection and more effective environmentally safer control measure. Several detection methodologies, including visual inspections, acoustic sensors (Potamitis, *et al.,* 2009), sniffer dogs (Nakash, 2000), and pheromone traps (Faleiro and Kumar, 2008) have been tested to assist quarantine efforts and identify infestations at early stages; however, each and every method has suffered certain logistic and implementation issues. Efforts to identify environmentally safe biological management of the RPW (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 provides a highly reliable differential responses towards any change in living organisms including plants. A number of proteins have been identified in *Vitis vinifera, Acca sellowiana,* and *Cyclamen persicum* using comparative proteomics which differentiate genotypes, organs, or involved in growth and development and responses to biotic and abiotic stresses (Winkelmann, *et al.* 2006; Marsoni, *et al.* 2008; Cangahuala-Inocente *et al.* 2009; Zhang *et al.* 2009). We selected improved version of two dimensional differential gel electrophoresis (2D-DIGE) for

differential proteomics study in RPW infested versus control plants. This highly sensitive fluorescence dye-based strategy increases the effectiveness of the proteomics technique by allowing multiplexing of different protein samples labeled with distinct fluorescent dyes and run on the same gel so as to avoid gel to gel variation (Timms, and Cramer, 2008). Our results revealed significant and reproducible differences in date palm leaves peptides upon infestation with RPW that could be used to devise an early detection method for removing infested plant to control further spread of this insect.

3.3 MATERIALS AND METHODS

3.3.1 Mechanical wounding and infestation with RPW treatment

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 of date palm cultivars were carried out as described previously (Lippert *et al*., 2007). Briefly, plants were separated into three groups. Group one was artificially infested with red palm weevil larvae, group two artificially wounded and third group without any treatment served as control. For artificial infestation each plant was infested with five 2^{nd} instar red palm weevil larvae by making holes in the stem using drill machine with 6-mm size bit. The wound was also created with same drill machine. These plants were then harvested after three days. Leaf tissues for differential proteomic study were taken from each tree and stored at -80^{o}C.

3.3.2 Protein extraction and SDS-PAGE

Leaves samples of infested, uninfested and artificially wounded date palm plants were taken after 3-days post-infestation for protein extraction. Leaves were removed with clean scissor from the plant and rinsed in distilled water. Proteins from control, infested and wounded date palm leaves (three replicate from each sample) were extracted using phenol/SDS extraction method as described by Gomez-Vidal *et al.* (2008) with minor modifications (Rasool *et al.* 2014). Briefly, 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), 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 minutes at 10,000 rpm at 4^{o}C. The upper phenolic phase was collected carefully without disturbing interphase and precipitated with five volumes of cold 0.1M ammonium acetate in methanol. The mixture was incubated at -20^{o}C for 30 min. Precipitated proteins were recovered by centrifugation at 1000 rpm for 5 min at 4^{o}C and then washed two times with cold methanol solution containing 0.1 M ammonium acetate and then two times with cold 80% v/v acetone. Each time, protein pellet was recovered by centrifugation at 8000 rpm for 5 minutes. Protein pellet was air-dried at room temperature for one hour and aliquot of each sample was suspended in 100 mM Tris buffer pH 8.0 and then mixed with equal volume of 2X 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 exactly as described by Laemmle (1970). After, electrophoresis, gel was stained with Commassie brilliant blue G-250 with

constant and gentle agitation for overnight. Upon destaining resolved protein fraction were visible in the form of light and dark bands.

3.3.3 Two dimensional (2D) differential gel electrophoresis

Protein samples were quantified by 2D quant kit (GE Healthcare, Germany) and labeled with CyDye DIGE Fluor minimal dyes according manufactures' recommendation (GE Healthcare, Germany) before electrophoresis. Briefly, 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 experimental design as shown in Table 1. Five IPG Immobiline DryStrips 24 cm pH 3-10 (GE Healthcare, Bucks UK) were rehydrated overnight in 450 µl 2DE rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 0.5 % pH 3-11 ampholytes (GE Healthcare, Bucks UK), 1% DTT, trace bromophenol blue). After rehydration, samples were focused using an Ettan IPGphor IEF unit (GE healthcare, Germany) according to manufacturer conditions. Further DryStrip cover oil was then pipetted across the surface to cover the IPG strip. Isoelectric focusing was performed using Ettan IPGphor IEF unit (GE Healthcare, Sweden) at 50µA per strip at 20^{o}C. After IEF, strips were equilibrated in equilibration buffers (2% SDS, 75 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 0.002% bromophenol blue) containing DTT (100mg/10ml buffer) or 2-iodoacetamide (250mg/10ml buffer), respectively, before second dimension separation of proteins on 5-20% SDS polyacrylamide gels in low fluorescent glass plates. The equilibrated strip

Table 1. Experimental design for 2D-DIGE. Three replicate from each control, infested and wounded protein samples were labeled and combined for 2D-DIGE.

Gel No.	**Cy2**	**Cy3**	**Cy5**
1	Pooled sample	Control 1	Wounded 1
2	Pooled sample	Control2	Wounded2
3	Pooled sample	Control3	Infested 3
4	Pooled sample	Infested 1	Wounded 3
5	Pooled sample	Infested 2	

was placed on the 5-20% 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). 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 six vertical unit (GE Healthcare, Little Chalfont, UK) at 15^{o}C for 1W per gel for 1h and then 2W per gel until the bromophenol blue dye reached the end of gel.

3.3.4 Image acquisition and analysis

The 2D gels were scanned using fluorescence gel scanner, Typoon imager (Trio) (GE, Healthcare, Sweden), using appropriate wavelengths and filters for Cy2, Cy3 and Cy5 dyes according to manufacturers' recommended protocol. The 2D gels images were analyzed using Progenesis sameSpot software version 3.3 (Nonlinear Dynamics Ltd, Newcastle Upon Tyne, U.K.). Differentially expressed peptides were evaluated using normalized protein spots in the Cy5 and Cy3 channels compared to the internal standard (Cy2). Spots of infested and wounded date palm samples were compared to control samples. One way analysis of variance (ANOVA) was used to calculate the fold difference values. A threshold level was set at 1.5 fold up- or down-regulation, at $p < 0.05$ level. Principal component analysis (PCA) was performed using Progenesis sameSpot software.

3.3.5 Protein identification by mass spectrometry

Differentially expressed peptides ascertained through 2D gel electrophoresis were identified and characterized by running a preparative gel using 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 days followed by rinsing in Milli Q water and stored until spots were picked and identified by Mass spectrometry. The differential protein spot after matching with reference gel were manually excised form Comassie stained preparative gels and digested with trypsin for MALDI-TOF analysis according to previously described methods (Alfadda *et al*., 2013). Following trypsin digestion peptides were extracted by adding 50 % acetonitrile/0.1 % Triflouroacetic acid followed by drying. 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 subjected to MALDI-TOF-MS (UltraFlexTrem, Bruker Daltonics, Germany) as described previously by Alfadda *et al*. (2013). MS data were interpreted by BioTools 3.2 (Bruker Daltonics, Germany) in combination with the Mascot search algorithm (version 2.0. 04) againt Swiss-Prot database for green plants. The identified protein was not accepted as correct until Mascot score is above 60.

3.4 RESULTS AND DISCUSSIONS

3.4.1 Evaluation of protein profiling by 2D-DIGE

Plants like humans are susceptible to pathogens like bacteria, mycoplasma, viruses, fungi, nematodes and protozoa. It has also been reported that phytopathogens elicit defense responses at molecular levels besides physiological and anatomical changes in plants (Jones and Dangl, 2006). This study was designed to investigate the date palm plant molecular responses subsequent to infestation with RPW. This pest severely damages the

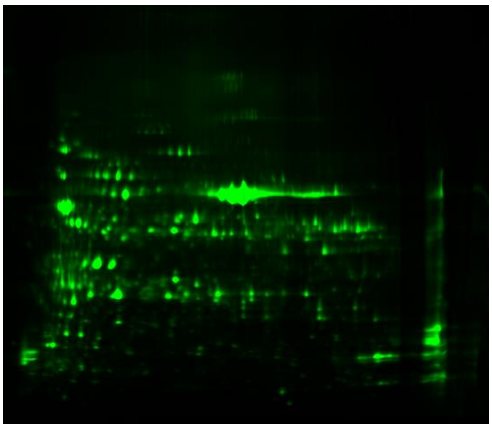
date palm trees ultimately leading to the death of the plant if not managed properly (Abraham *et al.* 2001).

Briefly, 2D DIGE was used to identify differential proteome changes among control, infested and wounded date palm samples. This powerful technique by passes the limitation associated with conventional 2-DE by introducing fluorescent reagents for protein labeling (difference gel electrophoresis or DIGE) as they provide higher sensitivity compared to normal staining and furthermore spot volume quantification is much more improved by the addition of internal standard and running multiple samples in the same gel (Alban *et al.* 2003). We also compared protein expression profile of RPW infested date palm with uninfested controls to identify specificity of molecular changes associated with pest infestation.

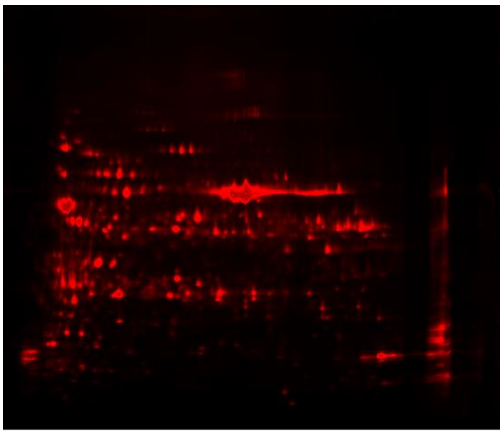
Date palm plant leaves from Control, infested and artificially wounded plants were alternatively labeled with either Cy3 or Cy5 dyes while internal standard was consistently labeled with Cy2 dye and internal standard contained equal amount of each sample present in the experimental design (Table 1). Five gels (a-e) were run by multiplexing the two samples in each gel along with internal standard except one gel contained 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 that biological variation. Furthermore, incorporation of 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 2D-DIGE gels were scanned using fluorescence gel scanner, Typoon imager (Trio) (GE Healthcare). The gels of 2D-DIGE are shown in Figure 1 revealing date palm control sample, artificially wounded and RPW

infested samples labeled with Cy dyes as indicated in Table 1. 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 SameSpots software. Our analyses revealed on the average, 745 proteins spots in each gel using 24cm immobilized pH gradient (IPG) strip, pH 3-11 by image analysis.

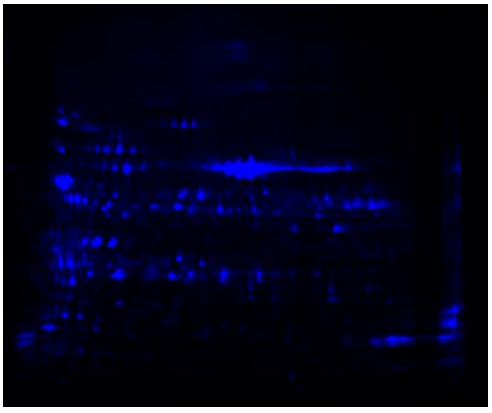
A. Cy3 (control-1)



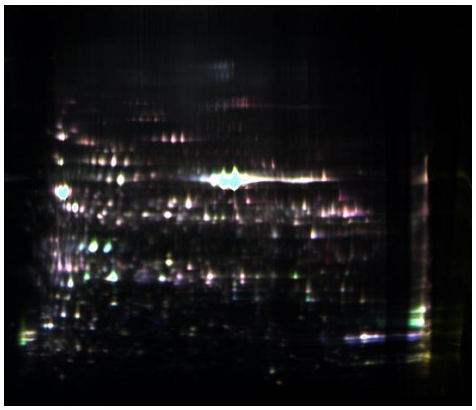
B. Cy5 (wounded-1)



C. Cy2 (Internal Standard)



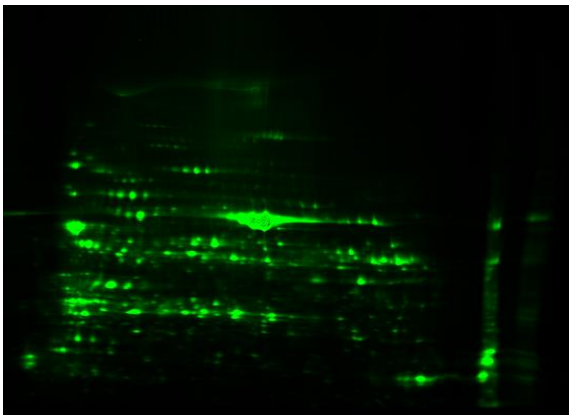
D. Merge Image



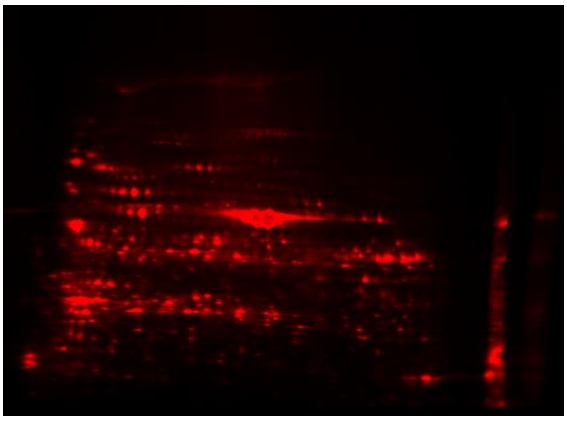
2D-DIGE Gel-1

Fig.1a. 2D-DIGE images of date palm proteins. The protein sample of control-1, wounded-1 and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by 2D-DIGE 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 pooled from all and labeled with Cy2 dye. D: Overlay gel of control, and wounded samples along with internal standard.

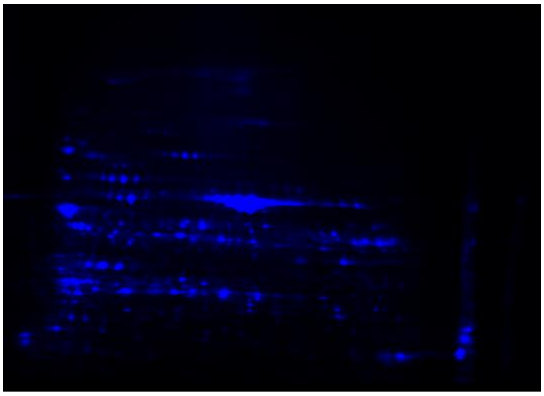
A. Cy3 (control-2)



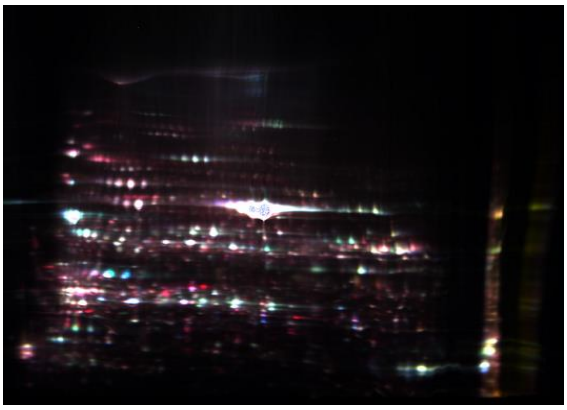
B. Cy5 (wounded-2)



C. Cy2 (Internal Standard)



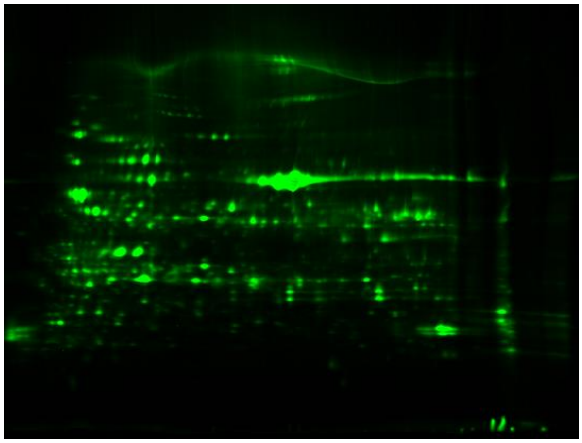
D. Merge Image



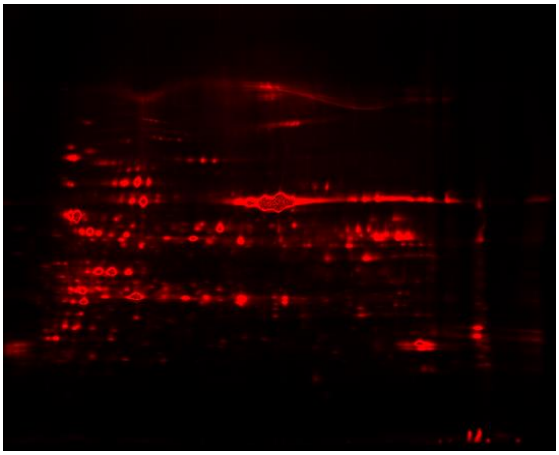
2D-DIGE Gel-2

Fig.1b. 2D-DIGE images of date palm proteins. The protein sample of control-2, wounded-2 and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by 2D-DIGE 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 pooled from all and labeled with Cy2 dye. D: Overlay gel of control, and wounded samples along with internal standard.

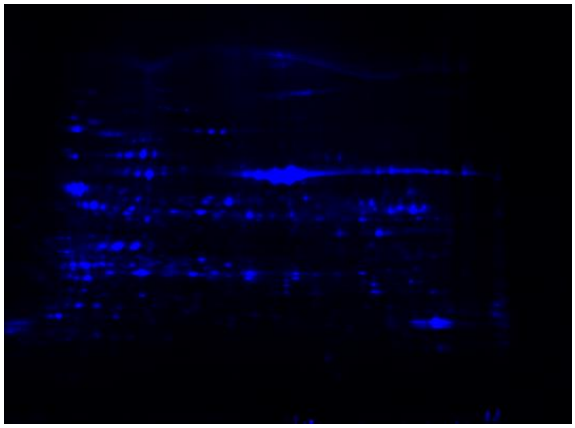
A. Cy3 (control-3)



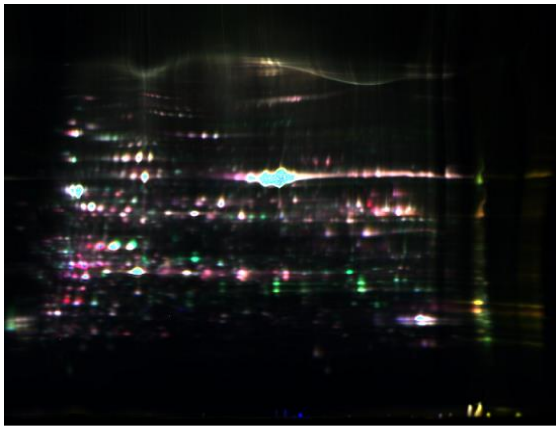
B. Cy5 (Infested-3)



C. Cy2 (Internal Standard)



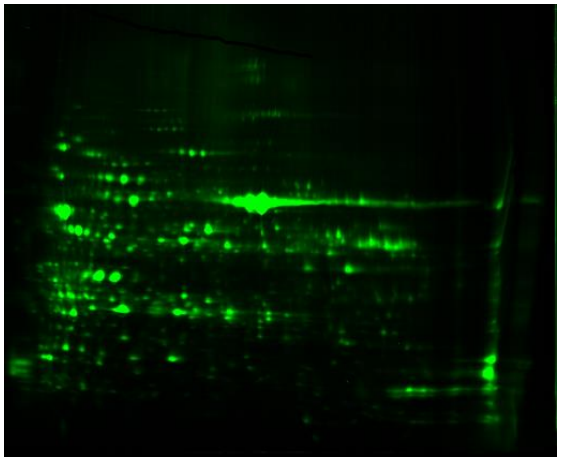
D. Merge Image



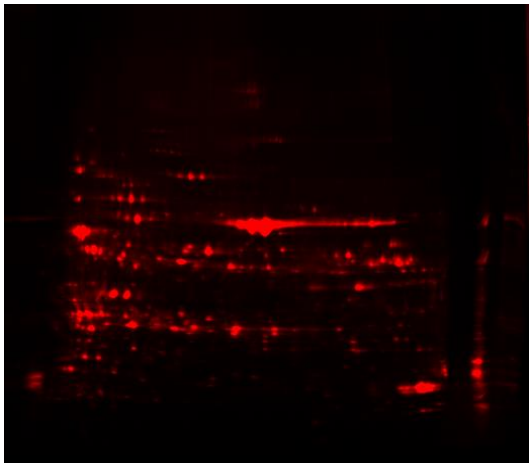
2D-DIGE Gel-3

Fig.1c. 2D-DIGE images of date palm proteins. The protein sample of control-3, Infested-3 and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by 2D-DIGE followed by image scanning. A: image of date palm control sample and labeled with Cy3 dye. B: image of date palm infested sample labeled with Cy5 dye. C: image of date palm sample pooled from all and labeled with Cy2 dye. D: Overlay gel of control, and infested samples along with internal standard.

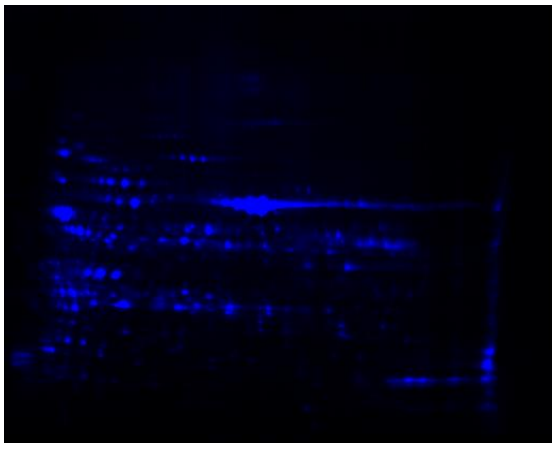
A. Cy3 (Infested-1)



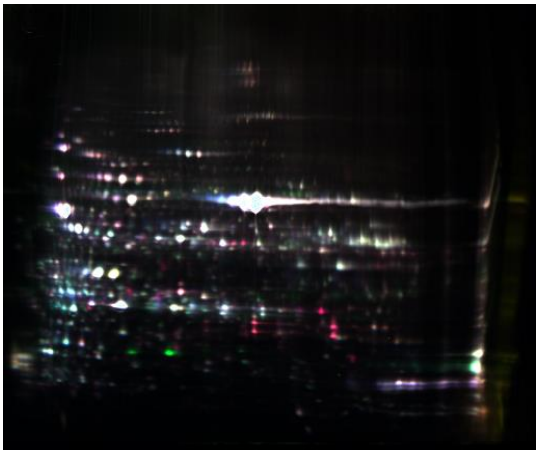
B. Cy5 (Wounded-3)



C. Cy2 (Internal Standard)



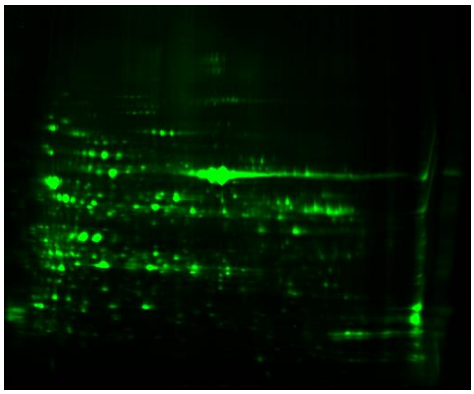
D. Merge Image



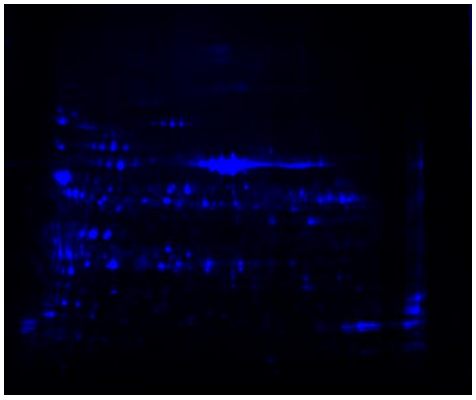
2D-DIGE Gel-4

Fig.1d. 2D-DIGE images of date palm proteins. The protein sample of Infested-1, Wounded-3 and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by 2D-DIGE followed by image scanning. A: image of date palm infested sample and labeled with Cy3 dye. B: image of date palm wounded sample labeled with Cy5 dye. C: image of date palm sample pooled from all and labeled with Cy2 dye. D: Overlay gel of infested, and wounded samples along with internal standard.

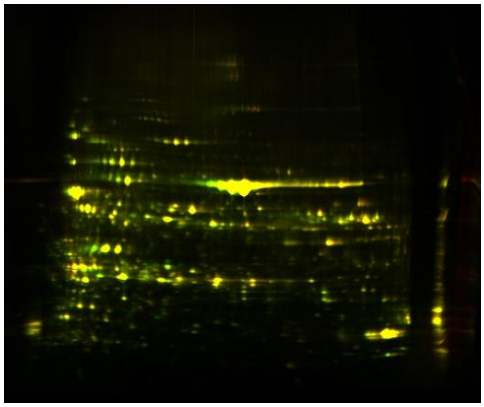
A. Cy3 (Infested-2)



B. Cy2 (Internal Standard)



D. Merged Image



2D-DIGE Gel-5

Fig.1e. 2D-DIGE images of date palm proteins. The protein sample of Infested-2 and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by 2D-DIGE followed by image scanning. A: image of date palm infested sample and labeled with Cy3 dye. B: image of date palm sample pooled from all and labeled with Cy2 dye. D: Overlay gel of infested, and with internal standard.

Figure (2) represents the reference gel showing differentially expressed spots used for Mass Spectrometric analysis.

Statistical analysis of the gels was computed between Control *vs* infested, Control *vs* wounded and wounded *vs* infested. Figure 3 shows the distribution of protein spots in 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 and wounded. Non-overlapping segment of the Venn diagram reveals that the numbers of significantly up-regulated spots in the infested sample compared to both wounded and control samples are 13 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 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.

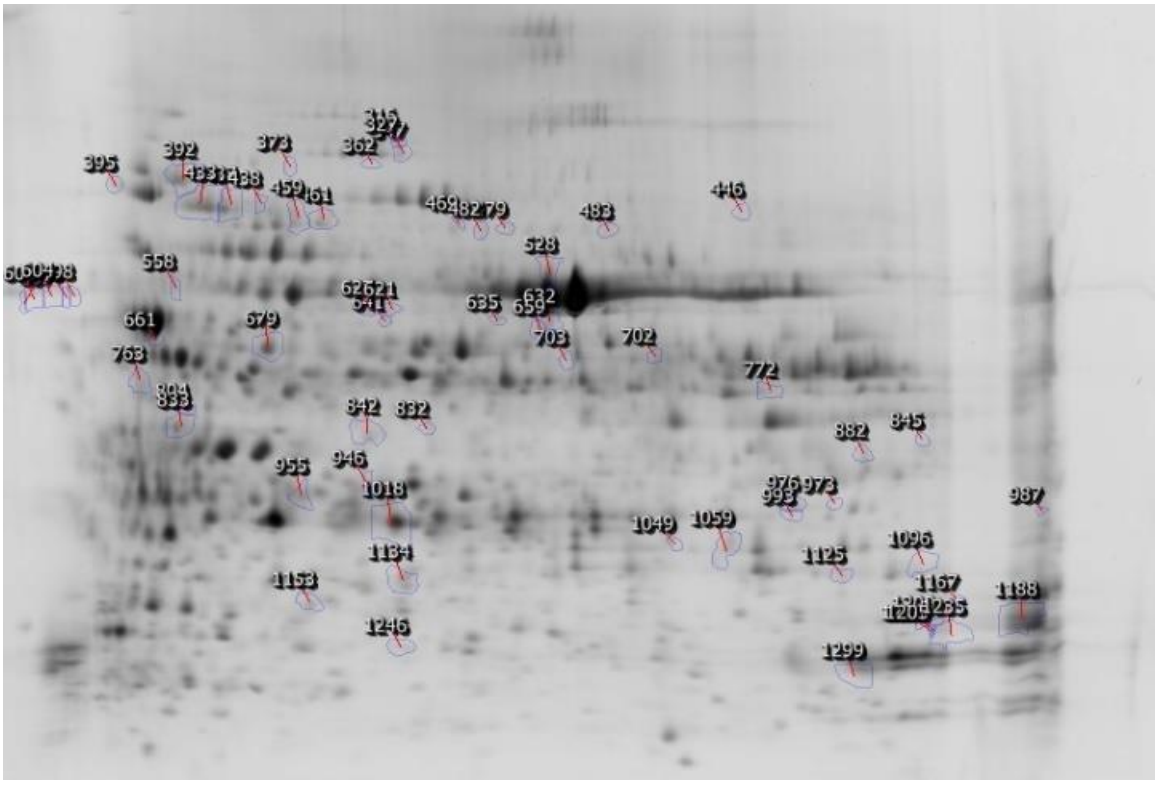


Fig. 2. Reference gel showing differentially expressed spots used for Mass Spectrometric analysis.

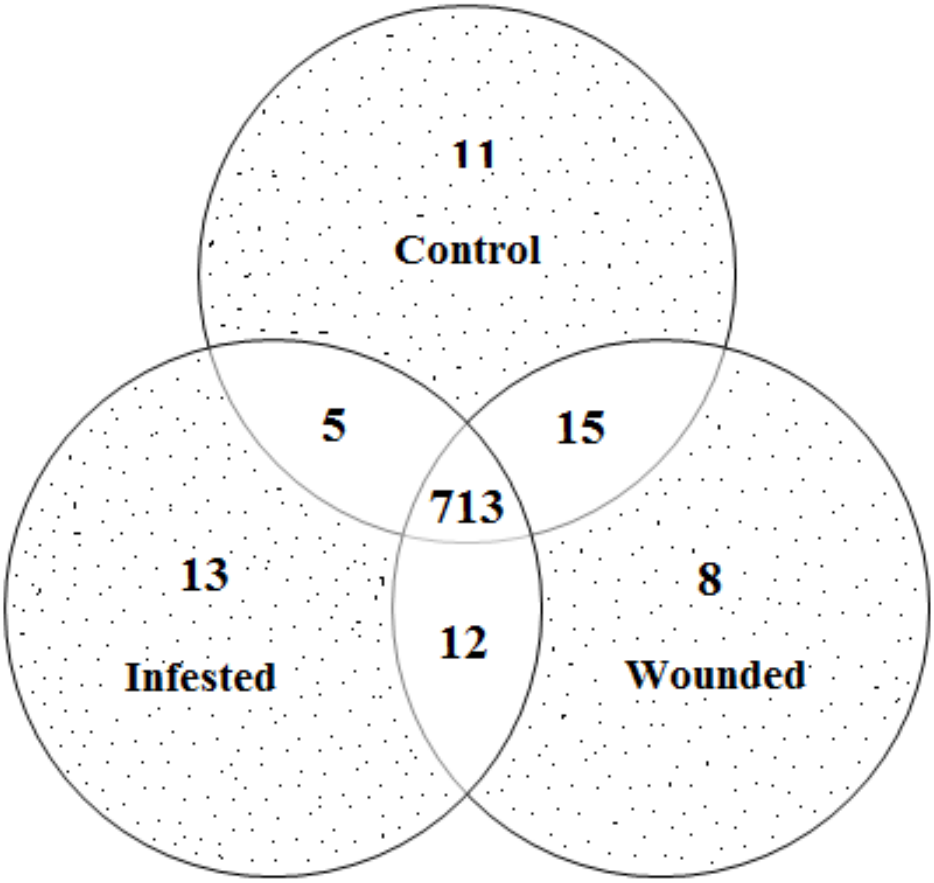


Fig. 3. Venn diagram for the relative distribution of proteins spots in control, mechanically wounded and RPW infested date palm samples. The non-overlapping segment of diagram represent the number of proteins which were significantly up-regulated (>1.5-fold) in the corresponding group when compared 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.

3.4.2 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, and stained by Colloidal Comassie blue G-250 and imaged (Figure 2). The 32 differential spots were excised from preparative gels, digested enzymatically with trypsin and identified by mass spectrometry. The collected Mass Spectrometric (MS) data were processed by BioTools3.2 (Bruker Daltonics, Germany) in combination with the Mascot search algorithm (version 2.0. 04) against green plants database. 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 to our great interest towards finding a highly reliable biomarker associated with RPW early infestation. To best of our knowledge, this is the first report of its type reporting differential protein in date palm after RPW infestation. Differentially expressed proteins were matched with specific proteins of *Arabidopsis thaliana* (20%), *Zea mays* (15%), *Solanum lycopesicum* (10%), *Solanum demissum* (10%), *Oryza sativa* subsp. Japonica (rice) (10%), *Phaseolus vulgaris* (bean) (5%), *Triticum aestivum* (wheat) (5%), *Tabebuia heterophylla* (Pink trumpet tree) (5%), *Calamus usitatus* (Palm tree) (5%), *Larrea tridentate* (Creosote bush), (5%), *Deppea grandiflora* (5%), *Beta vulgaris*(Sugar beet) (5%). The identified proteins were classified into 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 4. Majority of differentially expressed proteins subsequent to infestation with

Table 2. Differentially expressed proteins between controls, wounded and infested date palm leaves by MALDI-TOF peptide mass fingerprinting after 2D-DIGE.

Spot No.	FC (I)	FC (W)	Accession (uniprot)	Protein description	Function	p*I*	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↑	1.31↑	P11143	Heat shock 70 kDa protein	Stress response	5.22	70871	35	133	*Zea mays* (Maiz)
558	1.71↑	1.53↑	Q6L3X3	Putative late blight resistance protein homolog R1B-8	Hyper sensitive response Defense	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 response Defense	5.78	153116	20	57	*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	Photosynthesis Calvin 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	Cellolose 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 = RPW Infested samples, W = Mechanically Wounded samples, pI = Isoelectric point, MW = Molecular Weight.

RPW were stress related, and several others could potentially 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 while some other specially belonging to photosynthesis were down-regulated.

The seven differentially expressed peptides associated with RPW infestation (spot no 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 were identified and these proteins protect plants against various stresses. i.e. high temperature and pathogens by folding and unfolding of other proteins. These heat shock proteins were recognized according to their molecular weight. In our case, two differentially expressed peptides upon RPW infestation are heat shock peptides, Hsp 70KDa and Hsp 81-2. Hsp81-2 is also named as Hsp90. HSP family modulated in infested date palm were Hsp70 (spots 433,437 and 461) and Heat shock protein 81-2 also named as Hsp90 (392). The hsp70 is a ATP-dependent molecular chaperones mainly induced by heat or other abiotic stresses while others are not heat inducible and present under normal growth conditions in some tissues (Lee and Schöffl, 1996). They facilitate the folding process of newly synthesized proteins and minimize aggregation (Fink, 1999). A high level of HSP spot 461 spots 437 and 433 were observed in infested samples. Similar findings have been reported in other plants also (DeRocher and Vierling, 1994; Fink, 1999).

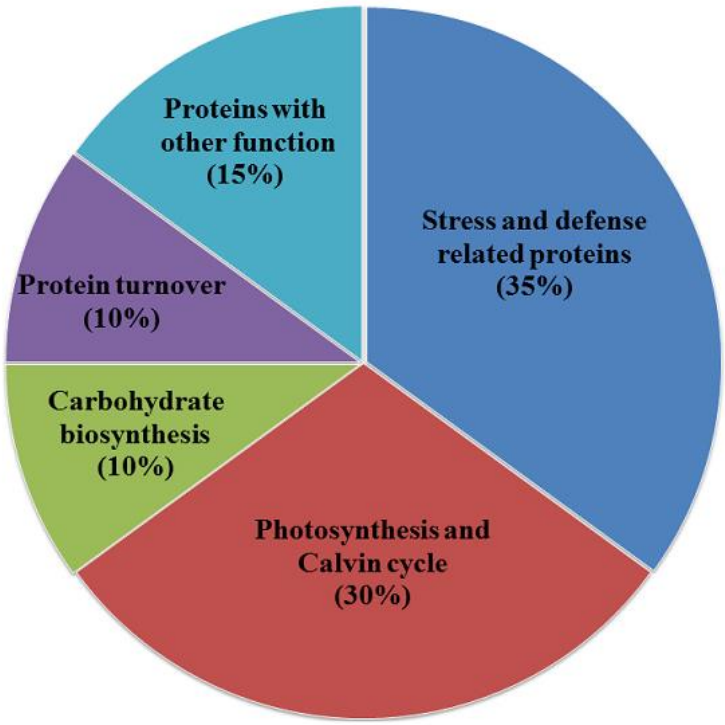


Fig. 4. A Pie chart presenting the classification of identified proteins according to their biological functions and are expressed in percentage.

The heat shock proteins of 90 kDa (Hsp90) are also molecular chaperones that promote folding, structural maintenance, and regulation of a subset of proteins involved in transduction of signals, cell cycle control, etc. Hsp90 also trigger growth and development of organisms involving conformational regulation of many regulatory proteins and protecting cells under stress (Kozeko, 2010). Hsp90 may have some role in disease resistance (Lu *et al.,* 2003). Some recent proteomics works also identified several Hsps modulation in response to different stress conditions pea (Curto *et al.,* 2006; Castillejo *et al*., 2010a) and triticale under a low N fertilization level (Castillejo *et al*., 2010b).

RPW infestation led to upregulation of two pathogen resistance proteins (spot 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/injury (Poupard *et al*., 2003; Tarchevsky *et al*., 2010). These are the resistance proteins that guard the plant against pathogen and ultimately stop the pathogen 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 RPW infestation.

The proteins spots (702, 528, 635, 661 and 1299) modulated upon infestation with RPW are related to photosynthetic machinery and identified as Ribulose bisphosphate carboxylase large chain. These proteins have very close Mr and pI values. These slightly variation could be attributed to posttranscriptional modification suggesting that these different member belonging to same functional family. The existence of these isoforms

with slight difference molecular weight and pI has been reported previously in date palm (Sghaier-Hammami *et al*., 2009; Marqués, *et al*., 2011) and in other species like Arabidopsis (Sghaier-Hammami *et al*., 2012). However, the expression level of these proteins is reduced in infested date palm sample and it 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) as reduction of photosyntetic activity lead to change resources from growth to defense (Bilgin *et al*., 2008). Another reason for this reduction is the hyper-response (HR) which leads to activation of numerous defense reactions as repression of photosynthesis-related genes during HR are also previously reported (Zou *et al*., 2005; Li *et al*., 2011).

A unique differentially expressed peptide spot (438) associated with RPW 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 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 peptide 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 involve 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 and Hicke, 2003). U-box domains look like the RING finger domain (Aravind and Koonin, 2000). The U-box domain is essential for the ubiquitin activity and 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 has also been reported (Ohi *et al*., 2003; Stone *et al*., 2003; Zeng *et al*., 2004). In plants, ubiquitination play 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 date palm defensive role. This ubiquitin protein ligase also play vital role in the regulation of a variety of cellular functions including cell cycle, transcription development, signal transduction and nutrient sensing (Jonkers and Rep, 2009). In

addition to this, it has just been reported that the proteolytic function of the ubiquitin-proteasome system regulate the virulence of pathogenic fungi (Lue and Xue, 2011). Therefore, appearance or up or down regulation of Ubiqutitin ligase in infested and wounded proteins suggest its strong role in infestation and have potential to serve as biomarker in early detection.

The spot no 627 exhibited homology to Glucose-1-phosphate adenylyltransferase small subunit and is a regulatory role for the biosynthesis of starch. The spot no 955 showed homology to Cellulose synthase-like protein D4 of *Arabidopsis thaliana* (Q9SZL9) is a Golgi-localized beta-glycan synthase that polymerize the backbones of noncellulosic polysaccharides (hemicelluloses) of plant cell wall.

The spot no 833 belong to DNA ligase 1 and matched with *Arabidopsis thaliana*, involves sealing nicks in double-stranded DNA during DNA replication, DNA recombination and DNA repair. Our proteomic study revealed that one 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, earliest cellular response in plants is an increase in reactive oxygen species (ROS), known as oxidative burst and this in turn lead to the activation of local and systemic resistance responses (Mendoza, 2011) resulted in cell wall reinforcement, programmed cell death and expression of defense genes. The superoxide radicals that produced are converted into H_2O_2 that triggers the HR in plants to kill the pathogen and moreover it induces the transcription of various resistance genes (Mellersh *et al*. 2002).

3.5 CONCLUSION

We provide first time a proof of concept that RPW infestation of date palm lead to molecular changes identified though proteomic methodology. Infestation with this highly damaging insect is a major threat in date palm growing countries including the Kingdom of Saudi Arabia where removal of infested trees has been recognized as the most effective strategies barring further spread. We have identified several molecular moieties to be used in future for developing a highly sensitive early infestation detection molecular test towards screening RPW infested date palm tree. Furthermore, our study has opened avenues for utilizing proteomic strategies in controlling phytopathogens, best varieties selection including several desirable traits identification in plant kingdom.

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Chapter FOUR

4 Molecular profiling of the date palm, *Phoenix dactylifera* L. infested with RPW, *Rhynchophorus ferrugineus* (Oliv.) (Curculionidae: Coleoptera) stem samples using 2D-DIGE analysis and MALDI-TOF

4.1 ABSTRACT

Plants like humans respond to wide range of biotic and abiotic stresses by developing different acquired defense mechanisms besides their innate defensive structures. Deciphering of earlier responses have the potential to be disease biomarkers in plants. Date palm mainly grown in the Gulf region is significantly threatened by infestation with RPW (*Rhynchophorus ferrugineus).* Concealed infestation cycle hinders quarantine measures and earlier molecular detection can help for removing infested plants to avoid further spread. This study explores differentially expressed proteins in the date palm stem tissues on infestation with RPW. A state of the art Two Dimensional Difference Gel Electrophoresis (2D-DIGE) and Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry has been employed to characterize peptides modulated in the date palm stem subsequent to infestation with RPW. Our analyses revealed 32 differentially expressed peptides associated with RPW infestation in date palm stem. Of importance, to identify RPW infestation specific peptides (I), artificially

wounded plants (W) were used as additional control besides uninfested plants, a conventional control (C). A constant unique pattern of differential expression in infested (I), wounded (W) stem samples compared to control (C) was observed. The up-regulated proteins showed relative fold intensity in order of I>W>C and down regulated spots trend as C>W>I, a quite intriguing pattern. Relatively, significant modulated expression pattern of a number of peptides in infested plants predicts the possibility of developing a quick and reliable molecular methodology for detecting plants infested with RPW. This study also reveals that artificially wounding of date palm stem almost affect the same proteins as infestation however relative intensity is quite lower than infested samples both in up and down regulated molecular peptide moieties. All 32 differentially expressed spots were subjected to MALDI-TOF analysis for their identification and we were able to match 21 proteins in the already existing databases. Furthermore, modulated proteins functionally belong to different physiological groups including: ion transport (33%), lipid biosynthesis (9%), protein folding (5%), plant defense (4%), ethylene signaling pathway (5%), protein folding (5%), carbohydrate metabolism (5%), proteolysis (5%), S-adenosylmethionine biosynthetic process (5%), nitrate assimilation (5%), porphyrin biosynthesis (5%), transcription (5%), lignin biosynthesis (4%) and unknown (5%) categories.

We believe that the protein molecules displaying modulated responses and especially those with up-regulated expression pattern in infested date palm samples will be helpful in developing diagnostic molecular markers for early detection of RPW infestation in date palm plants.

4.2 INTRODUCTION

The RPW (*Rhynchophorus ferrugineus*) has become the most destructive pest of date palm trees in several regions of the world including Saudi Arabia. Since its discovery in the Gulf region in 1980s, the insect is spreading rapidly and has been reported from almost every palm growing country in the World (Aldawood and Rasool, 2011). Bulk movement of date palm offshoots for planting is blamed to be the source of RPW invasion in the Middle East (Abraham *et al*., 1998). According to recent reports, RPW has been reported to infest 26 palm species belonging to 16 different genera worldwide (Dembilio and Jacas, 2012). Although, it is difficult to evaluate the overall actual global damages caused by RPW, however, only in Saudi Arabia just at 5% infestation, management and eradication of RPW in date plantation causes more than 8.69 Million USD, economic losses (El-Sabea *et al*., 2009).

It is also worth mentioning that RPW larval stage is most destructive and responsible for damaging the palm consuming date palm trunk tissue thus making it hollow (Faleiro, 2006; Kaakeh, 2005). This insect completes several generations within the same palm without any obvious symptoms until the tree collapses due to weakened stem (Rajamanickam *et al*., 1995; Avand Faghih, 1996). This cryptic infestation cycle of the RPW makes it difficult to detect diseased plants at earlier stages and severe decaying of the internal tissues lead to the death of the tree (Abraham *et al*., 1998; Gadelhak and Enan, 2005).

In Saudi Arabia, Ministry of Agriculture has launched a national campaign for controlling RPW to avoid losses inflicted on the production of dates. The campaign

includes removal of infested plants, pesticide application through injection and spraying in severely infested and newly infested areas, and the use of pheromone traps for monitoring and decreasing RPW populations (Mukhtar *et al*., 2011). It has been observed that infested plants can be recovered if infestation is detected at earlier stages however concealed infestation cycle of RPW is a major hindrance. Currently, available detection techniques including visual inspections, acoustic sensors (Mankin *et al.*, 2011; Potamitis *et al.*, 2009), sniffer dogs (Nakash *et al*., 2000), and pheromone traps (Faleiro and Kumar, 2008) are in practice to identify infestations at early stages. However, an effective and efficient technique with high throughput capability is still needed for the early detection of RPW.

We employed optimized proteomic methodology to identify earlier responses associated with RPW infestation in date palm based on scanty data utilizing plant genomics. Plants have evolved various innate and acquired defense mechanisms against visible/invisible injuries afflicted by insect pests. Innate or direct defense mechanisms in plants are specialized characteristics like thorns, trichomes, and primary and secondary metabolites (Kessler and Baldwin, 2002). Some herbivores feeding induce proteinase inhibitors in plants that prevent digestive enzymes required for insect's proper digestion (Tamayo *et al.*, 2000). Acquired defenses involve release of volatile organic compounds (VOCs) that attract arthropod predators and parasitoids to control herbivore populations Dicke and van Loon, 2000). Especially herbivores oral secretions discharged into plant tissues during feeding induce specialized responses (Felton and Tumlinson, 2008; Halitschke *et al*., 2001). The herbivores regurgitates and other oral secretions trigger plant defense related proteins (parallel to acquired immune system of mammals) or activate the plant

defense system releasing volatile compounds to attract predators (Korth and Dixon, 1997; Turlings *et al*., 1990). As the plant genomics/proteomics is in its earlier stages however, data started to emerge about this aspect (Bricchi *et al*., 2010). The insect plant interactions itself have great impact on plant defense responses (Kessler and Baldwin, 2002; Mewis *et al*., 2006).

Proteomics strategies have been extensively used for identifying infections/diseases among humans; however, its uses for plants have been relatively lesser. A few molecular studies involving plants encouraged us to embark on proteomics methodologies for saving beneficial date palm plant from RPW infestation. For example, proteome analysis of brittle leaf diseased date palm leaves when compared with that of their normal counterpart revealed quantitative differences in several proteins (Marqués *et al*., 2011). In another study, proteome analysis of brittle leaf disease affected date palm leaves indicated changes in the proteome at early disease stage where the decrease in Mn deficiency associated with MSP-33 kDa subunit protein was considered as brittle leaf disease biomarker (Sghaier-Hammami *et al*., 2012). Moreover, Gómez-Vidal *et al*., (2009) have evaluated the plant defense/stress, photosynthesis and energy metabolism related proteins, using 2-dimensional electrophoresis (2DE) proteomic techniques, that were differentially expressed in the date palm (*Phoenix dactylifera*) leaves in response to the attack by entomopathogenic fungi (*Beauveria bassiana, Lecanicillium dimorphum* and *L. cf. psalliotae*) as compared to control samples. In another study, 2DE of date palm sap revealed more than 100 protein spots of which 52 spots were identified. Some of the identified proteins were associated with *Saccharomyces cerevisiae* while others were related to vegetable proteins (Ben Thabet *et al*., 2010). Up to now, very few attempts

have been made on proteomics studies in the date palm. To our knowledge, the proteome analysis of the date palm in relation to changes induced by mechanical wounding and RPW infestation is conducted for the first time.

As such the objective of present study is to characterize the proteome changes occurring in date palm stem infested with RPW using 2D-DIGE and mass spectrometry for identifying the biomarker to be used in early detection of RPW for its effective management.

4.3 MATERIALS AND METHODS

4.3.1 Date palm material and infestation with RPW larvae

Tissue cultured date palm plants of Khudry cultivar were obtained from Al Rajhi Tissue Culture Laboratory, Riyadh, Saudi Arabia. These plants were divided into 3 groups, with three replicates each, and were then used for mechanical wounding and infestation with RPW as described previously (Lippert *et al*., 2007). Briefly, plants were separated into three experimental groups. Group one was artificially infested (I) with RPW larvae, group two artificially wounded (W) and third group was kept without any treatment as control (C). For artificial infestation each plant was introduced with 5 second instar RPW larvae by making holes in the stem using drill machine with 6-mm size bit. After treatment the stem part of the plants were wrapped up with fine steel mesh. The stem samples were taken after 3-days of infestation and stored at -80^{o}C until use.

4.3.2 Protein extraction and SDS-PAGE

Total proteins from control, infested and wounded date palm stem samples (three replicate from each sample) were extracted using phenol/SDS extraction method as described by Gomez-Vidal et al (2008) with minor modification leading to optimized protein isolation from date palm (Rasool *et al*., 2014). Briefly, stem tissues (one gram) were ground to fine powder in liquid nitrogen with pestle/mortar and suspended in 5 ml phenol and 5 ml dense SDS buffer (30% w/v sucrose, 2% w/v SDS, 0.1 M Tris-HCl, pH 8.0, 5% v/v 2-mercaptoethanol). After mixing and vortexing, mixture was centrifuged for 5 minutes at 10,000 rpm at 4^{o}C. The upper phenolic phase was collected and precipitated with five volumes of cold 0.1 M methanolic ammonium acetate. After incubating at -20^{o}C for 30 min, precipitated proteins were recovered by centrifugation at 1000 rpm for 5 min at 4^{o}C and then washed two times with cold methanol solution containing 0.1 M ammonium acetate and then two times with cold 80% v/v acetone. Protein pellet was recovered each time by centrifugation at 8000 rpm for 5 minutes. The final protein pellet was air-dried at room temperature and suspended in 100 mM Tris buffer pH 8.0 and the then added equal volume of 2X SDS-reducing buffer (100 mM Tris–Cl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol) containing 200 mM mercaptoethanol. SDS-PAGE analysis was carried out as described by Laemmle (1970). The gel was stained with Commassie brilliant blue G-250 with constant and gentle agitation for overnight.

4.3.3 Two dimensional difference gel electrophoresis (2D-DIGE)

The dried protein sample for 2D-DIGE was solubilized in rehydration buffer containing the chaotropic agent urea, alongside surfactants CHAPS and thiourea (7 M urea, 2 M

thiourea, 2% CHAPS w/v, 2% DTT, 0.5% IPG buffer, 0.002% bromophenol blue) by shaking at 150 rpm for 1 h at 25^{o}C. The insoluble residue was removed by centrifugation at 12,000 rpm for 10 minutes. The protein-containing supernatants were separated from insoluble debris. Protein concentration was measured using 2-D Quant kit (GE Healthcare, Little Chalfont, U.K.) according to the manufacturer's protocol and 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, U.K.) and solubilized in buffer (7M urea, 2M thiourea, 2% CHAPS, 30 mM Tris-Cl, pH 8.5) without DTT and IPG buffer and quantified again using 2-D Quant kit (GE healthcare). Protein was aliquoted to required amount (300 µg) and frozen.

After adjusting pH to 8.5, each protein sample was labeled with CyDye Flour minimal dyes (GE healthcare) according to manufacturer's recommendation. Briefly, 50 µg each protein sample was incubated with 400 pmol CyDye Flour minimal dyes on ice for 30 min in the dark. The control, wounded and infested samples were labeled alternatively with Cy3 or Cy5 (Table 1). Internal standard containing equal amount of proteins from each sample was labeled with Cy2. The reaction was stopped by adding 1.0 µl of 10 mM lysine solution and incubated 10 min on ice and proteins samples were combined according to experimental design as shown in Table 1.

For 2D-DIGE, IPG strip (24 cm. GE Healthcare) were rehydrated for 16 hours with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS w/v, 0.2% DTT, 0.5% IPG buffer, 0.002% bromophenol blue) containing the protein samples for each gel. Isoelectric focusing was performed with Ettan IPGphor3 IEF unit (GE Healthcare, Bucks UK) at 50µA per strip at 20°C according to following programme:1) 500V for 1 hour, 2)

1000V for 1h, 3) 8000V for 3h, 4) 8000 V for 45,000Vh. Strips were immediately equilibrated after Isoelectric focusing for 15 min in equilibration buffer 1 (50 mM Tris-HCl, pH 8.8; 6 M urea; 20% [v/v] glycerol and 2% [w/v] SDS) containing 2% DTT at room temperature under gentle agitation, and then by equilibration buffer 2 (50 mM TrisHCl, pH 8.8; 6 M urea; 20% [v/v] glycerol and 2% [w/v] SDS) containing 2.5% iodoacetamide. Second-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 5-20% polyacrylamide gradient gels using the Ettan DALT six vertical unit (GE Healthcare, Little Chalfont, U.K.) at 15^{o}C for 1W per gel for 1h and then 2W per gel until the bromophenol blue dye reached the end of gel. Polyacrylamide gradient gels were prepared on low fluorescence glass using 2D Optimizer (Nextgen Sciences).

4.3.4 Image acquisition and analysis

The 2D gels were scanned using fluorescence gel scanner, Typoon imager (Trio) (GE, Healthcare, Sweden), using appropriate wavelengths and filters for Cy2, Cy3 and Cy5 dyes according to manufacturer's recommended protocol. The 2D gels images were analyzed using Progenesis sameSpot software version 3.3 (Nonlinear Dynamics Ltd, Newcastle upon Tyne, U.K.). The differential expression was ascertained using normalized protein spots in the Cy5 and Cy3 channels compared to the internal standard (Cy2). The spots of infested and wounded date palm samples were compared to control samples. One way ANOVA was used to calculate the fold difference values and P-values. A threshold level was set at 1.5 fold up or down-regulation, at $p \leq 0.05$ level.

Table 1. Experimental design for 2D-DIGE. Three replicates from each control, infested and wounded protein samples were labeled and combined for 2D-DIGE.

Gel No.	Cy2	Cy3	Cy5
1	pooled sample	Infested R1	Control R3
2	pooled sample	Infested R2	Wounded R1
3	pooled sample	Infested R3	Wounded R2
4	pooled sample	Control R1	Wounded R3
5	pooled sample		Control R2

4.3.5 Protein identification by mass spectrometry

Preparative 2D gel was run using 700 µg total protein sample obtained by pooling all the samples present in the experimental design. The gel was fixed in ethanol (35%v/v), with phosphoric acid (2%v/v) for overnight and then washed three times with water for 30 min each time. Then the gels were incubated for 1 hour in methanol (34% v/v) containing ammonium sulphate (17%w/v) and phosphoric acid (3% v/v) for I hour and after that 0.5% g/L Commassie G-250 were added. The gels were stained for 5 days followed by rinsing in Milli Q water and stored until spots were picked and identified by mass spectrometry. Corresponding differential spots were matched to a colloidal commassie stained image of the preparative gel, which was first mapped to the reference image.

The differential protein spots were manually excised form commassie stained preparative gels and washed with solution containing 50 mM Ammonium bicarbonate. Then, the gels pieces were destained with 50 mM ammonium bicarbonate and 50 % acetonitrile followed by 100 % acetonitrile. After destaining, gels pieces were dried using vacuum centrifugation at 40^{o}C for 5 minutes. Dried Gel pieces were rehydrated and digested with 10 µl trypsin at a concentration of 2ng/µl (Promega, USA) in 25 mM NH_4HCO_3 pH 8.0 at 4^{0}C for 60 minutes and digestion was continued for an additional 16-24 hours at 37^{o}C. The digestion was stopped and digest mixtures was transferred to 0.5 ml tube and the peptides were extracted by adding 50% acetonitrile having 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) in

a positive mode. 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. The validity/accuracy of identified proteins was only accepted when the mascot score was ≥ 60.

4.4 RESULTS AND DISCUSSION

4.4.1 Date palm proteome analysis by 2D-DIGE

Plants respond to injuries/infestations and others abiotic stresses by activating a broad range of acquired defense system, including activation of pathogenesis-related (PR) genes both at local and systemic sites (Kessler and Baldwin, 2002), crosslinking of cell wall proteins, generation of reactive oxygen species (ROS) and local programmed cell death. This study reports the first proteomic analysis of date palm stem defense response against RPW infestation as well as mechanically wounded stem as the artificial infestation involves wounding experimental plants. The 2D-DIGE was used to explore the response of RPW infestation in date palm stem. For evaluating differential proteomic responses subsequent to infestation stem samples from infested control and artificially wounded plants were subjected to protein isolation followed by differential expression analyses. Proteins were extracted from stem samples using phenol-SDS extraction method (Gómez-Vidal *et al*., 2008). To minimize internal variations three replicates for each sample were used. The extracted proteins were quantified using 2D quant kit after solubilizing in 2D-rehyration buffer. 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 Commassie showed good reproducibility among replicates, consistent solubilization and reproducible extraction methods (Figure 1). Proteins extracted using this method was then evaluated by mini 2DE to assess reproducibility, isoelectric point and percentage of acrylamide selection of isolated proteins. A 125 µg of lysis buffer solubilized protein was used for mini 2DE experiments.

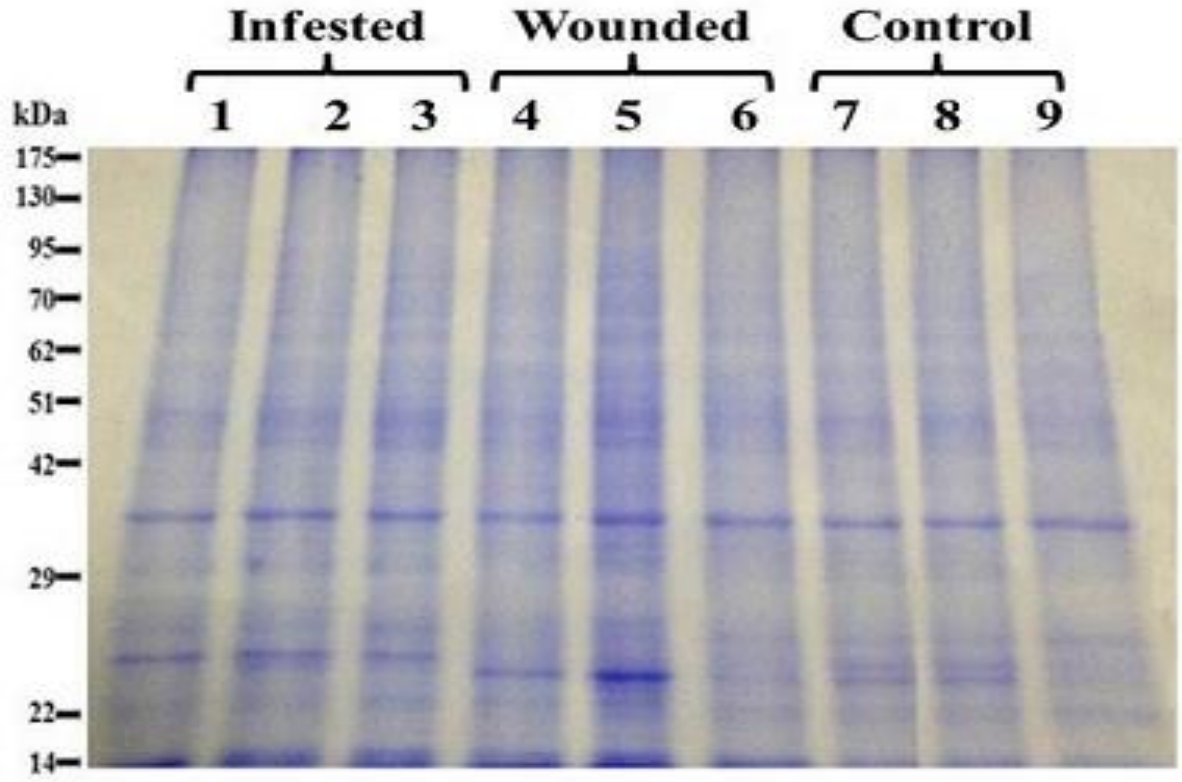


Fig. 1 Comparative protein expression profiling of the control, infested and wounded date palm samples using SDS-PAGE. Lanes 1-3 represent total cell proteins from 3-infested replicates, while lanes 4-6 represent proteins from wounded date palm samples, and lanes 7-9, represent proteins from control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

For determining isoelectric point (pI) ranging of isolated proteins, broad range immobilized pH gradient (IPG) strips pH 3-11 were used in the first dimension followed by separation with 12.5 % acrylamide second dimension gels. After separation in the second dimension, protein gels were stained with Commassie dye. Figure 2 shows protein profiles of stem with control and infested respectively.

Moreover, 2D DIGE (GE Healthcare, Bucks UK) was run to compare differences among control, infested and wounded samples. These samples were labeled with either Cy3 or Cy5 dyes while internal standard was consistently labeled with Cy2. Experimental design for 2D-DIGE experiment is shown in (Table 1). After labeling with Cy dyes, two samples were mixed with different combination along with internal standard and electrophoresed on the same gel except one gel contained single sample with internal standard. The representative gels of 2D-DIGE after scanning with fluorescence gel scanner, Typoon imager (Trio) (GE Healthcare) are shown in Figure 3. Progenesis Samespots software version 3.3 (Nonlinear Dynamics Ltd., UK) was used to statistically analyze the protein expression among control, infested and wounded samples. A total of 522 well-resolved protein spots were observed on each gel, out of them 32 spots showed statistically significant differences ($p \leq 0.05$, and intensity fold change ≥ 1.5) among expressions of proteins in either of this combination. Out of these 32 spots, 13 were found to be up regulated in the infested compared to control while 19 spots found to be down regulated in the infested samples compared with control (Figure 4). Interestingly, spots from the wounded samples showed the same trend of up or down regulation compared to control but fold change intensity is lower than the infested samples. However, some contrasting patterns were also observed in the infested and wounded samples that are of our interest.

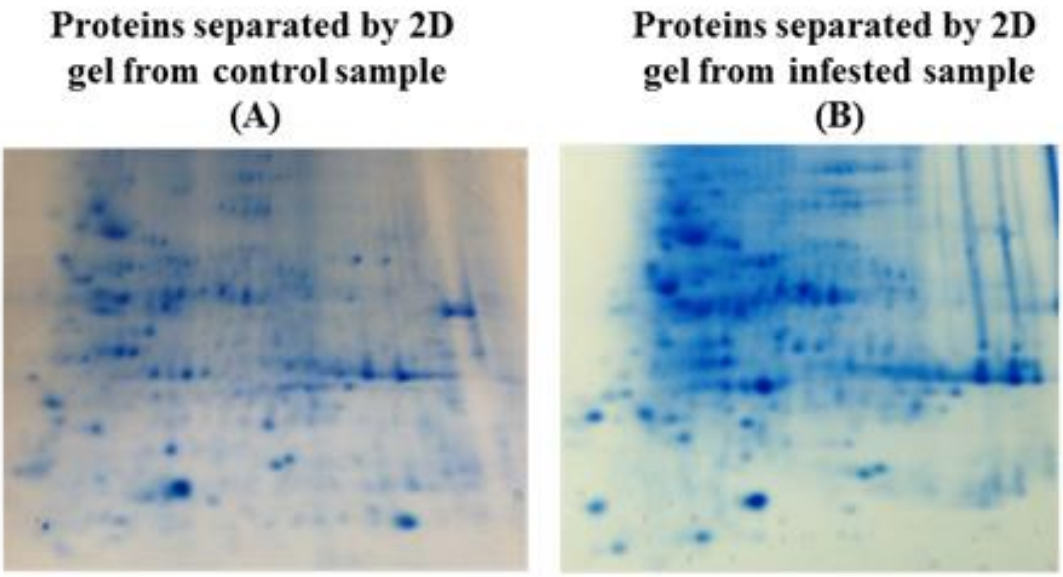


Fig. 2. Evaluation of protein samples isolated from date palm stem by mini 2D gels. 2D-PAGE of proteins from date palm stem. 7 cm IPG strip was used to separate the proteins solubilized in 2DE buffer in first dimension. 12.5 % acrylamide was performed to separate focused proteins. After separation, gel was stained with Coomassie. A: Proteins separated by 2D gel from control sample, B: protein separated by 2D from infested sample.

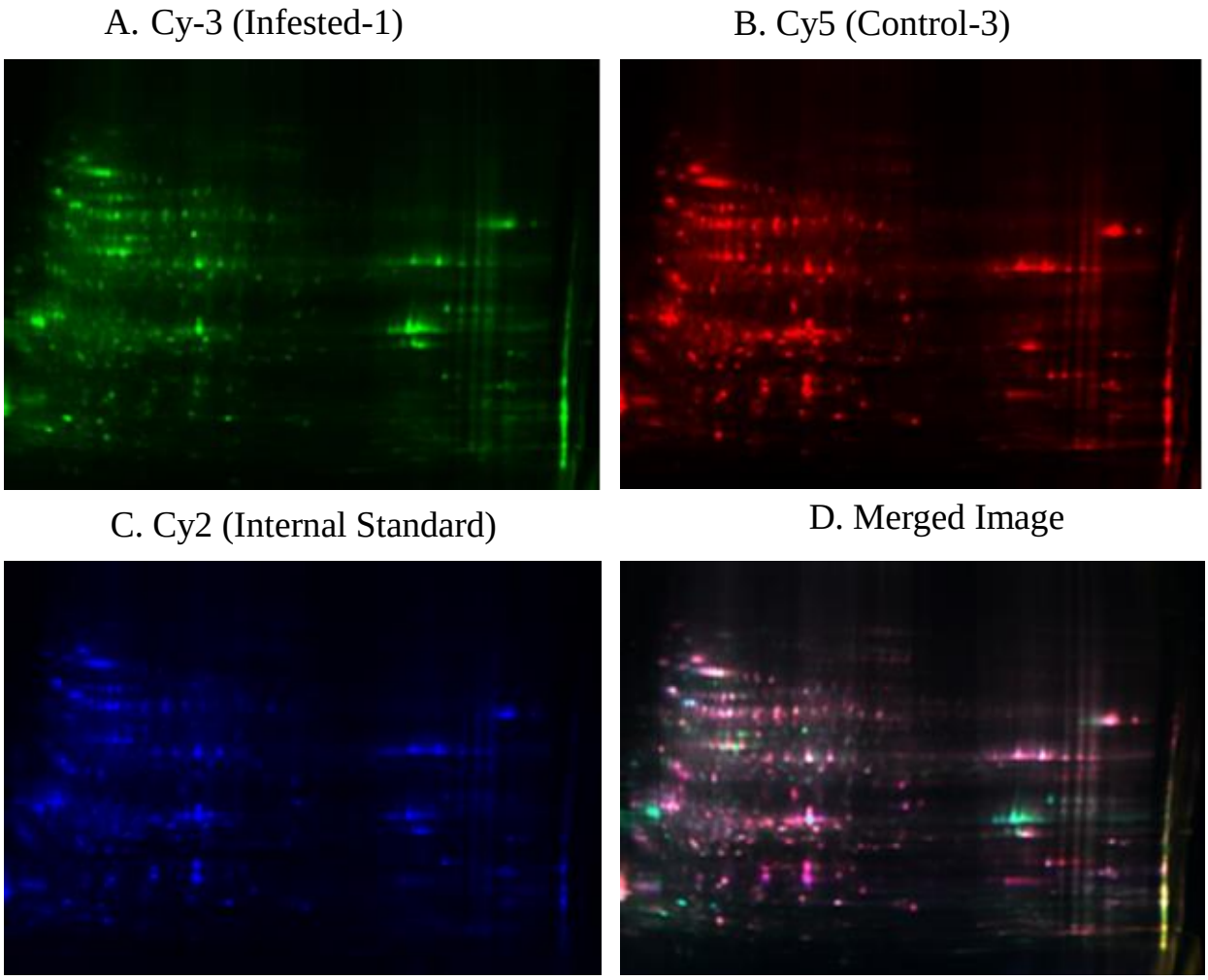


Fig.3a. 2D-DIGE images of date palm proteins. The protein sample of Infested-1, Control-3 and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by 2D-DIGE followed by image scanning. A: image of date palm infested sample and labeled with Cy3 dye. B: image of date palm control sample labeled with Cy5 dye. C: image of date palm sample pooled from all and labeled with Cy2 dye. D: Overlay gel of infested, and control samples along with internal standard.

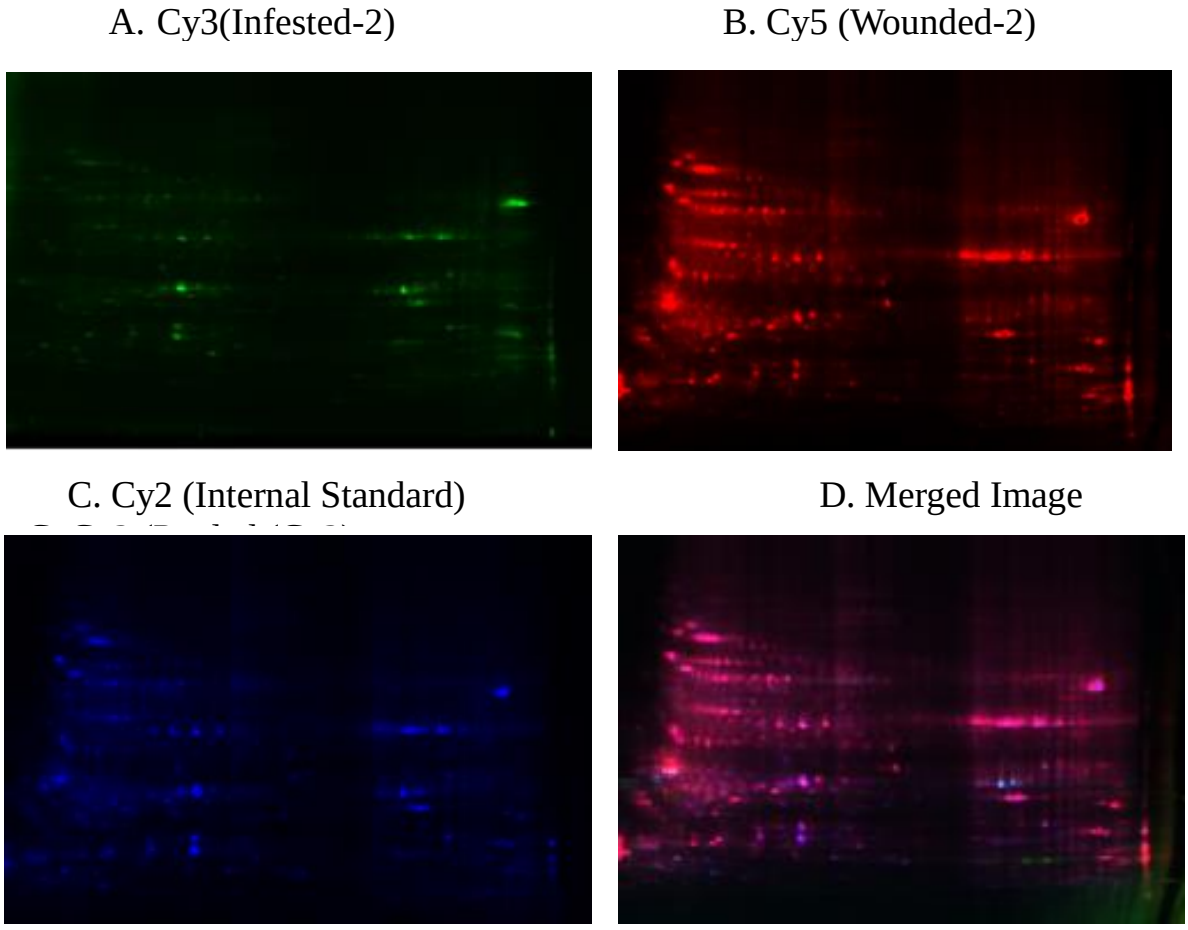
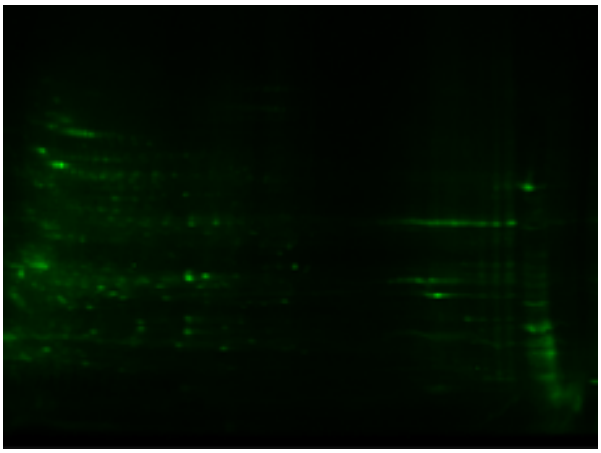
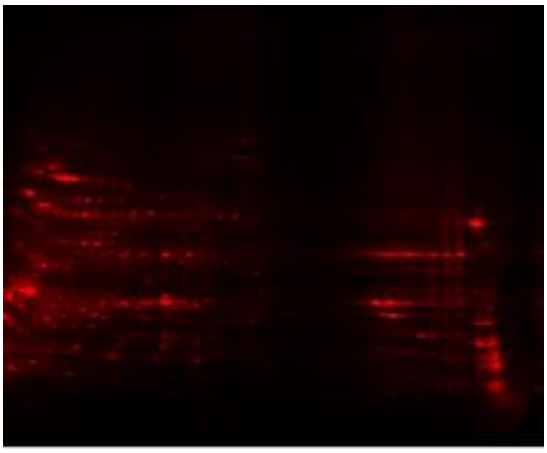


Fig.3b. 2D-DIGE images of date palm proteins. The protein sample of Infested-2, Wounded-1 and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by 2D-DIGE followed by image scanning. A: image of date palm infested sample and labeled with Cy3 dye. B: image of date palm wounded sample labeled with Cy5 dye. C: image of date palm sample pooled from all and labeled with Cy2 dye. D: Overlay gel of infested, and wounded samples along with internal standard.

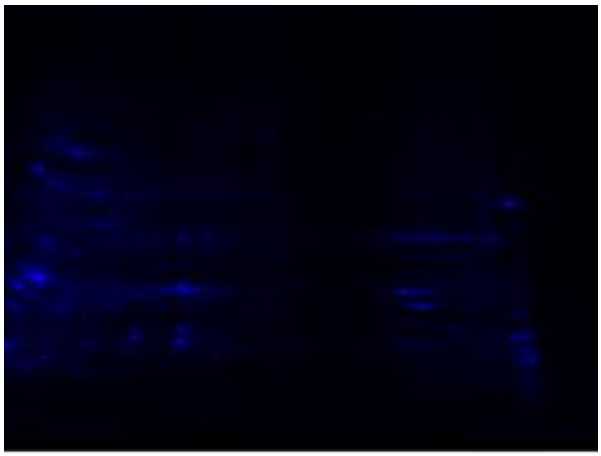
A. Cy3 (Infested-3)



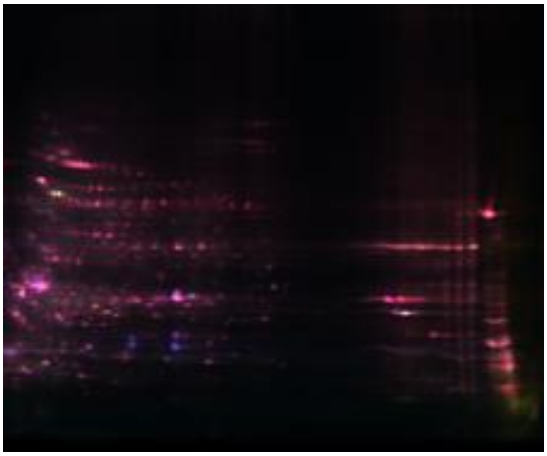
B. Cy5 (Wounded-3)



C. Cy2 (Internal Standard)



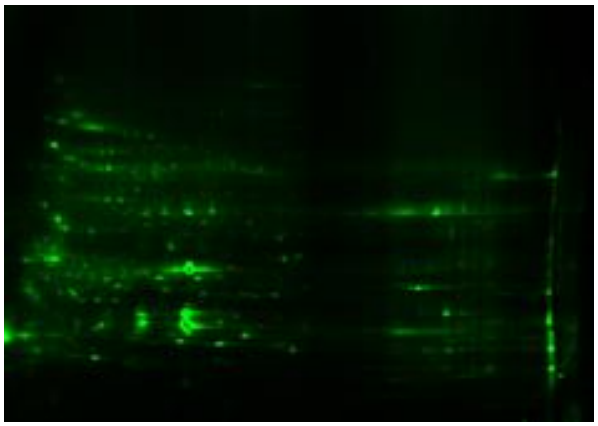
D. Merged Image



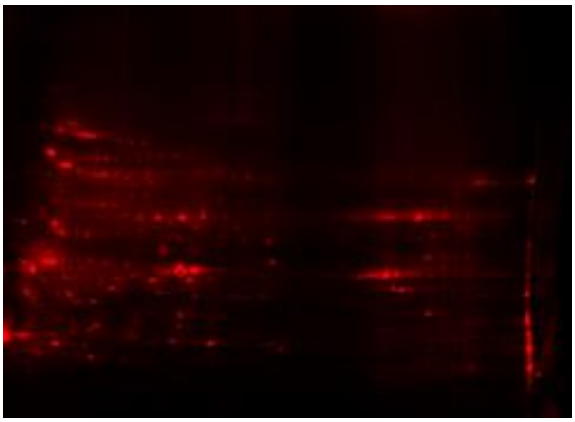
2D-DIGE Gel-3

Fig.3c. 2D-DIGE images of date palm proteins. The protein sample of Infested-3, Wounded-2 and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by 2D-DIGE followed by image scanning. A: image of date palm infested sample and labeled with Cy3 dye. B: image of date palm wounded sample labeled with Cy5 dye. C: image of date palm sample pooled from all and labeled with Cy2 dye. D: Overlay gel of infested, and wounded samples along with internal standard.

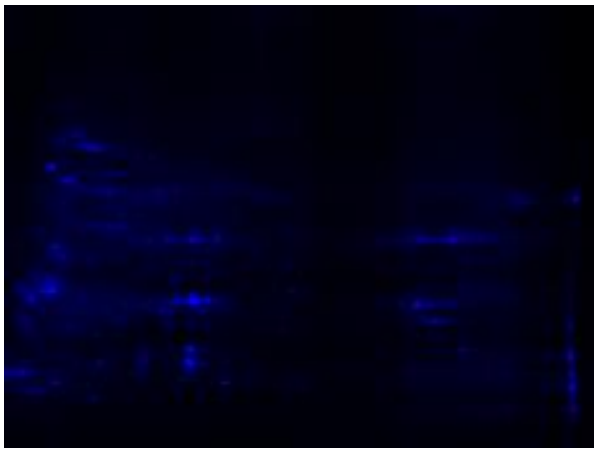
A. Cy3 (Control-1)



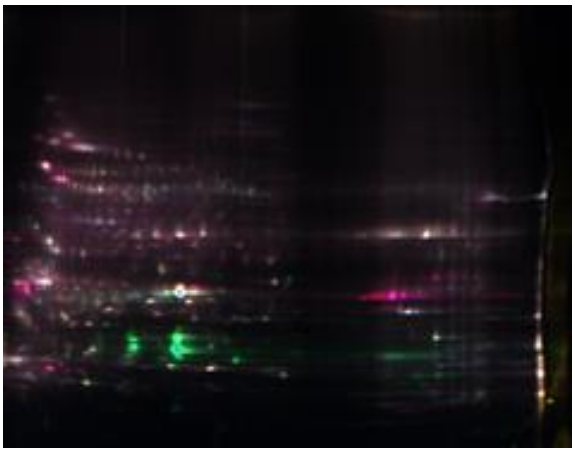
B. Cy5 (Wounded-1)



C. Cy2 (Internal Standard)



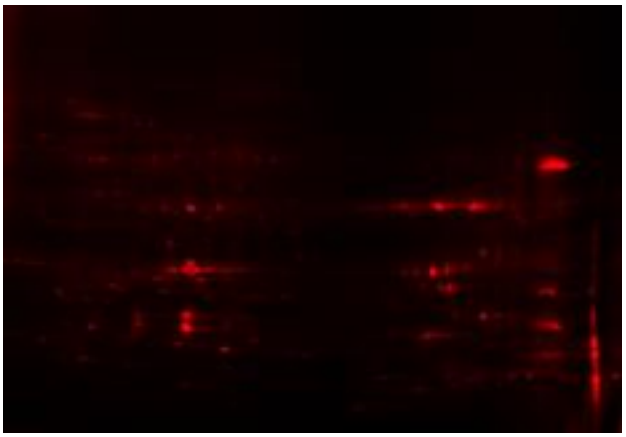
D. Merged Image



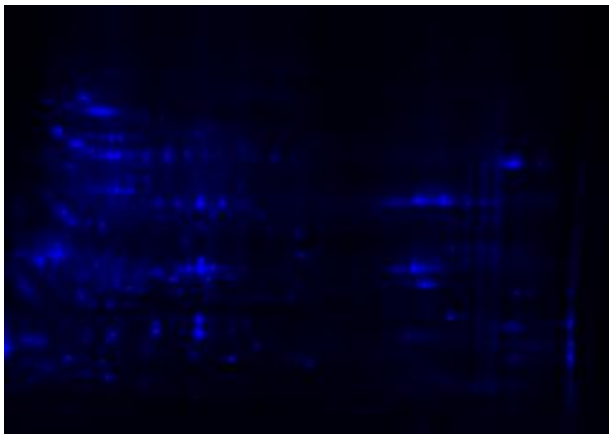
2D-DIGE Gel-4

Fig.3d. 2D-DIGE images of date palm proteins. The protein sample of Control-1, Wounded-3 and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by 2D-DIGE followed by image scanning. A: image of date palm control sample and labeled with Cy3 dye. B: image of date palm wounded sample labeled with Cy 5 dye. C: image of date palm sample pooled from all and labeled with Cy 2 dye. D: Overlay gel of control, and wounded samples along with internal standard.

A. Cy5 (Control-2)



B. Cy2 (Internal Standard)



D. Merged Image

2D-DIGE Gel-5

Fig.3e. 2D-DIGE images of date palm proteins. The protein sample of Control-3 and internal standard (pooled of all the samples) are individually labeled with Cy dyes, mixed together and separated by 2D-DIGE followed by image scanning. A: image of date palm control sample and labeled with Cy 5 dye. B: image of date palm sample pooled from all and labeled with Cy 2 dye. D: Overlay gel of control and internal standard.

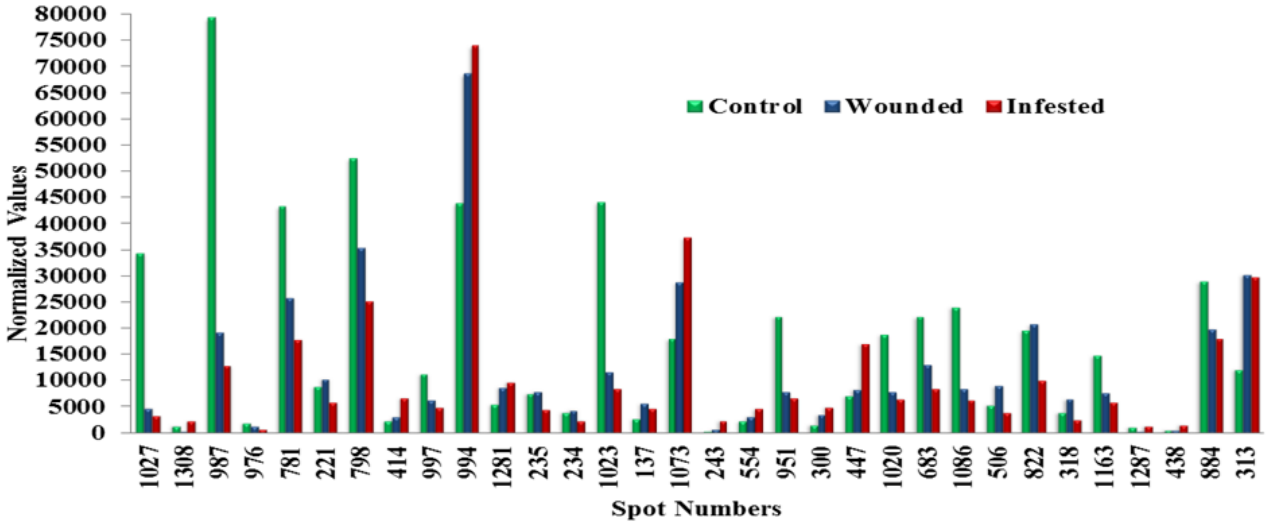


Fig. 4 Normalized spot volume occupied by differential protein spots of control (C), wounded (W) and infested (I) date palm stem samples.

Majority of the up regulated spots (spot no 414 243,554, 300, 447, 994, 1281, 1073, and 438) followed unique trend of up regulation like (I>W>C) except two spots (1308 and 1287) where the pattern was observed like (I>C>W). Similar to up regulation, almost the same trend was observed among majority of down regulated spots in reverse order C>W>I (spots nos. 1027, 987,976, 781, 798, 1023, 951, 1020, 683, 1086, 1163, and 884). However, some deviations were noticed in some spots (like 221, 235, 234, 506, 822, 318) where the expression pattern was observed as W>C>I except two spots (137 and 313) this pattern was like W>I>C. Our data clearly demonstrates that RPW infestation induces stronger defense responses in date palm than mechanical wounding, and these (stronger) responses could be exploited as molecular indicators for ascertaining infestation in date palm plants. The relative expression patterns are quite intriguing and previous reports also reports such trends in plants (Turlings *et al*., 1998; Duceppe *et al*., 2012).

4.4.2 Protein identification by mass spectrometry

Proteomic methodologies for differential expression is quite tedious in nature, however, it provides highly reliable information once a modulated peptide has been identified. Final identification subsequent to proteomic methodology involves identification procedures involving mass spectrometric analyses. A state of the art proteomic methodology MALDI-TOF used for the identification of proteins by peptide mass fingerprinting was employed to characterize peptides modulated in date palm stem subsequent to infestation with RPW. The above described 32 differentially expressed spots were excised from preparative gels, digested enzymatically with trypsin and subjected to MS analysis. Data were examined using BioTools3.2 (Bruker Daltonics, Germany) in combination with the

Mascot search algorithm (version 2.0. 04) against green plants database. Table 2 showed the spot number, their Swiss-Port accession numbers, protein description, function, theoretical pI, molecular weight, protein coverage (%), score, and matching organism for the differentially expressed proteins. MS results showed that identified 21 spots were matched to previously reported proteins found in databases. We were unable to characterize 11 protein spots as there was no protein matching proteins in the database. It is quite possible that date palm plant has some unique proteins not found in other plants. Once date palm genome is deciphered, these unique proteins will be of quite interest. In protein identification a small number of different spots showed the same identified proteins, indicating the presence of isoforms of specific gene or these were emerged due to post-translational modifications (PTMs) differing in molecular weight and pI. In our case V-type proton ATPase catalytic subunit A (spot no 221, 243), and probable glycerol-3-phosphate acyltransferase 8 (spot no 683 and 781) belong to these categories.

The identified proteins had shown a high homology especially with specific proteins of *Arabidopsis thaliana* (33%), whereas this homology ratio was 14.2% and 9.5% as compared to those of *Oryza sativa* (*subspecies Japonica*) (rice) and *Sorghum bicolor* (Sorghum), respectively. While a relatively low homology ratio (4.7%) was observed with each of *Zea mays*, *Solanum lycopesicum*, *Eucalyptus gunnii* (Cider gum), *Hevea brasiliensis* (Para rubber tree), *Citrus unshiu* (Satsuma mandarin), *Carica papaya* (papaya), *Medicago truncatula* (Barrel medic), *Spinacia oleracea* (Spinach), and *Hordeum vulgare* (Barley)**.**

Table 2. Differentially expressed proteins between control, wounded and infested date palm stem samples by MALDI-TOF peptide mass fingerprinting after 2D-DIGE.

Spot No.	FC (I/C)	FC (W/C)	Accession (uniprot)	Protein description	Function	pI	MW	Coverage	Score	organism
1308	1.60↑	3.03↓	P27161	Calmodulin	M: Calcium ion binding	4.18	16950	69	229	*Solanum lycopersicum* (Tomato)
781	2.42↓	1.67↓	Q5XF03	Probable glycerol-3-phosphate acyltransferase 8	Lipid biosynthesis	9.14	56345	25	60	*Arabidopsis thaliana* (Mouse-ear cress)
221	1.50↓	1.17↑	P49087	V-type proton ATPase catalytic subunit A	Ion transport	5.89	62198	25	62	*Zea mays* (Maize)
798	2.08↓	1.48↓	O04854	Caffeoyl-CoA O-methyltransferase	Lignin biosynthesis	5.02	28010	12	72	*Eucalyptus gunnii* (Cider gum)
414	2.77↑	1.28↑	P29685	ATP synthase subunit beta, mitochondrial	ATP hydrolysis coupled proton transport	5.98	60335	35	75	*Hevea brasiliensis* (Para rubber tree)
994	1.68↑	1.56↑	Q94FT8	Non-symbiotic hemoglobin 3	Iron and oxygen transport	9.83	18614	65	65	*Oryza sativa* subsp. japonica (Rice)
1281	1.76↑	1.58↑	Q94FT8	Non-symbiotic hemoglobin 3	Iron and oxygen transport	9.83	18614	83	62	*Oryza sativa* subsp. japonica (Rice)
137	1.66↑	1.99↑	P22953	Heat shock 70 kDa protein 1	Stress response, plant defense,	5.03	71712	38	88	*Arabidopsis thaliana* (Mouse-ear cress))
1073	2.07↑	1.50↑	Q9SVQ0	Ethylene-responsive transcription factor ERF062	Ethylene signaling pathway	9.46	44283	37	68	*Arabidopsis thaliana* (Mouse-ear cress))
243	5.31↑	1.77↑	Q9SM09	V-type proton ATPase catalytic subunit A	Hydrogen ion transport	5.29	68923	34	70	*Citrus unshiu* (Satsuma mandarin)
554	1.99↑	1.39↑	Q1PFG1	F-box protein At1g66490	Uncharacterized	8.75	43577	26	65	*Arabidopsis thaliana* (Mouse-ear cress))

300	2.98↑	2.13↑	Q39251	Actin-depolymerizing factor 2	Cytoskeletal protein	5.24	15963	36	65	*Arabidopsis thaliana* (Mouse-ear cress)
447	2.36↑	1.17↑	B1A986	NAD(P)H-quinone oxidoreductase subunit 4L, chloroplastic	Oxidoreductase activity, ATP synthesis coupled electron transport	9.65	11327	54	67	*Carica papaya (papaya)*
1020	2.81↓	2.35↓	Q9SKQ0	Peptidyl-prolyl cis-trans isomerase CYP19-2	B: protein folding	8.33	1868	48	63	*Arabidopsis thaliana* (Mouse-ear cress)
683	2.58↓	1.69↓	Q5XF03	Probable glycerol-3-phosphate acyltransferase 8	Lipid biosynthesis	9.14	56345	29	60	*Arabidopsis thaliana* (Mouse-ear cress))
506	1.36↓	1.65↑	Q8W0A1	Beta-galactosidase 2	Carbohydrate metabolic process	5.59	92630	19	62	*Oryza sativa* subsp. japonica (Rice)
822	1.92↓	1.06↑	C5X3M7	Putative uncharacterized protein Sb02g009233	Nucleic acid binding	5.6	14533	88	73	*Sorghum bicolor* (Sorghum)
318	1.48↓	1.62↑	A5C9D0	Putative uncharacterized protein	Proteolysis	9.41	119434	20	71	***S**orghum bicolor* (Sorghum)
438	2.24↑	1.02↑	A4PU48	S-adenosylmethionine synthase	Adenosylmethionine biosynthesis	5.59	43708	29	63	*Medicago truncatula* (Barrel medic))
884	1.61↓	1.45↓	P23312	S Nitrate reductase [NADH]	Nitrite assimilation	6.25	104703	68	62	*Spinacia oleracea* (Spinach))
313	2.44↑	2.47↑	Q42836	Delta-aminolevulinic acid dehydratase, chloroplastic)	Porphyrin biosynthesis	6.05	46639	46	80	*Hordeum vulgare* (Barley)

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.

Moreover, differentially expressed proteins had been characterized as: ion transport (33%), lipid biosynthesis (9%), protein folding (5%), plant defense (4%), ethylene signaling pathway (5%), protein folding (5%), carbohydrate metabolism (5%), proteolysis (5%), S-adenosylmethionine biosynthetic process (5%), nitrate assimilation (5%), porphyrin biosynthesis (5%), transcription (5%), lignin biosynthesis (4%) and unknown (5%) and were shown in Figure 5.

It is to be worth mentioning that a large number of proteins identified in this study belong to ion transport family of proteins that are stress related, while several others could potentially be involved in response to RPW infestation. These were V-type proton ATPase catalytic subunit A (*Zea mays* (Maize) (spot no 221 and 243). The spot 221 were suppressed 1.5 fold in the infested samples relative to control. However, spot 243 was much higher in infested samples (5.3 fold) compared to control. V-type proton ATPase is a proton pump present inside plant vacuole which plays an important role in plant salinity tolerance (Kluge *et al*., 2003; Barkla *et al*., 2009). V-type proton ATPase plays a crucial role in maintenance of ion homeostasis inside plant cells, acidifying compartments of the vacuole (Schnitzer *et al*., 2011; Silva and Gerós, 2009). It maintains electrochemical H^+-gradient to drive the transport of Na^+ into the vacuole lumen, compartmentalizing this toxic ion from the cytoplasm and maintaining low cytoplasmic Na^+ concentrations. Up regulation of this enzyme was also reported in response to stress in other plants as well (Silva *et al*., 2010; Qiuet *et al*., 2007; Kabala and Klobus, 2008; Otoch *et al*., 2001). The up regulation of enzyme V-type proton ATPase might provide insights into understanding the infestation of date palm and could be a useful marker for early diagnosis of RPW.

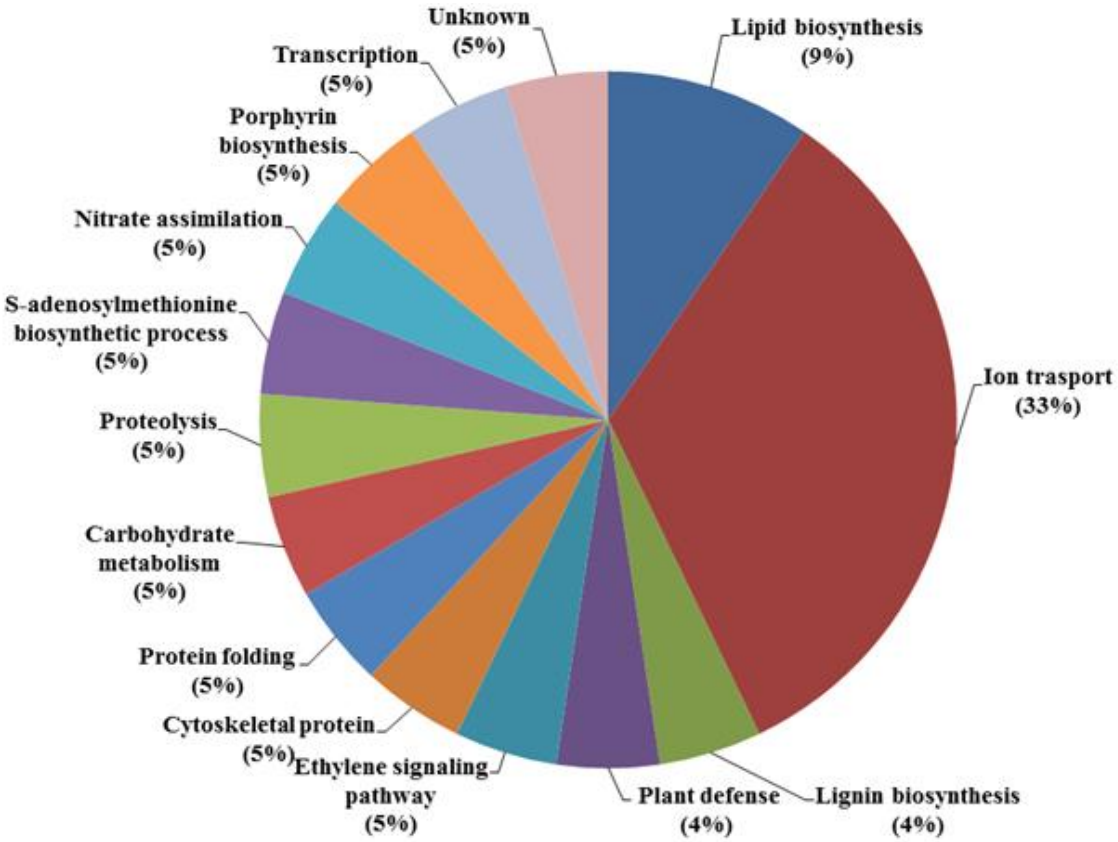


Fig. 5. A pie chart depicting the physiological classification of potentially identified proteins through Mass Spectrometry analysis.

Another spot (spot no 414) corresponded to ATP synthase subunit beta, mitochondrial identified as differential protein and up regulated in infested samples.

The expression of calcium binding protein, calmodulin (spot no 1308) increased (1.6 fold) in response to the infestation of RPW. Calmodulin is a calcium-binding messenger protein and regulates downstream functions in response to $Ca2^{+}$. It has several targets including ion channels, a large number of enzymes i.e. kinases, phosphatases, cytoskeletal proteins, synaptic proteins, cell cycle proteins (Yap *et al*., 2000; Hoeflich and Ikura, 2002; Kahl and Means, 2003; Calabria *et al*., 2008). Calcium activated calmodulin is reported to be involved in heat-shock signal transduction (Borisjuk *et al*., 1998).

Protein belonging to S-adenosylmethionine synthase (spot no 438) showed increased expression (2.2 fold) in infested sample relative to control. This enzyme catalyzes the synthesis of S-adenosylmethionine from methionine and ATP. Also, expression was up-regulated by 2.4 fold in spot no 313, a homologous to Delta-aminolevulinic acid dehydratase, chloroplastic) as compared to control. This enzyme is involved in the formation of porphobilinogen (von Wettstein *et al*., 1995). The expression of the spot number 137, homologous to heat shock 70 KDa proteins (HSP 70), was increased (1.66 fold) in the infested samples as compared to control. However, its expression was increased more in the wounded (~2 fold) relative to control. HSP 70 protein is a chaperone that assists the folding process of newly synthesized proteins and minimizes aggregation (Fink, 1999). A high expression of this protein under stress conditions has also been reported previously by other workers (Fink, 1999; De Rocher and Vierling, 1994). Several other HSPs have also been reported in response to different stress

conditions like because of pea and *Erysiphe pisi* interaction (Curto *et al*., 2006), pea and *Mycosphaerella pinodes* interaction (Castillejo *et al*., 2010a), or triticale under low N fertilization level (Castillejo *et al*., 2010b). The over-expression of these stress responsive proteins should not be surprising, is a natural defence mechanism that responds, as mentioned above, to any outer biotic/abiotic stress. The over expression of HSP 70 in present study could be a useful marker against RPW infestation.

The two spots (781 and 683) matching glycerol-3-phosphate acyltransferase, an essential enzyme for glycerolipid biosynthesis, were found to be down regulated in infested samples. Another down regulated spot (506) matched with protein Beta-galactosidase 2 was identified. This enzyme is responsible for non-reducing beta-D-galactose residues in beta-D-galactosides. Furthermore, the spot 1073 was identified as ethylene response factor (ERF1) and its expression was up-regulated (2.07 fold) in infested date palm sample relative to control. ERF1 is a member of novel family of plant-specific transcriptional factors in *Arabidopsis thaliana* (Nakano *et al*., 2006), and is activated by either ethylene (ET) or jasmonate (JA) and also activated synergistically by both hormones (Lorenzo 2003). ERF1 regulates defense response genes to the necrotrophic fungi *B. cinerea* and *P. cucumerina* by integrating ET and JA defense responses in *Arabidopsis* (Berrocal-Lobo *et al*., 2002; Lorenzo *et al*., 2003). ERFs have been reported to affect a number of developmental processes, and are also differentially adapted to biotic or abiotic stresses such as pathogen attack, wounding, extreme temperature, and drought (Ecker, 1995; Penninckx *et al*., 1996; O'Donnell *et al*., 1996). Our results suggest that ERF1 could be a key element in defense against RPW attack.

Another spot 884 matching to nitrate reductase was suppressed (1.6 fold) in date palm infested sample compared to control. This enzyme plays a key role in the synthesis of nitric oxide (NO) (Rockel *et al*., 2002), an important signaling molecule mediating physiological and developmental processes. Also, NO plays an important role in plant responses to biotic and abiotic stresses (Qiao and Fan, 2008; Corpas *et al*., 2011; Moreau *et al*., 2010). Furthermore, NO was also reported to modulates ethylene, salicylic acid, and jasmonic acid-signaling pathways and abscisic acid (ABA)-induced stomatal closure (Lamattina *et al*., 2003; Wendehenne *et al.*, 2004).

Another important protein identified as Nonsymbiotic hemoglobins 3 (spots no, 994 and 1281) found to be up-regulated in infested and wounded samples which are known to be induced in plants during hypoxic stress. Nonsymbiotic hemoglobin AHb1 plays an important role in NO detoxification in *Arabidopsis* by scavenging NO and reducing its emission under hypoxic stress. These proteins were reported to protect plants during hypoxia or other similar stresses (Hunt *et al*., 2002; Dordas *et al*., 2003a) and scavenge NO produced in stress conditions (Dordas *et al*., 2003b). Nonsymbiotic hemoglobin AHb1 plays an important role in NO detoxification in *Arabidopsis thaliana* by scavenging NO and reduces NO emission under hypoxic stress (Perazzolli *et al*., 2004).

A significant increase (2.98 fold) of actin depolymerizing factors (ADFs) (spot 300) was found in infested samples compared to control. ADF is a small actin-binding protein and involved in plant growth, development stress response and pathogen defense (Drobak *et al*., 2004; Staiger and Blanchoin, 2006; Hussey *et al*., 2006). The role of actin had also been reported in response to plant hormones and biotic or abiotic stresses (Solanke and Sharma, 2008; Drobak *et al*., 2004). The ADFs have been found related to plant

resistance to various pathogens in *Arabidopsis* and barley (Miklis *et al*., 2007; Clement *et al*., 2009; Tian *et al*., 2009). The energy produced by the depolymerization and polymerization of actin is used for the directional movement of cells which is necessary for wound healing, immune response, embryonic development and development of tissues (Pollard and Borisy, 2003). In this process a number of actin binding proteins such as profillin, actin depolymerizing factor (ADF)/cofilin, myosin, fibrin and villin are also involved.

Spot no 447 corresponding to NAD (P) H-quinone oxidoreductase subunit 4L, chloroplastic showed a significant increase (2.34 fold) in infested date palm samples as compared to control. NAD (P) H quinone oxidoreductase is an important enzyme that is involved in the detoxification of quinones and their derivatives (Radjendirane *et al*., 1998; Schuler *et al*., 1999; Gaikwad *et al*., 2001). Taken together, this enzyme increased to reduce oxidative stress and to detoxify toxic molecules produced by the stress.

The spot (1020) corresponded to peptidyl-prolyl cis-trans isomerase (PPIase) CYP19-2 was down regulated (2.8 fold) in the infested samples. This enzyme catalyses the cis-trans isomerisation process of proline residues during protein folding (Reimer and Fischer 2002). There are important groups of proteins, cyclophilin proteins which have this PPIase domains. Cyclophilins have been shown to involve in a wide range of cellular processes like stress tolerance (Andreeva *et al*., 1999), cell division (Schreiber, 1991), transcriptional regulation (Shaw, 2002), protein trafficking (Price *et al*., 1994), cell signaling (Freeman *et al*., 1996), pre-mRNA splicing (Horowitz *et al*., 2002) and molecular chaperoning (Weisman *et al*., 1996). Taken together, majority of identified

proteins was directly or indirectly related to defense of date palm and could be exploited for the early diagnosis of RPW infestation.

4.5 CONCLUSION

The date palm infestation with RPW is a key threat to date palm trees in the Kingdom of Saudi Arabia and other Gulf countries that could potentially wipe out this historical plant from this region. Our study is the first of its kind utilizing state of the art proteomic techniques to decipher molecular changes associated with RPW infestation to this plant, the date palm. Though our interest is to identify RPW specific molecular responses, however, it was intriguing to note that similar molecular moieties are up-regulated in artificial wounding as well as RPW infestation. However, relative modulation (down-regulation/up-regulation) is quite differential. We have also to go with further characterization of these responses. As both mechanical injury and RPW infestation are impinges upon the same molecular moieties of date palm stem. We have to establish certain baselines of proteomic changes for characterizing RPW specific molecular changes. This study mainly provides a proof of concept that stem, the hard part of date palm tree, is amenable to molecular analytical procedures for understanding infestation with RPW. Furthermore, it has opened new avenues for understanding proteome of this important tree. Techniques established and data generated will be crucial for date palm scientists in understanding diseases/infestation associated physiological process in this plant besides developing new cultivars.

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Chapter FIVE

5 General summary/ Future Plan/ Acknowledgements

5.1 General summary

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Oliv.) (Curculionidae : Coleoptera) is damaging date palm trees in several regions of the world. Infestation is highly concealed in nature. Applications of systemic pesticides through injection and spray to infested date palm trees have been partially effective in controlling RPW. However, after RPW infestation, destruction of the palm trees is the routine strategy to control spread of this insect. Presently, some detection techniques, including visual inspections, acoustic sensors, sniffer dogs, and pheromone traps have been tried out to detect RPW infestations at early stages; however, each technique has suffered certain logistic and implementation issues. A highly sensitive and reliable detection method of the infested plants is awaited to be applied in the field for early cure or removal of infested plants thus cur tailing further spread of this insect. Plants like other livings responds to external stimuli and as such RPW infestation associated responses of the host plant can be used for detection of pest infestation.

Therefore, we planned to identify the differentially expressed peptides of date palm possibly associated with RPW infestation. The study might be helpful in developing some molecular marker(s) for the early detection of RPW or its infestation. The objectives of our study were the molecular profiling of the date palm infested with RPW,

and identification and characterization of differentially expressed peptides using MALDI-TOF mass spectrometry analysis.

To achieve the purpose, efforts were first made to optimize a rapid and sensitive protocol for the isolation of high quality protein to be used in 2DE and 2D-DIGE from date palm samples, and then comparing differentially expressed peptides associated with RPW infestation of this plant using un-infested plants as control. Among several protocols we used for optimization phenol-simple buffer extraction with methanolic ammonium acetate precipitation (designated as protocol 3 in this study) yielded high quality protein.

In first experiment, 2DE protein analysis demonstrated both qualitative and quantitative differences between control and infested date palm leaves samples. To identify infestation specific responses artificially wounded trees were also used. 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 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 induce molecular changes manifested through differential expression of proteins.

In second experiment, RPW infestation-associated molecular changes were investigated in date palm leave samples i.e. protein expression differences were compared with the

healthy plants using highly sensitive 2D- DIGE followed by MALDI-TOF-TOF analysis. The 2D-DIGE results revealed qualitative and quantitative differences between control and RPW infested date palm samples. To identify infestation specific responses artificially wounded trees were also included in comparative expression profiling. Thirty two differential spots ($p \leq 0.05$ having ≥ 1.5 fold modulation) were subjected to mass spectrometry analysis and among these 22 spots were completely characterized. A detailed analysis revealed 7 as stress/defense related, 6 photosynthetic, 2 each from carbohydrate utilization system and protein degradation. Three peptides with other functions are also modulated. Proteins involved in date palm defense and stress response were up-regulated in infested and wounded sample while photosynthetic and other proteins were down regulated. This study suggests that RPW infestation modulate the plant defense responses through differential expression of proteins associated with defense, stress and photosynthesis and these proteins could help to identify RPW infestation at early stages.

In third experiment, we tried to explore differentially expressed proteins in the date palm stem tissues on infestation with the RPW. Our analyses revealed 32 differentially expressed peptides associated with RPW infestation in date palm stem. Of importance, to identify RPW infestation associated peptides (I), artificially wounded plants (W) were also used as additional control besides un-infested plants, a conventional control (C). A constant unique pattern of differential expression in infested (I), wounded (W) stem samples compared to control (C) was observed. The up-regulated proteins showed relative fold intensity in order of I >W>C and down regulated spots trend as C>W>I, a quite intriguing pattern. Relatively significant modulated expression pattern of a number

of peptides in infested plants predicts the possibility of developing a quick and reliable molecular methodology for detecting plants infested with date palm. This study also reveals that artificially wounding of date palm stem almost affect the same proteins as infestation however relative intensity is quite lower than infested samples both in up and down regulated spots. All 32 differentially expressed spots were subjected to MALDI-TOF analysis for their identification and we were able to match 21 proteins already existing in the databases. Furthermore, modulated proteins functionally belong to different physiological groups including: ion transport (33%), lipid biosynthesis (9%), protein folding (5%), plant defense (4%), ethylene signaling pathway (5%), carbohydrate metabolism (5%), proteolysis (5%), S-adenosylmethionine biosynthetic process (5%), nitrate assimilation (5%), porphyrin biosynthesis (5%), transcription (5%), lignin biosynthesis (4%) and unknown (5%) categories.

We believe that the protein molecules displaying modulated response and especially those with up-regulated expression pattern in infested date palm samples will be helpful in developing some diagnostic molecular markers for early detection of RPW infestation beneficial for manipulating this crucial problem of date palm wreckage.

5.2 Future Plan

We plan to design sequence-specific PCR primers based on promising differentially expressed peptide(s) identified by MALDI TOF. Then validation of the differentially expressed gene(s) will be done by PCR/ Quantitative RT PCR using cDNA as a template from the control and infested date palms, grown in the lab as well as in the field. Ultimately, we wish to develop a molecular marker associated with RPW for early detection of this date palm killing pest.

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