Feeding preference of *Rhynchophorus ferrugineus* (Oliver) (Coleoptera: Curculionidae) on different date palm cultivars and host biochemical responses to its infestation

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Research Paper

Abstract

To counter the insect infestation, plants respond with wide-ranging and highly dynamic biochemical reactions. Of these, the anti-oxidative activity is poorly understood. The red palm weevil (RPW) *Rhynchophorus ferrugineus* (Oliver), one of the most widespread pests in Pakistan, prefers to infest date palm *Phoenix dactylifera*. Our present study investigated the feeding preference of RPW to 11 different date palm cultivars and the results suggested that the Hillawi cultivar was most preferred. Greater infestation rate, fecundity and hatching rate were also recorded from Hillawi and Mozawati than other cultivars. No significant decreases were observed in chlorophyll a, chlorophyll b, total chlorophylls and carotenoids of RPW-infested Hillawi cultivar over un-infested control. In contrast, the contents of enzymatic antioxidants including phenols, proline, hydrogen peroxide, anthocyanin, malondialdehyde, ascorbic acid and glycine betaine showed a drastic increase after RPW infestation, and there was enhanced superoxide dismutase, peroxidase and catalase activities. Furthermore, we recorded the increase of total protein and sugar contents in RPW-infested date palms. These findings offer valuable insight into the antioxidative molecular mechanism of date palms under RPW attack and may contribute to the breeding of insect-resistant crops.

Introduction

Plants and herbivore insects have undergone co-evolution for more than 350 million years (War *et al.*, 2012), and it is widely known that biotic stress caused by insect infestation can affect the growth and development of host plants. As one of the key indicators of plant growth, photosynthesis is of vital importance. In most cases, insect infestation is associated with a reduction in photosynthesis. For example, the previous study explored the relationships between biotic damage and the expression of photosynthesis-related genes in eight plant species, which strongly supported the universal down-regulation of these genes and indicated that their expression was an adaptive response to biotic stress (Bilgin *et al.*, 2010). Also, the bug feeding led to a rapid and substantial decrease in photosynthesis in both *Brassica oleracea* L. var. *botrytis* and *Phaseolus vulgaris* L. leaves, and further in-depth studies demonstrated that *Murgantia histrionica* (Hahn) oviposition had the same effect (Velikova *et al.*, 2010). A similar investigation had been conducted in Scots pine, showing that the photosynthetic rate of oviposition-induced plants was remarkably lower than that of the control (Schröder *et al.*, 2005).

Conversely, to counter insect attacks, several defense responses mainly categorized as biochemical, molecular and behavioral aspects are induced after the stimulation of stress factors (Boller and Felix, 2009). In biochemical and molecular aspects, step-wise reactions generate (Erb *et al.*, 2012). First, plants perceive herbivore insect attack by recognizing alien infectious signals via conserved patterns of microbial molecules. After an attacker is recognized, different signaling cascades are activated and reprogrammed, mainly involving the transduction of cellular signals to downstream signaling molecules, and then the defense responses culminate. During this process, plants produce various defensive compounds necessary for their growth under the attack of herbivore insects (Schafer *et al.*, 2009). Among these defensive compounds, secondary metabolites, especially enzymatic antioxidants are of great concern (War *et al.*, 2020). There are numerous examples of antioxidants participating in defenses against insect herbivores, particularly, classical antioxidants such as phenols (Bi *et al.*, 1997), reactive oxygen species (ROS) (Kawano, 2003), proline (Qamar *et al.*, 2015), hydrogen peroxide (H_2O_2) (Levine *et al.*, 1994), malondialdehyde (Zhang *et al.*, 2008) and so on. Many solid foundations

have been laid that these non-enzymatic antioxidants were dramatically increased after insect herbivory (War *et al.*, 2012; Golan *et al.*, 2013; Akram *et al.*, 2017).

One of the important defense mechanisms of plants against herbivorous insect infestation is the activation of antioxidative enzymes. Among which, peroxidase, superoxide dismutase (SOD) and catalase play important roles (Rani and Jyothsna, 2010). SOD acts in removing superoxide anion-free radicals (Sudhakar *et al.*, 2001). Surveys such as that conducted by Yang *et al.* (2017) have shown that the activity of SOD increased in response to *Nilaparvata lugens* (Stål) infestation (Yang *et al.*, 2017). In contrast, peroxidases are monomeric hemoproteins within cells and commonly existed in plants. A numbers of processes acting in direct or indirect role in plant defense are regulated by peroxidases (War *et al.*, 2012). For example, transgenic tobacco, tomato and tree species with tobacco anionic peroxidase overexpression conferred resistance to several insects (Dowd and Lagrimini, 1997). Also, it has been previously observed that *Spodoptera frugiperda* (JE Smith) and *Blissus occiduus* (Barber) infestation resulted in elevated peroxidase activity in corns and buffalo grass, respectively (Heng-Moss *et al.*, 2004; Chen *et al.*, 2009). In addition to peroxidase and SOD, catalase also functions in plant defense. In catalase-deficient *Arabidopsis thaliana* (Heynh), the expression of defense-related genes drastically decreased (Vandenabeele *et al.*, 2004). Besides, the fluctuation of total soluble protein and sugar important for plant growth and maintenance also affects its defense response against insect herbivory, and much evidence showed that the increase in protein and sugar contents mediated plant resistance to herbivore infestation (de Souza Cândido *et al.*, 2011; Bolouri Moghaddam and Van den Ende, 2012).

As one of the most important functional and dietetic fruit crops in arid and semi-arid regions outspreading from the Arabian Peninsula to North Africa and the Middle East, the date palm *Phoenix dactylifera* L. has been cultivated for thousands of years (Memon *et al.*, 2015), and its production dramatically increased more than 100-fold (Chao and Krueger, 2007). Among all date palm producing countries, Pakistan ranked seventh, indicating its economic importance (Nadeem *et al.*, 2019). Being rich in nutrition, date palms provide a perfect choice for insect feeding (Haider *et al.*, 2018). Among the pests infecting date palms, *Rhynchophorus ferrugineus* (Oliver) (red palm weevil (RPW)) is the most common one. It belongs to the Curculionidae of Coleoptera, and is an invasive forest pest that seriously endangers various plants. It was reported that the RPW originated from India and broken out in many places, including North Africa, Middle East and Southern Europe during the past few decades (Chao and Krueger, 2007). The RPW was identified as the first category serious pest by the Food and Agriculture Organization and caused severe injury to the date palms. Surveys conducted by Mohamed *et al.* (2019) showed that the infestation of RPW inside the trunk of date palms led to its collapse within few months (Mohamed *et al.*, 2019). Moreover, the RPW invasion caused an annual loss of $1.74–8.69 million in the East of the Kingdom of Saudi Arabia (El-Sabea *et al.*, 2009). In Pakistan, RPW was identified and characterized in four provinces, Punjab, Sindh, Khyber Pakhtunkhwa and Baluchistan, also posing a huge threat to agriculture (Mujahid *et al.*, 2018).

Several lines of evidence have suggested that the RPW prefers to feed on date palms. No such survey has been conducted to investigate the preference of RPW to different cultivars. The host plants exhibit a diversity of defense responses upon RPW infestations, including changes in antioxidants and antioxidase activities. Hereby, the present study first aims at comparing the feeding preference of RPW to 11 date palm cultivars, and then analyzes the biochemical responses of date palms under RPW infestation. It is expected that this study enhances our understanding of the biochemical mechanism of date palms under RPW attack, and further contributes to its control.

Materials and methods

Feeding preference analysis

Fifty grams fresh leaves of 11 commercial date palm cultivars, Hillawi, Mozawati, Kechanr, Aseel, Khudravi, Shamrani, Dhaki, Zaidi, Zeri, Kobra and Denda, were collected from grown orchards and used for olfactometer assay. The feeding preference experiment was conducted using the Inlet Odour Source System. Briefly, fresh date palm pieces of cultivars and the control (Kobra and Denda cultivars) were kept on each side of the two-arm olfactometer. The test RPW was collected from a previous indoor-maintained population and 40 gravid female adults of 15 days old were used for the behavioral analysis. After 10 min, the insects trapped in each side of an olfactometer were collected and counted. The attraction rate of cultivar '*X*' (%) was calculated as '(the number of RPW in cultivar X – ram/the total number of RPW in two-arms) × 100'. We counted the number of laying eggs of RPW on each cultivar and calculated the hatchability (%) as follows '(the hatched eggs/total laying eggs) × 100'. Each measurement was repeated three times.

Fecundity and egg hatchability

The new leaves of 11 cultivars were collected and separately kept in 11 plastic cages with a dimension of 35 cm × 40 cm × 60 cm, respectively. Three leaves were kept in pots of water to keep humid per cage. Then, ten gravid females and two male weevils were released into these cages. After 1 week, the adults were removed. To establish number of eggs and hatchability, the eggs laid by RPW females and hatched larvae on each cultivar were counted, respectively. We calculated the hatchability (%) as follows '(the hatched larvae/total laid eggs) × 100'. Each measurement was repeated three times.

Sample preparation

RPW were collected from the Punjab province of Pakistan and reared in an artificial climate incubator. Briefly, 15 RPW first-instar larvae within 24 h hatching were fed on the fresh leaves of date palm cultivar Hillawi, and the healthy date palms of the same age without RPW infestation were used as control. After 30–35 days, the infested and uninfested leaves of date palms were collected and chopped into small pieces for subsequent biochemical analysis, respectively.

Determination of biochemical substance contents

Total soluble protein

Total soluble protein of date palm leaves was extracted from 0.5 g leaves. Briefly, a fresh sample was ground in 5 ml of 50 mM cooled phosphate buffer (pH 7.8), and the homogenate was centrifuged at 15,000 rpm for 15 min. The supernatant was used for soluble protein determination. We measured the soluble proteins using

the Bradford method (Bradford, 1976). A total of 100 μl supernatant was transferred into a microfuge tube and mixed with 1 ml of Bradford reagent. The sample was incubated at 37°C for 10–15 min followed by absorbance measurements at 595 nm using the Picodrop spectrophotometer (Hitachi-U-2001, Japan). Each measurement was repeated three times.

Total soluble sugar

Total soluble sugar was measured using the calorimetrical method according to the previous description (Loewus, 1952). Briefly, 0.5 g leaves were ground in 80% ethanol and vortexed followed by incubation for 30 min at 80°C. The mixture was centrifuged at 10,000 rpm for 15 min, the supernatant was mixed with 2 ml anthrone and incubated for 7 min at 80°C. The absorbance was measured at 620 nm and the contents of total soluble sugars were calculated based on the standard curve. Each measurement was repeated three times.

Chlorophylls and carotenoids

We homogenized 0.5 g fresh leaves in 5 ml 80% acetone and filtered the samples using a filter. The absorbance of the homogenate was respectively measured at 645 and 663 nm. The contents of chlorophyll a, chlorophyll b and total chlorophylls were calculated according to the following formulas: chlorophyll a $= (12.7 \times OD_{663} - 2.69 \times OD_{645}) \times V \times W/1000$, chlorophyll b $= (22.9 \times OD_{645} - 4.68 \times OD_{663}) \times V \times W/1000$, total chlorophylls $= (20.2 \times OD_{645} + 2.69 \times OD_{663}) \times V \times W/1000$. Here, '$V$' means the volume and '$W$' represents the weight of fresh leaves. The carotenoid contents were determined as described by Arnon (1949). Each measurement was repeated three times.

Proline

A total of 0.5 g fresh leaves were homogenized with 3% sulfosalicylic acid and filtered with a filter. We mixed 1 ml filtrate with 1 ml 41.67 mg ml^{-1} ninhydrin solution dissolved in 30 ml glacial acetic acid. The mixture was vortexed and heated at 100°C for 1 h, and the reaction was stopped by ice bath. Then, 4 ml toluene was added and the mixture was incubated for 15–20 min. The absorbance was measured at 520 nm and the proline was estimated based on the previous method (Bates *et al.*, 1973). Each measurement was repeated three times.

H₂O₂ and anthocyanin

A total of 0.5 g fresh leaves were crushed in 5 ml 0.1% trichloroacetic acid (TCA) solution, and the homogenate was transferred into a microfuge tube and then centrifuged at 12,000 rpm for 15 min. A total of 500 μl supernatant was mixed with an equivalent potassium phosphate buffer (pH 7) and 1 ml 1 M potassium iodide solution. We measured the absorbance at 390 and 465 nm. The concentrations of H_2O_2 and anthocyanin were calculated, respectively, based on the reported method (Velikova *et al.*, 2000). Each measurement was repeated three times.

Phenols

A total of 0.5 g leaves were ground in 5 ml 80% acetone and centrifuged at 12,000 rpm for 15 min. A total of 100 μl supernatant was transferred into a 10 ml microfuge tube filled with 1 ml double-distilled water (ddH₂O). Then, 2.5 ml Folin–Ciocalteu and 20% Na_2CO_3 were added and vortexed for 5–10 s. After 20 min of incubation, the absorbance was measured at 750 nm. The standard curve was prepared using the tannic acid method. Total phenol contents were estimated according to the previous

description (Julkunen-Tiitto, 1985). Each measurement was repeated three times.

Malondialdehyde

A total of 0.1 g fresh leaves were collected and homogenized in 1 ml 5% TCA. The homogenate was centrifuged at 12,000 rpm for 15 min. For every aliquot, 1 ml 20% TCA containing 0.5% thiobarbituric acid was added, and the mixture was incubated at 95°C for 30 min. The absorbance was measured at 532 nm. Malondialdehyde was calculated based on the previous method (Heath and Packer, 1968). Each measurement was repeated three times.

Ascorbic acid

A total of 0.25 g leaves were ground in 10 ml of 6% TCA. Then, 4 ml extract was mixed with 2 ml 2% 2,4-dinitrophenyl hydrazine and 10% thiourea solution dissolved in 70% ethanol. The solution was incubated at 100°C for 5 min and then 5 ml of 80% H_2SO_4 was added. The absorbance was recorded at 530 nm. Each measurement was repeated three times.

Glycine betaine

Glycine betaine was measured according to the description (Di Martino *et al.*, 2003). Briefly, 50 mg of leaves were ground into fine powder in liquid nitrogen followed by a dilution of 2 ml ddH₂O. After 24 h of incubation, the homogenate was centrifuged and the supernatant was eluted by High-performance liquid chromatography using a cationic exchange column (Dionex Hypersil SCX, 5 μm, 250 × 4.6 mm) with a flow rate of 1 ml min^{-1}. The eluent phase was 95% 0.05 M sodium phosphate buffer (pH 3.7) and 5% methanol. The eluted glycine betaine was detected by measuring the absorbance of 195 nm and the pure glycine betaine solution was used as a standard.

Enzymatic determination of antioxidant enzymes

For extracting antioxidant enzymes, 0.5 g frozen leaves were ground in 5 ml of 50 mM cooled phosphate buffer. The homogenate was centrifuged at 1500 rpm for 15 min at 4°C and the supernatant was used for enzymatic activity assay.

Superoxide dismutase

A total of 20–50 μl of enzyme extract was mixed with 3 ml of 50 μM nitro-blue tetrazolium, 1.3 μM riboflavin, 13 mM methionine, 75 nm ethylenediaminetetraacetic acid and 50 mM phosphate buffer. The mixture was irradiated under fluorescent lamps at 78 μmol s^{-1} m^{-2} for 15 min. The absorbance was read at 560 nm and the activity of SOD was measured according to the previous description (Giannopolitis and Ries, 1977). Each measurement was repeated three times.

Catalase and peroxidase

A total of 0.1 ml enzyme extract was mixed with a 3 ml catalase reaction solution dissolved in 50 mM phosphate buffer (pH 7.0) and 5.9 mM H_2O_2. Similarly, 0.1 ml enzyme extract was mixed with 3 ml peroxidase reaction solution dissolved in 50 mM phosphate buffer (pH 5.0), 20 mM guaiacol and 40 mM H_2O_2. The absorbance was determined at every 20 s, and the activities of catalase and peroxidase were respectively calculated. One-unit catalase or peroxidase activity was defined as the absorbance change per minute. Each measurement was repeated three times.
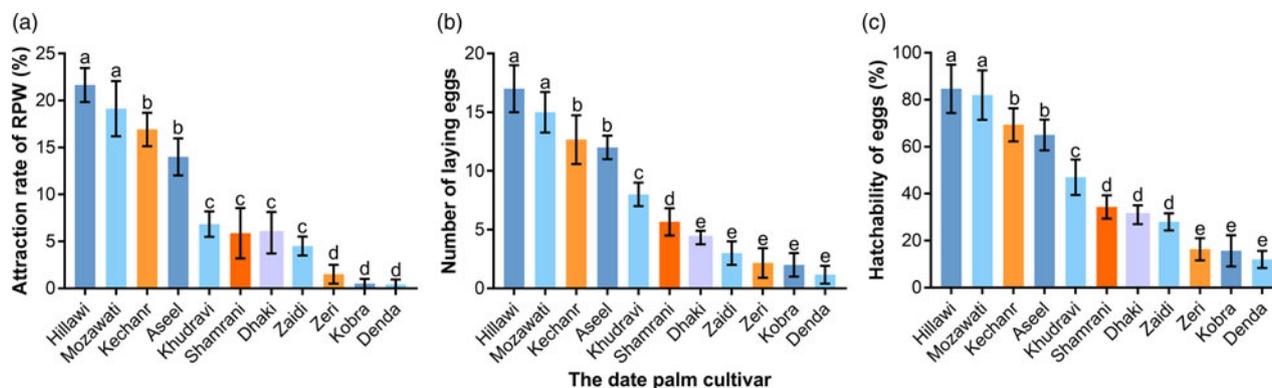
Figure 1. Feeding preference of RPW to 11 cultivars. (a) The feeding preference of RPW to 11 cultivars based on two-arm olfactometer assay ($n = 40$). (b) The number of laying eggs of RPW on 11 cultivars ($n = 10$). (c) The hatchability of RPW eggs on 11 cultivars ($n = 10$). The data were analyzed using the Scott Knott test and all statistical differences were marked with different letters (a, b, c, d, e). The results were shown as mean ± SD and each measurement was repeated three times.
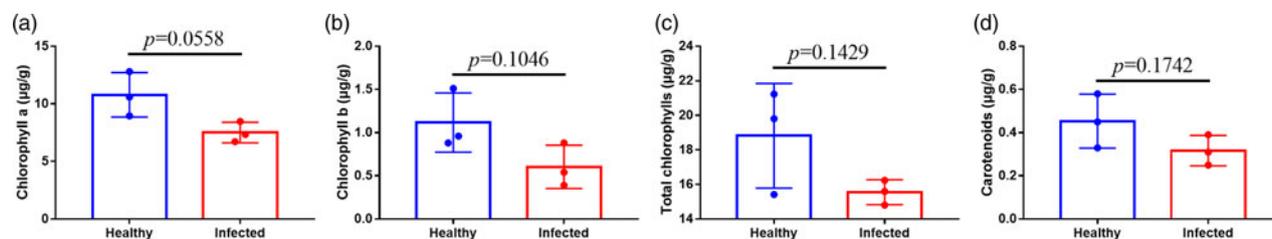

Figure 2. Comparisons of chlorophyll a (a), chlorophyll b (b), total chlorophylls (c) and carotenoid contents (d) between RPW-infested and healthy date palms. The data were analyzed using an unpaired two-tailed Student's t test and $P > 0.05$ indicated no statistical significance. The results were shown as mean ± SD and each measurement was repeated three times ($n = 3$).

Data analysis

The attraction rates, amount of egg-laying, hatchability of 11 cultivars (expressed as an average percentage), biochemical substance contents and antioxidase activity data were respectively imported into the statistical computing software Data Processing System v9.50 for analysis (Tang and Zhang, 2013). All figures were plotted by using GraphPad Prism 7.0 (GraphPad, San Diego, USA).

Results

RPW preferred to infest the Hillawi cultivar

The results obtained from fig. 1a showed that RPW preferred to infest the Hillawi and Mozawati among all 11 date palm cultivars, showing higher attraction rates of 21.66 and 19.13%, respectively and significantly differed from those of Kechanr, Aseel, Khudravi, Shamrani, Dhaki, Zaidi, Zeri, Kobra and Denda. In contrast, the Denda cultivar showed a lowest sensitivity to RPW with a mere 0.43% attraction rate. Among all 11 cultivars, the attraction rates of Mozawati, Kechanr and Aseel were above 10% and those of Khudravi, Shamrani and Dhaki were higher than 5%. In the following, we compared the fecundity and egg hatching rates of RPW fed on these cultivars. Analogous to the above results, the highest amount of egg laying and hatching rate with 17 and 84.67% were respectively recorded in Hillawi (fig. 1b, c). Conversely, RPW did not prefer to lay eggs on the Denda cultivar (average 1.17) and its hatching rate (12.00%) was relatively low, showing less attraction to this pest. The results in this section indicated that RPW preferred to infest the Hillawi and Mozawati cultivars. Considering that the variety Hillawi was the most heavily infested source for weevils and the largest planting

cultivar in the Punjab province of Pakistan, the next section, therefore, moved on to discuss the RPW infestation-induced biochemical responses of Hillawi date palm.

RPW infestation did not impair the host photosynthesis

As shown in fig. 2a, RPW infestation did not lead to a remarkable decrease in chlorophyll a content compared to the healthy date palms ($P = 0.0558$). Also, no significant difference in chlorophyll b content ($0.60\,\mu g\,g^{-1}$) was revealed in infected date palms compared with the healthy one ($1.12\,\mu g\,g^{-1}$), but we indeed recorded a reduction trend (fig. 2b). Next, we determined the total chlorophyll and carotenoid contents, both showing no obvious changes after RPW infestation (fig. 2c, d). Together these results provided ample evidence that date palms did not impair the capacity of photosynthesis upon RPW infestation.

RPW infestation increased host antioxidant levels

To figure out the effect of RPW infestation on host anti-oxidative defense responses, we determined the levels of antioxidants in both infested and healthy date palms. Apparently, the contents of these antioxidants including total phenols, proline, H_2O_2, anthocyanin, malondialdehyde and glycine betaine increased by 1.53–2.48-fold with significant differences except for ascorbic acid content (fig. 3a–f). Among these results, the largest change was observed in proline level, that was, an average of $8.60\,\mu mol$ g^{-1} in the infested date palms compared with an average of $3.47\,\mu mol\,g^{-1}$ in healthy plants (fig. 3b). Conversely, we recorded a relatively low fold change in malondialdehyde content, namely, $8.77\,\mu mol\,g^{-1}$ in the infested date palms and $5.74\,\mu mol\,g^{-1}$ in
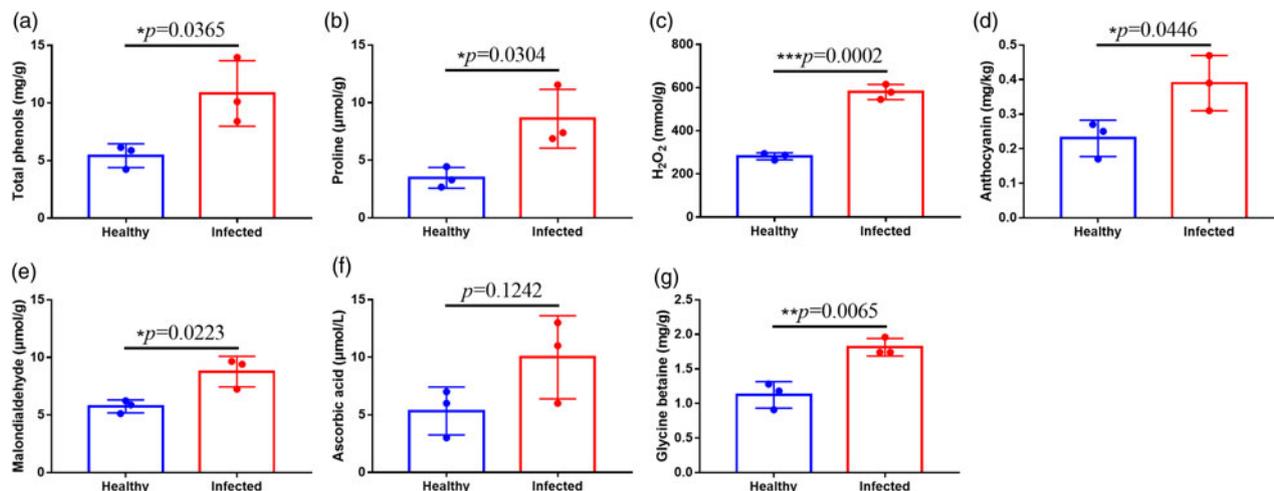
Figure 3. Comparisons of total phenols (a), proline (b), H_2O_2 (c), anthocyanin (d), malondialdehyde (e), ascorbic acid (f) and glycine betaine (g) between RPW-infested and healthy date palms. The data were analyzed using an unpaired two-tailed Student's t test and all statistical significances were marked with asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The results were shown as mean $\pm$ SD and each measurement was repeated three times ($n = 3$).
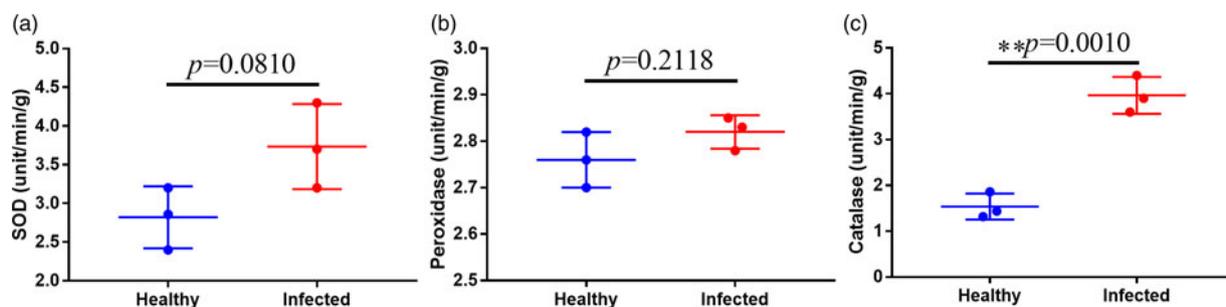

Figure 4. Comparison of activities of SOD (a), peroxidase (b) and catalase (c) between RPW-infested and healthy date palms. The data were analyzed using an unpaired two-tailed Student's t test and the statistical significance was marked with asterisks (**$P < 0.01$). The results were shown as mean $\pm$ SD and each measurement was repeated three times ($n = 3$).

healthy ones (fig. 3e). In summary, RPW infestation remarkably activated the anti-oxidative defense responses of host plants by increasing the antioxidant levels.

RPW infestation increased host antioxidase activity

This section of the survey was concerned with the underlying regulatory mechanism of activated antioxidative defense responses in host plants. Therefore, the activities of three antioxidases were evaluated. As the results presented, the activity of SOD slightly increased by 1.32-fold after RPW infestation, although there was no significant difference (fig. 4a). Analogous to SOD, the RPW-infested date palms did not show a striking increase in peroxidase activity (2.82 units min^{-1} g^{-1}) compared with healthy ones (2.76 units min^{-1} g^{-1}) (fig. 4b). However, upon RPW infestation, the enzymatic activity of catalase in infested date palms reached 3.97 units min^{-1} g^{-1} relative to the 1.54 units min^{-1} g^{-1} in healthy date palms with $P < 0.01$ (fig. 4c). Overall, these results indicated that the activation of host plant antioxidative defense response was due to the elevation of antioxidase activity.

RPW infestation elevated host soluble protein and sugar levels

In the final part of this survey, we were concerned about the soluble protein and sugar levels in date palms. The results obtained

from fig. 5a showed that total soluble proteins of infested host plants dramatically increased to 7.84 mg g^{-1}, and a lower level of 5.86 mg g^{-1} was observed in healthy date palms with $P < 0.01$. In contrast, there was no significant difference in total soluble sugars between RPW-infested (39.67 mg g^{-1}) and healthy hosts (32.33 mg g^{-1}) (fig. 5b).

Discussion

Herbivorous insects are one of the most speciose groups in invertebrates. They have the capability to infest almost all the greeneries. Accordingly, the attraction of different cultivars to insects varies a lot. In the date palm and RPW model, the preference of RPW to different date palm cultivars has not been figured out. Therefore, the first set of analyses examined the odor preference response of RPW to 11 date palm cultivars, and significant stronger attractions of Hillawi and Mozawati cultivars to RPW were recorded. Further closer inspection showed that RPW prefers to lay eggs on Hillawi and Mozawati in line with the higher hatchability rates. However, considering that the variety Hillawi was the most heavily infested source for weevils and the largest planting commercial cultivar in the Punjab province of Pakistan. Therefore, our subsequent analysis mainly focused on the Hillawi cultivar. Notwithstanding these limitations, these findings are rather encouraging in providing novel insight into the indoor raising of RPW.
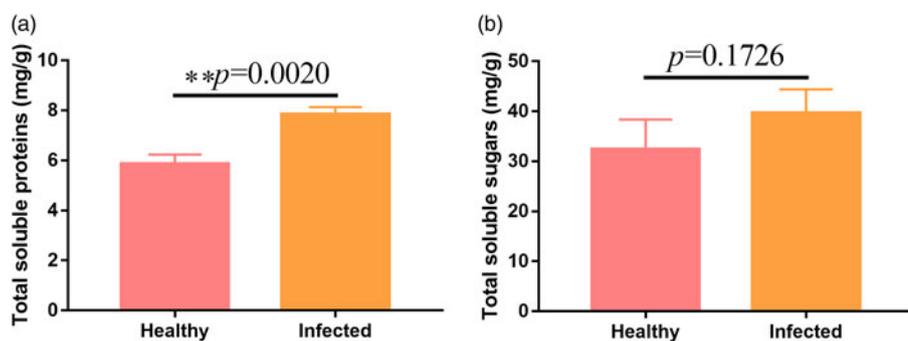
Figure 5. Comparisons of total soluble proteins (a) and sugars (b) between RPW-infested and healthy date palms. The data were analyzed using an unpaired two-tailed Student's *t* test and the statistical significance was marked with asterisks (**$P < 0.01$). The results were shown as mean $\pm$ SD and each measurement was repeated three times ($n = 3$).

Defoliating insects consume mineral contents in the leaves of plants, resulting in the reduction and inhibition of pigment biosynthesis (Jakobs *et al.*, 2019). Hence, we compared the contents of photosynthetic pigments in RPW-infested hosts with that of control. Contrary to expectations, the contents of chlorophyll a, chlorophyll b, total chlorophylls and carotenoids did not show remarkably decreases after RPW infestation. Despite we did not record a significant repaired capability in these photosynthetic pigments, the downward trends were quite clear. A previous study demonstrated that these pigments remarkably decreased after infestation by gall forming psyllids (Golan *et al.*, 2015). Also, Moussa *et al.* (2012) revealed that the aphid attack caused a serious reduction in host photosynthetic pigment content (Moussa *et al.*, 2012). To further verify the effects of RPW infestation on plant photosynthesis, an important point involving long-term and multipoint detection should be undertaken. Additionally, the transcriptome analysis implied that the debilitating near-term losses in photosynthetic capacity were caused by down-regulation of photosynthesis-related genes (Bilgin *et al.*, 2010). Thus, future studies on this point are therefore recommended.

Our next research depicted an apparent biochemical response of date palms after artificially infested with RPW, showing a significant increase in total phenol contents. Comparison of these findings with the earlier study confirmed the herbivore-medicated antioxidative defensive response correlated with the phenol accumulations (Hasim and Yusuf, 2017). Besides, proline, H_2O_2, anthocyanin, malondialdehyde, ascorbic acid and glycine betaine are universal non-enzymatic antioxidants not only essential for scavenging ROS, but also modulating many fundamental functions in plants under stress conditions (War *et al.*, 2012; Akram *et al.*, 2017). Our results strongly supported these arguments, showing that these contents were low under normal conditions, but increased in plants after RPW infestation. The potential relationship between significant increased antioxidants induced by herbivorous insects and elevated anti-oxidase activities had been revealed (War *et al.*, 2012). Thus, we assayed the enzymatic activities of SOD, peroxidase and catalase. In accordance with the above results, the enhancement of SOD, peroxidase and catalase activities was recorded, consistent with other research revealing that psyllid-infested leaves of eucalyptus showed significantly increased SOD and catalase activities (Khattab and Khattab, 2005). Taking all the results together, we confirm that RPW infestation activated the host defensive response.

There is growing evidence that the herbivore infestation affects the total soluble protein and sugar levels of host plants (Peumans and Van Damme, 1995; Hogenhout and Bos, 2011). To throw light on this issue, the average level of total soluble proteins between RPW-infested and healthy plants was compared. As expected, there was an elevated level of total soluble proteins after RPW infestation, probably contributing to its defensive role. Besides, it was also well-documented that sugars are involved in plant defense responses under biotic stress (Bolouri Moghaddam and Van den Ende, 2012). Therefore, we estimated the total soluble sugars and an increasing trend was recorded in RPW-infested date palms, corresponding to that biotic stress resulted in an increased level of total soluble sugars (Gómez-Ariza *et al.*, 2007). In contrast, the herbivore-induced concentration of sugars in the leaves of jasmonate biosynthesis-deficient *Nicotiana attenuata* plants significantly reduced, thus weakening its resistance to *Manduca sexta* and leading to increased *M. sexta* growth (Machado *et al.*, 2015). However, our results failed to explain the defense mechanism and further research should be undertaken.

Altogether, this research aims to examine the biochemical responses of host date palms to RPW infestation. The most obvious finding was that RPW induced the activation of host antioxidative defense response, complementing those of earlier studies, which indicated that the host plants evolved numerous biochemical and molecular processes to compensate for various environmental damages during the process of mutual confrontation between plants and herbivore insects. These findings, while explorative and preliminary, contribute in several ways to our understanding of plant defense system to resist attackers and provide a basis for breeding insect-resistant crops.

Conflict of interest. None.

References

Akram NA, Shafiq F and Ashraf M (2017) Ascorbic acid-a potential oxidant scavenger and its role in plant development and abiotic stress tolerance. *Frontiers in Plant Science* **8**, 613.

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* **24**, 1.

Bates LS, Waldren RP and Teare I (1973) Rapid determination of free proline for water-stress studies. *Plant and Soil* **39**, 205–207.

Bi J, Felton G, Murphy J, Howles P, Dixon R and Lamb C (1997) Do plant phenolics confer resistance to specialist and generalist insect herbivores? *Journal of Agricultural and Food Chemistry* **45**, 4500–4504.

Bilgin DD, Zavala JA, Zhu J, Clough SJ, Ort DR and Delucia EH (2010) Biotic stress globally downregulates photosynthesis genes. *Plant, Cell & Environment* **33**, 1597–1613.

Boller T and Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology* **60**, 379–406.

Bolouri Moghaddam MR and Van den Ende W (2012) Sugars and plant innate immunity. *Journal of Experimental Botany* **63**, 3989–3998.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Chao CT and Krueger RR (2007) The date palm (*Phoenix dactylifera* L.) overview of biology, uses, and cultivation. *HortScience* **42**, 1077–1082.

Chen Y, Ni X and Buntin GD (2009) Physiological, nutritional, and biochemical bases of corn resistance to foliage-feeding fall armyworm. *Journal of Chemical Ecology* **35**, 297–306.

de Souza Cândido E, Pinto MFS, Pelegrini PB, Lima TB, Silva ON, Pogue R, Grossi-de-Sá MF and Franco OL (2011) Plant storage proteins with antimicrobial activity: novel insights into plant defense mechanisms. *The FASEB Journal* **25**, 3290–3305.

Di Martino C, Delfine S, Pizzuto R, Loreto F and Fuggi A (2003) Free amino acids and glycine betaine in leaf osmoregulation of spinach responding to increasing salt stress. *New Phytologist* **158**, 455–463.

Dowd P and Lagrimini L (1997) *The Role of Peroxidase in Host Insect Defense. Advances in Insect Control*. London: Taylor and Francis Ltd.

El-Sabea AM, Faleiro J and Abo-El-Saad MM (2009) The threat of red palm weevil *Rhynchophorus ferrugineus* to date plantations of the Gulf region in the Middle-East: an economic perspective. *Outlooks on Pest Management* **20**, 131–134.

Erb M, Meldau S and Howe GA (2012) Role of phytohormones in insect-specific plant reactions. *Trends in Plant Science* **17**, 250–259.

Giannopolitis CN and Ries SK (1977) Superoxide dismutases: I. Occurrence in higher plants. *Plant Physiology* **59**, 309–314.

Golan K, Rubinowska K and Górska-Drabik E (2013) Physiological and biochemical responses of fern *Nephrolepis biserrata* (Sw.) Schott. to *Coccus hesperidum* L. infestation. *Acta Biologica Cracoviensia Series Botanica* **55**, 93–98.

Golan K, Rubinowska K, Kmieć K, Kot I, Górska-Drabik E, Łagowska B and Michałek W (2015) Impact of scale insect infestation on the content of photosynthetic pigments and chlorophyll fluorescence in two host plant species. *Arthropod–Plant Interactions* **9**, 55–65.

Gómez-Ariza J, Campo S, Rufat M, Estopà M, Messeguer J, Segundo BS and Coca M (2007) Sucrose-mediated priming of plant defense responses and broad-spectrum disease resistance by overexpression of the maize pathogenesis-related PRms protein in rice plants. *Molecular Plant–Microbe Interactions* **20**, 832–842.

Haider MS, Khan IA, Jaskani MJ, Naqvi SA, Mateen S, Shahzad U and Abbas H (2018) Wheat TaCRT1 contributes to drought tolerance. *Pakistan Journal of Botany* **50**, 1069–1076.

Hasim NN and Yusuf N (2017) Hydrogen peroxide, lipid peroxidation and total ion leakage in coconut cultivar 'Pandan' and 'Matag' infested with red palm weevil (*Rhynchophorus ferrugineus*). *Malaysian Applied Biology* **46**, 221–228.

Heath RL and Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* **125**, 189–198.

Heng-Moss T, Sarath G, Baxendale F, Novak D, Bose S, Ni X and Quisenberry S (2004) Characterization of oxidative enzyme changes in buffalograsses challenged by *Blissus occiduus*. *Journal of Economic Entomology* **97**, 1086–1095.

Hogenhout SA and Bos JI (2011) Effector proteins that modulate plant–insect interactions. *Current Opinion in Plant Biology* **14**, 422–428.

Jakobs R, Schweiger R and Müller C (2019) Aphid infestation leads to plant part-specific changes in phloem sap chemistry, which may indicate niche construction. *New Phytologist* **221**, 503–514.

Julkunen-Tiitto R (1985) Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. *Journal of Agricultural and Food Chemistry* **33**, 213–217.

Kawano T (2003) Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. *Plant Cell Reports* **21**, 829–837.

Khattab H and Khattab I (2005) Responses of Eucalypt trees to the insect feeding (gall forming psyllid). *International Journal of Agricultural and Biological Engineering* **7**, 979–984.

Levine A, Tenhaken R, Dixon R and Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79**, 583–593.

Loewus FA (1952) Improvement in anthrone method for determination of carbohydrates. *Analytical Chemistry* **24**, 219.

Machado RA, Arce CC, Ferrieri AP, Baldwin IT and Erb M (2015) Jasmonate-dependent depletion of soluble sugars compromises plant resistance to *Manduca sexta*. *New Phytologist* **207**, 91–105.

Memon MIN, Noonari S, Kalwar AM and Sial SA (2015) Performance of date palm production under contract farming in Khairpur Sindh Pakistan. *Journal of Biology, Agriculture and Healthcare* **5**, 19–27.

Mohamed AA, Pathak MR, Farooq M and White JA (2019) Study of gut associated bacteria of red palm weevil (*Rhynchophorus ferrugineus*) – a destructive pest of date palm in the kingdom of Bahrain. *Arab Gulf Journal of Scientific Research* **37**, 39–40.

Moussa SF, Salman A and Bakry M (2012) The negative effects of *Parlatoria blanchardii* (Targ.) infestation on the morphological and chemical characters of certain varieties leaflets of date palm trees at *Luxor governorate*, Egypt. *Egyptian Academic Journal of Biological Sciences. A, Entomology* **5**, 169–181.

Mujahid M, Ahmad JN, Arif MJ, Nazir J and Giblin-Davis RM (2018) Molecular identification and phylogenetic analysis of distinct geographical populations of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) in Pakistan. *International Journal of Agriculture and Biology* **20**, 1997–2004.

Nadeem M, Qureshi TM, Ugulu I, Riaz MN, Ain QU, Khan ZI, Ahmad K, Ashfaq A, Bashir H and Dogan Y (2019) Mineral, vitamin and phenolic contents and sugar profiles of some prominent date palm (*Phoenix dactylifera*) varieties of Pakistan. *Pakistan Journal of Botany* **51**, 171–178.

Peumans WJ and Van Damme E (1995) Lectins as plant defense proteins. *Plant Physiology* **109**, 347.

Qamar A, Mysore K and Senthil-Kumar M (2015) Role of proline and pyrroline-5-carboxylate metabolism in plant defense against invading pathogens. *Frontiers in Plant Science* **6**, 503.

Rani PU and Jyothsna Y (2010) Biochemical and enzymatic changes in rice plants as a mechanism of defense. *Acta Physiologiae Plantarum* **32**, 695–701.

Schafer ZT, Grassian AR, Song L, Jiang Z, Gerhart-Hines Z, Irie HY, Gao S, Puigserver P and Brugge JS (2009) Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature* **461**, 109–113.

Schröder R, Forstreuter M and Hilker M (2005) A plant notices insect egg deposition and changes its rate of photosynthesis. *Plant Physiology* **138**, 470–477.

Sudhakar C, Lakshmi A and Giridarakumar S (2001) Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. *Plant Science* **161**, 613–619.

Tang QY and Zhang CX (2013) Data Processing System (DPS) software with experimental design, statistical analysis and data mining developed for use in entomological research. *Insect Science* **20**, 254–260.

Vandenabeele S, Vanderauwera S, Vuylsteke M, Rombauts S, Langebartels C, Seidlitz HK, Zabeau M, Van Montagu M, Inzé D and Van Breusegem F (2004) Catalase deficiency drastically affects gene expression induced by high light in *Arabidopsis thaliana*. *The Plant Journal* **39**, 45–58.

Velikova V, Yordanov I and Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science* **151**, 59–66.

Velikova V, Salerno G, Frati F, Peri E, Conti E, Colazza S and Loreto F (2010) Influence of feeding and oviposition by phytophagous pentatomids on photosynthesis of herbaceous plants. *Journal of Chemical Ecology* **36**, 629–641.

War AR, Paulraj MG, Ahmad T, Buhroo AA, Hussain B, Ignacimuthu S and Sharma HC (2012) Mechanisms of plant defense against insect herbivores. *Plant Signaling & Behavior* **7**, 1306–1320.

War AR, Buhroo AA, Hussain B, Ahmad T, Nair RM and Sharma HC (2020) Plant defense and insect adaptation with reference to secondary metabolites. In Mérillon J-M and Ramawat KG (eds), *Co-Evolution of Secondary Metabolites*. Cham, Switzerland: Springer, pp. 795–822.

Yang L, Han Y, Li P, Li F, Ali S and Hou M (2017) Silicon amendment is involved in the induction of plant defense responses to a phloem feeder. *Scientific Reports* **7**, 1–9.

Zhang S-Z, Hua B-Z and Zhang F (2008) Induction of the activities of antioxidative enzymes and the levels of malondialdehyde in cucumber seedlings as a consequence of *Bemisia tabaci* (Hemiptera: Aleyrodidae) infestation. *Arthropod–Plant Interactions* **2**, 209–213.