The role of volatile organic compounds in the interactions between leafhopper *Arbordiahussaini* (Hemiptera: Cicadellidae) and grapevine leaves

Youssif Ali Mohammed [1], Qasim Ahmed [1*] and Mokhtar AbdulsattarArif[2]

[1]Department of Plant Protection, College of Agricultural Engineering Sciences, University of Baghdad, Baghdad, Iraq
[2]Plant Protection Directorate, Ministry of Agriculture, Baghdad, Iraq
[*]Corresponding Author email:qasim.h@coagri.uobaghdad.edu.iq

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Abstract

The leafhopper *Arbordiahussaini* can seriously destroy grapevines and is susceptible to the infestation of leafhopper. Volatile organic compounds (VOCs) released from uninfested and infested Kamali grape leaves were identified in this study. VOCs released from leaves were collected by the HS-SPME technique and analyzed by GC-MS. The results indicated that the infested leaves produce 19 VOCs; some VOCs were absent in uninfested because of the grape leafhopper infestation. VOCs were different between uninfested and infested leaves. The Olfactometer results showed leafhoppers preferred uninfested and infested leaves compared to filtered air treatment. Moreover, *A. hussaini* preferred infested leaves compared to uninfested ones. Our findings imply that these variations in VOCs are a crucial mediator in insect-plant interactions and may be employed in future research to pinpoint the primary substances that might be used as a lure for tracking and managing *A. hussaini*.

Keywords: *Vitis vinifera*, Plant-insect interaction, lure, kairomone, semiochemical, IPM, *Arbordiahussaini*

Introduction

The grape trade has developed in the last decades, as the circulation and consumption of its products have become worldwide [1]. Grapes have multiple uses, as their fruits are eaten fresh, such as table grapes, or consumed as dried fruit, and can be used in other industries, such as making juice and wind drink [2]. For the summer of 2019, Iraq is expected to produce 420,466 tons of grapes across an area of 12,044 hectares [3].

Grape trees are exposed to different insect pests, including leafhopper, grape moths, thrips, Cicadas and other pests. Leafhopper is one of the main pests that infest the leafy group part of grapevines [4]. The damaged leafhopper caused yellow spots on the feeding areas by sucking plant sap of grapevine leaves, then the spots turned to brown spots with the progress of the leafhopper infestations leads to drying and falling off of grape leaves. Moreover, the presence of leafhoppers can alter the physical and chemical composition of grape leaves and their quantities of chlorophyll and other nutrients, which leads to a deterioration in production quantity and quality [4-5]. Furthermore, during the feeding process, leafhoppers can transfer pathogens such as bacteria and viruses from infested to healthy grapevines [6].

Plants have developed communication systems based on producing volatile organic compounds (VOCs) released as a response to the infestation. These compounds act as olfactory signals for other organisms, such as insects, to locate their host and VOCs can be influenced by the quantity and quality of compounds by insect infestation [7-8]. Volatile compounds have a great potential to affect the structure and dynamics of ecosystems by inducing indirect resistance to host plants [9]. The feeding or egg-laying behavior of arthropods can also stimulate the plant to produce VOCs, which can then be used by natural enemies such as parasitoids and predators to locate infested plants [10].

Although there have not been several studies on how leafhopper species choose their hosts, utilizing semiochemicals to deter insects from important crops, plants offer available alternatives to chemical management [11]. Analysis of the volatile organic chemicals released by leafhopper-infested and uninfested grapevine leaves indicated quantitatively and qualitative differences between infested and uninfested grapevine leaves. Olfactory signals significantly affect the behavioral of leafhoppers and they use as visual cues [11]. Although volatile substances may elicit a response from different species of leafhoppers and serve as a supplement to visual, gustatory, and auditory cues to host plant location [12].

In this context, the grape leafhopper *Arbordiahussaini* is reported in Iraq and affects many plant families. Grapevine has a high level of leafhopper infestation [4,13]. Therefore, the study aimed to evaluate the role of the VOCs from infested and uninfested grapevine leaves and their effect on the presence and behavior of the grape leafhopper *A. hussaini*.

Materials and Methods

Insect rearing

Apterous adults of *A. hussaini* were collected from an infested Kamali grape orchard located in Salah al-Din Province/ Dujayl District (Latitude: 33° 50' 48.01" N, and Longitude: 44° 14' 3.98" E). In wood cages measuring 25 × 25 × 40 cm with 10 cm diameter mesh covered with made holes for ventilation; *A. hussaini* laboratory colony was formed and kept in a climate-controlled room (25 ±1 ˚C, 50 ±5% relative humidity (RH), and photoperiod 16:8, L:D). A 20% honey solution was used to feed the colony. The third generation was used in the experiments.

The adults and nymphs of leafhopper feed on grapevine by sucking plant sap and causing leaves damage. In this context, the undamaged and 50 -70% of damaged grape leaves were taken from random threeyears age seedlings for VOCs analysis. Newly emerging adults and nymphs were used. Moreover, NPK (20:20:20) fertilizer was provided, and irrigation was applied every three days for infested and uninfested grape seedlings. The study was conducted at the College of Agriculture, University of

Baghdad's Entomology laboratory at ambient temperature. The study was carried out from February to July 2022.The insect was diagnosed by the Natural History Museum, and the grape plant was also diagnosed by the Directorate of Horticulture and Forestry, Ministry of Agriculture.

Plant Volatile Collection

The plant volatiles was collected separately from each uninfested and infested grape leaves with a mixture of males and females of *A. hussaini* and replicated using clusters of ten fresh leaves taken randomly from the top of the grape branch of the Kamali grape cultivar. For twenty hours, the VOCs collection from uninfested or infested leaves was carried out using a glass jar (15x15x20 cm) separately covered with aluminum foil. After that, the solid phase microextraction (HS-SPME) type polydimethylsiloxane (PDMS) fibre inserts into the headspace of the glass jar for 30 min and then removed and the fibre was injected into GC-MS. Each experiment was repeated three times.

Chemical analysis

The HS-SPME was used for the VOCs extracts from the grape leaves of Kamali cultivar. VOCs were analyzed using 7820A Agilent gas chromatography (GC) coupled with Agilent 5977E mass spectrometry (MS). For each grape extract sample, HS-SPME fibre was injected in splitless mode using helium as the carrier gas into a DB-35 MS (30m 250m 0.25m) column. Temperatures for the injector and detector were 250 and 280 C, respectively. The GC oven was preheated to 50°C for one min before being raised to 250°C at a rate of 15°C per min. At 70 eV, electron impact ionization spectra were recorded, capturing mass spectra ranging from 40 to 550 amu. The peak area of each identified compound was determined for quantification purposes. Based on comparing RI and mass spectra with those reported in the NIST library and literature and the alkane C7-C40 standard, compounds were identified [14].

Behavioral Bioassay

The response of adult *A. hussaini* to plant VOCs was examined using a glass Y-tube olfactometer. The Y-shaped tube olfactometer had a 2 cm inner diameter and had two arms that were 15 cm long for each and connected to the test sources as well as the body of the tube was 7 cm long. Before entering the source of the VOCs, the airflow was pushed by an air pump and directed via an activated charcoal purifier and then to distillate water in the flask bottle to filter the air and raise the humidity. The airflow in the olfactometer was 1 ml per min at the end of each arm and was calibrated using a flow meter (Figure 1). Above the Y-tube olfactometer, the white light was positioned in the center. Adult *A. hussaini* specimens were inserted one by one into the center tube. Every insect was monitored for five minutes. The preference of adults for uninfested or infested leaf grapes was assessed. Each experiment was carried out three times by using 50 adults of *A. hussaini* in each experimental treatment group [14].

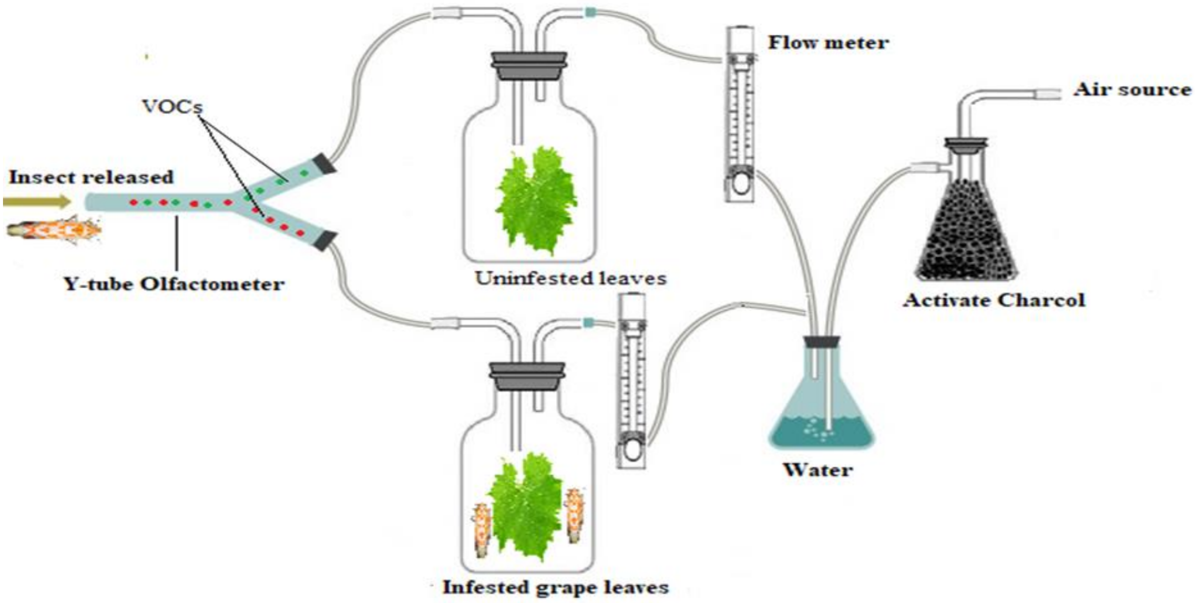

Figure (1): The diagram of the glass Y-tube olfactometer where the grape leafhopper *Arbordiahussaini* was released individually and exposed to VOCs blends released from uninfested and plants infested with *A. hussaini* as shown by the green and red dots.

Statistical analysis

The mean of the chemical compound was analyzed using the online software MetaboAnalyst version 5 for heat map and principal component analysis (PCA) analysis. Variable importance in projection (VIP) plot, commonly used in partial-Least Squares Discriminant Analysis (PLS-DA), ranks the VOCs based on their importance in discrimination between the uninfested and infested grape leaves. VIP score is a weighted sum of squares of the PLS loadings. The amount of explained Y-variance in each dimension influenced the weights. Descriptive analysis and expressed by percentage were present with standard deviation (SD) [15]. Data from open Y-shaped olfactometer experiments were analyzed with Chi-Square ($\chi 2$) tests. Statistical analyses were performed using the SPSS software version 26 (IBM Company, New York, USA).

Results and Discussion

Chemical analysis of Volatile Organic Compounds (VOCs)

The quantities of volatile organic compounds emitted from grape leaves with and without the grape leafhopper *A. hussaini* infestation are shown in Table (1). A total of 19 VOCs were identified by GC-MS in both uninfested and infested grape leaves with *A. hussaini*. Several volatile compounds released from infested grape leaves were detected by GC-MS analysis but they were not detected in the uninfested ones, such as ethanol, pentanone, 1-hexenol, pentenol, non-enone, ethoxypropanol and (E)-2-hexenal. However, some VOCs were highly concentrated in the uninfested than infested grape leaves for instance diethyl acetal, 2-hexenol and 1,5-octadien-3-ol. On the other hand, the VOCs that were identified by GC-MS were in high peak area percentages in the infested grape leaves than uninfested ones such as propanoic acid, hexanone, 3-hexenal and linalool.

Table (1): The concentrations of VOCs released from uninfested and infested leaves of Kamali grape variety with leafhopper *Arbordiahussaini*. The peak area percentage was presented with standard deviation (SD).

RT[1]	KI [2]	Compound names	Uninfested Area%±SD [3]	infested area%±SD
3.367	715	ethanol	0.00	4.55±0.99
3.489	725	pentanone	0.00	2.74±3.48
3.925	762	propionic acid	3.00±3.09	9.82±2.07
4.091	776	butanol	11.18±9.73	14.18±4.68
4.226	788	1-hexenol	0.00	68.67±21.07
4.5	811	hexanone	4.79±0.70	12.01±9.99
4.647	822	pentenol	0.00	10.84±18.72
5.192	866	diethyl acetal	24.77±10.18	13.60±0.88
5.543	894	3-hexenal	2.06±2.08	10.86±5.34
5.648	903	nonenone	0.00	2.68±1.48
5.848	920	ethoxypropanol	0.00	3.86±1.53
6.042	936	(E)-2-hexenal	0.00	9.46±4.26
6.409	967	2-hexenol	31.17±10.67	18.51±4.77
7.748	1087	1,5-octadien-3-ol	22.63±8.87	17.54±3.99
8.83	1194	linalool	11.34±1.50	15.10±7.16
10.349	1360	Gerniol	16.60±3.80	18.90±1.33
11.697	1521	beta-caryophyllene	6.67±1.68	8.00±0.23
12.849	1671	geranyl isovalerate	3.68±1.13	4.29±0.21
13.868	1813	(E)-farnesol	3.08±0.68	3.18±0.50

[1]RT= retention time; [2] KI= Kovats retention index; [3] SD= standard deviation

The results from Figure (2 a,b) were shown by the analysis component principal (PCA). The extent of the variance and compatibility between the VOCs from uninfested and infested grape leaves were explained by variances that showed in the brackets as presented PC1 (81.4%) and PC2 (9.9%) and weighted by the average of the original variable for both uninfested and infested grape leaves. The PCA's Figure (2,a) analysis observed that there are two groups of coefficients (the first group was represented in light green with three replicates of uninfested grape leaves and the second group was represented in pink with three replicates for infested grape leaves). However, Figure (2,b) explained the PCA biplot between the distribution and selected volatile com-pounds within each group of uninfested and infested leaves to test different centering of com-pounds for each group.

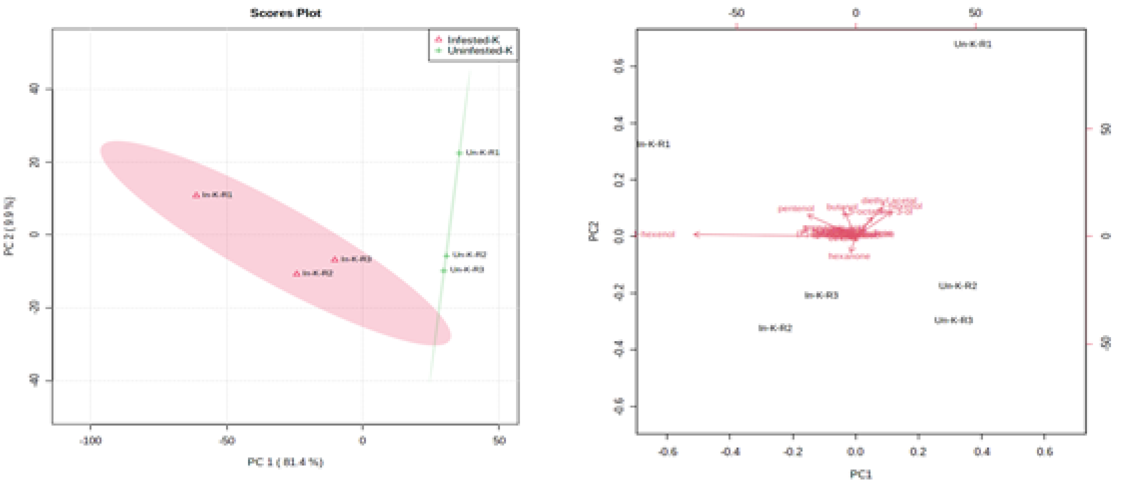

Figure (2): Shows the score plot between two groups of VOCs that were released from uninfested and infested grape leaves with grape leafhopper *Arbor-diahussaini*. (a) The explanation of variance was shown in brackets and, (b) PCA bi-plot between the selected PCs displayed the centering test of VOCs released from different groups of uninfested and infested grape leaves.

The results of Figure (3) show the VIP scores (Variable Importance in Projection Plot), which displayed significant features with analyzed VOCs found by PLS-DA in descending order of significance. According to the leafhopper *A. hussaini*, the graph displays the relative contribution of VOCs to the variation between grape leaves that are not infested and those that are infested. Given the high VIP score by colors, it is clear that the VOCs had a considerable impact on the group division. If the metabolite concentration is higher, represented by red or lower, represented by blue in the uninfested vs. infested leaves VOCs, it is shown on the graph's right side by the dark red and dark blue boxes. The analyzed VOCs are ranked in the plot of Figure (3) based on the variable importance ratings (VIP). The same VOCs were shown to be significant by PLS-DA as they were by paired t-test. The following VOCs 1-hexenol, 2-hexenol, diethyl acetal and pentenol were significant, with a VIP cut-off of around. Only one feature, 1-hexenol, received a VIP score of more than 3.5, indicating that it is of the utmost relevance to the whole model.

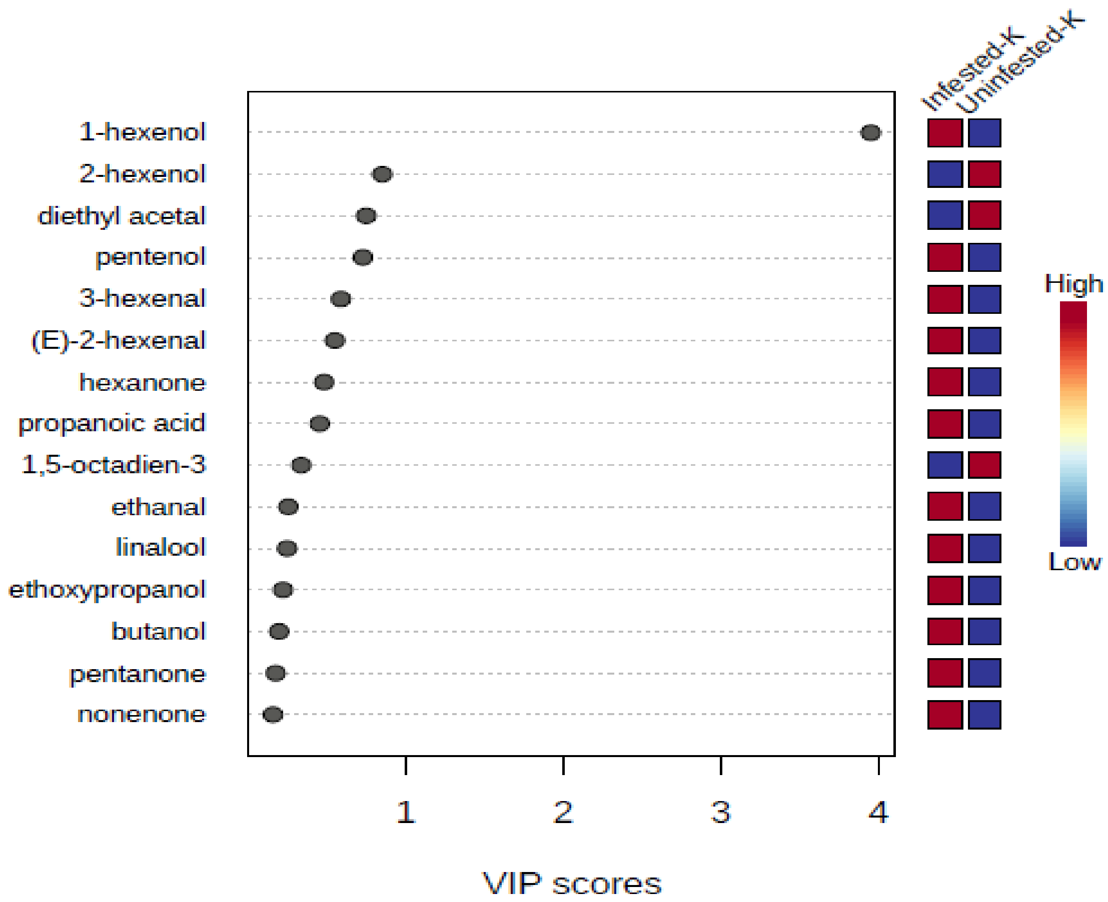

Figure (3): Colorful boxes on the right side depict the relative amounts of the relevant metabolite in each group under study and the characteristics detected by PLS-DA for VOCs produced from grape leaves by VIP scores that were calculated for each chemical.

Furthermore, the heatmap displayed the results of hierarchical cluster analysis conducted independently on both treatments uninfested and infested grape leaves with grape leafhopper *A. hussaini* and variables of VOCs for the 19 volatile compounds. Values importance varies ranging from >2 (highest value, in red color) to < −2 (lowest values, in blue color) and VOCs numbers are those found in Table 1. The hierarchical cluster analysis was conducted on only treatments showing the 2 distinctive clusters displayed in (Figure 4) for clarity purposes, and the data were centered and scaled. The heatmap is obtained by analyzing the 19 volatile compounds listed in Table (1) that differentially accumulated among treatments and comparing their chemical abundance in infested versus uninfested grape leaves. Interestingly, all the detected VOCs were significantly affected by the infestation of grape leafhopper *A. hussaini* or metabolites. Overall, we found that those infested leaves with leafhopper among VOCs showed a higher number of differential VOCs with higher chemical abundance (red color). In comparison, a lower number of different compounds with lower chemical abundance (green color) were observed in the uninfested grape leaves with leafhopper.

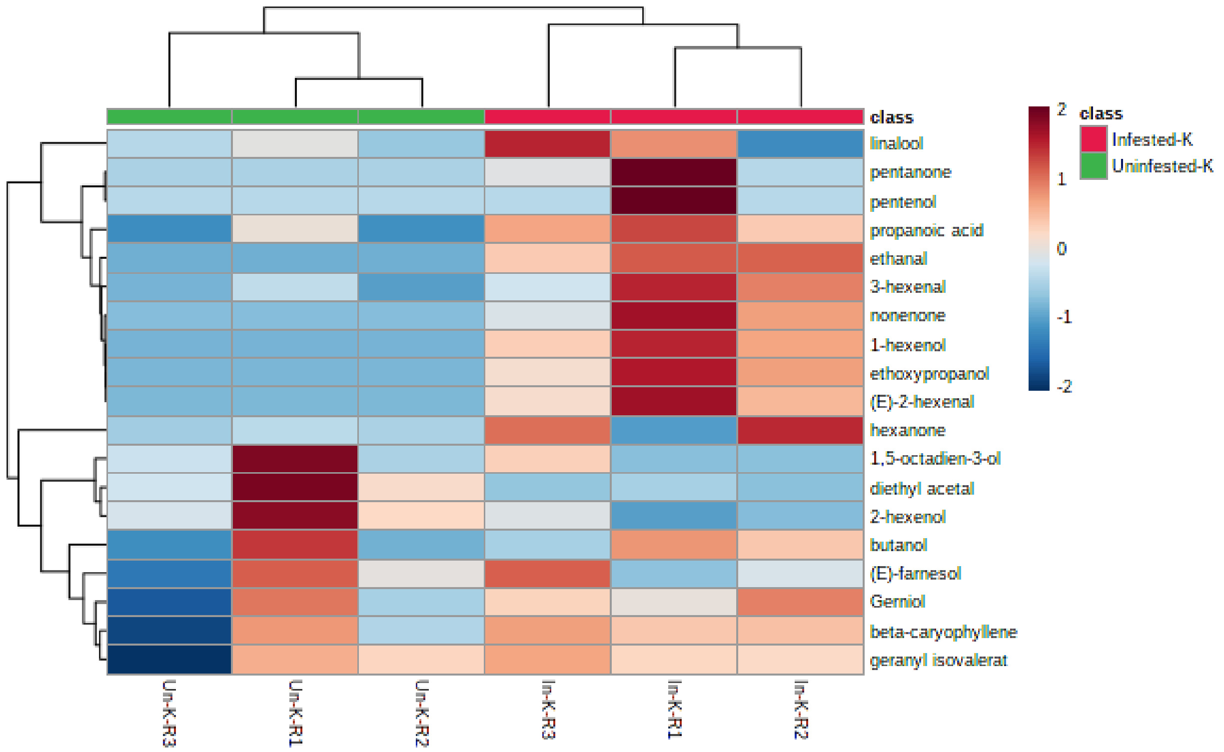

Figure (4): Hierarchical clustering heat map of differential volatile compounds produced by uninfested and infested grape leaves with grape leafhopper *Arbordiahussaini*. Each column represents the samples obtained from the replicate of each uninfested and infested treatment. The red and blue colors in the heat map indicate higher and lower chemical abundance, respectively. Treatments are displayed according to the color scale (class) shown in the legend at the top right. Data are presented as individual values from each biological replication.

The Kamali cultivar of grapevines was used in this study to assess how the phloem-feeding leafhopper *A. hussaini* reacted to VOCs emission from uninfested and infested grapevines. The leafhopper's behavioural responses varied significantly regarding the infested and uninfested grapevine VOCs. Our study provides evidence that grape leaves release different VOCs and their quantity and quality increase due to infestation by *A. hussaini*. These compounds act as a chemical signal by which the insect can locate and attract to host plant [16]. Our results indicated that the identification of VOCs using GC-MS analysis showed 19 VOCs produced from uninfested and infested grape leaves.

In our analysis, we assumed that grape leaves damaged by leafhoppers would emit a variety of volatile compounds based on leafhopper feeding activities. Comparable results were seen in Tasin et al. [17] who reported the behavioral responses of grapevine moth *Lobesiabotrana* females to different volatiles from grapevine and attracted to infested grapevines. Our results indicated that the identification of VOCs using GC-MS analysis showed several volatile chemicals produced from uninfested and infested grape leaves. These VOCs including ethanal, pentanone, 1-hexenol, pentenol, nonenone, ethoxypropanol, and (E)-2-hexenal were not identified in the uninfested leaves. However, some VOCs, like diethyl acetal, 2-hexenol, and 1,5-octadien-3-ol, were concentrated more in the uninfested grape leaves than the infested ones. Propanoic acid, hexanone, 3-hexenal, and linalool were among the VOCs detected by GC-MS in higher peak area rates in the infested grape leaves than in the uninfested ones. In our analysis, we made the assumption that grape leaves damaged by leafhoppers would emit a variety of volatile compounds based on leafhopper feeding. Xin et al. [18] investigated the volatile chemicals released from tea green that varied to leafhopper *Empoascavitis* infestations and chooses suitable host plants by detecting the emission level of (3Z)-hexenyl acetate from the plant. Moreover, eight VOCs have been released from the grapevine Isabella cultivar, including 1-heptanol, 1-octanol, 2-hexanol, 2-nonanone, beta-pinene, camphene, cis-3-hexenyl acetate, and phenethyl alcohol, which may play in plant defense have been noted as being of interest. The distinctive properties of the chemicals in the Isabella cultivar may contribute to this plant's well-known tolerance to primary infestations 19. In contrast, some VOCs including methyl salicylate, alpha and beta-farnesene, and (E)-beta-caryophyllene had a negative impact on egg deposition. According to Markheiser et al. [20], female antennae are probably better able to recognize these chemicals than other female extremities.

One of the key elements influencing the nature of the mixture of volatile organic compounds emitted by plants is the type of plants and the level of insect infestations. Due to this context, we characterized the volatile chemical properties released by uninfested and infested grapevine of Kamali variety with grape leafhopper *A. hussaini* and the VOCs amounts increased when the grapevines is infested by insect pests or mechanically injured.

Behavioral Bioassay of Y-tube Olfactometer

The results of a glass Y-shape olfactometer bioassay on the response of grape leafhopper *A. hussaini* are described in Figure (5) with the Kamali grape cultivar. Three experiments were done which were (1) a comparison between uninfested leaves and clean air, (2) a comparison between infested and clean air, and (3) a comparison between infested and uninfested grape leaves. The comparison olfactometer results between uninfested leaves and clean air indicated that the grape leafhopper *A. hussaini* was significantly attracted and favored toward uninfested grape leaves, where the percentage of attraction was 80%, while the percentage of attraction to filtered air was 20% ($\chi2$ =108, df = 1, $p < 0.05$, n = 300). However, in the comparison between infested leaves and filtered air, the percentage of the attraction of grape leafhopper to infested leaves was 83.33%, whereas the attraction of grape leafhopper to filtered air treatment was 16.67% ($\chi2$ =133, df =1 $p < 0.05$, n =300). The results showed a significant response of the grape leafhopper *A. hussaini* that attracted to the infested leaves treatment with the percentage of its attraction was 80% compared with uninfested leaves, which was 20% ($\chi2$ =108, df = 1, p < 0.05, n = 300).

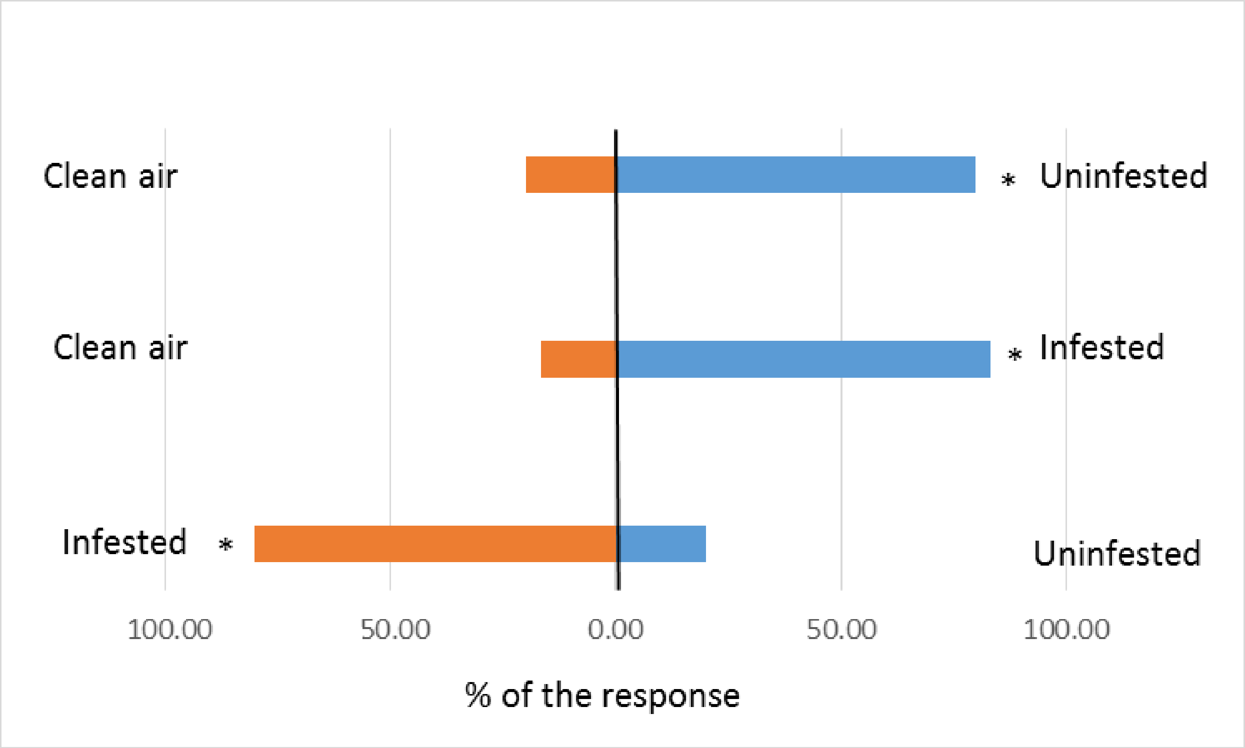

Figure (5): Behavioral response of grape leafhopper *Arbordiahussaini* to VOCs released from uninfested and infested grape leaves with *A. hussaini*. *=refereed to significant difference at (P-value) P<0.05 in the Chi-square ($\chi2$) test.

The current study showed the olfactometer bioassay, the response of *A. hussaini* to uninfested and infested leaves was higher compared to filtered air. The attraction of leafhopper *A. hussaini* to infested or uninfested treatments was the evidence of insects could recognize their host by using chemical cues because of the VOCs released during leafhopper feed activities [21-22]. Additionally, *A. hussaini* were attracted to infested grape leaves with a higher percentage than attracted to the uninfested leaves, perhaps because they were affected by the release of VOCs. Markheiser et al. [20] explained that *L.botrana* and *Eupoeciliaambiguella* could attract the VOCs that are released from infected grapevines by different pests. The VOCs found in the Isabella grape cultivar may be involved in a variety of physiological, defensive, and other processes, including plant defense systems, insect attraction and repulsion, and interactions and communication within and between plants and other species of insects [19]. Similar results were also observed with the grape moth *L. botrana* and the African fig fly of *Zaprionusindianus* that respond to damaged and infested host plants [23-24]. Our investigation confirmed that VOCs were released from grape leaves by comparing the VOCs emitted from both uninfested and infested grape leaves, and these results were supported by an olfactometer bioassay. The current investigation suggested that using volatile compounds might be a promising way for pest control.

Arbordiahussaini is a leafhopper that can significantly damage grapevines. The current study detective volatile organic compounds (VOCs) that are produced from uninfested and infested Kamali grape leaves. As a result of the grape leafhopper infestation, the results showed that the afflicted leaves produce 19 VOCs, and uninfested and infested leaves with leafhoppers had different VOCs levels. According to the Olfactometer results, *A. hussaini* preferred infested and uninfested grape leaves over the filter air treatment. Our results suggest that these variations in VOCs are an essential mediator in insect-plant interactions and may be exploited in future studies to identify the key compounds that might be used as a lure for locating and controlling *A. hussaini*.

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