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Q.Ahmed[1*], M.Agarwal[2], A.Alsabte[1], A.B. Aljuboory[1] and Y.Ren[2*]

[1]Agricultural Engineering Sciences, Baghdad University, Al-Jadriya, Iraq
[2]School of Veterinary and Life Sciences, Murdoch University, South Street, Murdoch 6150, WA

[*]Corresponding author E-mail: qasim.h@coagri.uobaghdad.edu.iq

Abstract. Volatile organic compounds (VOCs) from uninfested and infested broccoli plant samples with green peach aphid *Myzus persicae* were investigated by headspace solid microextraction (HS-SPME) combined with gas chromatography mass spectrometry (GC-MS). Overall, 25 compounds were identified in uninfested and infested broccoli plants. The HS-SPME combined with GC-MS analysis of the volatiles described the differences between the infested and uninfested broccoli plant samples. Based on peak area from the GC-MS analysis, the VOCs from infested broccoli were significantly greater than that from uninfested broccoli, such as D-limonene, Undecane, 3,4-dimethyl-, Heptane, alpha-Pinene, Oxalic acid, Citronellol, Tridecane, n-Decanoic acid, Cyclopentane, pentyl- and n-Hexadecanoic acid compared with volatiles released from uninfested broccoli. The results of Y-tube olfactometer showed that *Myzus persicae* were significantly attracted by the VOCs released from infested broccoli plants, more than uninfested plants or clean air. The percentage of aphid choice was 70% towards infested broccoli, and 10% were attracted to the clean air choice and uninfested plants. With the comparison between clean air and uninfested plants, the aphids were attracted by 84% for broccoli, while 7% were attracted towards clean air. Comparing between infested and uninfested, the aphid attracted by 26.6% for infested broccoli, versus 30% for uninfested broccoli. The preferences of *Aphidus colemani* to the infested, uninfested plants with *M. persicae* and compared with clean air were measured. The results indicated that parasitoids could discriminate the infested broccoli and significantly respond to the plant odour and attractive by 100% for the parasitoid towards infested broccoli plants.

1. Introduction:

The green peach aphid, *Myzus persicae* (Hemiptera: Aphididae), has a worldwide distribution, and is considered a serious pest that has caused damage to hundreds of horticultural species of plants in more than 66 families [1]. The aphid mainly exists in young plant tissues, causing reduced leaf size, delayed growth of the plant and reduced yield [2]. The damage can be caused in two ways: feeding on plant sap by sucking, and also acting as a virus transmission on the plant (indirect damage) [3]. *M. persicae* is considered a common pest insect of the Cruciferae (*Brassica*ceae) family, and sucks plant sap leading to yellowing and curling of plant leaves. Similarly, excretion of honeydew by aphids affects plant photosynthesis and encourages fungal growth and also transmission of plant viruses from one crop to another [4]. Broccoli is commonly attacked by several insect pests, such as species of aphids like turnip aphid *Lipaphis erysimi*, cabbage aphid *Brevicoryne brassicae* and green peach aphid *M. persicae*, which economically damage these crops [5]. Moreover, aphids can cause an economic effect

on a crop at levels of population densities that would decline the yield and affect marketability by physical contaminations with cast skins of aphid and honeydew [5].

Plants VOCs play an important role in plant–insect interactions by prompting insect communication and plant defence [6]. As a plant response to feeding of green peach aphid, is to release odours in the form of VOCs. The VOCs have an important role in plant–insect interactions because they can be used as an attractive agent for natural enemies to locate their host [7]. Solid Phase Microextraction (SPME) was presented by [8] who reported the SPME had become a useful tool in organic analytical chemistry combined with GC-MS because it was easy to use and has a rapid extraction procedure that does not need organic solvents [9]. Broccoli attacked by aphids may emit volatile compounds that attract parasitoid wasps or predators [10-11].

Aphidius colemani (Hymenoptera, Braconidae) is endoparasitoids of many species of aphids and attack *M. perisecae* [12]. The parasitoid lay their eggs in aphid and their young larvae consume the insides of the body of aphid, eventually pupating within its body and turning aphid to mummy. The parasitoid pupa then emerges as an adult to begin the new life cycle [13]. When the aphids feed on the plant leaves, the plant produces blends of volatiles as a response to the infestation by aphids, releases volatile compounds in different quantities and qualities from damaged Brassica plants, and these differences in the VOCs can attract both other pests and natural enemies [12]. The parasitoid wasps *A. colemani* is specific to green peach aphids, and the parasitoid females use VOCs cues to detect and locate where aphids are feeding and lay eggs into hosts [14].

In Y-tube olfactometer tests, [15] reported no attraction of the parasitoid *Diaeretiella rapae* to the cabbage leaves. The heavy population of *M. persicae* on the plant can accumulate wasps, while the uninfested plant sees few parasitoids come to the plant because wasps failed to locate the healthy plant [16].

The study aimed first, to determine the VOCs released from broccoli plant between healthy and infested plants by green peach aphids *M. persicae*; second, to elucidate the responses of Y-tube olfactometer to the green peach aphids *M. persicae* and their parasitoid *A. colemani* when broccoli plants are not infested, and when it is infested by aphids and affected by plant VOCs.

2. Materials and Methods:

2.1. Plants materials

Broccoli (*Brassica oleracea* L. var. *italica*) seeds were sown in a 90 mm square pot filled with commercial potting soil mixture and grown under greenhouse conditions at 23–25°C, 60–70% relative humidity and L16: D8 light cycle. When the broccoli reached to 5–7 leaves stage and used for all experiments. Green peach aphid was reared on broccoli plants in cages made from anti-insect white mesh with external dimensions of 40 cm x 40 cm x 40 cm.

2.2. Aphids and parasitoids colonies

Myzus persicae were obtained from the Department of Primary Industries and Regional Development, Entomology Branch (Western Australia) and maintained on potted broccoli seedlings in a glasshouse that were placed into large cages (210 cm x 90 cm) covered by anti-aphid mesh and provided with a control light system set at L16: 8D photoperiod, at the glasshouse temperature 23–25°C, located at Murdoch University (Western Australia).

Aphidus colemani (Hymenoptera, Braconidae) were commercially obtained from Biological Services (South Australia) as mummies and maintained on potted broccoli plants with *M. persicae* as hosts. Mummies of wasps were removed from the plant leave and placed in open 9 cm petri dishes inside a small cage 40 cm x 40 cm x 40 cm, in glasshouse conditions (23–25°C, 60–70% RH, 16:8 L:D) until emergence. Then the parasitoids were allowed to mate in the cage for one day with provided 50% honey solution for feeding. After that, the parasitoid was held individually in glass vials (one wasp per vial) and a small piece of cotton attached to the vial cap for the drop of 50% honey solution to feed the parasitoid until tested. Female wasps were used for the Y-shape olfactometer choice test [16-17].

2.3. Headspace VOCs extraction

The analysis of volatiles was focused on broccoli for infested and uninfested plants with the green peach aphid. Broccoli were placed individually into 4 L glass jars, and one plant in each jar was

analysed. For each glass jar, a 5 mm port was drilled into the side, into which a septa (20633 Thermogreen® LB-2 Septa, plug) was placed and used for collection of infested and uninfested plant VOCs. Aluminium foil 100 m x 44 cm (Vital Packaging Company) was used to carefully cover and wrap the surface of the top of the plant pot, and the glass jar placed upside down on the plant. VOCs were extracted from samples, which were infested and uninfested broccoli plants with *M. persicae*. Headspace technique analyses were used with three replicates in all experiments, for profiling and characterisation of VOCs from plants. Three phase fibres 50/30 μm divinylbenzene/ carboxen/ polydimethyl siloxane (PDMS/ CAR/ DVB; Sigma-Aldrich, Australia, catalogue number 57347-U) coating was selected for volatiles released from infested and uninfested plants. For optimising various conditions, the sealing time was optimised to 2.30 hours under laboratory temperature 25±1°C, and the SPME fibre was exposed to the headspace of the samples by inserting the SPME into the jar through the septum for two hours to extract the VOCs, which characterised the optimum extraction time. The desorption time of SPME fibre was 5 min in the GC injection port. The SPME was used because it is a fast, simple and modern tool for GC-MS analysis.

2.4. VOCs analysis by GC-MS

The HS-SPME was performed on a gas chromatography mass spectrometer (GC Agilent GCMS 7820A) equipped with MS detector 5977E (Agilent Technologies, USA) and a DB-35ms column (30 m x 250 μm x 0.25 μm) (Santa Clara, CA 95051, USA). The fibre was desorbed in the splitless injector 270°C of GCMS with other operation conditions. The initial temperature of the column was 50°C and held for 2 min, then increased to 250°C at 5°C min-1 and held for 5 min at 250°C. Helium gas (He) was used as a carrier and supplied by (BOC Gas, Sydney, Australia) and the flow rate of the column was 1:1 mL min-1, while the splitless was 20 mL min-1 at 1.5 min and the total GC-MS run time was 45 min. The calibration of the SPME fibre was done by injecting the n-alkanes standard C7–C30. HS-SPME/GC-MS analysis of the VOCs were identified by using AMDIS software version 2.72 and the US National Institute of Standards and Technology (NIST) 2014 MS database. The VOCs were confirmed by comparing GC retention time data with those of authentic standards or from the published literature [18].

2.5. Olfactometer set up:

A glass Y-tube olfactometer was used to determine the responses of *M. persicae* and its parasitoid *A. colemani*, to each of the following pairs of treatments. For the aphid responses, the test was (1) infested (broccoli plant infested with *M. persicae*) versus clean (filter) air; (2) non-infested versus clean air; and (3) infested versus non-infested plants. For the test of parasitoid *A. colemani*, (1) infested plant versus clean air; (2) non-infested plant versus clean air; and (3) infested versus non-infested plants. Bioassays to compare their olfactory responses to VOCs released from healthy plants versus clean air or infested plants with *M. persicae* versus healthy plants.

Glass Y-tube olfactometer with a 7 cm arm length and 2 cm internal diameter, ground glass fitting for the air that passed 200 mL min-1 through each arm, controlled by air flow meter (SCFH AIR, Dwyer Instruments, Michigan City, IN 46360, USA). Each arm tube was connected to a glass chamber (2 L desiccator). Couples of blend VOCs (released from different plant treatments) were presented in a sealed glass chamber (2 L each) at the end of either arm. The compressed air was filtered by using activated charcoal passed through two glass chambers, before the treatment plant could be introduced, and then the air passed through the olfactometer. After assembly, the olfactometer was left to stabilise for 15 min prior to use.

2.6. Bioassays

A single aphid or single parasitoid was introduced into the main arm of olfactometer and pushed 1–2 cm inside the main arm. Each aphid or wasp was given up to 3 min in the olfactometer to respond. Once an individual moved beyond 2 cm and into one of the Y-tube arms, it was considered to have made a choice for the conforming plant treatment in that arm. Non-responders that did not make a choice in 5 min were discarded and excluded from the statistical analysis.

Three replicates and 30 adults of wingless aphid *M. persicae* were assayed for each replicate, and each aphid was tested only one time. Every 10 aphids were assayed, the volatile treatment resources were removed, and all glass vessels cleaned with ethanol, then washed with water and oven dried at 100°C

for a minimum of 30 min. For the comparison, three replicates were carried out on different days using new aphids and fresh infested and non-infested plants. All plant resources were the same age and same size.

The same procedure above was carried out for the parasitoid *A. colemani*. Also, three replicates were used for the parasitoids with 15 wasps for each replicate and wasps were used only once. Throughout experiments, after all 15 wasps were assayed for each replicate, the apparatus was cleaned with water and ethanol after 5 replicates, then dried and heated in the oven at 60°C for more than 30 min. Statistical significance between wasp responses to pairwise combinations of plant treatments was determined using Chi-Square tests at the 5% level.

2.7. Statistical analysis

To identify the differences in the emission of volatile compounds between healthy and infested broccoli by aphids, all peak area analyses were performed with MetaboAnalyst software for P-value, principal component analysis (PCA and PLS-DA) and the hierarchical clustering heatmaps [19]. Differences in the results were compared by using the least significant difference test ($P \leq 0.05$) for determining the means between infested and healthy plants. The peak area was divided by 100,000 for every single compound that obtained from GC-MS. The data of the Y-tube olfactometer bioassays were analysed for preference (aphid M. persicae and the parasitoid A. colemani choice between two treatments tested) using the Chi-Square goodness of fit test by using SPSS software version 24.0.

3. Results and discussion:

3.1. VOCs released from infested and uninfested broccoli

Analysis of the volatiles of broccoli induced by *M. persicae* for the infested and uninfested plant treatments showed significant differences. Several compounds were present in all samples that were trapped by SPME and identified by GC-MS. Plants damaged by *M. persicae* can change in plant odour emission, and the volatiles of samples were significantly higher than uninfested plants. Results from the volatile profiling are taken from the headspace of infested or uninfested broccoli *B. oleracea italica* by SPME. Table 1 shows the differences between uninfested and broccoli infested with *M. persicae*; with 25 VOCs identified in both plant samples by HS-SPME and GC-MS. VOC emission from all samples and the identity of volatiles confirm that infestation of broccoli with aphids significantly increased the peak area of 10 volatile compounds which were released in high amount, such as D-limonene, Undecane, 3,4-dimethyl, Heptane, alpha-Pinene, Oxalic acid, Citronellol, Tridecane, n-Decanoic acid, Cyclopentane, pentyl- and n-Hexadecanoic acid. The peak area was 7.20, 9.74, 26.38, 42.08, 6.13, 24.59, 8.59, 5.24, 5.28 and 65.76 respectively, compared with the volatile compounds that were not released from uninfested broccoli. These compounds were released highly after the infestation of the plant with *M. persicae*. The response of infested broccoli can be reduction in the peak area odour released by plants for some volatiles; 2-Propenamide, Naphthalene, 2-methyl, Benzyl Benzoate, n-Butyric acid, 2-ethylhexyl ester, Ethyl 2,2-diethoxypropionate and Nonyl alcohol.

The VOCs that released from infested *Brassica* plants by *M. persicae* showed many compounds comparing with uninfested plants and reported by previous studies [20-21]. In the current study, volatile compound profiles of uninfested and infested broccoli plants with *M. persicae* were compared to show the differences between treated plants and used as idintification tools for the infestation. [21] reported that a comparison of volatile compounds identified from uninfested and aphid infested plants from several *Brassica* plants. The VOCs can be released by an intact and healthy *Brassica*ceous plant in large amounts [22]; however, the VOCs of Limonene, Undecane, 3,4-dimethyl-, Heptane, alpha-Pinene, Oxalic acid, Citronellol, Tridecane, n-Decanoic acid, Cyclopentane, pentyl- and n-Hexadecanoic acid were detected from infested broccoli. These compound were found in the headspace of infested broccoli and can be involved in attracting beneficial insects as a response to the aphid infestation [23-24]. Thus, the selection of SPME in the extraction of volatile compounds from uninfested and infested broccoli plants with *M. persicae* was based on the peak areas of all compounds identified in the treatments.

Table (1**)** Volatile compounds detected in the headspace for uninfested and broccoli infested with M. persicae by using solid phase microextraction (SPME)

No	Compound name	RT[a]	Uninfested broccoli Area±SD[b]	Infested broccoli Area±SD	*P-value*
1	Camphor	5.13	5.31±3.83	16.90±3.00	0.099
2	Decanal	5.30	9.08±0.57	34.17±16.97	0.581
3	Octanedioic acid	6.64	4.60±4.06	6.52±2.84	0.232
4	D-limonene	9.75	ND c	7.20±1.74	0.015*
5	Undecane, 3,4-dimethyl-	10.15	ND	9.74±0.18	0.521
6	Heptane	10.24	ND	26.38±13.24	0.013*
7	alpha-Pinene	13.05	ND	42.08±26.92	0.012*
8	n-Butyric acid 2-ethylhexyl ester	14.31	14.00±2.15	18.05±0.78	0.017*
9	1,14-Tetradecanediol	16.59	1.74±0.17	8.69±0.01	0.076
10	Oxalic acid, 2-isopropylphenyl octyl ester	18.38	ND	6.13±1.09	0.010*
11	Citronellol	20.27	ND	24.59±6.41	0.007*
12	Nonyl alcohol	21.35	71.02±4.96	6.14±4.45	0.013*
13	Cyclandelate	23.89	14.05±1.77	5.07±3.70	0.018*
14	3-Oxobutan-2-yl 2-methylbutanoate	24.24	3.29±1.83	4.27±0.36	0.645
15	Tridecane	24.53	ND	8.59±0.87	0.706
16	Hexadecanedioic acid	27.97	7.06±0.40	8.50±1.30	0.427
17	Benzene, 1-butylheptyl-	33.44	10.94±0.67	4.49±1.53	0.011*
18	n-Decanoic acid	33.78	ND	5.24±2.25	0.050*
19	Cyclopentane, pentyl	36.01	ND	5.28±0.62	0.007*
20	n-Hexadecanoic acid	37.35	ND	65.76±21.14	0.022*
21	Ethyl 2,2-diethoxypropionate	38.24	16.62±0.65	ND	0.005*
22	n-Butyric acid, 2-ethylhexyl ester	39.40	20.53±3.44	ND	0.019*
23	Benzyl Benzoate	39.47	1.99±0.81	ND	0.182*
24	Naphthalene, 2-methyl	39.95	3.21±0.04	ND	0.007*
25	2-Propenamide	40.42	3.13±0.17	ND	0.005*

a = RT indicated to the retention time of compounds; b = SD referred to the standard deviation of peak area; c = ND referred to not detected; *indicated to the significant different 5%.

3.2. Olfactometer assays of the parasitoid

Results of the laboratory experiments using Y-tube olfactometer bioassays showed the response of the aphids *M. persicae* and the parasitoid *A. colemani* to the uninfested and infested broccoli plants by 30 individual aphids and 15 individuals per replicate of parasitoid. These results indicated that green peach aphids in broccoli were significantly more attracted to the VOCs released from infested plant rather than clean air. The percentage of aphids attracted to the infested broccoli plant was 70% versus 10% (Chi-Square (χ2)= 14.44, df=1 and P<0.0005) (Figure 1). Results showed that *M. persicae* were significant different in the preference broccoli, with more attracted to the healthy plants than clean air. The percentage of attracted aphids was 84% versus 7% to the broccoli plant (χ2 = 19.59, df=1 and P=0.0005). The results showed broccoli volatiles have no significant effect. The percentage of aphid numbers attracted towards infested broccoli plants was 57%, versus attracted 30% to healthy plant (χ2 = 2.46, df=1, and P=0.117).The attraction of parasitoids *A. colemani* to volatiles released by broccoli plants where they were given a choice between healthy and infested plants. *A. colemani* parasitoids were significantly more attracted to volatiles from plants infested with green peach aphids compared with clean (Figure 2). The frequency of parasitoid attraction was 93.33% and 100% towards the infested broccoli plant versus 7% and 20% towards the clean air for parasitoids *A. colemani* (χ2 = 11.26, df= 1 and P=0.001). The statistical analysis showed that parasitoids were significantly attracted

to the infested plant. However, there was no differences between attracted wasps for the odours released from a healthy plant and clean air, there were no responses for both parasitoids *A. colemani* to the health plant odour versus clean air. On percentage, 4.44% of *A. colemani* wasps were attracted to volatiles released from uninfested plants, versus 7% for both parasitoids headed for clean air treatment, while the percentage of no responses of parasitoids was 88.86% ($\chi 2$ = 19.20, df= 2 and P=0.001). 86.67% of the *A. colemani* responded towards infested broccoli versus 9% of the *A. colemani* attracted to healthy plants ($\chi 2$ = 10.28, df= 1 and P=0).

The results of Y-tube olfactometer bioassays confirmed the results of aphids *M. persicae* and the parasitoids *A. colemani* were influenced and attracted to volatiles produced by brocooli plants. These wasps significantly preferred, and were attracted to, volatiles from aphid infested plants over uninfested plants. The use of Y-tube olfactometer to test the response of aphid *M. persicae* to the host broccoli plant indicated that *M. persicae* was influenced by the volatiles released *B. oleracea* var. *italica* and were significantly attracted to both healthy and infested plants when compared with clean air choice.

This result confirms past study [25] that show aphids find their host plants by plant odour as well as visual cues. Moreover, the attraction of aphids to the plant volatiles using olfactometer has been reported in experiments testing plant odour against aphids and their host-finding ability [25]. Our results showed that aphids tended to be attracted to both damaged and undamaged plants. Our observation that plant compounds can explain the variance in attraction by aphids and also plant volatile compounds can increase in response to feeding [26-27].

Results from the olfactometer studies demonstrated that parasitoids respond to the plant volatiles and that *A. colemani* respond to the odour released from infested plants. The tested parasitoids are responsive to plant volatiles significantly when compared with a clean air treatment. This finding is consistent with [28]. The preference of *A. colemani* showed no response of parasitoid attraction to clean air and uninfested broccoli plant; while, the parasitoids noted statistically a significant non-response. [29] explain that the attraction of parasitoids can be significantly higher to the infested plant and attack aphids feeding on the same plant as the origin of the mummy offered. The results are consistent with [30] who showed parasitoid *A. colemani* could be attracted to volatiles released from *Brassica juncea* and preferred plants damaged by green peach aphids rather than plants damaged by *M. persicae* and *Plutella xylostella* caterpillars.

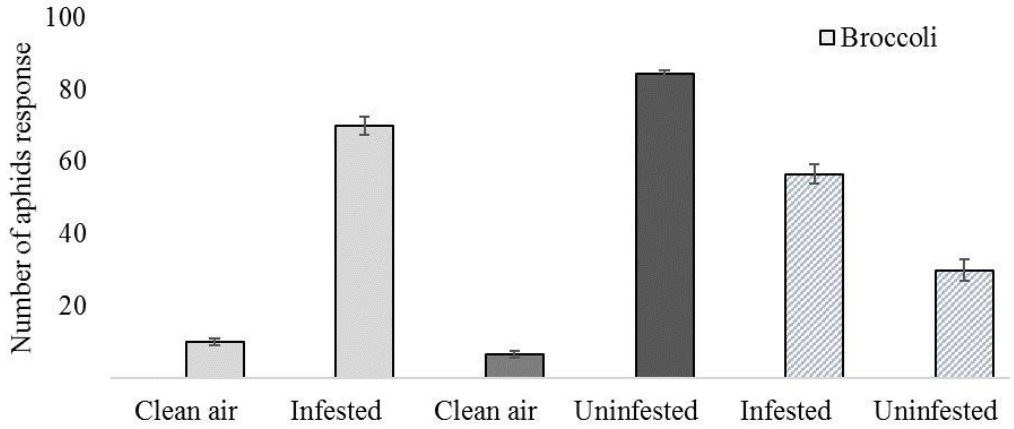

Figure (1) Olfactory response of *M. persicae* in Y-tube olfactometer experiments to volatiles released from infested and uninfested broccoli. Different colors referred to different treatments (uninfested versus infested plants; uninfested versus clean air; infested versus clean air).

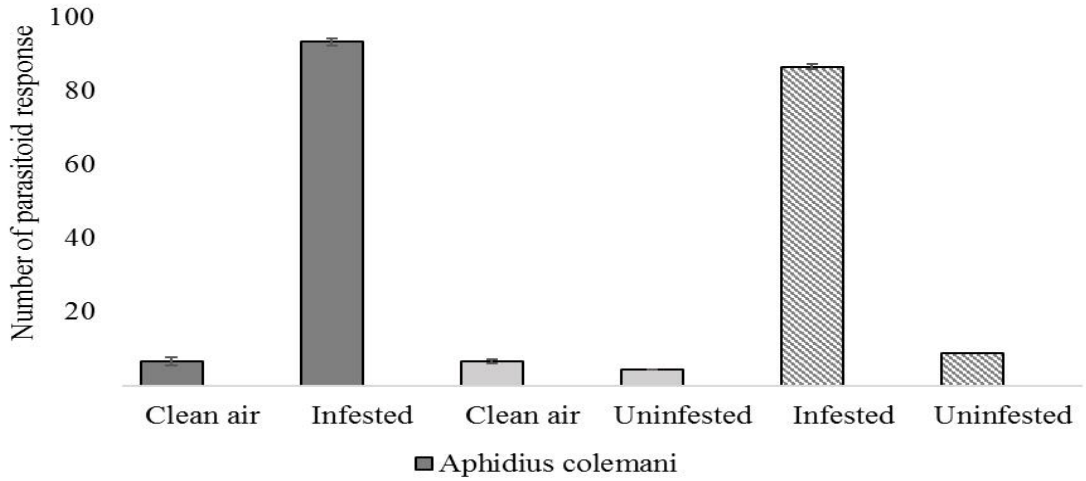

Figure (2**)** Olfactory response of two parasitoids *A. colemani* in Y-tube olfactometer experiments to volatiles released from infested and uninfested broccoli. Different colors referred to different treatments (uninfested versus infested plants; uninfested versus clean air; infested versus clean air).

4. Conclusion:

The HS-SPME with GC-MS analysis for the volatiles described the differences between the infested and uninfested broccoli tested crops and their role in attracting natural enemies of aphids. Collection of volatiles from broccoli occurred by using HS-SPME to detect volatiles compounds between uninfested and plants infested with M. persicae and examined the attraction of natural enemies. A total of 25 VOCs were identified in broccoli plant treatments, by using HS-SPME combined with GC-MS. To detect and locate hosts it is believed that A. colemani, such as many parasitoids, rely on odours released from infested plants as a response to aphids feeding. The results indicated that the preferences of A. colemani to infested plants with M. persicae compared with uninfested plants and clean air by using an olfactometer. Thus, we believe that aphid parasitoids can find damaged plants and then detect aphids on the plant by plant odour. It is likely that the natural enemies' search for aphid infestation may start before landing on the uninfested plant, because parasitoids will first find a damaged plant and then begin searching for aphids. For this reason, many aphid parasitoids efficiently search for damaged plants where aphids will be present as explained by [14].

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