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ARTICLE

Chemically Mediated Interactions between Grapevine, Aphid, Ladybird, and Ant in the Context of Insect Chemical Ecology

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ABSTRACT

This study simplifies the complex relationship among grapevine plants, aphids, ladybirds, and ants, which is essential for effective pest management and ecological balance. This study investigated the impact of aphid attacks and the presence of ants and ladybirds on the volatile compounds profile released into the chemosphere of the community consisting of the common vine *Vitis vinifera*, the aphid *Aphis illinoisensis*, the ladybird *Coccinella undecimpunctata*-and the ant *Tapinoma magnum*. This study aims to analyze the volatile compounds emitted by the grapevine and surrounding insects in response to these intricate interactions. The extraction of volatile organic compounds (VOCs) was carried out using closed-loop stripping (CLS) and then analyzed via gas chromatography-mass spectrometry (GC-MS) and principles coordinated analysis (PCA) was performed. The grapevine was exposed to different types and order of treatments, including non-infested, aphid-infested, aphid-infested with ant, aphid-infested with ladybird, and various combinations of ant and ladybird. After the aphid attack, the outcomes uncovered massive alterations in the volatile compound profiles. Infested grapevine displayed distinct emissions of germacrene D, an alcohol, and an alkene compared to non-infested plants. The characteristic VOC profile was the share of infested grapes in the presence of ants, with benzene derivatives and sesquiterpenes dominating the components. The coexistence of ladybirds with ants and aphids resulted in a different volatile profile characterized by elevated levels of aldehydes, ketones, α-farnesene, and its hydroxy derivative. It was concluded that the emission of VOCs into the chemosphere of the grapevine communities varied qualitatively and quantitatively depending on the level of the relationship complexity within each community in response to the infestation of grapevines by aphids, the presence of ladybirds as natural predators, and the presence of ant as protector. The grapevine's status-dependent compounds can serve as indicators of infestation status and contribute to non-destructive early-stage diagnosis of the aphid.

KEYWORDS

Aphids; *Aphis illinoisensis*; volatile organic compounds; chemosphere; pest management; gas chromatography-mass spectrometry; closed-loop stripping

Nomenclature

V. vinifera	*Vitis vinifera*
A. illinoisensis	*Aphis illinoisensis*
C. undecimpunctata	*Coccinella undecimpunctata*
T. magnum	*Tapinoma magnum*
VOCs	Volatile organic compounds
CLS	Closed-loop stripping
GC–MS	Gas chromatography-mass spectrometry
RT	Retention time
G1, G2, G3, G4, G5, G6	Groups 1, 2, 3, 4 5, and 6, respectively
PCA	Principal component analysis
TPC1	The tripartite community consists of grapevine leaves + *A. illinoisensis* + *T. magnum*
TPC2	The tripartite community consists of grapevine leaves + *A. illinoisensis* + *C. undecimpunctata*
QPC1	The quadripartite community consists of grapevine leaves + *A. illinoisensis* + *T. magnum* + *C. undecimpunctata*
QPC2	The quadripartite community consists of grapevine leaves + *A. illinoisensis* + *C. undecimpunctata* + *T. magnum*
°C	Degree Celsius
h	Hour
µL	Microliter
mL	Milliliter
min	Minute
m/z	**M** stands for mass and **Z** stands for charge number of ions
amu	Atomic mass unit
eV	Electron volt
|r|	Absolute value describes the distance from zero that a number is on the number line, without considering direction

1 Introduction

The value of grapevine (*Vitis vinifera*) at the farm gate is around 80 billion dollars worldwide, making it a valuable fruit crop [1]. *V. vinifera* is the second most important economic fruit in Saudi Arabia after the date. The Taif Governate produces 3000 tons of *V. vinifera*, a special grape characterized worldwide by its taste and shape [2]. It is one of the most common grape species in the Vitaceae family and belongs to the genus *Vitis*. Phenolic compounds, aromatic compounds, flavonoids, proanthocyanins, and stilbenoids are among the significant chemicals found in grapevine [3,4]. Grapevines are at the center of complex communities and relationships in trophic networks, with a wide number of organisms residing both above and below the ground. Some of these organisms, such as rhizobacteria, mycorrhizal fungi, or entomophagous arthropods, are beneficial, while others, such as microbial pathogens or phytophagous arthropods, are detrimental to grape production [5]. The production of grapes is restricted by a variety of biotic and abiotic factors, including pathogens, arthropod pests, increasing global temperature, and drought [6,7]. Pests have the power to influence plant growth and development by feeding directly, spreading viruses or bacteria indirectly, or changing plant biochemistry [8]. Hemipteran insects, which have piercing or sucking mouthparts, bypass the direct breakdown of plant material and instead extract plant materials by ingesting liquids, either through external digestion of cell contents or feeding on phloem or xylem [9]. Plant growth or development may be significantly impacted by these insects, either

through the removal of resources or the introduction of viruses. The use of salicylic acid has been suggested for insects that feed by sucking or piercing to induce direct plant defenses [10]. There are many indirect defenses that can be initiated, such as plant hormone changes, beneficial insect attraction, and volatile chemical release [11]. Plant hormones can be released to alter plant growth, metabolism, and photosynthetic rate when sucking insects are present [12–14].

Beneficial insects' attraction decreases the effect of sucking insects, as they eat or parasitize parasitize insects feeding on plants [15]. In horticulture, aphids are considered among the most destructive pests [16]. The aphid-ant relationship is a typical example of symbiosis due to their common association with ants for protection [17]. Aphids are the target of attacks from various predators, including ladybirds and lacewing larvae [18,19]. Ladybirds are regarded as one of the most prominent hunters of aphids and coccids worldwide. This greatly underestimates the diversity of their biology, and maximizing the impact on their prey is also essential for modern conservation. Additionally, indigenous natural enemies are being utilized for biological control instead of the classical method of introducing foreign species [20].

Ladybirds are among the most beneficial insects in the vineyard [21]. Both young and adult larvae are active feeders; young larvae try to penetrate their prey and suck out all the nutrients and components that keep them alive [22]. In contrast, larvae or adult ladybirds help engulf moth eggs, some pathogens, and small insects; they also survive on pollen and nectar in and near vineyards [20].

In response to insect infestation, there has been limited research on VOCs in grapevines. However, one study did demonstrate changes in grapevines inflicted with phylloxera (*Daktulosphaira vitis* Fitch) [23]. Infected grapevines have higher levels of certain compounds, including methyl salicylate and benzaldehyde. In plants attacked by Hemiptera, as well as under stressful abiotic conditions, the levels of numerous salicylic pathway compounds are known to increase. Insinuating that these plants usually respond defensively to sucking insects' attacks [24]. The study by Alotaibi et al. [25] defined aphid species chemically upon mechanical stress, and *A. illinoisensis* was distinguished by a group of VOCs, specifically esters, benzenoids, and monoterpenes.

Nonetheless, key physiological, biochemical, and subatomic viewpoints of extraordinary edge development exist; how grapevines respond to environmental pressure and deal with biotic and abiotic stresses is ambiguous [26].

The volatile profile of a system can indeed undergo significant alterations due to various biotic factors, such as the introduction or removal of organisms, including symbionts, as well as abiotic changes encompassing temperature, pH, humidity, light exposure, geographical location, and seasonal variations, among others [27].

The purpose of this study is to enhance our knowledge of the chemical ecology of the pest (aphids) and their interactions with other organisms and the environment such as the host plant (grapevine), the natural enemy (ladybird), and pest protector (ant). Nonetheless, it also offers valuable insights that can be used to develop effective pest management strategies. Specifically, by discerning how volatile profiles respond to such alterations, we can identify candidate compounds for use in pest trapping and repellent applications. This comprehensive examination aimed to inform the development of eco-friendly pest management strategies, including utilizing chemical signals released by the grapevine, and the aggregation pheromone of the aphid for pest trapping, employing the defence compounds released by the grapevine in the presence of the aphid for pest repellence, and harnessing alarm pheromones released by aphid in the presence of the natural enemy ladybird as a means of pest repellent. This study explored the potential heightened impact of chemical signals released by ladybirds in the presence of both the aphid and its protector compared to those released in the presence of the aphid alone without a protector for pest

repellence purposes. Finally, the potential use of aggregation pheromones produced by prey (aphids) as a means to attract natural enemies for pest control was investigated [28,29].

2 Materials and Methods

2.1 Insect Samples

Individuals of the species *Aphis illinoisensis*, *Tapinoum magnum*, and *Coccinella undecimpunctata* with accession numbers MZ091377, ON149799, and ON149796, respectively (National Center for Biotechnology Information: NCBI repository), were collected from Taif city and used in this study [30].

2.2 Molecular Identification

For molecular identification of the collected insects, DNA extraction was accomplished using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) as described elsewhere. The mitochondrial cytochrome oxidase gene (COI) was amplified with PCR, using the primer set (LCO1490) (F-50-GGTC AACAAATCATAAAGATATTGG-30) and (R HCO2) (R-50-TAAACTTCAGGGTGACCAAAAAATC A-30). The PCR reaction was performed as described in our previous study [30]. The assembled sequences were deposited in GenBank with the following accession numbers (*A. illinoisensis*: MZ091377, C. *undecimpunctata*: ON149796, and *T. magnum*: ON149799).

2.3 Experiment Workflow

Six groups (n = 24) were set up in 2 L glass desiccators-style chambers (Fig. 1) to minimize VOC loss according to the following criteria:

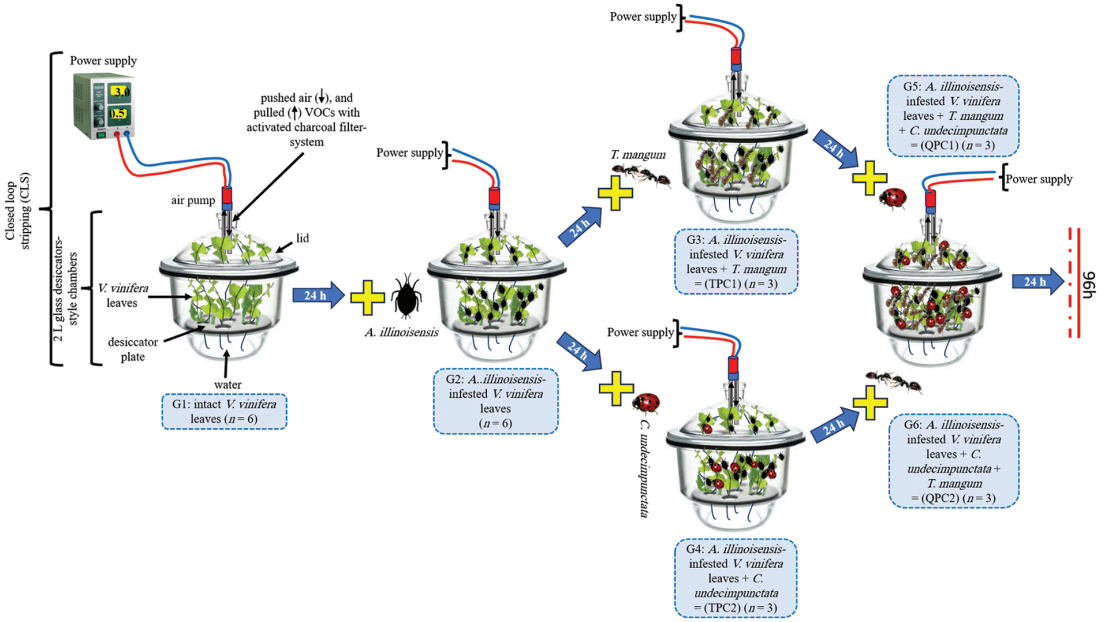

Figure 1: Experimental setup using closed-loop stripping (CLS) to collect VOCs from the chemosphere of six treatments (G1–G6). The experiment workflow started with intact grape leaves (G1) and ended with quadripartite communities (G5: QPC1, G6: QPC2) passing through G2: aphid-infested grape leaves, G3: TPC1, and G4: TPC2

Group 1 (G1) included a noninfested grapevine (*Vitis vinifera*), in which an intact branch 20 cm tall was placed. Following G1 were 50–60 *A. illinoisensis* aphids added to form Group 2 (G2), which included

infested grapevine plants. 20 *T. magnum* ants (G3), or 10 *C. undecimpunctata* eleven-spot ladybird females (G4) were added to G2 to collect VOCs after 24 h of exposure. Thus, G3 and G4 introduced tripartite communities (TPC1 and TPC2, respectively). G5 (QPC1), and G6 (QPC2) introduced quadripartite communities after adding 10 ladybirds *C. undecimpunctata* females to G3 and 20 ants *T. magnum* to G4, respectively. Each group was subjected to three independent biological replicates, and G1, and G2 were initiated with six replicates.

2.4 Headspace Analysis

2.4.1 Airborne Metabolite Collection

The different groups were subjected to headspace extraction using closed-loop stripping (CLS) (Fig. 1) [31,32]. Adsorbent-activated carbon traps were cleaned beforehand by rinsing with methanol and then ethyl acetate and drying at 80°C for 2 h. Airborne metabolites were collected during the continuous circulation of headspace air into the interior of the closed chambers. The enclosed volume of air containing the VOCs emitted by the different treatment groups was circulated with vacuum pumps (model no. DC6/18 F; Fürgut GmbH, Germany). The plants in each group were monitored for 24 h. After 24 h, the activated carbon traps were immediately eluted with 60 µL (3 × 20 µL) of ethyl acetate.

2.4.2 Gas Chromatography-Mass Spectrometry (GC–MS) Analysis Conditions

Samples were analyzed with a GC (580 Clarus) coupled to MS (560S Clarus) (Agilent Technologies, Santa Clara, USA). One microliter of each sample was injected into the GC port in split mode. The GC had a nonpolar Elite-5ms capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies). The GC parameters for the analysis were as follows: The carrier gas was helium at a constant flow rate of 1 mL/min. The injection predwell time was set at 0.1 min (hot needle injection). The injector temperature was 220°C. One microliter was injected into the split mode at a ratio of 1:5. The temperature program of the oven was set at 40°C for 3 min. Then, the temperature was increased from 2.8°C/min to 130°C, followed by an increase of 2.4°C/min to 180°C, where it was ramped up to the final temperature of 250°C for 6°C/min. The total GC run time was 67 min. The MS parameters were set as follows: electron energy = 70 eV, trap current emission = 100 µA, source temperature = 150°C, and transfer line temperature = 280°C. The mass spectrometric detector was operated in scan mode (m/z 40–450 amu). The components were identified by comparing their mass spectra with those of Wiley09 and the National Institute of Standard Technology (NIST 14) mass spectral database. The strategy of identifying compounds based on reverse matching for untargeted analysis was adopted by Alsufyani et al. [33]. All the chromatograms were checked manually, and the area of each peak was normalized by the sum of all the peak areas in the sample's chromatogram. The means of replicates were used for further statistical analysis. Airborne metabolites from different samples were collected and subjected to advanced ecological statistical analysis. The unconstrained discriminant principal component analysis (PCA) was applied to the raw data.

2.4.3 Principal Component Analysis (PCA)

PCA was carried out using XLSTAT 2023 (XLstst.exe, version 25.1.1407; Lumivero, Denver, Colorado) [34]. Before processing the data by PCA, preprocessing was performed, which consisted of calculating the average data for each group of samples and the absence of any compound considered to be zero.

The resulting first two principal axes, F1 and F2, as well as sample coordinates, were then imported into SigmaPlot (version 11.0, Systat Software, USA) for graphical illustration. The biomarkers were screened for significant correlation coefficients, with PCA axes Compounds having a correlation coefficient ($|r| \geq 0.1$) with one of the two PCA axes retained as significantly characteristic compounds.

3 Results

Comprehending the plant's chemical environment before and after it has been infested with agricultural pests, taking into account the presence or absence of natural enemies or protectors will aid in pest prevention, control, and resistance. In this study, we extracted VOCs emitted in the chemosphere, where 33 airborne metabolites stimulate the beneficial and detrimental interactions between grapevine and *A. illinoisensis* as key entities and the rest of the surrounding organisms either a pest-protector *T. magnum* (TPC1), or a pest-natural enemy *C. undecimpunctata* (TPC2), or all of them (QPC1, 2) (Table 1, Fig. 2A), without exposure to plant, or insect tissue using CLS. The activated charcoal traps used in CLS allowed us to cover a broad range of airborne metabolites that differed across the six treatments of grape *V. vinifera*. According to the specifications of our study, we are confident that the chemosphere compounds were released either from *V. vinifera* or *A. illinoisensis*, *T. magnum*, or *C. undecimpunctata* or were released due to interactions between those organisms.

Table 1: VOCs (n = 33) were released into the chemosphere before and after grapevine infestation by *A. illinoisensis*, in the presence or absence of *T. magnum* and *C. undecimpunctata* (24 samples). Experiments were conducted under laboratory conditions, and metabolites were extracted by CLS and analyzed by GC-MS

Substances classes	Biomarkers	RT (min)	Compounds	Grapevine treatments						Number of samples
				G1 (n = 6)	G2 (n = 6)	G3 (n = 3)	G4 (n = 3)	G5 (n = 3)	G6 (n = 3)	
Benzenoids	Bio1	8.83	O-Xylene**	0	0	+	0	0	0	1
	Bio4	11.3–11.4	Propylbenzene??	0	0	+	0	0	0	1
	Bio5	11.6–11.7	1-Ethyl-3-methylbenzene??	0	0	+	0	0	0	1
	Bio7	12.05–12.16	Mesitylene*	+	0	0	0	0	0	1
	Bio9	13.17–13.22	1,5-Dimethylbenzene??	0	0	+	0	0	0	1
Total of benzenoids				**1**	**0**	**4**	**0**	**0**	**0**	
Aldehydes & ketones	Bio2	10.01–10.34	2-Methyl-4-heptanone?	0	0	0	0	0	+	1
	Bio6	11.88–11.94	Benzaldehyde*	0	0	+	0	0	0	1
	Bio8	12.96–13.34	6-Methyl-5-hepten-2-one?	0	0	0	0	+	+	2
	Bio14	18.90–18.95	Nonanal* (GLV)	0	0	0	+	+	0	2
	Bio19	23.99–24.04	Decanal?	0	0	0	0	+	+	2
Total of aldehydes & ketones				**0**	**0**	**1**	**1**	**3**	**3**	
Terpenes	Bio3	10.40–10.50	α-Pinene?	0	0	+	0	0	0	1
	Bio12	15.96	Tricyclene?	0	0	+	0	0	0	1

(Continued)

Table 1 (continued)

Substances classes	Biomarkers	RT (min)	Compounds	Grapevine treatments						Number of samples
				G1 (n = 6)	G2 (n = 6)	G3 (n = 3)	G4 (n = 3)	G5 (n = 3)	G6 (n = 3)	
	Bio24	27.04–27.09	Citral*	0	0	0	0	0	+	1
	Bio25	33.69–33.72	Caryophyllene*	0	+	+	+	+	+	5
	Bio26	34.18	α-Cubebene*	0	0	+	0	0	0	1
	Bio27	35.27–35.32	β-Farnesene*	0	+	+	+	0	0	3
	Bio28	36.41–36-44	Copaene?	0	0	+	0	0	0	1
	Bio29	36.45	Germacrene D?	0	+	0	0	0	0	1
	Bio30	37.77	α-Farnesene?	0	0	0	+	+	+	3
	Bio31	38.17–38.19	α-Cadinene?	0	0	+	0	0	0	1
	Bio33	45.20–45.28	7-Hydroxyfarnesene?	0	0	0	0	+	+	2
Total of terpenes				**0**	**3**	**7**	**3**	**3**	**4**	
Easters	Bio10	13.97–14.20	Hex-3-enyl acetate? (GLV)	+	+	0	+	+	0	4
	Bio11	14.36–14.47	Hexyl acetate?	0	0	0	0	+	0	1
	Bio16	23.00–23.08	3-Hexenyl isobutyrate??	+	+	0	+	+	+	5
	Bio17	23.19–23.25	Methyl salicylate*	0	0	+	+	0	0	2
	Bio18	23.55	2-Hexenyl butanoate?	0	0	0	0	+	0	1
	Bio21	25.23–25.28	3-Hexenyl-α methylbutrate?	+	0	0	0	+	0	2
	Bio22	25.80–25.83	3-Methyl hexyl butanoate?	0	0	0	0	0	+	1
	Bio23	26.36	2-Phenylethyl benzene acetate	0	0	+	0	0	0	1
	Bio32	41.35–41.40	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate??	+	+	0	0	0	0	2
Total of esters				**4**	**3**	**2**	**3**	**5**	**2**	
Alkanes & alkenes	Bio13	18.60–18.65	4,5-Dimethylnonane?	0	0	0	+	+	+	3
	Bio15	19.28–19.37	4,8-Dimethylnona-1,3,7-triene?	0	+	0	+	+	0	3
Total of alkanes & alkenes				**0**	**1**	**0**	**2**	**2**	**1**	
Alcohols	Bio20	24.37	2,6-Dimethylocta-3,5,7-trien-2-ol??	0	+	0	0	0	0	1

(Continued)

Table 1 (continued)

Substances classes	Biomarkers	RT (min)	Compounds	Grapevine treatments						Number of samples
				G1 (n = 6)	G2 (n = 6)	G3 (n = 3)	G4 (n = 3)	G5 (n = 3)	G6 (n = 3)	
Total of alcohols				**0**	**1**	**0**	**0**	**0**	**0**	
Total biomarkers				**5**	**8**	**14**	**9**	**13**	**10**	

Note: **1:** Noninfested grapevines, **G2:** aphid-infested grapevines, **G3:** aphid-infested grapevines + Ant (TPC1), **G4:** aphid-infested grapevines + ladybirds (TPC2), **G5:** aphid-infested grapevines + ant + ladybirds (QPC1) and **G6:** aphid-infested grapevines + ladybirds + ant (QPC2). GLV: Green leaf volatile. The compounds were identified by the NIST library and RT (tentative identification). *Several key compounds were verified by comparison with authentic standards. **probably a contaminant from the aeration system. The metabolites marked with a "?" had a reverse match between 990–850 and those marked with a "??" had a reverse match between 800 and 700. (0): not detected, (+): detected.

3.1 Overview of the Compounds Detected in the Chemosphere

Thirty-three compounds were detected in the chemosphere of the different *V. vinifera* grapevine treatments extracted by CLS, as shown in Fig. 1.

For non-target analysis, these compounds were tentatively identified (most of which were alkanes, aldehydes, esters, benzene derivatives, and sesquiterpenes) (Fig. 2B). The chemosphere constituents of the noninfested grapes were benzene derivatives (2.28%) and esters (97.7%). After infestation by *A. illinoisensis*, the chemosphere of the aphid-infested grape plants released alkanes (25.1%), terpenes (26.6%), esters (42.1%), and alcohol (6.1%), but no benzene derivatives or aldehydes were detected. After the ants were added to the infested grape plants, the chemosphere profile of the tripartite community (TPC1) consisted of terpenes (41%), benzene derivatives (50.8%), and esters (8.1%), but no alcohol or aldehydes were detected. The tripartite community of the infested grape and ladybird (TPC2) profile consisted of alkanes (20.3%), terpenes (40.8%), aldehydes (9.6%), and esters (29.1%), but no alcohol or benzene was detected. The quadripartite communities (QPC1,2) were carried out in different orders. When ladybirds were added to TPC1, the chemosphere profile of QPC1 consisted of alkanes (6.5%), terpenes (63.7%), aldehydes (13.5%), and esters (16.2%), but no alcohol or benzene was detected. Additionally, when the ant was added to TPC2, the chemosphere profile of QPC2 contained the same compounds but in different percentages: alkanes (3.01%), terpenes (43.7%), aldehydes (50.5%), and esters (2.6%) (Fig. 2B). Overall, sesquiterpenes were identified only after infestation as seen in Fig. 2C. Thus, this specific sesquiterpene blend, with a retention time of 33.69 to 45.28 min (Table 1), can be used as an indicator for the infestation status of grapevine by A. *illinoisensis*. Germacrene D (bio #29) was detected 24 h after infestation by *A. illinoisensis* (G2), and disappeared after that (i.e., G3-G6), unlike caryophyllene (bio #25) which remained in all infested groups (G2-G6). The alarm pheromone *β*-farnesene (bio #27) was identified in G2–G4 but vanished in QPCs (G5, G6). The highest percentage recorded was the share of the isomer α-farnesene (bio #30) in the QPC1 (G5), followed by QPC2 (G6), and TPC2 (G4), respectively, while the presence of farnesene derivativ7-hydroxy farnesenene (bio #33) was limited to the QPCs (G5, G6). The presence of *T. magnum* in the infested grapevine (TPC1-G3) was indicated with three sesquiterpenes, namely α-cubebene (bio #26), copaene (bio #28), and cadinene (bio #31). To simplify the complicity of the profile emissions of VOCs, the multivariate principal coordinates analysis (PCA) was implemented on the raw GC-MS dataset. PCA found biomarkers in the chemosphere that are distinctive for grapevine treatments, depending on whether compounds are present, absent, or abundant in chemosphere profiles.

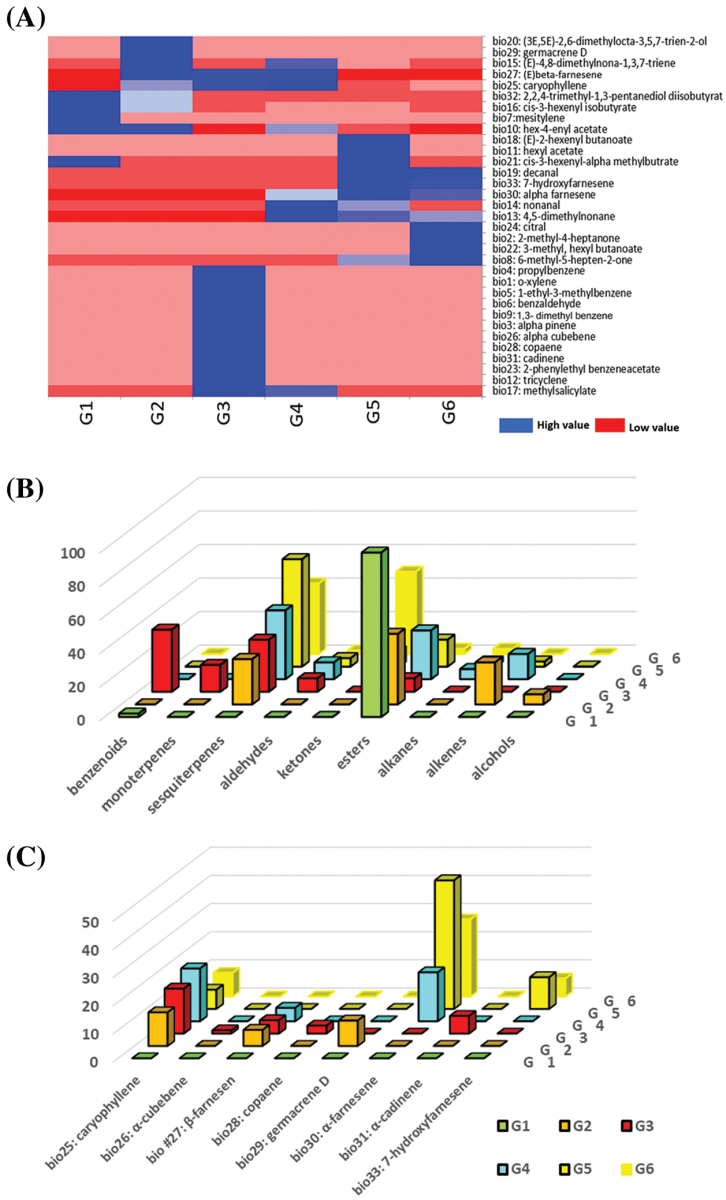

Figure 2: (A) The heatmap of the average percentage of VOCs released into the chemosphere of the six grapevine treatments (G1–G6) according to the GC–MS analysis. The percentages ranged from 0% to 16.3%. Smaller values are shown as red cells and higher values are shown as blue cells in the horizontal direction. Metabolite identification was accepted if the quality varied between 50% and 99%. (B) Column chart represents the emissions percentage of VOCs released in the chemosphere of six grapevine treatments. (C) Column chart shows the distribution of sesquiterpenes in the chemosphere of six grapevine treatments. G1 (light green): intact grapevine, G2 (orange): *A. illinoisensis*-grapevine, G3 (red): *A. illinoisensis*-grapevine + *T. mangum* (TPC1), G4 (light blue): *A. illinoisensis*-grapevine + *C. undecimpunctata* (TPC2), G5 (yellow with black frame): *A. illinoisensis*-grapevine + *T. mangum* + *C. undecimpunctata*, respectively (QPC1), G6 (yellow without frame): *A. illinoisensis*-grapevine + *C. undecimpunctata* + *T. mangum*, respectively (QPC2)

3.2 Biomarkers Extraction for Group Separation

The statistical analysis of PCA (Fig. 3A) revealed that there are significant differences between VOCs in the chemosphere as a result of quantitative or qualitative estimation (Figs. 2A, 3B).

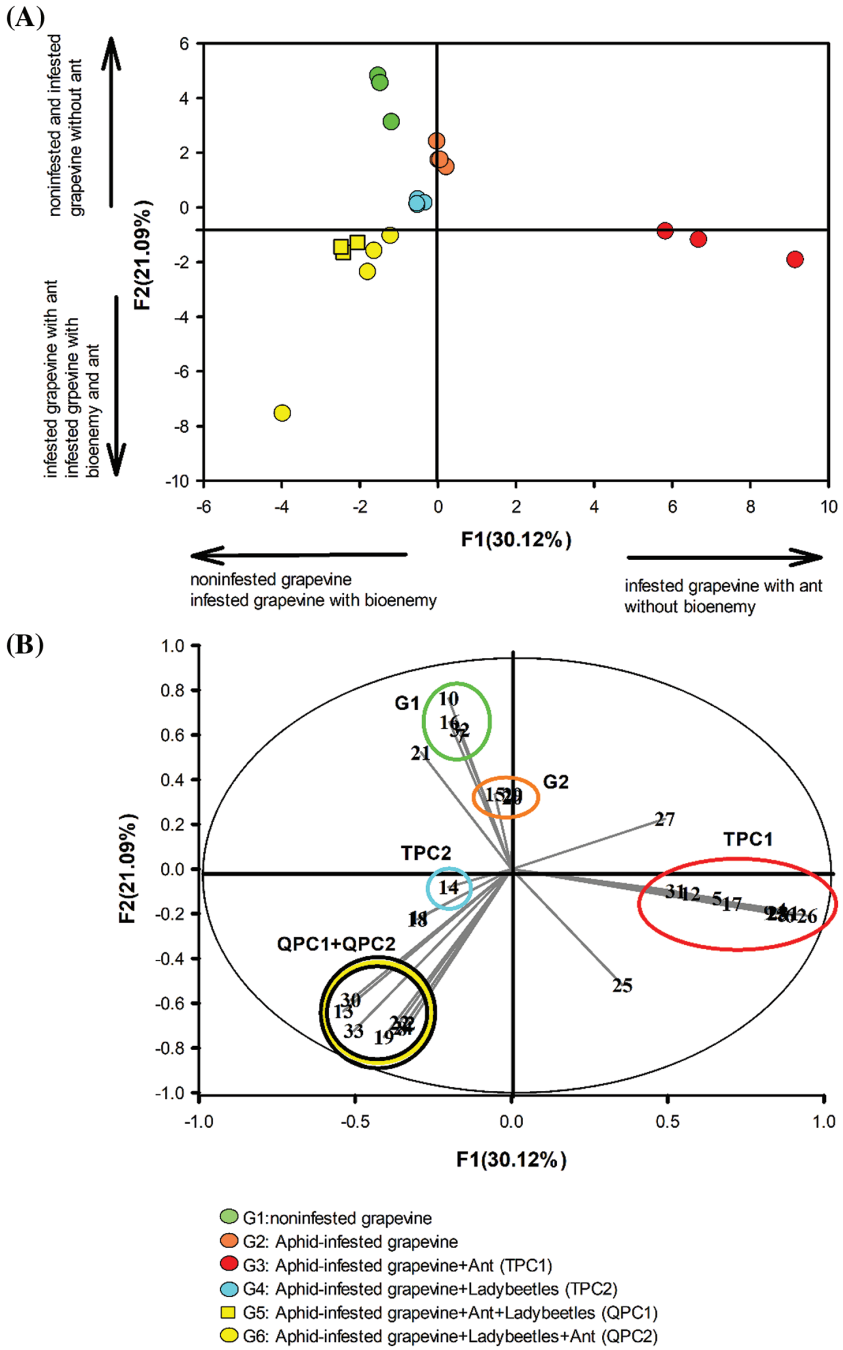

Figure 3: The principal component analysis (PCA) of the chemosphere compounds (n = 33) released by the noninfested grapevine (G1), infested grapevine (G2), tetrapartite communities (G3, G4), and quadripartite communities (G5, G6) analyzed by GC/MS. (A) The first two axes, F1 and F2, which represent 30.21% and 21.09%, respectively, of the variables, are plotted. PCA demonstrated the separation of the samples

Figure 3 (continued)
based on the organisms present in the groups, which affected the presence and absence of chemosphere compounds. (B) Scaled vectors of the chemosphere compounds (ID numbers) were significant ($|r| > 0.2$) for the separation of the groups. Quadrant communities G5, and G6 were regarded as one group with the same biomarkers by both axes F1 and F2. The numbers refer to the biomarkers in Table 1 and Fig. 1

As shown in Fig. 3A, F1 and F2 (with eigenvalues of 9.94% and 6.96%, respectively) represented 30.12% and 21.09%, respectively, of the variabilities. These findings suggest that F1 and F2 are two variables that contain useful information for distinguishing different groups within the dataset. Such sample distribution provides insights into the relationships and differences between the groups based on the patterns or characteristics captured by these variables (Fig. 3B). Taking the distribution of samples by PCA into account (Fig. 3A), it is clear that the separation via the F1 axis is fully dependent on the infestation status of the grapevine. Thus, the infestation status introduced by the infested grapevine with the aphid-tending ant (G3: TPC1) was separated from the noninfested (G1), as well as the biotreated grapevine by *C. undecimpunctata* (G4–G6: TPC1, TPC2, QPC1, QPC2). Notably, G1 and G2 stood apart from the groups containing ant (G3: TPC1, G5: QPC1, and G6: QPC2), along the F2 axis. The separation of these groups via F2, particularly concerning the presence or absence of *T. mangum*. The separation of five groups via F1, and F2 (Fig. 3) emphasizes the intricate chemical coordination governing the interactions among grapevine plants, aphids, ants, and ladybirds. These findings unravel the complexity of ecological relationships and chemical communication mechanisms, accentuating the crucial roles of specific VOCs in shaping plant responses to infestations and the presence of natural protectors. The chemical and statistical results in Table 1, along with Fig. 3 indicate that the grapevine's status-dependent compounds can differentiate between five out of six communities, as presented in Fig. 4.

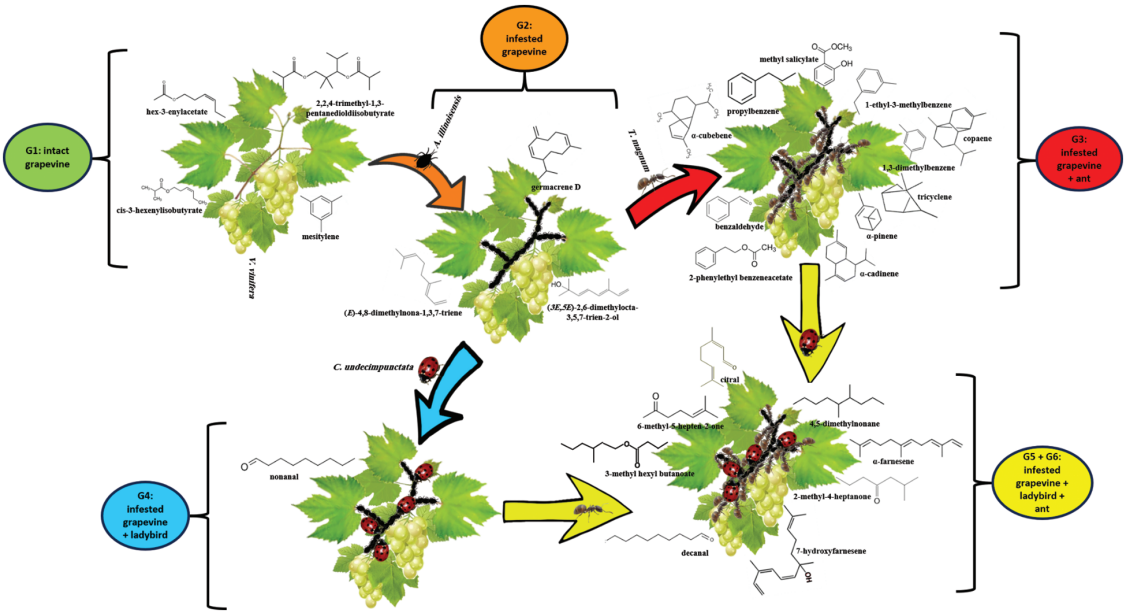

Figure 4: The chemosphere compounds released by grapevine before and after *A. illinoisensis* infestation were analyzed by CLS-GC/MS and subjected to PCA analysis, with G1–G6 being referred to as Fig. 1

3.3 Intact Grapevine (G1)

In noninfested grape plants (G1), VOCs represented by mesitylene (bio #7), hex-3-enyl acetate (#10), 3-hexenyl isobutyrate (#16), and 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (#32) were detected in the chemosphere (Figs. 3B, 4). It's worth noting that these VOCs either vanished or decreased significantly after infestation by *A. illinoisensis* (Table 1, Fig. 2).

3.4 Grapevine–Aphid Interaction (G2)

This system describes the interaction between the grapevine and *A. illinoisensis*. Grapevine plants are responding to aphids, which are adept sap feeders, by emitting VOCs into the chemosphere. Three significant biomarkers serve as indicators of infestation such as 4,8-dimethylnona-1,3,7-triene (bio #15), 2,6-dimethylocta-3,5,7-trien-2-ol (#20), and caryophyllene (#25) (Figs. 3B, 4). Any change in this community as a result of adding biotic variables affected the chemosphere profile, as noted in other communities (Fig. 3A).

3.5 Grapevine-Aphid-Ant Interaction (TPC1)

The presence of *T. magnum* in this community was indicated by the availability of bio benzenoids (bio #4, #5, #9), an aldehyde (#6), monoterpenes (#3, #12), sesquiterpenes (#26, #28, and #31), and esters (#17, #23) in the chemosphere (Table 1, Figs. 2A, 3B, 4). This unique VOC composition of this group can be attributed to the combined effect of grapevine, *A. illinoisensis*, and *T. magnum*, and was never detected in other communities even in low concentrations except for bio #17 (Table 1).

3.6 Grapevine-Aphid–Ladybird Interaction (TPC2)

The interaction between predators and prey is exemplified by the attack on *A. illinoisensis* by *C. undecimpunctata*, which could be attributed to the only significant biomarker, nonanal (bio #14) (Figs. 3, 4).

3.7 Grapevine-Aphid-Ant-Ladybird Interaction (QPC1/QPC2)

The quadripartite communities consist of all four organisms of the study's interest regardless of the adding order of *T. magnum* or *C. undecimpunctata* (QPC1, QPC2) were considered as one group by PCA (Fig. 3) and characterized by a ketone (bio#2), an aldehyde (#19), one monoterpene (#24), and sesquiterpenes (#30, #33), an ester (#22), and an alkane (#13). Despite the nonsignificant differences observed by the heatmap (Fig. 2A) and the column chart (Fig. 2B) show differences between the two quadrant communities.

4 Discussion

4.1 Understanding the Chemical Ecology of A. Illinosensis-Infested Grapevine

In this complex network of interactions in the grapevine-aphid-ant-ladybird system, the exact balance between ecological dynamics and chemical signaling is important in structuring the ecosystem's stability and functioning [35]. The intricate relationships among these organisms reflect their complex interplay from ecological and chemical perspectives.

The significant alternation of chemosphere among grapevine treatments as was noticed in Table 1, Fig. 2 demonstrated that VOCs play many roles in plant defense mechanisms, and attract insects [36,37]. Benzenoids and esters were detected as VOCs in the chemosphere of intact *V. vinifera* leaves (G1) as predominant classes. Similar results were seen in Rodríguez-Declet et al. [38], with alcohol and hydrocarbon as the most prominent class emitted from leaves of grapevine cv. *Isabella*. Exposure to various biotic variables, like *A. illinosensis*, *T. magnum*, and *C. undecimpunctata*, resulted in significant qualitative and quantitative alterations in the emitted VOCs in the chemosphere (Table 1, Fig. 2). Aphid, proficient sap feeders, initiate an ongoing response in grapevine plants by emitting VOCs into the

chemosphere [1,39] as clearly witnessed in all other groups (G2–G6) (Table 1, Fig. 2). After infestation with *A. illinosensis* (G2), the presence of sesquiterpenes including the alarm pheromone (bio #27: β-farnesene), alkenes, and the absence of benzenoids distinguished this community. These VOCs act as chemical signals, alerting both the plant and other organisms to the presence of a stressor. The plant's release of these compounds is an immune response to herbivore attacks, stimulating chemical defenses and attracting natural enemies, including the ladybird [40]. Initially, *A. illinosensis*-infested grapevine (G2) was the site of detection for the alarm pheromone (*β*-farnesene-bio #27), and it persisted in TPC1(G3), and TPC2 (G4) after *T. magnum*, and *C. undecimpunctata* addition, respectively, whereas *β*-farnesene was absent in both quadripartite communities (QC1,2) (Table 1, Fig. 2A,C, Fig. 3B). In addition, the emissions of another farnesene isomer (α-farnesene, bio #30), and its hydroxyl derivative (7-hydroxy farnesene, bio #33) were exclusively observed in all *C. undecimpunctata*-associated treatments (G4–G6), and QPCs (G5, G6), respectively (Table 1, Fig. 2A,C, Fig. 4). Although the reproductive organs of grapes emit α-farnesene, which acts as a cue for grape moth oviposition [41], further investigations are required to determine its biological functions under the study's conditions. *β*-farnesene can be produced by attacked plants as a semiochemical that attracts natural enemies, or it can be generated by aphids as an alarm pheromone [42]. Aphids release *β*-farnesene that is too low for predators to detect over a certain distance because each aphid releases nanograms, from 1 to 2, and up to 50 [42]. Therefore, the predator's ability to detect this volatility from a distance and before aphids are disturbed is limited due to *β*-farnesene emissivity in the presence of danger [43]. Thus, we can argue that the detected *β*-farnesene in TPC2 (Table 1, Fig. 2A) is an *A. illinosensis*-induced grapevine volatile, as is also the case in G2, and TPC1. Notably, a study by Verheggen et al. [44] revealed that the ant *Lasius niger* utilizes *β*-farnesene in low amounts to find aphid colonies and facilitate communication between aphids and ants, which could be a reflection of the situation between *A. illinosensis*-*T. magnum* in TPC1. The absence of *β*-farnesene in QPC1, and QPC2 (Table 1, Fig. 2A,C) is likely to be attributed to (1) the experiment's workflow depicted in Fig. 1 involved collecting VOCs from quadripartite communities after 96 h, which is long enough for *β*-farnesene volatilization to decline exponentially, as discussed comprehensively by Vandermoten and coauthors [45], (2) larger predator, such as ladybird, consumes its prey in its entirety, it can effectively stop an aphid's alarm signal (*β*-farnesene), and it's important to keep in mind that different predators feed differently, and this could affect the duration and strength of the signal as reported by Schwartzberg et al. [43]. These results support the notion that *β*-farnesene is a biomarker for *A. illinoisensis*-infested grapevine. *β*-farnesene was recently identified as a biomarker of *A. illinoisensis* due to exposure to mechanical stress [25]. Caryophyllene (bio #25) was detected in all infested groups regardless of the existence or the absence of ant and ladybird (G2–G4), but with less amount in QPCs (G5, G6) (Table 1, Fig. 2A,C). According to Alotaibi and coauthors [25], caryophyllene is present in the headspace profile of *A. illinoisensis* after mechanical stress. A study by Howard et al. [46] confirmed that plant caryophyllene has a detrimental effect on ant species. This is a sesquiterpene that is widely present in plants [47] and produced by multiple terpene synthases [48]. It is notable for its contribution to plant defense, which involves repelling spider mites and attracting herbivorous enemies either above or below ground [49]. The increase in aphid parasitoids in damaged tomato plants was attributed to one of the highest concentrations of caryophyllene released in aphid-infested tomatoes [50]. Similarly, in maize, caryophyllene attracts natural enemies of herbivores [48], and *Aphidius colemani*, a parasitic wasp of *Aphis gossypii*, was attracted to caryophyllene, and it was observed that it could have negative impacts on aphid fitness [51]. According to Fig. 2C, the decrease in caryophyllene release in QPCs (Fig. 2C) is probably due to aphid effectors that could alter multiple plant defense tools, including the emission of VOCs, as demonstrated in other plant-aphid interactions [52]. *β*-farnesene can be suppressed by the other VOCs in the chemosphere, including caryophyllene and germacrene D [53]. In our study, germacrene D (bio #29) was detected only in the chemosphere of *A. illinosensis*-infested grapevine (G2) (Fig. 2A,C). The separation of G3 was significantly influenced by the sesquiterpenes α-cubebene (bio #26), copaene

(bio #28), and α-cadinene (bio #31) (Fig. 2A,C, Figs. 3, 4). Staudt et al. [54] identified α-cubebene as an aphid-induced VOC. Cadinene and copaene were reported as induced sesquiterpenes that deter whitefly *Bemisia tabaci* from settling on its host plants [55]. Germacrene D, α-cubebene, copaene,α-farnesene, α-cadinene, and 7-hydroxy farnesene were neither detected in the headspace profile nor as significant biomarkers of mechanically stressed *A. illinoisensis* [25].

Green leaf volatiles (GLVs) have a variety of biological functions, including herbivore defense, plant priming, pathogen defense, aroma, and flavor according to a review by Ul Hassan et al. [56]. Hex-3-enyl acetate (bio #10), and nonanal (bio #14) were two GLVs detected in the current study (Table 1, Fig. 2A). Hex-3-enyl acetate was identified in high concentration in intact grapevine (G1), and all treatments except for group-associated *T. magnum*, i.e., TPC1, QPC1 (very low concentration), and QPC2 (Table 1, Fig. 2A). Many studies shed light on the repelling effect of alkyl acetate esters on certain species of ants such as *Tetramorium tsushimae* [57], and *Solenopsis invicta* [58]. The disappearance of this compound in ant-containing treatments in this study and the anti-ant detection observed in previous studies provide a new avenue of research into this relationship. Nonanal was determined after 24 h of treating infested groups by *C. undecimpunctata*, represented by TPC2, QPC1, unlike TPC1, where *C. undecimpunctata* remained for 48 h (Table 1, Fig. 2A). Nonanal is a source of attraction for ladybird species (*Harmonia axyridis*) as outlined by Xiu et al. [59].

The phenylpropanoid pathway product methyl salicylate (bio #17) was detected in TPC1 (Table 1, Fig. 2A), as a significant biomarker (Figs. 3, 4), whereas benzaldehyde (bio #6) was detected in TPC1, 2 (Table 1). These two volatiles were also detected in the headspace profile of mechanically stressed *A. illinoisensis* by Alotaibi et al. [25]. Plant defense against pathogens and certain herbivores, particularly aphids, can be induced by methyl salicylate [60]. Lacewing, an aphid predator, is attracted to benzaldehyde [61].

4.2 Diagnosis of Grapevine Infestation by A. illinoisensis

The use of a specific ecological statistic tool (Fig. 3) simplified our understanding of complex relationships that occur during grapevine infestation by *A. illinoisensis*. This comprehension applies to non-destructive early-stage diagnosis of the aphid organism. Furthermore, it can aid entomologists in tracking the status of infestation chemically and precisely by detecting the significant biomarkers that resulted from PCA analysis (Fig. 3), as explained in Fig. 4. Focusing on the significant biomarkers in each chemosphere and the differences in their profile emissions can be beneficial for aphid management. If there are three esters (i.e., hex-3-enylacetate, 2,2,4-trimethyl-1,3-pentanedioldiisobutyrate, and cis-3-hexenylisobutyrate) and one benzene derivative (mesitylene) in abundance (Fig. 4), it can be concluded that the grapevine is noninfested by aphid (G1). Benzenoids and certain esters are crucial for plant signaling and defense mechanisms [62]. In comparison, the appearance of germacrene D, (*E*)-4,8-dimethylnona-1,3,7-triene, and (3*E*,5*E*)-2,6-dimethylocta-3,5,7-triene-2-ol (Fig. 4) is the rapid and most reliable indicator of *A. illinoisensis* infestation in grapevine (G2). The presence of *T. mangum* in the infested grapevine can be indicated by two monoterpenes, three specific sesquiterpenes, and six benzene derivatives, including three alkyl benzenes, 2-phenylethyl benzeneacetate, benzaldehyde, and methyl salicylate (Fig. 4). Aphids emit honeydew, a sugary secretion relayed by ants as a food source [63]. In response, ants protect aphids [64,65]. The chemical cues emitted by aphids and their honeydew guide ants to aphid colonies, establishing a chemical dialogue that coordinates this symbiotic relationship [66,67]. The detection limit could be the cause of the lack of n-alkanes in TPC1, as ants rely on aphid cuticular n-alkanes to identify aphid species [68]. In that regard, complex mutualism between honeydew-producing hemipterans and ants, manifesting both ecologically and chemically is important for ecology and evolution in terrestrial habitats [69], but they can also lead to pest infestations in agriculture [69,70]. The chemical signals between these two organisms serve as communication channels vital for

maintaining this mutualism [71]. Simultaneously, ants are usually active predators of various agricultural pests, can enhance the quality of soil, and can manage certain plant pathogens [70,72]. Due to the mutualistic interaction between *T. magnum* and *A. illinoisensis*, the deterrent interaction between grapevine and *A. illinoisensis*, in addition to the interaction between grapevine and *T. magnum*, TPC1 was significantly separated from the other groups (Fig. 3A). The sole evidence of *C. undecimpunctata* presence in the infested system (TPC2) is nonanal (Figs. 3B, 4). As exemplified by *C. undecimpunctata*, ladybirds act as significant aphid predators, playing a crucial role in natural pest control [73]. Chemical signals released by aphid-infested plants attract ladybirds to the scene, optimizing their predatory efficiency [74]. This interplay between chemical signals and predator-prey interactions emphasizes the importance of chemical communication in regulating aphid populations and structuring ecosystem dynamics [75]. The quadripartite communities (QPCs) were distinguished by three aldehydes (citral, decanal), two hepta-ketones (2-methyl-4-heptanone, 6-methyl-5-hepten-2-one), alarm pheromone isomer and its derivative (α-farnesene, 7-hydroxyfarnesene), an ester (3-methyl hexyl butanoate), and a methyl alkane (4,5-dimethylnonane) (Fig. 4). The coexistence of aphids, ants, and ladybirds further increases the complexity of the interactions, due to the multi-relationships which take place within this community [76,77]. Chemical communication intricately shapes aphid feeding behavior, custodial actions, and ladybird predation [78]. The resulting modulation of volatile compounds creates a subtle and intricate volatile profile within the ecosystem [79]. The presence of both ladybirds and ants appeared to affect the composition of these compounds, possibly signifying their correlated impact on plant-insect interactions [80]. By combining the interactions, a dynamic equilibrium defines the grapevine-aphid-ant-ladybird complex. The response of grapevine to aphid attack triggers a cascade of chemical signals, influencing ladybird behavior and altering ant activities in response to the chemically transformed environment [81]. This intricate interplay of ecological and chemical interactions emphasizes the system's resilience and the complexity of its defense mechanisms [82]. Despite the discussed interactions which are portrayed by grapevine-aphid (G2), aphid-ant (TPC1), and aphid-ladybird (TPC2), the ant-ladybird interactions have a significant impact on the VOC emissions and can manifest both directly and indirectly as demonstrated by Majerus et al. [77]. Ants' behavior towards ladybirds may impact ladybird predation activities, influencing their efficiency in controlling aphid populations [83]. In contrast, ants might perceive ladybirds as potential disruptors of their mutualistic association with aphids, prompting them to deter or eliminate ladybirds [84]. These interactions emphasize the complex interplay of chemical cues in shaping community dynamics.

For confirmation, biomarkers have to be evaluated through electrophysiological and behavioral studies to determine their bioactivity. It's worth considering that the combination of VOCs can result in a unique behavioral reaction to specific compounds. For example, (Z)-3-hexen-1-ol was only attractive in the olfactory sensor during the compound testing, despite being less active than the complete mixture [85]. At levels close to the natural release rates, each compound was capable of repelling according to the dose-response measurements in the olfactometer for all 15 compounds. The complete mixture was once again attractive when these levels were used to reassemble it.

We collected the VOCs in an enclosed space with limited natural airflow, which could lead to a faster rate of volatile emission into the chemosphere, compared to an open, natural system where air currents could cause a faster rate of volatile emission into the chemosphere. In our opinion, the natural dynamics are represented by the observed dynamics of VOC volatilization in this study. Our design enabled a nonbiased qualitative and quantitative comparison of VOC emissions between the chemosphere of six different treatments of grapevine *V. vinifera*, despite any slight differences in natural dynamics. These findings will greatly enhance our understanding of the chemical ecology of *A. illinoisensis*, and thus contribute to aphid management by getting use of the characteristic biomarkers of each infestation status that the *A. illinoisensis*-infested grapevine goes through. Variations in climate, soil, and different aphid

species in other areas could impact the observed interactions. Increasing the sample size and replication will be considered in further studies to enhance the statistical robustness of the results. More ecological and long-term researches are required to improve and develop sustainable management strategies.

5 Conclusion

This study revealed notable changes in volatile compound profiles due to aphids, and the associated organisms during the grapevine infestation. The distinct biomarkers are a key determinant of the infestation status of grapevine by *A. illinoisensis*. The chemosphere often differs between the five statuses of the grapevine. Thus, the study results can determine the status of the grapevine, whether it is intact, infested by *A. illinoisensis*, infested in the presence of ants, infested in the presence of ladybirds, or infested in the presence of ants and ladybirds. These differences in chemosphere would ideally be used for (1) non-destructive early-stage diagnosis of the aphid, (2) sustainable management of ant-aphid associations, and (3) understanding of the success of predator-prey dynamics for biological control. This study provides valuable insight into grapevine protection and effective aphid management approaches which are decisive in constructing biomarker-based decision support systems for the control of aphids.

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