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Comparison of the volatiles composition between healthy and buprestid infected *Juglans regia* (Juglandaceae)

Comparación de la composición de los volátiles de *Juglans regia* (Juglandaceae) sanos e infestadas por un escarabajo bupréstido

YAQIN CUI[1]; SHUQING KONG[2]; XINHAI LIU[3]; SUICUN LIU[4]

[1] Post-Doc. Shanxi Academy of Forestry Sciences, Taiyuan, Shanxi, 030012, China, *sxtycyq@foxmail.com*, https://orcid.org/0000-0001-9764-8425. [2] Department head. The Bureau Forestry of Linfen City, Linfen, Shanxi, 041000, China, *lflyjstgz@163.com*, https://orcid.org/0000-0002-8786-0892. [3] Master. Ulanqab Gardens Bureau, Ji'ning District of Ulanqab, Ulanqab, Inner Mongolia Autonomous Region, 012000, China, *175601540@qq.com*, https://orcid.org/0000-0003-4243-3798. [4] Prof. Shanxi Academy of Forestry Sciences, Taiyuan, Shanxi, 030012, China, *13934131208@126.com*, https://orcid.org/0000-0001-7090-9621.

Abstract: *Meliboeus ohbayashii primoriensis* (Coleoptera: Buprestidae) is an important pest of the walnut tree *Juglans regia* (Juglandaceae), but the volatiles mediating this plant–herbivore interaction are unknown. In this study, volatiles emitted by healthy *J. regia* and by plants infested with *M. ohbayashii primoriensis* (Coleoptera: Buprestidae) were obtained by a dynamic headspace method and analyzed by gas chromatography-mass spectrometry. We identified 26 major compounds and compared the volatile composition of healthy and buprestid-infected *J. regia*. Green leaf volatiles were detected in all damaged plants, including the monoterpenoids β-phellandrene and (*E*)-β-ocimene, the sesquiterpenoids (-)-β-bourbonene, β-ylangene, and (*E*,*E*)-α-farnesene, the alcohols linalool, myrtenol, and (*E*)-(-)-pinocarveol, the ketones (*E*)-pinocamphone and (*Z*)-pinocamphone, and the ester methyl salicylate. The major volatiles detected in healthy plants were β-pinene (36.26 %), α-pinene (23.81 %), D-limonene (12.03 %), sabinene (8.63 %), and β-myrcene (4.35 %). The main volatiles from *M. ohbayashii primoriensis* larva-infested plants were β-pinene (37.82 %), α-pinene (20.36 %), D-limonene (14.71 %), germacrene D (5.24 %), sabinene (4.52 %), and β-phellandrene (3.80 %). These results enrich our understanding of volatiles of healthy plants and plants infested with *M. ohbayashii primoriensis*. Furthermore, they provide a theoretical basis and scientific foundation for integrated pest management and for effective ecologically sustainable pest control strategies.

Keywords: *Juglans*, *Meliboeus ohbayashii primoriensis*, volatile compounds, plant–herbivore interaction, Coleoptera, Buprestidae.

Resumen: *Meliboeus ohbayashii primoriensis* (Coleoptera: Buprestidae) es una plaga importante del nogal *Juglans regia* (Juglandaceae), pero se desconocen los compuestos volátiles que median esta interacción planta-herbívoro. En este estudio, se obtuvieron los volátiles emitidos por plantas de *J. regia* tanto sanas como infestadas con *M. ohbayashii primoriensis* (Coleoptera: Buprestidae), mediante un método de espacio de cabeza dinámico y se analizaron por cromatografía de gases-espectrometría de masas. Se identificaron 26 compuestos principales y se comparó la composición volátil de *J. regia* sana e infectada con bupréstido. Se detectaron volátiles de hojas verdes en todas las plantas dañadas, incluidos los monoterpenoides β-felandreno y (*E*)-β-ocimeno, los sesquiterpenoides (-)-β-bourboneno, β-ylangeno y (*E*,*E*)-α-farnesano, los alcoholes linalool, mirtenol y (*E*)-(-)-pinocarveol, las cetonas (*E*)-pinocamfono y (*Z*)-pinocamfono, y el éster salicilato de metilo. Los principales volátiles detectados en plantas sanas fueron β-pineno (36,26 %), α-pineno (23,81 %), D-limoneno (12,03 %), sabineno (8,63 %) y β-mirceno (4,35 %). Los principales volátiles de las plantas infestadas de larvas de *M. ohbayashii primoriensis* fueron β-pineno (37,82 %), α-pineno (20,36 %), D-limoneno (14,71 %), germacreno D (5,24 %), sabineno (4,52 %), y β-felandreno (3,80 %). Estos resultados enriquecen nuestra comprensión de los volátiles de plantas sanas e infestadas con *M. ohbayashii primoriensis*. Además, proporcionan una base teórica y una científica para el manejo integrado de plagas y para estrategias efectivas, ecológicamente sostenibles, para el control de plagas.

Palabras clave: *Juglans*, *Meliboeus ohbayashii primoriensis*, compuestos volátiles, interacción planta-herbívoro, Coleoptera, Buprestidae.

Corresponding author: Suicun Liu, Prof. Shanxi Academy of Forestry Sciences, Taiyuan, Shanxi, 030012, China, *13934131208@126.com*, https://orcid.org/0000-0001-7090-9621.

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Introduction

The walnut *Juglans regia* L. (1753) (Juglandales: Juglandaceae) is an economically important cultivated trees species. *J. regia* is widely distributed in China (north to Heilongjiang Province, south to Yunnan Province and Guizhou Province, west to Xinjiang Uygur Autonomous Region, and east to Shandong Province, Liaoning

Province). It has nutritional and medicinal value (Ma *et al*. 2006; Xu *et al*. 2006; Wang *et al*. 2006; Zhou and Lv 2006); therefore, the walnut is classified as a strategic species for human nutrition and is included in the FAO priority list of plants (Gandev 2007).

The main herbivore affecting *J. regia* is *Meliboeus ohbayashii primoriensis* (Alexeev, 1979) (Coleoptera: Buprestidae) (Wang *et al*. 2018). *M. ohbayashii primoriensis* is univoltine and overwinter as larvae on branches, with pupation beginning in mid to late April and adult emergence in early May to early July. Female adults lay eggs on leaf scars of *J. regia*. The larvae are distributed on the branches of 2- to 3-year-old plants and mainly cause damage to branch bark. Adult *J. regia* feed on leaves. Although the species does not kill host plants immediately, it can reduce water and nutrient flow, resulting in leaf loss and reduced fruit yields and thereby contributing to tree mortality (Wang and Li 1993; Chen *et al*. 2015; Wang *et al*. 2015).

Plant volatiles, used for chemical signaling, affect insect behavior and can contribute to interactions between plants and insects. Specific plant volatiles can help insects locate a plant and select an oviposition site or can serve as repellents (Heil and Ton 2008; Hiltpold *et al*. 2010; Gish *et al*. 2015). Many recent studies have focused on the behavioral responses of insects to host volatile emissions; both the quantitative and qualitative characteristics of herbivore-induced changes in the emission of volatiles have attracted increasing attention (Nottingham *et al*. 1991; Musetti and Neal 1997; Kessler and Baldwin 2001; Mauck *et al*. 2010; Ramadan *et al*. 2011; Tamiru *et al*. 2012). The emerald ash borer locates its host, *Agrilus planipennis* (Fairmaire, 1888) (Coleoptera: Buprestidae), by plant volatile emissions (Rodriguez-Saona *et al.* 2006; Grant *et al.* 2010). It is specifically attracted by (3*Z*)-lactone and (3*Z*)-hexenol from leaves of host plants (Groot *et al*. 2008; Ryall *et al*. 2012; Ryall *et al*. 2013). Prior work has evaluated volatiles from healthy walnut leaves, husks, and kernel oils (Farag 2008; Abdallah *et al*. 2015; Sarles *et al*. 2017) as well as the behavioral response of pests to volatiles of healthy plants, such as *Rhagoletis completa* (Cresson, 1929) (Diptera: Tephritidae), *Cydia pomonella* (L., 1758), (Lepidoptera: Tortricidae), *Amyelois transitella* (Walker, 1863) (Lepidoptera: Pyralidae), and *Batocera horsfieldi* Hope, 1839 (Coleoptera: Cerambycidae) (Yang *et al*. 2011; Román *et al*. 2015; Sarles *et al*. 2017). However, studies of the volatiles of *J. regia* infested with *M. ohbayashii primoriensis* are lacking. We compared the emission of volatiles from *J. regia* infested with *M. ohbayashii primoriensis* and healthy control plants. The objective of this study was to improve our understanding of host plant volatiles and to provide a basis for the development of ecologically sustainable strategies for herbivore control.

Materials and methods

Plant material. An infested *J. regia* fruit garden of 26.67 hm^2, located in Yonghe County, Shanxi, China, was studied. Ten individuals of *J. regia* were chosen for headspace collection. All plants were 20 years old with an average diameter at breast height of 19.9 cm, tree height of 6-8 m, and crown diameter of 5-6 m.

Herbivore material. *J. regia* was widely infested with *M. ohbayashii primoriensis*. Larvae were observed at densities of 10–15 larvae/tree. Volatile compounds were collected from walnuts infested with *M. ohbayashii primoriensis*.

Headspace collection. At 9:00 am each day, volatiles were collected from healthy and herbivore-infested plants using a dynamic headspace method following a procedure similar to that described by Bäckman *et al*. (2001). Each aerial part was covered with an oven bag (48.26 cm × 23 cm × 1.27 cm; Reynolds, Richmond, CA, USA). The air was first exhausted from the bag, and the bag was then refilled with air percolated through an activated charcoal filter. Plastic bags were used only once to avoid contamination. For sampling, air was cycled through the oven bag, which was connected to a glass tube (length, 10.60 cm; inner diameter, 0.35 cm; outer diameter 0.85 cm) and filled with 200 mg of Porapak Q (80/100 mesh; Waters Corporation, Milford, MA, USA). Plant volatiles were sampled for 3 hours using a portable battery-operated air sampler with a constant flow of 100 ml/min. Subsequently, the glass tubes were taken to the laboratory and washed five times with 200 µl of dichloromethane to extract samples into 2 ml clear screw vials with patch Borosilicate (9 mm, CNW). The vials were kept at -20 °C until analysis.

GC-MS analysis. A Shimadzu 2010 (Shimadzu, Tokyo, Japan) gas chromatograph was equipped with a split-splitless auto-injector (model AOCi) and an auto sampler (model AOC-20i), and a MS-QP 2010 (Shimadzu) series mass selective detector was used for sample analysis. The analytical conditions were as follows: GC: column, Rtx-5MS (30.0 m × 0.25 mm × 0.25 µm; J&W, Palo Alto, CA, USA); carrier gas, helium; flow rate, 1.01 ml/min; column temperature, 40 °C held for 2 min, then at the rate of 6 °C / min to 180 °C (held for 2 min), and 15 °C/min to 270 °C. MS: analytical mode, full-scan; mass range, m/z 50-400.

Identification of volatile compounds. Retention indices (RI) were calculated based on retention times of n-alkanes, which were injected after volatiles under the same conditions. Relative contents were calculated based on GC peak areas without correction factors. The volatile compounds were identified by comparing mass spectral data with those of authentic samples in a mass spectra library (Turbo Mass ver. 5.4.2, NIST11), and the compounds were confirmed by comparing RI values with published data (Davies 1990; Farag 2008; Abdallah *et al.* 2016).

Statistical analysis. All measurements were obtained in six replicates and statistical analyses were performed using Microsoft Excel 2007. Results are presented as averages ± SEM.

Results

Volatile compound detection. The volatile compounds identified from healthy plants and plants infested by *J. regia* are listed in Table 1. We identified 26 major volatile compounds. With respect to green leaf volatiles, we detected the monoterpenoids β-phellandrene and (*E*)-β-ocimene, the sesquiterpenoids (-)-β-bourbonene, β-ylangene, (*E*,*E*)-α-farnesene, the alcohols linalool, myrtenol, and (*E*)-(-) -pinocarveol, the ketones (*E*)-pinocamphone and (*Z*)-pinocamphone, and the ester methyl salicylate in all herbivore-infested plants.

Healthy *J. regia*. The volatile constituents of the aerial parts of *J. regia* were mainly classified as terpenoids (93.54 %) (monoterpenoids (86.03 %) and sesquiterpenoids (7.51 %)), esters (2.62 %), alcohols (1.43 %), ketones (1.08 %), and aromatic hydrocarbons (0.23 %). The most abundant components were β-pinene (36.26 %), α-pinene (23.81 %), D-limonene (12.03%), sabinene (8.63 %), and β-myrcene (4.35 %).

Buprestid beetle-infested (herbivore-infested plants) *J. regia*. Buprestid beetle-infested volatiles were mainly classified as terpenoids (95.37 %) [monoterpenoids (84.67 %) and sesquiterpenoids (10.70 %)], alcohols (2.11 %), ketones (0.97 %), esters (0.70 %) and aromatic hydrocarbons (0.03 %). The most abundant components were β-pinene (37.82 %), α-pinene (20.36 %), D-limonene (14.71 %), germacrene D (5.24 %), sabinene (4.52 %), and β-phellandrene (3.80 %). Additionally, we detected the monoterpenoids β-phellandrene and trans-β-ocimene, sesquiterpenoids β-ylangene, (-)-β-bourbonene, and (*E,E*)-α-farnesene, alcohols linalool, myrtenol, and (*E*)-(-)-pinocarveol, ketones (*Z*)-pinocamphone and (*E*)-pinocamphone, and ester methyl salicylate in herbivore-infested plants.

In addition to these qualitative differences, beetle infestation induced quantitative changes in the proportions of terpenoids. In particular, β-pinene, D-limonene, germacrene D, and (*E*)-β-farnesene contents were higher and α-pinene, sabinene, β-myrcene, camphene, and longifolene contents were lower in infested plants than in healthy plants.

Table 1. Compositional variation in healthy (H) and buprestid beetle-infestation (AR) *Juglans regia.*

No.	Compounds	RI[a] sample	RI stand.	Relative content ± SE (%)	
				H	AR
	Monoterpene hydrocarbons				
1	α-Pinene	939	940	23.81 ± 0.20	20.36 ± 0.22
2	Camphene	947	950	0.95 ± 0.05	0.31 ± 0.04
3	Sabinene	973	973	8.63 ± 0.22	4.52 ± 0.18
4	β-Pinene	976	978	36.26 ± 0.25	37.82 ± 0.20
5	β- Myrcene	991	989	4.35 ± 0.21	2.90 ± 0.20
6	D-Limonene	1027	1029	12.03 ± 0.08	14.71 ± 0.21
7	β-Phellandrene	1031	1031	- [b]	3.80 ± 0.19
8	(*E*)-β-Ocimene	1050	1052	- [b]	0.25 ±0 .09
	Sesquiterpene hydrocarbons				
9	(-)-β-Bourbonene	1384	1384	- [b]	0.40 ± 0.03
10	Longifolene	1398	1399	1.22 ± 0.04	0.77 ± 0.05
11	β-ylangene	1428	1428	- [b]	0.42 ± 0.01
12	l-Caryophyllene	1434	1435	1.52 ± 0.02	- [b]
13	(*E*)-β-Farnesene	1440	1440	2.13 ± 0.02	3.50 ± 0.05
14	(*E,E*)-α-Farnesene	1458	1458	- [b]	0.37 ± 0.03
15	Humulene	1454	1454	0.38 ± 0.01	- [b]
16	Germacrene D	1490	1491	2.26 ± 0.02	5.24 ± 0.04
	Esters				
17	(*Z*)-3-Hexen-1-ol acetate	992	993	2.62 ± 0.05	0.58 ± 0.02
18	Methyl salicylate	1190	1190	- [b]	0.12 ± 0.01
	Alcohols				
19	Eucalyptol	1059	1060	1.43 ± 0.04	1.75 ± 0.02
20	Linalool	1082	1084	- [b]	0.27 ± 0.02
21	(*E*)-(-)-Pinocarveol	1131	1132	- [b]	0.04 ± 0.06
22	Myrtenol	1191	1189	- [b]	0.05 ± 0.03
	Ketones				
23	(*E*)-Pinocamphone	1164	1164	- [b]	0.12 ± 0.08
24	(*Z*)-Pinocamphone	1165[c]	1165c	- [b]	0.81 ± 0.06
25	m-Ethylacetophenone	1242[c]	1244c	1.08 ± 0.09	0.04 ± 0.08
	Aromatic hydrocarbons				
26	Indane	1047[c]	1048[c]	0.23 ± 0.02	0.03 ± 0.01

[a] Observed retention index. [b] Not detected. [c] Compounds for which RI values have not been previously reported in the literature. Compounds were verified by comparison with an authentic standard when possible and compared to internally generated database values. When standards were not available, retention indexes and/or mass spectra were only compared with internally generated data and/or with NIST (NIST02).

Discussion

Herbivore attacks increase the emission of volatiles (Kessler and Baldwin 2001). The release of volatiles can improve plant resistance by attracting natural enemies (parasitoids and predators) of the herbivore as an indirect defense mechanism (Metcalf and Kogan 1987; Dicke 1994; Turlings and Benrey 1998; Kessler and Baldwin 2001).

Terpenoids are the largest class of natural products. Terpenoid phytoalexins play important roles in attacking herbivores by direct and indirect defense mechanisms (Mumm and Hilker 2006; Gershenzon and Dudareva 2007). The volatiles D-limonene, sabinene, α-pinene, (*E,E*)-α-farnesene, (*E*)-β-ocimene, and methyl salicylate, which we detected in our experiments, are very important compounds for the attraction of herbivore enemies (Hardie *et al*. 1994; Ament *et al*. 2010).

The relative β-pinene content was higher after herbivore attack than in healthy plants. Inversely, α-pinene contents were lower in infested plants than in healthy plants. The ratio of α-pinene to β-pinene was higher in healthy *J. regia* than in plants infested with *M. ohbayashii primoriensis*. Ning *et al*. (2006) concluded that a low ratio of α-pinene to β-pinene indicates declining host health, consistent with our observations of a relatively low ratio in infested plants. However, further research is needed to verify whether the condition of host plants could be determined by the ratio of α-pinene to β-pinene.

We detected eucalyptol in healthy plants. Eucalyptol, linalool, myrtenol, and (*E*)-(-)-pinocarveol were present in all herbivore-infested plants. Alcohol compounds play an important role in the attraction of herbivores (Kessler and Baldwin 2001). We detected (*Z*)-3-hexen-1-ol acetate and methyl salicylate in herbivore-infested plants, both of which contribute to the attraction of herbivore enemies (Hardie *et al*. 1994; James 2003).

We analyzed the volatiles emitted by healthy *J. regia* and plants infested with *M. ohbayashii primoriensis*. We detected qualitative and quantitative differences in the volatile profiles, with particularly notable variation in the terpenoids composition. Dynamic headspace coupled with GC/MS was suitable for the qualitative and quantitative investigation of volatiles in walnut. This approach can be used for further investigations of plant-derived attractants of herbivore enemies, volatile compositions, and ecologically sustainable strategies for the control of pests.

Conclusion

Our results expand our understanding of volatiles for the location, manipulation, or alteration of the chemical ecology of the environment to protect or improve walnut, a valuable natural resource.

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Author contribution

Yaqin Cui's main task for this paper is data collection, analysis and article writing work.

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Xinhai Liu's main task for this paper is the modification and arrangement of this article.

Suicun Liu's main task for this paper is the necessary modifications for the submission and fully responsible for the tasks of this article.