

# TWO KEY VOLATILES OF CHINESE WHITE PINE (*PINUS ARMANDII*) (PINALES: PINACEAE: PINOIDEAE) PHLOEM RESIST INVASION BY CHINESE WHITE PINE BEETLE (*DENDROCTONUS ARMANDII*) (COLEOPTERA: CURCULIONIDAE: SCOLYTINAE)

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**Abstract.** *Pinus armandii* phloem is the key tissue in which *Dendroctonus armandii* live and breed. Although previous research examined the volatiles in *P. armandii* phloem, the dynamic changes and functions of these volatiles were never revealed. This study detected the changes in *P. armandii* phloem volatiles at each stage of infestation from healthy to dead trees and tested the toxicity of these volatiles against *D. armandii*. It revealed that (1) the weight of females was significantly greater than that of males, and females were more tolerant to host volatiles than males. (2) Limonene and myrtenol of *P. armandii* phloem volatiles played the key roles in resisting the invasion of *D. armandii*. The first increased the percentage of limonene together with other volatiles killing *D. armandii* in the resistant period of *P. armandii*, and the second synthesised myrtenol to further resist the invasion of *D. armandii* in the retreat period of *P. armandii*. These observations highlight the differences in the resistance of males and females to toxicity from the volatiles and the difference in the toxicity of different volatiles to *D. armandii*, revealing the defensive system of *P. armandii* phloem to provide a theoretical basis for the control and management of *D. armandii*.

**Keywords:** host volatiles, toxicity, host defence, limonene, myrtenol

## Introduction

The Chinese white pine beetle (*Dendroctonus armandii*) is the most destructive forest pest in the Qinling Mountains and Ta-pa Mountains of China (Chen et al., 2010). Unlike red turpentine beetle (*Dendroctonus valens*) and mountain pine beetle (*Dendroctonus ponderosae*) that invade weakened pine trees, and only infest healthy trees during outbreaks (Sun et al., 2013; Krause et al., 2018), *D. armandii* is a primary pest of healthy Chinese white pine (*Pinus armandii*) (Hu et al., 2016). *D. armandii* has two generations per year at elevations lower than 1700 m a.s.l, three generations in two years between 1700 and 2150 m a.s.l, and one generation per year in areas higher than 2150 m a.s.l (Ning et al., 2019). Females invade first and overcome the tree's resistance, drill tunnels, and release pheromones to attract males (Dai et al., 2015). The successful invasion of *D. armandii* is often followed by colonization of secondary pests (such as *Ips acuminatus*,

*Ips sexdentatus*, *Hylurgops longipilis*, *Tomicus piniperda* and *Trypodendron lineatum*) in the weakened pines, and the infected *P. armandii* will die in two years (Chen et al., 2007). Since 1970, more than  $3 \times 10^8$  m<sup>3</sup> of *P. armandii* trees (older than 30 years) have been harmed by *D. armandii* (Xie and Lv, 2012), and even young *P. armandii* trees were found to have been invaded by *D. armandii* (Chen et al., 2015).

Insect pheromones play a vital role in the management of bark beetles (Pureswaran et al., 2008; Perkins et al., 2015; Gillette et al., 2009). Integrated pest management measures focused on the identification of pheromones have been performed for *D. armandii* in recent years. Aggregation pheromones are considered to be a key factor in the success of insect invasion and colonization (Faccoli and Stergulc, 2008; Blazenec and Jakus, 2009). Frontalin +  $\alpha$ -pinene is an aggregation pheromone released by virgin females and mated males of *D. armandii* (Zhao et al., 2017a), and myrtenal may be an aggregation pheromone produced by females of *D. armandii* to exert aggregation effects on other females (Zhao et al., 2019). The anti-aggregation pheromones of bark beetles, such as verbenone in *D. ponderosae* and *D. valens*, and toxic host terpene have been used to protect pine species from bark beetles (Gillette et al., 2006). Verbenone has been detected using gas chromatography and mass spectrometry (GC-MS) analyses of the hindguts of female beetles and the fumes emanating from *P. armandii* logs naturally attacked by *D. armandii* (Xie and Lv, 2012; Chen et al., 2015). In our previous study, verbenone was verified as an anti-aggregation pheromone based on electrophysiological (EAG) and Y-tube laboratory assays. In addition, field trials indicated that the addition of verbenone to the bait used to trap *D. armandii* significantly decreased the efficiency of field trapping (Zhao et al., 2017b). *trans*-Verbenol is a pheromone of western pine beetle (*Dendroctonus brevicomis*), southern pine beetle (*Dendroctonus frontalis*), *D. ponderosae* and Douglas-fir beetle (*Dendroctonus pseudotsugae*) (Byers et al., 1984; Chiu et al., 2018; Pureswaran et al., 2004), but it has not been found to have a clear role in the pheromone ecology of *D. armandii* (Zhao et al., 2017b). Quantities of potential semiochemicals identified in extracts of the hindguts of female *D. armandii*, include *cis*-verbenol, *trans*-verbenol,  $\alpha$ -pinene,  $\beta$ -caryophyllene, 3-carene, verbenone, myrcene and limonene (Xie and Lv, 2012).

*Leptographium qinlingensis* is a symbiotic fungi of *D. armandii* that assists in the invasion of the host tree by blocking flow of water and resin, thereby reducing host defenses. Metabolites biosynthesized by *L. qinlingensis* that may be phytotoxic to *P. armandii* seedlings, include 6-methoxymethyleugenin, maculosin and cerevisterol (Li et al., 2012).

Some volatiles of *P. armandii* were tested and applied to traps in the field. Myrtenol was found to be a kind of *P. armandii* volatile and did not exhibit significant toxicity towards *D. armandii* (Zhao et al., 2019). Myrtenol was produced by infected *P. armandii* after *D. armandii* invasion and had significant toxicity towards *D. armandii*, especially females (Zhao et al., 2019).  $\alpha$ -Pinene, camphene,  $\beta$ -pinene, myrcene, 3-carene, limonene and longifolene were detected from *P. armandii* logs with or without *D. armandii* attack (Chen et al., 2015).

The pine defence against bark beetles is multifaceted and temporally dynamic (Franceschi et al., 2005). Resin flow can kill invading bark beetles (Strom et al., 2002; Hood and Sala, 2015; Kane and Kolb, 2010). In addition, pines produce defence volatiles, such as monoterpenes, sesquiterpenes, diterpenes, and phenolics (Raffa et al., 2017), and the invasion of bark beetles can increase the concentration of host phloem volatiles (Kolb et al., 2019). Other defence measures include the physical defence

provided by bark and water (Arango-Velez et al., 2016; Erbilgin et al., 2017; Kolb et al., 2019). Resin flow and terpene release are known means of defence in response to bark beetles and their symbiotic fungi (Arango-Velez et al., 2018; Roth et al., 2018). For example, ponderosa pine can undergo induction of both resin flow and phloem terpenes in response to bark beetle attack (Kolb et al., 2019). Current research has mainly concentrated on *P. armandii* trees in a single state, such as a healthy or infested tree. The kinds of volatiles produced during different stages of host condition from fully healthy to fully infested is not clear. Whether new volatiles are synthesized specifically to resist insect invasion is also unknown. There has been no study on the dynamic changes of volatiles during the whole process of *P. armandii* resistance to bark beetle invasion. The purpose of this study was to examine the changes in host volatile production during the invasion process of *P. armandii* from healthy to dead and to test the toxicity of these volatiles against *D. armandii* to obtain more specific knowledge of the autonomic chemical defence process of *P. armandii*.

## Materials and methods

### Sampling

The study sites were located on the southern slope of the middle Qinling Mountains, Ningshan County, Shaanxi, China, and mainly occurred in Huoditang Forest Farm (33°18'-33°28'N, 108°21'-108°39'E) and Pingheliang Forest Farm (33°22'-33°34'N, 108°24'-108°36'E). The two forest farms were chosen because they were severely affected by *D. armandii*. The investigated *P. armandii* trees were distributed widely inside the two areas.

For convenience, the defence process of *P. armandii* against invasion by *D. armandii* was divided into four periods: healthy period, resistance period, retreat period and withered period. Healthy period was defined by the lack of resinous pitch tubes in *P. armandii* trunks. The resistance period was defined by resinous pitch tubes in *P. armandii* trunks; resinous pitch tubes are comprised of colloidal liquid containing some dead *D. armandii*. Dry frass is usually a sign of successful invasion by bark beetles (Gillette et al., 2006). The retreat period was defined by a change in the resinous pitch tubes to dry frass, and a change in the needles from green to yellow. The withered period was defined by *D. armandii* mating and production of the next generation, the needles becoming withered and yellow, and some limbs dropping out of the trees.

Phloem samples were collected from *P. armandii* trunks with a small sickle (length × width = 22 cm × 6 cm, custom-made by a blacksmith) in June 2019. Phloem was collected from healthy *P. armandii* and infested areas of different period (resistance, retreat and withered periods) of attacked trees, and the beetles and frass were cleared from the infested phloem. These phloem samples of every period were collected from five different trees of the period subjected to five repetitions. Glass culture dishes with phloem samples were placed in an outdoor cooler (4 °C) and transported to the laboratory.

### Collection and identification of host volatiles

We collected five 1.5 g samples of phloem from each of the four periods and placed each sample in a separate 50 ml vial for volatile collection. The volatiles released from these phloem samples were collected passively by headspace solid-phase microextraction (HS-SPME) (Chai et al., 2012; Keenan et al., 2012). An SPME fibre

coated with a 75- $\mu\text{m}$  film of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, Sigma-Aldrich, Bellefonte, PA, USA) was exposed to the headspace of each vial/phloem sample for 3 min. The split ratio of the phloem samples was 10:1. The fibre was selected due to its suitability for gases and compounds with a low molecular mass. Prior to use, the fibre was preconditioned in the injection port of the gas chromatograph at 270 °C for 60 min. Extracts were analysed using a DB-5 MS column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ ) (Thermo Fisher Scientific Company, Shanghai, China). The SPME fibre was injected directly into the injection port at a temperature of 250 °C for 3 min. The temperature of the GC oven was maintained at 40 °C for 2.5 min, increased to 240 °C at a rate of 6 °C/min and maintained at 240 °C for 10 min. The flow of helium (carrier gas) was 1.0 mL/min. Compounds were identified by comparison of their retention times and mass spectra with those in the NIST and Varian libraries. Furthermore, the retention times and mass spectra of  $\alpha$ -pinene,  $\beta$ -pinene, myrcene, limonene, 3-carene and longifolene detected during the GC-MS analysis were also compared with the purchased standards.

### Insects

*P. armandii* logs invaded by *D. armandii* larvae and pupae were felled in the Pingheliang Forest Farm (33°28'12.1"N, 108°29'26.2"E) in July and August 2019. The logs were transported to the Huoditang Forest Farm experiment base, placed in a greenhouse, covered by a thin stainless-steel net (bore diameter  $\leq$  0.8 mm) and watered to keep the bark moist. Once emerging beetles appeared in the net, active beetles were collected and analysed on the same day using toxicity assays (Light, 1983; Zhang et al., 2006).

### Chemicals

The chemicals used in this study are listed in *Table 1*.

**Table 1.** The purity of host volatiles and companies (with addresses) from which these chemicals were purchased

Volatile	Purity	Company	Address
(-)- $\alpha$ -Pinene	$\geq$ 99.0%	sigma	Shanghai, China
(+)- $\alpha$ -Pinene	Analytical standard	sigma	Shanghai, China
(-)- $\beta$ -Pinene	Analytical standard	sigma	Shanghai, China
(+)- $\beta$ -Pinene	Analytical standard	sigma	Shanghai, China
Myrcene	$\geq$ 99.0%	Yuanye Bio-Technology Co., Ltd	Shanghai, China
S-(-)-limonene	Analytical standard	sigma	Shanghai, China
R-(+)-limonene	Analytical standard	sigma	Shanghai, China
(+)-3-carene	Analytical standard	sigma	Shanghai, China
(+)-longifolene	$\geq$ 99.0%	Yuanye Bio-Technology Co., Ltd	Shanghai, China

### Toxicity assays

Certain volatiles from the GC-MS analysis of *P. armandii* phloem were chosen for toxicity assays (*Table 1*). Camphene was in a solid state at room temperature and was not chosen for the toxicity assays. The toxic effects of *trans*-verbenol, verbenone, myrtenol, and myrtenal on *D. armandii* have previously been explored (Zhao et al., 2017b, 2019)

and were not examined in this study. The toxicity of (-)- $\alpha$ -Pinene, (+)- $\alpha$ -pinene, (-)- $\beta$ -pinene, (+)- $\beta$ -pinene, myrcene, *S*-(-)-limonene, *R*-(+)-limonene, (+)-3-carene and (+)-longifolene towards *D. armandii* was tested using a method used previously for *D. ponderosae* (Reid et al., 2017; Chiu et al., 2017). A 1.5 × 1.5 cm filter paper was placed in a 20 mL glass vial as the reagent carrier. Different dosages of the test volatiles were applied to the filter paper using a micro-dispenser. Beetles in the control group were exposed to untreated filter paper for 24 h. Each glass vial contained only one *D. armandii*, and the glass vials were sealed after the beetles were placed inside. Before the beetle was put into the glass bottle, they were weighed. Preliminary toxicity tests were conducted with each volatile to refine an intermediate range of dosages from lethal to non-lethal, to more accurately determine an LC<sub>50</sub> (Table 2). The *D. armandii* beetles were maintained in glass vials for 24 h. After that, *D. armandii* were considered to have died if they showed no limb movements after the glass vial was shaken. Each *D. armandii* was weighed again to calculate their weight loss. Each concentration (contain all concentration in the preliminary experiments and the experiments of LC<sub>50</sub>) of each tested volatile compound was tested with 40 *D. armandii* individuals (20 females and 20 males). To eliminate potential bias of different treatment in results, beetles collected at different times were used for all the assays in a completely random manner.

**Table 2.** The determination doses of different volatiles for females and males

Volatile	Sex	Volatile dosage (μL)									
		0	0.2	0.4	0.7	1	1.5	2	4	10	15
(-)- $\alpha$ -Pinene	Male	√		√		√	√	√			
	Female	√		√		√	√	√			
(+) - $\alpha$ -Pinene	Male	√		√	√	√		√			
	Female	√			√	√	√	√			
(-)- $\beta$ -Pinene	Male	√		√		√	√	√			
	Female	√		√		√	√	√			
(+) - $\beta$ -Pinene	Male	√		√		√	√	√			
	Female	√		√		√	√	√			
Myrcene	Male	√						√	√	√	√
	Female	√						√	√	√	√
<i>S</i> -(-)-limonene	Male	√		√	√	√		√			
	Female	√		√	√	√		√			
<i>R</i> -(+)-limonene	Male	√	√	√		√		√			
	Female	√		√	√	√		√			
(+) -3-carene	Male	√		√	√	√		√			
	Female	√		√	√	√		√			
(+) -longifolene	Male	√						√	√	√	√
	Female	√						√	√	√	√

### Statistical analysis

The probit analysis method was used to determine the LC<sub>50</sub>. One-way ANOVA was used to test for differences in the mean weights of males and females. Multiple comparisons (LSD-*t* test) were used to test the difference in mean % of limonene, mean weight loss, mean weight loss % and death rate %. The probit analysis, one-way ANOVA and multiple comparisons ( $P < 0.05$ ) were run in SPSS (1999).

## Results

### Collection and identification of host volatiles

In the GC-MS analysis of the phloem during four different *P. armandii* periods (healthy period, resistance period, retreat period and withered period), eleven host volatiles were detected (Table 3). Eight host volatiles ( $\alpha$ -pinene, camphene,  $\beta$ -pinene, myrcene, 3-carene, myrtenol, verbenone, longifolene) were detected in all four periods. *trans*-Verbenol was only detected in the resistance period, and myrtanol was only detected in the retreat period. The percentage of limonene in the total phloem volatiles was similar during the healthy and retreat periods, significantly increased during the resistance period, and absent in the withered period (Fig. 1). Over thirty volatiles (accounting for 18%-30%), including pentadecane, naphthalene, tetradecane, octadecane, and acenaphthene, were also detected. Because these volatiles were not major volatiles (such as  $\alpha$ -pinene) or were not markedly changed in the different periods (such as myrtanol) in the preliminary analysis, they were not subjected to further analysis.

**Table 3.** Mean (SE) percentage composition of identified volatiles emitted from phloem of *Pinus armandii* during different stages of infestation by *Dendroctonus armandii*. Five replicate samples were analysed for each period

	Healthy period (n = 5)	Resistance period (n = 5)	Retreat period (n = 5)	Withered period (n = 5)
$\alpha$ -Pinene	77.46 $\pm$ 3.72(5)	65.69 $\pm$ 24.71(5)	61.41 $\pm$ 11.34(5)	60.07 $\pm$ 9.41(5)
Camphene	1.74 $\pm$ 0.92(5)	1.45 $\pm$ 0.77(5)	10.72 $\pm$ 5.79(5)	21.31 $\pm$ 18.75(3)
$\beta$ -pinene	5.10 $\pm$ 0.56(5)	11.42 $\pm$ 7.23(5)	13.35 $\pm$ 4.65(5)	11.99 $\pm$ 6.81(5)
Myrcene	5.44 $\pm$ 1.23(5)	5.17 $\pm$ 3.30(5)	2.1 $\pm$ 1.47(5)	8.06 $\pm$ 3.13(4)
Limonene	8.81 $\pm$ 2.65(5)	15.08 $\pm$ 5.53(5)	9.24 $\pm$ 4.22(5)	ND <sup>b</sup>
3-Carene	1.75 $\pm$ 1.61(5)	0.09 $\pm$ 0.02(5)	0.8 $\pm$ 0.74(5)	5.12 $\pm$ 4.16 (4)
<i>trans</i> -Verbenol	ND <sup>b</sup>	0.01 $\pm$ 0.01(5)	ND <sup>b</sup>	ND <sup>b</sup>
Myrtenol	0.025 $\pm$ 0.015(2)	0.02(1)	0.13 $\pm$ 0.11(2)	0.017 (1)
verbenone	0.028 $\pm$ 0.011(5)	0.03 $\pm$ 0.02(5)	0.14 $\pm$ 0.13(5)	0.044 $\pm$ 0.027(4)
Myrtanol	ND <sup>b</sup>	ND <sup>b</sup>	0.04 $\pm$ 0.03(3)	ND <sup>b</sup>
Longifolene	1.38 $\pm$ 0.72(5)	1.05 $\pm$ 1.02(5)	2.18 $\pm$ 1.02(5)	1.17 $\pm$ 0.69(5)

<sup>a</sup>The values shown are the means  $\pm$  SE, and the values in parentheses are the number of samples in which the component was identified

<sup>b</sup>ND indicates that the pheromone was not detected

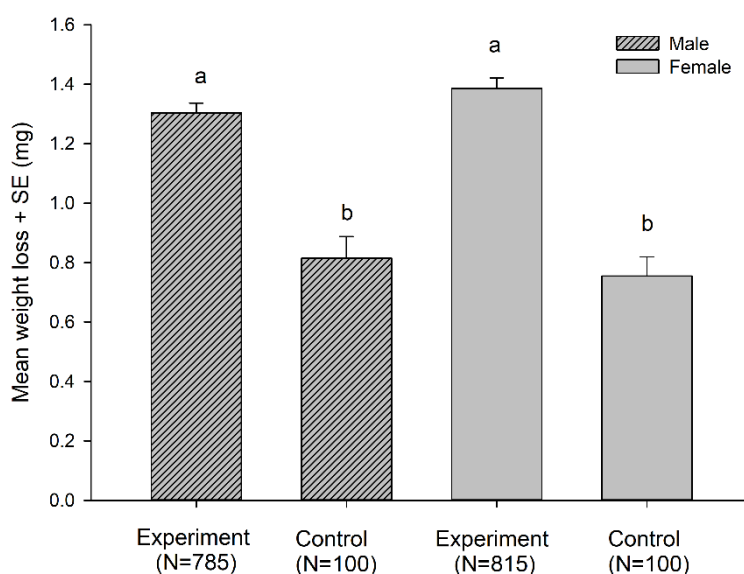
### Toxicity assays

The mean weight ( $\pm$  SE) of female *D. armandii* (9.74  $\pm$  0.05) was marginally but significantly higher than that of males (9.17  $\pm$  0.05) (ANOVA,  $F = 60.39$ ,  $df = 1798$ ,  $P < 0.01$ ). After the toxicity assays, each beetle was re-weighed and the percentage weight loss was calculated. There was a significant difference between the experiment group and the control group, the beetles exposed to host volatiles lost more mass than control beetles (Fig. 2). Although there was no significant difference between experiment females and males, the females lost more mass than males (Fig. 2). Males

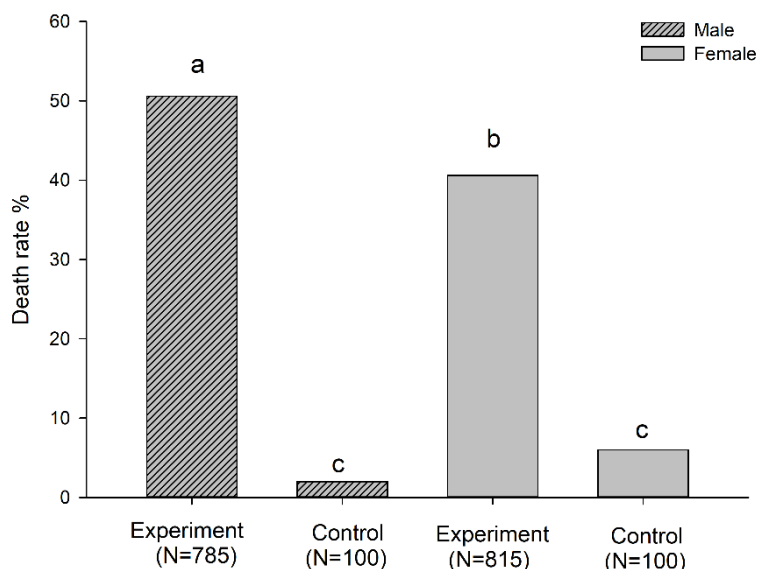
(14.14 ± 0.33) lost a higher percentage of weight than females (13.80 ± 0.33), but the difference was not significant (LSD-*t*,  $P = 0.467$ ). After 24 h of volatile treatment, the death rate was calculated for the experiment and control groups. The death rate of experiment males was significantly higher than that of control males, the death rate of experiment females was significantly higher than that of control females (Fig. 3). There was a significant difference between experiment females and experiment males, and the experiment males had a higher death rate than females (Fig. 3).



**Figure 1.** Mean % ± SE of limonene in four *P. armandii* periods. Different letters indicate a significant difference (LSD;  $p < 0.01$ ), and the same letter indicates no significant difference



**Figure 2.** Mean weight loss + SE (mg) of *D. armandii* males and females after 24 h exposure to various phloem volatiles on filter paper (experimental group) or untreated filter paper (control group). Means with different letters differed significantly (LSD,  $P < 0.01$ ). Numbers in parentheses are the numbers of beetles measured



**Figure 3.** Percentage mortality of *D. armandii* exposed for 24 h to various dosages of phloem volatiles on filter paper (experimental group) or untreated filter paper (control group). Percentage mortality with different letters differed significantly (LSD,  $P < 0.01$ ). Numbers in parentheses are the numbers of beetles measured

The  $LC_{50}$  values for nine host volatiles at 24 h of vapour exposure to *D. armandii* were measured. The range of  $LC_{50}$  values was from 17 to  $> 750$ , revealing the differences in toxicity of different host volatiles (Table 4). *R*-(+)-Limonene was the most toxic host volatile to both females and males, followed by (+)-3-carene and *S*-(-)-limonene. This ranking was consistent in females and males. The next most toxic volatiles were (+) and (-) of  $\alpha$ -pinene and (+) and (-) of  $\beta$ -pinene. Myrcene and (+)-longifolene were the two lowest toxicity host volatiles. *R*-(+)-Limonene, (+)-3-carene, *S*-(-)-limonene, (-)- $\alpha$ -pinene, (+)- $\alpha$ -pinene, (+)- $\beta$ -pinene and myrcene were more toxic to males than to females. (-)- $\beta$ -Pinene and (+)-longifolene were more toxic to females than to males (Table 4).

**Table 4.** The lethal concentration of elect phloem volatiles necessary to kill 50% of *D. armandii* males and females after 24 h exposure

Male		Female	
Volatile	$LC_{50}$ ( $\mu\text{L/L}$ )	Volatile	$LC_{50}$ ( $\mu\text{L/L}$ )
<i>R</i> -(+)-limonene	17	<i>R</i> -(+)-limonene	32
(+)-3-carene	25	(+)-3-carene	39
<i>S</i> -(-)-limonene	42	<i>S</i> -(-)-limonene	49
(-)- $\alpha$ -Pinene	60	(+)- $\alpha$ -Pinene	68
(+)- $\alpha$ -Pinene	61	(-)- $\beta$ -Pinene	70
(+)- $\beta$ -Pinene	62	(+)- $\beta$ -Pinene	74
(-)- $\beta$ -Pinene	73	(-)- $\alpha$ -Pinene	82
Myrcene	399	Myrcene	507
(+)-longifolene	$> 750$	(+)-longifolene	682

The probit analysis method was used to determine the  $LC_{50}$  in SPSS (1999). Less than 50% of the males died at all test doses of (+)-longifolene



## Discussion

Female *D. armandii* are responsible for locating and invading hosts, overcoming tree defence, building tunnels and oviposition, and males are mainly responsible for mating. Comparison of the body weights of male and female individuals revealed that the mean weight of female *D. armandii* was significantly higher than that of males. This is in line with their respective roles. Females take on more work and need more energy. After exposure to host volatiles, the weight loss (absolute values and percentages) of both sexes were significantly different between the experiment and control groups (Fig. 2). This demonstrated that the host volatiles have significant toxicity to female and male *D. armandii*. The death rate in the beetles exposed to host volatiles was significantly higher in males than in females, indicating that females are more resistant to the toxic effects of the tested pine volatiles. The LC<sub>50</sub> values for 24 h of exposure to each tested host volatile except (+)-longifolene were higher in females than in males, further indicating that females are more resistant to the toxic effects of pine volatiles than males. Toxicity is usually affected by an individual organism's weight, and females were significantly larger than males; this could at least partially explain why the LC<sub>50</sub>s for females were higher than those for males.

In previous research with *D. ponderosae*, beetle gender had no significant effects on body weight or toxicity of most monoterpenes (Chiu et al., 2017; Reid et al., 2017). A possible reason for the difference may be because at low population densities, *D. ponderosae* colonizes weakened pine and colonizes and kills healthy pine trees when beetle populations are at high densities (Krause et al., 2018; Coops et al., 2008). Whereas *D. armandii* mainly invades healthy pine greater than 30 years old (Chen et al., 2010). Due to the differences in the health status of these hosts, it can be concluded that female *D. armandii* consume more energy than female *D. ponderosae* to resist host pine resistance during invasion. At the same time, female *D. armandii* need stronger anti-host defence abilities than female *D. ponderosae* to ensure successful invasion. Female *D. armandii* have evolved significant differences from male *D. armandii*. In *D. valens*, the host defence chemicals, mainly volatile  $\alpha$ -pinene, were found to influence its feeding behaviour and gut bacterial community structure (Xu et al., 2016). In other *Dendroctonus* spp. (*Dendroctonus rhizophagus*, *D. brevicomis*, *D. frontalis*, *Dendroctonus rufipennis*), although the host volatiles were identified and applied in behavioural research (Cano-Ramírez et al., 2012; Sullivan, 2005; Ryall et al., 2013), the toxicity of the host volatiles was not determined.

*Pinus contorta* and *Pinus banksiana* are hosts of *D. ponderosae*. Limonene was detected as one of the main volatiles in *P. contorta* and *P. banksiana*, accounting for  $5.26 \pm 0.85\%$  and  $4.77 \pm 0.99\%$  of the total volatiles (Clark et al., 2014). The toxicity of (-)-limonene to *D. ponderosae* was the strongest among a few test host volatiles, including ((-)- $\beta$ -phellandrene, (+)-3-carene, myrcene, terpinolene, (-)- $\alpha$ -pinene, (+)- $\alpha$ -pinene, (-)- $\beta$ -pinene, (+)- $\beta$ -pinene, (-)-limonene and (+)-limonene), and (+)-limonene was the second most toxic (Chiu et al., 2017). The LC<sub>50</sub> of (-)-limonene and (+)-limonene were 49 and 89  $\mu\text{L/L}$ , respectively, for *D. ponderosae*. Compared with our research results (Tables 3 and 4), the percentage of limonene in healthy *P. armandii* was higher than in *P. contorta* and *P. banksiana*; at the same time, the LC<sub>50</sub> of limonene was lower in *D. armandii* than in *D. ponderosae*. These results show that *P. armandii* has advantages in resisting bark beetle invasion compared with *P. contorta* and *P. banksiana*. Although limonene was not detected in more host survival states, limonene was the main volatile in the *D. ponderosae* host and was the most toxic host volatile.

The percentage dosage may increase in the host resistance period and increase host defence. However, this still needs further research. Limonene was also the main volatile found in *Picea abies* (the host of *Ips typographus*) (Persson et al., 1996), *Pinus tabuliformis* (the host of *D. valens*) (Chen et al., 2019), and *Pinus ponderosa* (the host of *D. brevicornis*) (Kolb et al., 2019). Adding limonene to the standard lure ([1:1:1 blend of (+)-alpha-pinene, (-)-beta-pinene, and (+)-3-carene]) decreased the response of *D. valens* but not significantly (Sun et al., 2004). While the toxicity and the existential state of limonene were not further considered in these studies, if limonene was the main host defence against invasion of *Dendroctonus* spp., more related data is needed for other bark beetle species.

Myrtenol was present in very low amounts (<0.2%) during all periods of *P. armandii* invasion by *D. armandii* colonization (Table 3). (1R)-myrtenol was converted into both (1R)-myrtenal and (1R)-myrtanol using a *P. abies* suspension culture (Lindmark-Henriksson et al., 2004). Based on the research result, myrtenol may be the raw material of myrtanol in the retreat period of *P. armandii*. In our previous study, myrtanol was found to be toxic to *D. armandii*, especially females (Zhao et al., 2019). Myrtanol was produced in the retreat period of *P. armandii*; at this time, the resin flow stopped, and females released pheromones to attract males for mating. At the same time, the females were the most numerous sex, the number of males gradually increased. The toxicity of the generated myrtanol was stronger in females than in males (Zhao et al., 2019), this was consistent with the bark beetle population proportion in the retreat period of *P. armandii*.

Based on these studies, the chemical defence of *P. armandii* has gradually become clear. After the first female *D. armandii* tunnels into the *P. armandii* trunk, the tree responds with host defences (the resistance period). Two lines of defence in the *P. armandii* phloem were launched to resist the invasion of *D. armandii*. At first, *P. armandii* secreted resin to mount a physical attack on *D. armandii*. The concentration of host phloem volatiles was increased (Kolb et al., 2019), and at the same time, the % dosage of the key volatile limonene in the phloem rapidly increased, leading to *D. armandii* death. If the first defensive line was overcome, *P. armandii* entered the retreat period. The resin production was hindered by *L. qinlingensis*, and the % dosage of limonene was lowered. At this time, the second defensive line was initiated. *P. armandii* used volatiles (perhaps myrtenol) to synthesize the toxic compound myrtanol to resist *D. armandii*, especially females. If the second defensive line was overcome by *D. armandii*, limonene and myrtanol became absent in the phloem. *P. armandii* then entered the withered period and was unable to resist invasion.

## Conclusion

In summary, this research provided evidence that females were stronger and were significantly better than males at resisting the toxicity of host volatiles. The differences between *D. armandii* and other *Dendroctonus* spp. shows that distinct bark beetle management must be used for the former. During the *D. armandii* invasion process, *P. armandii* phloem organized two defensive lines to resist the invasion of *D. armandii*. Furthermore, our results indicated that the percentage of limonene was elevated from the healthy period to the resistance period and decreased from the resistance period to the retreat period, while limonene was absent in the withered period. What triggers these changes in limonene is still not clear. Myrtanol was produced in the retreat period

and disappeared in the withered period. Myrtranol was produced after the first line of defence was broken, but the factors that resulted in its disappearance are not clear. Further research is needed to determine the biosynthetic mechanisms of limonene and myrtranol in *P. armandii*. Furthermore, whether limonene and myrtranol were toxic to the fungi carried by *D. armandii* was not clear. The defence of *P. armandii* against fungi is also worthy of further study.

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