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REGULAR PAPER

# Effect of needle damage on the release rate of Masson pine (*Pinus massoniana*) volatiles

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**Abstract** The effect of needle damage on the release rate of Masson pine (*Pinus massoniana* Lamb.) volatiles was examined. Needles were continuously damaged by mechanical damage (MDP) or by feeding of pine caterpillar (*Dendrolimus punctatus*) larvae (LFP); undamaged pine was used as a control (UDP). Volatiles were collected before damage, and at 16, 24, 40, 48, 64, 72, 88 and 96 h post-damage, and analyzed. The analyses revealed that 19 compounds identified as constitutive volatiles from UDP were terpenes and green leaf odors. The release rate of volatiles from MDP or LFP was higher than that from UDP. At 96 h post-damage, emission from MDP or LFP returned to the same level as that of UDP. Some volatiles, including sabinene, ocimene, limonene-1,2-epoxide, linalool, linalool acetate, germacrene D-4-ol, farnesol, and (*E*)-4,8-dimethyl-1,3,7-nonatriene were induced by mechanical damage and/or larval attack. Furthermore, the release rate of linalool acetate, farnesol, or (*E*)-4,8-dimethyl-1,3,7-nonatriene from LFP was higher than that from MDP. Based on an exact estimation of the proportion of damaged pine needles, a significant linear correlation between the release rate of total volatiles identified and the proportion of damaged needles was found in the case of LFP but not MDP.

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## Introduction

Masson pine (*Pinus massoniana* Lamb.) is one of the most widely planted conifers in Asia. Zhao et al. (1995) identified the volatiles of Masson pine needles by simultaneous distillation extraction and found that about 75% of the volatile fraction obtained was terpenes (including α-pinene, camphene, β-pinene, limonene, and β-caryophyllene); other trace volatile components included hemelene, terpinyl acetate, α-muurolene, γ-cadinene etc. Similar results were reported by Hao and Ha (2000) and Zhang et al. (2002), and Shen et al. (2006) applied a steam distillation method to study the essential oils from Masson pine needles. Qin et al. (2006) reported the relative abundance and enantiomeric composition of the main monoterpene hydrocarbons (α-pinene, camphene, β-pinene, limonene, and β-phellandrene) in intact Masson pine needles using a headspace sampling method. All these studies agree that terpenes dominate in the volatiles from Masson pine needles, with small amounts of other components.

The pine caterpillar, *Dendrolimus punctatus* Walker (Lepidoptera: Lasiocampidae), is one of the major pests threatening Masson pine wood production (Hou 1987; Dao 1990). Being the preferred host tree for *D. punctatus*, damaged Masson pine continuously releases a large amount of volatile compounds into the surrounding air , thus influencing the interaction between tree and herbivore. Using a TCT-GC/MS (thermal-desorption cold trapping-gas chromatograph-mass spectrum) technique, Ren et al. (2005) suggested that when Masson pine was artificially

damaged or attacked by *D. punctatus* larvae, the relative content of volatiles, with the exception of α-pinene, increased with time.

However, it remains unclear whether there is a significant difference between pine volatiles induced by larval attack or by artificial damage, and whether the release rate of volatiles is correlated with the proportion of pine needles damage. Investigating the precise results of various types of damage will be helpful in understanding the interaction between pine and caterpillar, and in pest management of *D. punctatus*. In this study, volatiles released from undamaged and damaged Masson pines suffering different proportions of needle damage were collected using the headspace sampling method and analyzed.

## Materials and methods

### Pine and larvae

Masson pines were transferred from Gao'an County (N 28°25′, E 115°22′, Jiangxi Province, China) to Beijing (N 39°55′, E 116°28′) in February 2003, and were planted in pots (50 cm ID and 45 cm height) filled with topsoil transported from Gao'an. To avoid frost damage, the pines were kept in a greenhouse from early November to late April of the next year. During the vegetative growth period of pines (May–October), the pines were placed in a nursery near the greenhouse. The pines were irrigated weekly and fertilized every season (NLF-1, Beijing Zhongnong Zhicheng Factory, Beijing, N:P:K = 12:12:12). Two years later, these pines were growing normally and were used for testing. *D. punctatus* cocoons were collected from Gao'an in May 2005, and then kept in a large wood-structure cage covered with nylon gauze for eclosion and egg-laying. Batches of 200 newly hatched larvae were placed in a 5,000 ml glass container covered with cotton gauze. The larvae were reared with fresh Masson pine needles (about 200 g per glass per day) to the third instar stage and then batches of 50 larvae were each reared in a container to the fifth instar stage. All containers with larvae were kept in a climate chamber (14 light:10 dark cycle, 26 ± 0.5°C, and 75 ± 5% relative humidity). The fifth instar larvae used for experiments were isolated and starved for 12 h.

### Method of estimating the proportion of needle damage

Because no fine-tuned method of estimating the proportion of needle damage has previously been reported, a new method was designed in detail as follows. A small pine branch with fresh needles was weighed ($W_B$); this pine branch and one starved fifth instar larva were placed in a 5,000 ml glass container; the stem end was wrapped with moist cotton to prolong the survival time of the pine branch. This procedure was replicated 30 times. Then, all glass containers were covered with cotton gauze and kept under laboratory conditions. After 24 h, portions of the stem without needles ($W_S$), remaining needles ($W_R$) and non-ingested needles (needles cut by larvae but not ingested by larvae, $W_N$) were weighed. The feces in each container was collected, dried at 60°C for 48 h to constant weight ($W_F$), and weighed.

Assuming that the fresh weight of needle ingested by larvae ($W_I$) is correlated linearly with the dried weight of feces ($W_F$):

$$W_I = K \cdot W_F + C \quad (1)$$

where $K$ and $C$ are constants.

The weight of needle damage ($W_L$) and the initial weight of needles ($W_O$) were estimated or calculated by:

$$W_L = W_I + W_N \quad (2)$$

$$W_O = W_L + W_R = W_B - W_S \quad (3)$$

The proportion of needle damage ($P_L$) by larvae or artificial damage was calculated by:

$$P_L = W_L/W_O \times 100\% \quad (4)$$

### Wound treatments and collection of volatiles

The experiment was carried out during the main vegetative growth period of pine in 2005. In another study of the diurnal rhythm of volatile compounds from Masson pine, we found that the emissions and contents of pine needle volatiles were similar at 0900 hours and 1700 hours in July (unpublished data). Thus, artificial damage were applied during 0830–0900 hours and 1630–1700 hours (which is the habitual feeding time of caterpillar larvae) and the sampling times were at 0900 hours and 1700 hours each day.

Pines of similar size (about 2.5 m in height, 9 years old) were treated in one of three ways. Treatment 1: one pine branch was infested with 80 larvae at 1630 hours on 7 July (0 h) and the larvae allowed to attack continuously for 64 h (LFP), and then all larvae were removed from the branch. Treatment 2: artificial damage, ≈15% of total needles on a pine branch was sheared down from 0800 to 0830 hours on 8 July (16 h). The needle-cutting was finished in 0.5 h. The needle segment was 0.3–0.5 mm and 6–8 segments were damaged. The amount of needle damage was almost equivalent to that of LFP. Thereafter, similar needle damage was made at 24, 40, 48, and 64 h (MDP). Treatment 3: Undamaged pine was used as a control (UDP). Each treatment was replicated with four pines.

During the treatment time, feces (dried at 60°C for 48 h to constant weight) and non-ingested needle segments from LFP and needle segments from MDP were collected and

weighed. After finishing volatile collecting, all residual needles on the damaged branch of LFP and MDP and total needles on the sampling branch of UDP were plucked off and weighed.

Volatiles were collected at nine timepoints using the headspace sample method (Qin et al. 2006; Su et al. 2007). In detail, each damaged or undamaged pine branch was enclosed in a cooking bag (35 cm × 43 cm, Reynolds, Richmond, VA ; which gave out few volatiles under high temperature and strong light intensity). The opening of the bag was bundled around the stem with a cotton line and the bag volume kept ca. 3 l. The bag was connected with an air inlet pipe (silica gel pipe) and an air outlet pipe. The inlet pipe was clamped and the air in the bag was first quickly extracted using a vacuum pump (QC-1, Institute of Labor Protection, Beijing, China) from the outlet pipe. Then, the outlet pipe was clamped and the inlet was connected to a glass container filled with activated carbon, and the purified air that was filtered through activated carbon container was pumped into the bag fully. Afterward, the outlet pipe was connected to an adsorbent column and volatile-collecting began. Each column used to collect sample was a Teflon tube (15 cm × 0.3 cm, ID) containing ≈150 mg Porapak Q adsorbent (mesh 80–100, Supelco, Bellefonte, PA). Both ends of the column were stoppered with polypropylene glass wool (Supelco). The column was wrapped with aluminum foil to protect the light-sensitive Porapak Q from direct sunlight during the sampling period. The volatiles from the pine branch were collected for 0.5 h at a flow rate of ca. 200 ml $min^{-1}$. The sampling times were before-treatment (0 h), and 16, 24, 40, 48, 64, 72, 88, and 96 h post-treatment (PD).

## Analysis and identification of volatiles

In the laboratory, each sampling column was extracted with 600 µl hexane (HPLC grade). Heptanoic acid ethyl ester solution (2 µl; 25 mg $ml^{-1}$, as internal quantification standard) was injected with each extracted sample. The volume of solution sample was concentrate to 200 µl. All volatile samples were stored at 8°C until analyses. Chemical analyses employed an HP Agilent 6890N gas chromatograph coupled with an HP 5973N mass selective detector (GC-MS). The GC was equipped with a DB-WAX (60 m × 0.25 mm × 0.25 µm). The injector temperature was 200°C. Samples (2 µl) were injected manually. The oven temperature was 50°C for 2 min before being increased at 5°C $min^{-1}$ to 200°C, and then held for 5 min. Helium was used as the carrier gas at a controlled flow rate of 21 cm $s^{-1}$. The temperature of the ion source was 230°C, and the quadruple was kept at 150°C. Full scan spectra (30–300 amu) were generated at 70 eV. Components were preliminarily identified by searching the NIST02 library spectra (Agilent Technologies, Santa Clara, CA) in the data system of Xcalibur (Finnigan; http://www.thermo.com/), checking the retention index, and comparing mass spectra of authentic reference compounds (most compounds were purchased from Sigma-Aldrich Chemie, Taufkirchen, Germany, and Acros Organics, Morris Plains, NJ ; some compounds, including longifolene, α-copanene, β-elemene, α-muurolene, myrtenal, farnesol and (*E*)-4,8-dimethyl-1,3,7-nonatriene were kindly provided by Z. Chenghua and M. Xianzuo). Selective characteristic ions were used for further determination. All quantifications were calculated on calibrated GC-FID peak areas and peak heights.

## Data quantification and statistical analysis

In a preliminary study, we found that few volatiles were released from pine stem (the release rate from pine stem was <5% of that from pine needles), thus the volatile emission from pine stem was ignored. The amount of the individual volatile compound ($A_i$) released was calculated by the following equation described by Su et al. (2007):

$$A_i = \frac{V_s N_s S_i}{S_s} \tag{5}$$

where $A_i$ = the released amount of individual compound ($i$); $V_s$ = the volume of internal standard injected; $N_s$ = the concentration of the internal standard injected (W/V); $S_i$ = the integral area of individual compound ($i$) according to the chart of total ionic current; $S_s$ = the integral area of the internal standard according to the chart.

The amount of total identified volatile compounds released ($A_T$) was calculated by:

$$A_T = \sum_1^n A_i \tag{6}$$

The release rate of the individual volatile compound expressed as a rate on a fresh mass and time basis was calculated by the equation:

$$R_i = \frac{A_i}{TW_O(1 - P_L)} \tag{7}$$

where $R_i$ = the emitting rate of individual compound ($i$); $T$ = the sampling time (in this study, $T = 0.5$ h); $W_O$ = the needle weight of sampling branch, $P_L$ = the proportion of needle damage.

The release rate of total identified volatile compounds ($R_T$) was calculated by:

$$R_T = \sum_1^n R_i \tag{8}$$

An average of four replicates followed by standard deviation (mean ± SD) is presented for the release rate,

**Table 1** Proportion of needle loss estimated and the fresh weights of total needles on treated and control Masson pine branches. *LFP* Pines attacked by Masson caterpillar larvae (*Dendrolimus punctatus*), *MDP* pines damaged mechanically by human treatment, *UDP* undamaged pine (control)

| Pine sample | Proportion of needle damage[a] | | | | | | $W_O$ (g)[b] |
|---|---|---|---|---|---|---|---|
| | 0 | 16 h | 24 h | 40 h | 48 h | 64 h | |
| LFP | 0 | 17.5 ± 2.7 | 30.2 ± 5.1 | 47.1 ± 6.7 | 58.3 ± 8.3 | 73.7 ± 11.2 | 35.5 ± 2.6 a |
| MDP | 0 | 15.4 ± 2.3 | 28.3 ± 3.7 | 46.8 ± 6.1 | 60.6 ± 8.2 | 77.5 ± 10.6 | 36.1 ± 2.9 a |
| UDP | 0 | 0 | 0 | 0 | 0 | 0 | 34.9 ± 2.1 a |

[a] Average percentage of needle loss on treated pines of four replicates followed by standard deviation (mean ± SD)

[b] $W_O$: average fresh weight of total pine needles on the sampling branches of four replicates followed by standard deviation (mean ± SD); the values of $W_O$ were compared and values with the same lowercase letters are not significantly different ($P > 0.05$) after *F*-test

relative content of individual volatile compound, and total volatiles from damaged and undamaged trees. The release rate of volatiles was compared by ANOVA and *t*-test and *F*-test with software SPSS 10.0. results were considered significant at the level of $P = 0.05$.

## Results

### Estimated proportion of needle damage

There was a significant linear relationship between the fresh weight of needle ingested by larvae ($W_I$) and the dried weight of larva feces ($W_F$) ($df = 29$, $W_I = 3.0176\ W_F - 0.1120$, $R^2 = 0.9033 > R_{0.001}{}^2 = 0.554$) with the constant $K = 3.0176$ and $C = 0.1120$. From Eqs. 1–3, the proportions of needle damage ($P_L$) of LFP and MDP were estimated accurately (Table 1).

### Volatiles from pines

Nineteen volatile compounds were identified to be the main volatile constitutes of UDP (Fig. 1). The volatiles mainly included green leaf odors, terpenes (e.g., monoterpenes and sesquiterpenes) and their derivatives. Most of the wound-induced volatiles of MDP or LFP were similar to the volatile constitutes from UDP. Some trace volatiles, such as γ-terpinene, α-copanene, β-elemene, α-muurolene and myrtenal, were found in samples collected from LFP.

### Release rate of pine volatiles

The release rate of volatiles from treated and control pines is shown in Fig. 2. The release rate peak of MDP appeared at 24 h post-damage (PD24h), i.e., much earlier than that of LFP (at PD64h). Nevertheless, the maximum release rate of MDP (12,015 ng $g^{-1}$ $h^{-1}$) was lower than that of LFP (19,400 ng $g^{-1}$ $h^{-1}$) (*t*-Test, df = 3, $P = 0.2305$). When the larval attack on LFP ceased at PD64h, volatile emission from LFP decreased rapidly. However, the emission of

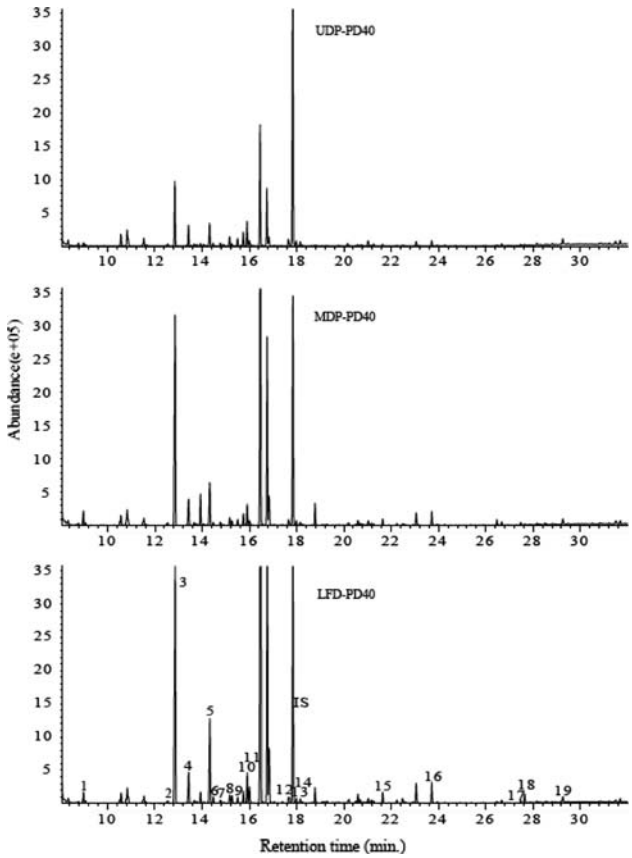



**Fig. 1** The total ion spectrum of volatiles emissions from mechanically damaged Masson pine (MDP), pine attacked by feeding *Dendrolimus punctatus* larvae (LFP), and undamaged pine (control, UDP) at 40 h post-damage. Compounds: *1* hexanal, *2* tricyclene, *3* α-pinene, *4* camphene, *5* β-pinene, *6* myrcene, *7* (*Z*)-3-hexenol, *8* α-phellandrene, *9* terpinene, *10* limonene, *11* β-phellandrene, *12* terpinolene, *13* nonanal, *14* α-pinene oxide, *15* unknown alcohol, *16* bornyl acetate, *17* longifolene, *18* β-caryophyllene, *19* α-humulene, *IS* heptanoic acid ethylester (internal quantification standard)

MDP decreased from PD24h onward, despite the fact that the mechanical damage continued until PD64h. The difference was probably due to the different means of damage.

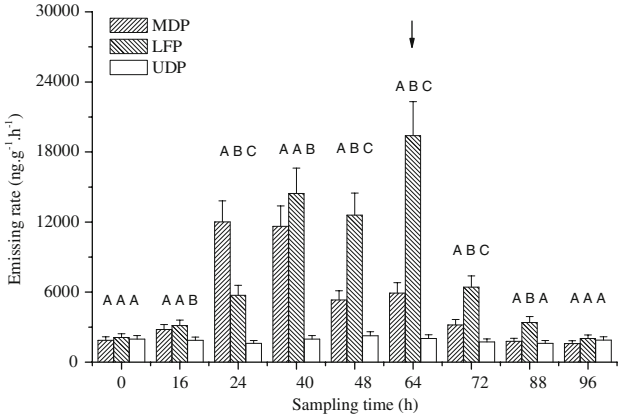



**Fig. 2** The release rate of volatile compounds released from MDP, LFP, and UDP. The *vertical arrow* indicates that the damage (larvae feeding and mechanical damage) on treated breaches ceased after 64 h. Values with the same upper case letter in the same sampling time are not significantly different ($P > 0.05$) after *F*-test

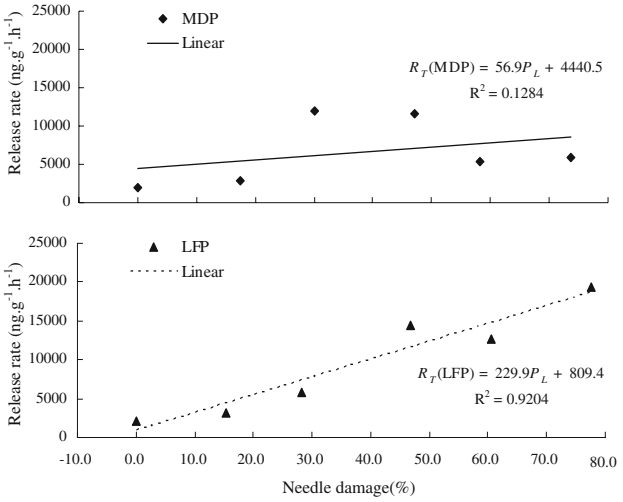



**Fig. 3** Relationship between release rate of total identified volatiles ($R_T$) and the percentage of needle damage of Masson pine ($P_L$) MDP and LFP samples. The linear regression equation was $R_T = kP_L + C$, $df = 5$, $R_{0.05}{}^2 = 0.7545$, $R$ (effect of damaged and non-damaged pine needle on the tropism)$_{0.01}{}^2 = 0.8745$

### Effect of needle damage on release rate of pine volatiles

The relationship between the release rate of volatiles from damaged pine (LFP or MDP) and $P_L$ was simulated by a linear regression equation (Fig. 3). The release rate clearly increased significantly with $P_L$ of LFP ($R_T = 229.9 P_L + 809.4$; df = 5, $R^2 = 0.9204 > R_{0.01}{}^2 = 0.8745$), but no significant correlation was found for MDP.

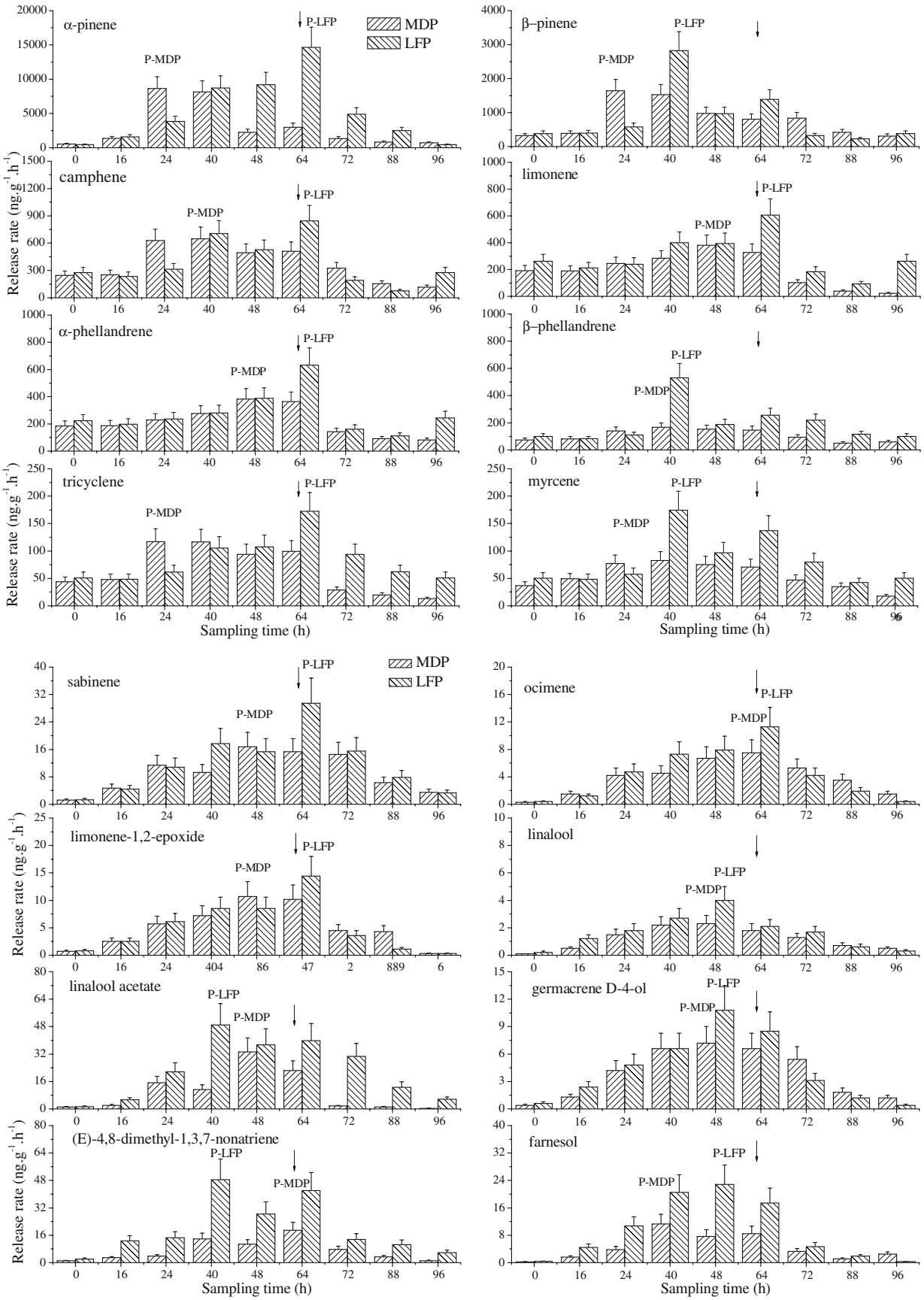
### Release rate of induced volatiles

When the pine was damaged, either by caterpillar larvae or artificial shearing, volatiles such as sabinene, ocimene, limonene-1,2-epoxide, linalool, linalool acetate, germacrene D-4-ol, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and farnesol were induced. Moreover, the emission peaks of most induced volatiles of the LFP sample were higher than those of the MDP sample (Fig. 4).

## Discussion

When damaged, plants release more and/or different volatile compounds than undamaged plants (Dicke et al. 1990; Turlings et al. 1990; Michelozzi 1999; Takabashi et al. 1991; McCall et al. 1993; Loughrin et al. 1994, 1995; Zagatti et al. 1997; Kessler and Baldwin 2001). Volatile compounds, especially monoterpenes, can be induced by biotic (e.g., herbivore attack, insect damage) or abiotic (mechanical) wounding (Lewinsohn et al. 1991). In the present study, there was greater emission of volatiles, especially some trace compounds, from damaged pine than from undamaged controls. In general, induced volatile compounds function as defensive compounds; the emission of large quantities of stored volatiles following wounding can be an important source of biogenic hydrocarbons and may contribute to changes in air chemistry (Staudt et al. 1997), especially when pine is damaged heavily (Litvak and Monson 1998). A large amount of volatiles probably influences the behavior of pine caterpillar moths and their natural enemies. This might explain why immigrant pine caterpillar moths rarely lay eggs on injured Masson pines (Zhao and Yan 2003; Huang et al. 2006), but the mechanism is still unclear.

Both the emission peak time and maximum release rate of volatiles from LFP and MDP were significantly different. The difference in volatiles released in response to insect attack and mechanical damage indicates that oral secretions play a key role. Two factors are involved in the release of volatiles following insect attack. One is the influence of the mechanical damage caused by insects chewing, and the other, which is more important, is insect oral secretions, as they likely contain some active components. Mechanical damage destroys plant cells, some stored volatiles are emitted, thus increasing the release rate of volatiles significantly (Mithöfer et al. 2005). The oral secretions of insects play an important role in the emission of induced volatiles when plants are infested by insects (Mattiacci et al. 1995; Alborn et al. 1997). When insects attack plants, besides mechanical damage, the effective components in the oral secretions of the insect bind to receptors on the plant cell membrane, activating specific signal cascades to release still more volatiles, such as constitutive (*Z*-3-hexenol, bornyl acetate, and *β*-caryophyllene) and induced (such as ocimene, linalool,


α-pinene
β–pinene
camphene
limonene
α-phellandrene
β–phellandrene
tricyclene
myrcene
sabinene
ocimene
limonene-1,2-epoxide
linalool
linalool acetate
germacrene D-4-ol
(E)-4,8-dimethyl-1,3,7-nonatriene
farnesol
MDP
LFP
P-MDP
P-LFP
Release rate (ng.g-1.h-1)
Sampling time (h)

◀ **Fig. 4** Release rates of volatiles from MDP and LFP Masson pine at different sampling times. The *arrowheads* indicate that damage (larvae feeding and mechanical damage) to treated breaches ceased after 64 h. *P-MDP* and *P-LFP* indicate the peaks of volatile emission of MDP and LFP pines, respectively

germacrene D-4-ol, DMNT, and farnesol) volatiles, thus the release rate of volatiles from pine attacked by larvae is larger than that from mechanically damaged pine (Lewinsohn et al. 1991; Loughrin et al. 1995; Litvak and Monson 1998; Kessler and Baldwin 2001). Furthermore, it must be remembered that mechanical damage happens only once, while insect attack causes continuous destruction of plants.

Schürmann et al. (1993) suggested that monoterpenes such as α-pinene, β-pinene, myrcene and limonene were distributed in resin ducts and mesophyll from needles of Norway spruce. The main monoterpenes were formed directly from photosynthetic carbon completely and rapidly within 20 min in Mediterranean pine (Loreto et al. 1996). Large increases in the volatilization of monoterpenes following wounding were previously observed in conifers (Litvak and Monson 1998) and in Masson pine (Ren et al. 2005). In our study, ≈85% of the volatiles from undamaged pine were monoterpenes, but this percentage was >95% when the pine was damaged, whether through artificial damage or by larval attack. The emission of monoterpenes induced by mechanical damage or pine caterpillar larvae was much stronger than in the control. For example, α-pinene and β-pinene were released at 8,625 and 1,645 ng $g^{-1}$ $h^{-1}$, respectively, from MDP (PD24h) and at 14,650 and 1,395 ng $g^{-1}$ $h^{-1}$, respectively, from LFP (PD64h). The emissions of volatiles induced by various damage decrease gradually after a rapid increase, but did not cease even after the damage stimulus had ceased (PD64h). What causes the reduction of volatiles release from damaged Masson pine is less clear. Loreto et al. (2000) supported a hypothesis stating that the reduction in emission may reflect the time during which the monoterpene reservoirs (resin ducts) are emptied. This time depends on both the compound volatility and the internal pool size. Litvak and Monson (1998) observed that the pool size decreased in wounded needles of several conifers. In our study, a few volatile monoterpenes were reduced rapidly and ceased almost completely 24–48 h post-damage, while the abundant monoterpenes first increased and then reduced gradually over a long period of time (>64 h).

Two plant volatile elicitors, volicitin (Alborn et al. 1997) and β-glucosidase (Mattiacci et al. 1995) have been isolated from caterpillar herbivory oral secretions. The release of many volatiles depends on such elicitors. Myrcene, *trans*-ocimene, 3-hexen-1-ol acetate, and indole have been regarded as chemically induced (De Moraes et al. 1998; Röse et al. 1996). Some of the volatiles, such as (*Z*)-3-hexenyl acetate, decanal, 3-hexenyl isovalerate, nonanal ocimene, and 2-cyanobutane, can be induced not only by insect attack but also by mechanical damage; some, such as 2,6-dimethyl-1,3,5,7-octatetraene, caryophyllene, and isovaleronitrile, cannot be induced by mechanical damage but only by insect attack (Hu et al. 2004). In the present study, some volatile compounds, such as sabinene, ocimene, limonene-1,2-epoxide, linalool, linalool acetate, germacrene D-4-ol, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and farnesol were induced by both mechanical damage and insect attack, but their release rate following insect attack was higher than by mechanical damage, thus indicating that the oral secretions of the insects magnified the emission of volatiles.

An interesting finding of this study was that the release rate of volatiles induced by larvae attacking increased with the proportion of needle damage, and indeed a significantly linear correlation was found. Further investigations will be carried out to determine whether this phenomenon is related to the oral secretions of the pine caterpillar, i.e., whether the oral secretions of caterpillar larvae regulate the release rate of pine volatiles in an as yet unknown way. The development of a mechanical device (MecWorm; Mithöfer et al. 2005) has enabled us to overcome previous experimental constraints in separating physical and chemical signals originating from insects during herbivory. This will facilitate our understanding of whether ,and to what extent, herbivorous insects manipulate plant defenses, a central question in the study of plant–insect co-evolution.

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