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Different headspace profiles in wild crucifer species in response to *Plutella xylostella* herbivory and exogenous jasmonic acid application

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Abstract Although exogenous treatment of plants with jasmonic acid (JA) may result in induced responses similar to plant defences induced by herbivory, few studies have compared the details of insect herbivory and JA-mimicked responses. We compared volatiles of two crucifer species, *Cardamine impatiens* and *Lepidium virginicum*, in response to *Plutella xylostella* larval feeding and exogenous application of JA, over the entire period of time when induced changes were detectable. Significant differences in the composition and timing of volatiles occurred between herbivory and JA treatments in both plants. The quantity of nitrile and isothiocyanate released in response to herbivory was significantly larger than that upon JA treatment. In each of the two plant species, most volatile components were emitted immediately upon larval feeding and their quantity dropped rapidly once feeding ceased. In contrast, the emission of volatiles in response to JA treatment lasted for a longer period of time, and the maximum emission rate was recorded 2 and 3 days after JA treatment in *L. virginicum* and *C. impatiens* respectively. These findings are discussed in the context of signal-transduction pathways and mechanisms involved in induced emissions of plant volatiles, as well as induced defences mediated by plant volatiles.

Key words Brassicaceae, herbivory, induced response, jasmonic acid, *Plutella xylostella*, volatile emission

Introduction

Plants have evolved various induced defence responses against herbivores. A major signal-transduction pathway involved in induced plant defence against herbivores is the octadecanoid pathway (Farmer & Ryan, 1992), and the central component of this pathway is jasmonic acid (JA). It is well known that the endogenous concentration of JA in plants increases in response to herbivore feeding, and this increase mediates the accumulation of secondary metabolites that hamper the performance of herbivores (Farmer & Ryan, 1992) as well as the production of volatiles that attract parasitoids or predators of herbivores to plants (Kessler & Baldwin, 2001; Van Poecke & Dicke, 2002; Ament *et al.*, 2004). With the rapid recognition of the key role of JA in mediating plant defences against herbivores, ecologists have utilized chemical elicitors, such as JA or methyl jasmonate (MeJA), to mimic the induced responses of plants to herbivore attack, and examined the effects of induced responses on herbivores or their natural enemies (Dicke *et al.*, 1999; Thaler, 1999; Thaler *et al.*, 2001, 2002; Bruinsma *et al.*, 2007, 2009), and estimated fitness or costs of induced response (Van Dam & Baldwin, 1998; Cipollini, 2007). Some ecologists also indicated that chemical elicitors of JA-dependent defences may provide tools to be used in behavioral manipulation for pest management (Thaler *et al.*, 2001).

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Given the complex spatial, temporal and oral secretion-related nature of insect herbivory, it is unlikely that a single elicitor or treatment can fully mimic the stimulus of insect feeding (Schmelz *et al.*, 2003). Plants are exposed to various types of herbivores, and may respond differently to different herbivores. In this process, the differential composition of oral secretions from herbivores may help plants to distinguish between attacks by different herbivores (Dicke & Hilker, 2003; Voelckel & Baldwin, 2004). For example, feeding by different larval instars of the same herbivore species even induced plants to release different volatile blends (Takabayashi *et al.*, 1995).

Recent studies at the molecular level have indicated that gene expression patterns induced by herbivory and exogenous JA may differ (Arimura *et al.*, 2000; De Vos *et al*., 2005; Broekgaarden *et al.*, 2007), and such differences were also reflected in some physiological processes (Ozawa *et al.*, 2000; Halitschke *et al.*, 2000; Schmelz *et al.*, 2003). Differences in the nature of induced responses in plants by different treatments may be important. For example, in lima bean plants, the blend of volatiles induced by *Tetranychus urticae* feeding is similar to that induced by exogenous JA, although qualitative and quantitative differences exist (Dicke *et al.*, 1999). However, the absence of methyl salicylate, which is induced by *T. urticae* herbivory but not by JA application, is a major determinant of the differential attraction of natural enemies of *T. urticae* to spider-mite-infested and JA-treated plants (De Boer *et al.*, 2004). Thus, before JA or MeJA can be exploited in pest control, it is necessary to know how the responses induced by herbivory and exogenous JA or MeJA differ. However, few attempts have been made to compare plant responses, especially continuous responses over a period of time, induced by insect herbivory and JA (but see Dicke *et al.*, 1999; Degenhardt & Lincoln, 2006).

In an earlier study addressing the induced resistance in a range of wild crucifers, we found that induced responses in these brassicaceous plants, as measured in terms of oviposition preference by their common herbivore, the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), differed between herbivory and JA treatments, and we speculated that herbivory and JA treatment also result in differences in volatile emissions (Zhang *et al.*, 2008). Here, we investigated the volatile profiles of two wild brassicaceous species in response to *P. xylostella* larval feeding and JA application, over the entire period of time when induced changes were detectable. Our results demonstrate that larval feeding and JA application result in significant differences in the composition and timing of volatile emissions by the plants.

Materials and methods

Insects and plants

The *Plutella xylostella* culture was originally established from insects collected on a cabbage farm in a suburb of Hangzhou, China in October 2004 and maintained on common cabbage, *Brassica oleracea* L. var. *capitata*, cultivar Jing-feng No. 1, in a temperature-controlled room at 26 ± 1°C, 60%–70% RH and 14 : 10 L : D photoperiod.

Seeds of two wild brassicaceous species, *Lepidium virginicum* (Linn.) and *Cardamine impatiens* (Linn.) (Brassicales: Brassicaceae), were collected from natural populations in a suburb of Hangzhou in 2005. All experiments utilized plants grown from seeds in 350-mL pots in a mixture of peat, perlite, vermiculite and organic fertilizer. The plants were maintained in greenhouses under ambient temperature and humidity and natural light. When plants had grown to the vegetative stage of 6–8 fully expanded leaves, they were transferred to a temperature-controlled room (26 ± 1°C, 60%–70% RH and 14 : 10 L : D photoperiod) for the experiments. Only clean, undamaged plants were used.

Plant treatment

The plants were carefully removed from their pots, and the soil and undisturbed root system were fully covered with aluminum foil. Preliminary tests had indicated that this treatment had little effect on plant growth and volatile collection. The plants with their roots and surrounding soil packed within aluminum foil were kept for 2–3 days in a temperature-controlled room before they were treated by one of the following methods.

(i) **Herbivore damage**. We infested each test plant with 10 third instar *P. xylostella* larvae and allowed them to feed freely on the plants for 24 h. In a previous study, we demonstrated that this level of herbivory was effective in inducing behavioral responses by the test insect to the plants (Zhang *et al*., 2008). During this period of feeding the plants with larvae were used for headspace collection for 24 h, and the larvae and their feces were then removed. Thereafter volatiles were continuously collected from the damaged plants until no volatiles were detected by gas chromatography. Intact plants, that received no treatment, were used as controls.

(ii) **Application of jasmonic acid (JA).** JA (Sigma-Aldrich, St Louis, MO, USA) was dissolved in 2 mL of acetone and dispersed in water (containing 0.5% Tween 20) to achieve a 1 mmol/L aqueous JA solution. We liberally sprayed the foliage of each plant with 5 mL of

JA solution with a hand sprayer. In a previous study, we demonstrated that this method and dose of JA application was effective in inducing behavioral responses by the test insect to the plants (Zhang *et al*., 2008). Treated plants were used for headspace collection 1–2 h after JA application. The headspace of JA-treated plants was continuously collected until no volatiles were detected by gas chromatography. We sprayed intact plants with 5 mL of water (containing 2% acetone and 0.5% Tween 20) and used these plants as controls.

Headspace−collection system

Air was first pushed through a charcoal filter to eliminate impurities, then through a glass container with water to humidify it, and a flow-meter to measure and regulate the air flow. The moist and clean air then entered a glass cylinder (16 cm diameter, 35 cm high) at 300 mL/min. To create a laminar flow, the air was forced through a glass frit at the top of the cylinder. Approximately 4 cm above the bottom, there was a 25-mm horizontal female ground-glass connector for a collection trap. The traps were glass tubes (10 cm long, 5 mm diameter) that contained 30 mg of 80/100 mesh Super-Q (Altech Assoc., Deerfield, IL, USA). The air passing over the plant was pulled through the Super-Q adsorbent mesh, and vented out.

Headspace collection and analysis

All experiments were conducted in a temperature-controlled room (26 ± 1°C, 60%–70% RH and 14 : 10 L : D photoperiod). Volatiles from JA- or herbivore-treated plants and their corresponding control plants were collected for each 24-h interval until no compounds were detectable by gas chromatography. We also collected volatiles from a blank cylinder to check whether the system was clean. After each collection, traps were rinsed with 200 mL of methylene chloride, and 1500 ng of *n*-octane and nonyl acetate (Sigma, St Louis, MO, USA) were added as internal standards. Each treatment was replicated three times.

Analyses were carried out with a Hewlett-Packard (HP) 6890 series gas chromatograph (GC) equipped with an HP-5 (30 m−0.32 mm inside diameter, 0.25 μm film thickness) column in splitless mode and a flame ionization detector (FID). Of each sample, a 3-μL aliquot was analyzed. Helium (1 mL/min) was used as carrier gas. Following injection, the column temperature was maintained at 40°C for 4 min, increased to 220°C at 8°C/min, and held at 220°C for 5 min and 30 s. Data were analyzed with HP GC Chemstation software and the detected volatiles were quantified based on comparison of their peak areas with those of internal standards.

For identification of compounds, selected samples were also analyzed by GC-MS (mass spectrometry), using an HP 6890 gas chromatograph coupled to an HP 5973 mass spectrometer. For the gas chromatography separation, the same HP-5 column was used with helium (1 mL/min). The injector was held at 250°C, and the temperature program was the same as used in gas chromatography (described above). The MS was used in electron impact ionization mode (70 eV). Volatile compounds were identified by comparison of GC retention times and mass spectra with those of commercially available standards and by comparison of mass spectra with spectra of the National Institute of Standards and Technology (NIST) database.

Results

Chemical analysis of volatiles emitted from *L. virginicum* and *C. impatiens* showed that undamaged plants released only trace amounts of compounds that were only just detectable by gas chromatography; in contrast, both herbivore-treated and JA-treated plants emitted more compounds than undamaged plants (Figs. 1 and 2). There were apparent qualitative as well as quantitative differences in volatile composition of blends emitted by herbivore-treated and JA-treated plants (Figs. 1–3).

Headspace composition

Upon larval feeding *L. virginicum* plants emitted mainly two compounds, benzyl nitrile and benzyl isothiocyanate, which accounted for 95% of the total volatile emission. These two compounds are hydrolysis products of glucosinolates. JA-treated *L. virginicum* mainly emitted an unknown compound and benzyl isothiocyanate (Fig. 1).

Upon larval feeding *C. impatiens* plants emitted several volatiles, which were roughly divided into two groups: (i) hydrolysis products of glucosinolates (e.g., isopropyl isothiocyanate) that accounted for 71% of the total amount emitted, and (ii) terpenoids [(*E*)-β-ocimene, (*Z*)-β-ocimene and α-farnesene] (Fig. 2). In contrast, JA-treated *C. impatiens* plants mainly emitted terpenoids (Fig. 2).

Timing of volatiles

During the first 24 h, that is, during larval feeding, both *L. virginicum* and *C. impatiens* emitted the highest diversity of compounds and also the largest amount of volatiles

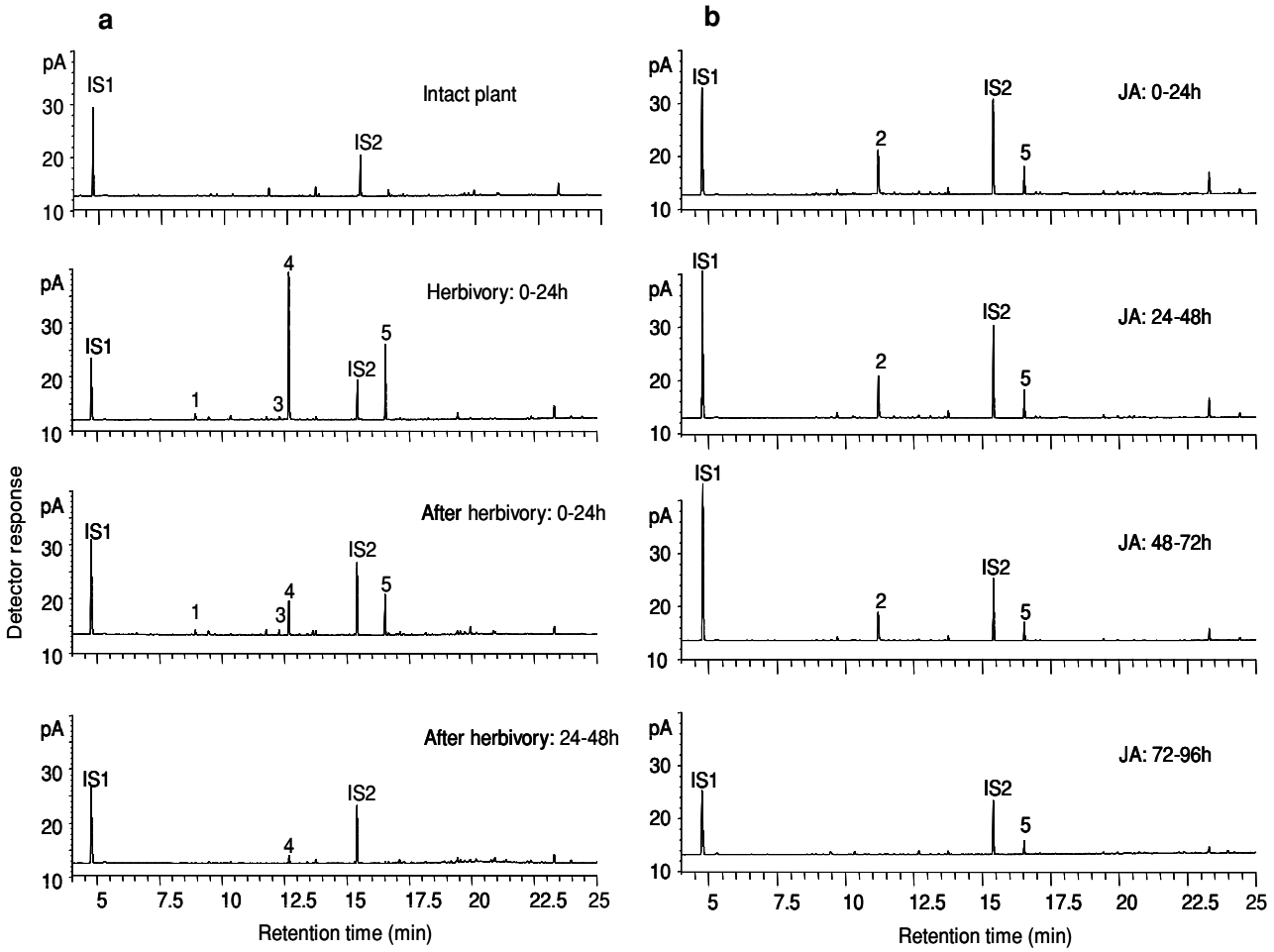

Fig. 1 Representative examples of gas chromatograph flame ionization detector (GC-FID) chromatograms of volatiles collected from *L. virginicum* plants before, during and at different time intervals after 24 h of damage by larvae of *P. xylostella* (a) and at different time intervals after application of jasmonic acid (JA) (b). Peak numbers represent: 1, unknown; 2, unknown; 3, benzyl isocyanate; 4, benzyl nitrile; 5, benzyl 1-isothiocyanate. The internal standards were *n*-octane (IS1) and nonyl acetate (IS2).

(Fig. 3). During the next 24 h, that is, after removal of the larvae from the plants, the headspace compositions of *L. virginicum* and *C. impatiens* plants changed little, but the total amount of volatiles rapidly declined; 48 h after removal of larvae, most volatile compounds were hardly detectable (Figs. 1a, 2a, and 3).

In contrast, upon JA treatment both *L. virginicum* and *C. impatiens* emitted the main compounds with a lower emission rate (Figs. 1b, 2b, and 3). The total amounts of volatiles emitted by *L. virginicum* and *C. impatiens* gradually increased, and reached a maximum at 2 and 3 days after JA treatment, respectively (Fig. 3). Thereafter, the amounts decreased and were hardly detectable 5 days after JA treatment (Figs. 1b, 2b, and 3).

The significant differences in timing of volatile release between herbivory and JA treatments were also reflected in the emission patterns of individual compounds (Fig. 3).

Discussion

For both *L. virginicum* and *C. impatiens*, the composition of the volatile blend induced by *P. xylostella* larval feeding differed from that induced by JA application (Figs. 1 and 2). The distinct compounds were nitriles and isothiocyanates, which were clearly emitted from herbivore-treated plants but hardly from JA-treated plants (Figs. 1 and 2). These differences in volatile composition may be due to different impacts on plants by insect herbivory and JA. As a chemical elicitor, JA induces plant responses in the absence of mechanical damage. In contrast, insect

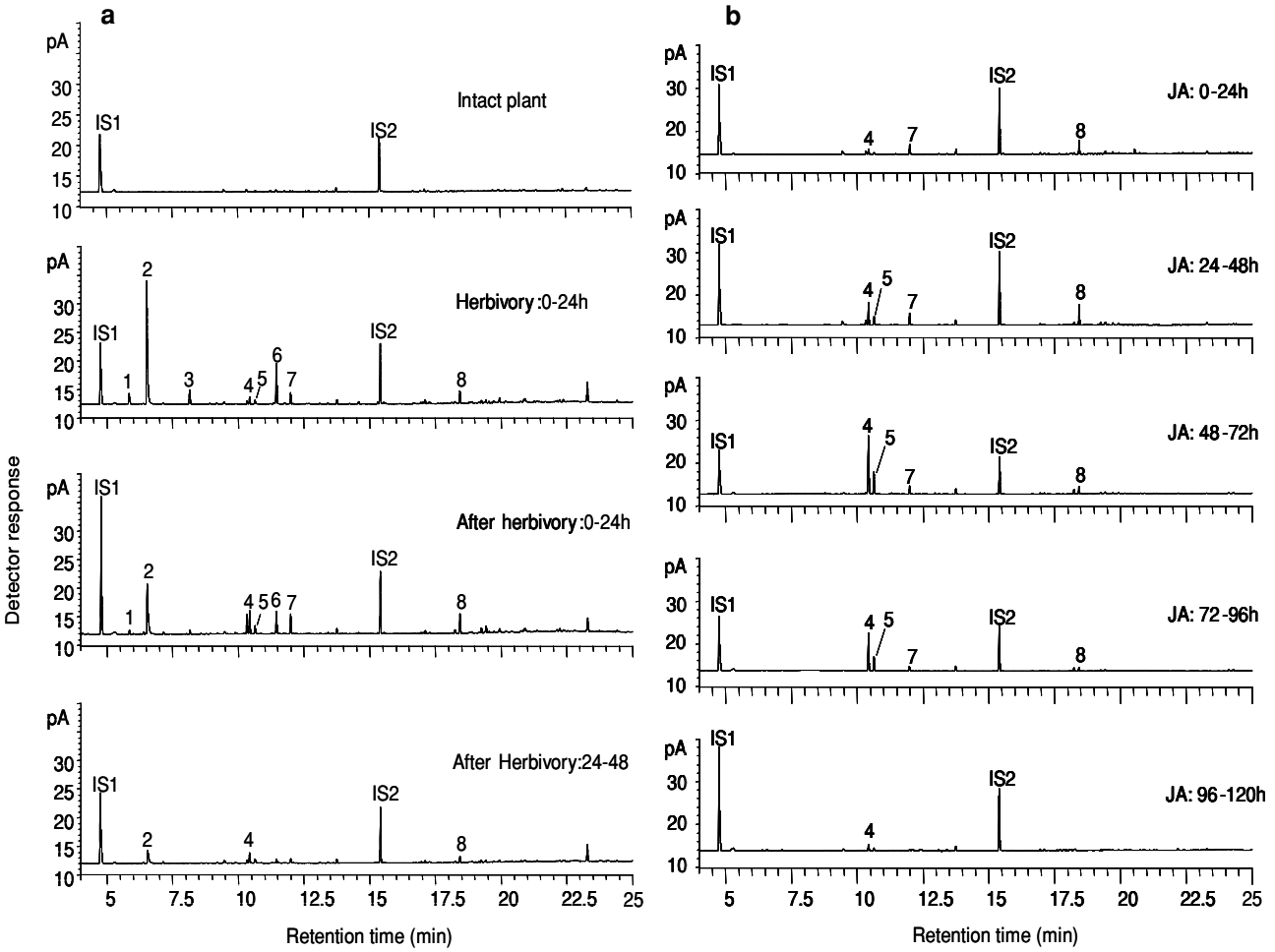

Fig. 2 Representative examples of gas chromatograph flame ionization detector (GC-FID) chromatograms of volatiles collected from *C. impatiens* plants before, during and at different time intervals after 24 h of damage by larvae of *P. xylostella* (a) and at different time intervals after application of jasmonic acid (JA) (b). Peaks numbers represent: 1, isopropyl isothiocyanate; 2, unknown; 3, unknown; 4, (Z)-β-ocimene; 5, (E)-β-ocimene; 6, unknown; 7, unknown; 8, α-farnesene. The internal standards were *n*-octane (IS1) and nonyl acetate (IS2).

herbivory causes physical damage and an interaction with oral secretion-derived compounds (Turlings *et al.*, 1990; Mattiacci *et al.*, 1995). Wounding plays an important role in eliciting the release of volatiles. It is well known that once crucifer plants are damaged by herbivory, glucosinolates are degraded in a reaction catalyzed by myrosinases, thereby resulting in the production of toxic compounds such as nitriles and isothiocyanates, and wounding is a prerequisite in this reaction (Bones & Rossiter, 1996). Some compounds in herbivore oral secretions have been shown to elicit an endogenous JA burst and/or herbivore-induced volatiles in some plant species (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Halitschke *et al.*, 2001). Herbivory can also induce more than one signal-transduction pathway. For example, Ozawa *et al.* (2000) reported that volatiles from lima bean induced by methyl salicylate (MeSA) and JA were more similar to *T. urticae*-induced volatiles than those induced by JA alone, and thus they inferred that production of volatiles from lima bean infested by *T. urticae* were mediated by the SA and JA signaling pathways. In maize, JA and ethylene signaling pathways mediate the emission of volatiles induced by beet armyworm, *Spodoptera exigua* (Schmelz *et al.*, 2003). In *Nicotiana attenuata* where JA-dependent pathways dominate in defences against herbivores (Baldwin *et al.*, 1997), exogenous MeJA-induced responses differed from those elicited by the attack of *M. sexta* larvae (Halitschke *et al.*, 2000). In *Arabidopsis* a molecular

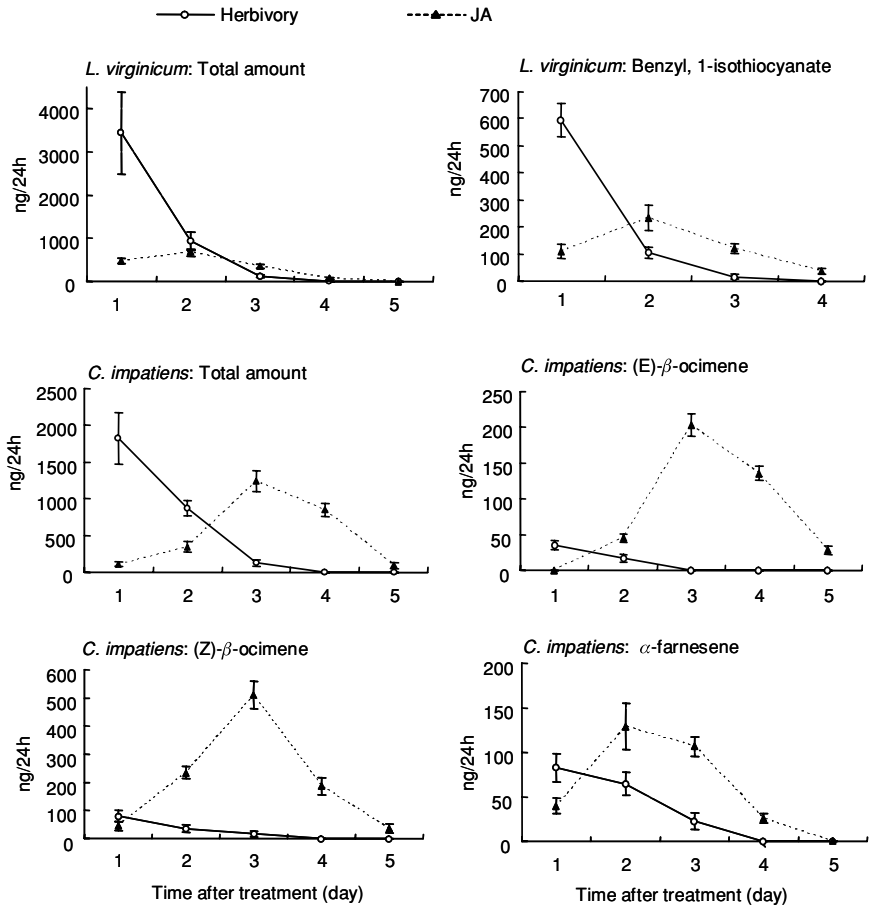

Fig. 3 Comparison of emission rates of total amount or individual compounds per experimental day (mean ± SE) in response to herbivory or jasmonic acid (JA) application in *L. virginicum* and *C. impatiens* over the entire collection period ($n = 3$).

genetic approach showed that both JA and SA mediate the emission of herbivore-induced plant volatiles (Van Poecke & Dicke, 2002).

Our results also indicate that the emission of volatiles from *L. virginicum* and *C. impatiens* induced by *P. xylostella* larval feeding was more rapid and transient than that induced by exogenous JA. Herbivore-induced volatiles may function as induced direct defence by reducing herbivore oviposition or feeding (De Moraes *et al.*, 2001), and meanwhile as induced indirect defence by attracting parasitoids and predators, the natural enemies of the attacking herbivores (Dicke *et al.*, 1990, 2009; Turlings *et al.*, 1990; Kessler & Baldwin, 2001; Van Poecke & Dicke, 2002). In the present study, after initial larval feeding both *L. virginicum* and *C. impatiens* rapidly emitted isothiocyanates, which have been demonstrated to be toxic to *P. xylostella* larvae (Li *et al.*, 2000). Meanwhile, *C. impatiens* also emitted (*E*)-β-ocimene and α-farnesene, which are known to attract natural enemies of herbivores (Dicke *et al.*, 1990; Du *et al.*, 1998; Vuorinen *et al.*, 2004). Hence, the rapid reactions of *L. virginicum* and *C. impatiens* suggest that they can rapidly defend themselves against herbivores, possibly before the herbivore can do substantial damage. Similar rapid reactions to herbivory by emitting volatiles have been reported in many plant species (Turlings *et al.*, 1998; Scascighini *et al.*, 2005).

In contrast, the release of volatiles induced by JA occurred at a lower emission rate that lasted for a relatively longer period of time compared to that in response to herbivory. The time of maximum emission rates induced by JA differed between the two plant species: 48 h after JA application for *L. virginicum*, and 72 h for *C. impatiens* (Fig. 3). This difference in the timing of responses to JA treatment between plant species should be considered in future research on JA-induced defences in plants as it might indicate that plant species differ in their reaction speed to phytohormones.

Chemical elicitors of JA-dependent defences have been proposed as a tool in pest management strategies against a broad range of herbivores (Thaler *et al.*, 2001). One important aspect of JA-induced defences is the release of volatiles, which are known to attract parasitoids or predators of herbivores (Dicke *et al.*, 1999; Thaler, 1999; Gols *et al*., 1999). However, evidence obtained so far indicate that JA-induced volatiles differ from those induced by herbivory not only in the composition of volatiles (Dicke *et al.*, 1999; Ozawa *et al.*, 2000; Halitschke *et al.*, 2000; Schmelz *et al.*, 2003), but also in the timing of emission (current study). Given that herbivore-induced volatiles function as reliable and specific chemical cues for parasitoids and predators, an important question is whether different compositions of volatiles induced by JA interferes with the host-location process of natural enemies in natural ecosystems. Gols *et al.* (2003) reported that a pretreatment of the plants with a low JA dose (0.1 mmol/L) followed by a low-density spider mite infestation enhanced the attraction of its acarine predator *P. persimilis* to plant volatiles compared to attraction to volatiles from plants that were only infested with spider mites and without a pretreatment with JA. The application of this low JA dose by itself did not affect the behavior of the predators. Thus, the low-dose JA application primed (Frost *et al.*, 2008) the plant for enhanced herbivory-induced emission of predator attractants. The application of higher JA doses to enhance the attraction of natural enemies is not advisable because this would result in a longer-lasting emission of induced volatiles at high rates that result in the attraction of carnivorous arthropods to places without herbivores (Dicke *et al.*, 1999). This may result in a negative associative learning, where natural enemies would associate the plant volatiles with the absence of prey (Drukker *et al.*, 2000). Therefore, detailed research on the effects of chemical elicitors such as JA in a tritrophic context is required to examine their potential as tools in pest management.

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