Comparing induction at an early and late step in signal transduction mediating indirect defence in *Brassica oleracea*

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Abstract

The induction of plant defences involves a sequence of steps along a signal transduction pathway, varying in time course. In this study, the effects of induction of an early and a later step in plant defence signal transduction on plant volatile emission and parasitoid attraction are compared. Ion channel-forming peptides represent a class of inducers that induce an early step in signal transduction. Alamethicin (ALA) is an ion channel-forming peptide mixture from the fungus *Trichoderma viride* that can induce volatile emission and increase endogenous levels of jasmonic acid (JA) and salicylic acid in plants. ALA was used to induce an early step in the defence response in Brussels sprouts plants, *Brassica oleracea* var. *gemmafera*, and to study the effect on volatile emission and on the behavioural response of parasitoids to volatile emission. The parasitoid *Cotesia glomerata* was attracted to ALA-treated plants in a dose-dependent manner. JA, produced through the octadecanoid pathway, activates a later step in induced plant defence signal transduction, and JA also induces volatiles that are attractive to parasitoids. Treatment with ALA and JA resulted in distinct volatile blends, and both blends differed from the volatile blends emitted by control plants. Even though JA treatment of Brussels sprouts plants resulted in higher levels of volatile emission, ALA-treated plants were as attractive to *C. glomerata* as JA-treated plants. This demonstrates that on a molar basis, ALA is a 20 times more potent inducer of indirect plant defence than JA, although this hormone has more commonly been used as a chemical inducer of plant defence.

Key words: Alamethicin, Brussels sprouts, *Cotesia glomerata*, jasmonate, parasitoid host-location behaviour, peptaibol, octadecanoid pathway, salicylate, volatile emission.

Introduction

Plants are attacked by a variety of herbivores and have evolved a wide range of strategies to defend themselves (Agrawal, 1998; Karban and Baldwin, 1997). Direct defence strategies affect the herbivore itself and indirect defence strategies affect the herbivore by attracting the herbivore’s enemies, such as predators or parasitoids (Turlings et al., 1990; Dicke, 1999; Dicke et al., 2003). Herbivore-induced plant volatiles play an important role in the attraction of predators and parasitoids, which make use of these volatile signals to locate their prey or host. The emission of plant volatiles can be induced by herbivore feeding and oviposition (Arimura et al., 2005; Hilker and Meiners, 2006; Bruinsma and Dicke, 2008), and has been recorded for >23 plant species from 13 families (Dicke, 1999). Signal transduction of herbivore-induced plant defences is mainly mediated by pathways centring around three plant hormones: jasmonic acid (JA), salicylic acid (SA), and ethylene (Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002; Dicke et al., 2003). Manipulation of the levels of these hormones using inducers or inhibitors allows the investigation of the importance of these hormones for plant responses and insect behaviour in a controlled manner and in the absence of differences in visual cues resulting from feeding damage.
Plant responses to different types of attack can be highly specific. Mechanical wounding elicits a response different from that elicited by herbivore feeding, and even different herbivore species, herbivore instars, and duration of feeding will result in different responses (overview given by Bruinsma and Dicke, 2008). Early events in insect–plant interactions, responsible for recognition of the attacker and triggering signal transduction, take place within the first seconds to minutes after attack, and involve changes in membrane potentials, Ca\(^{2+}\) signalling (spatial and temporal changes in cytosolic Ca\(^{2+}\) concentrations), and production of reactive oxygen species (White, 2000; Maffei et al., 2007). Oral secretions from eight Lepidopteran larvae (including *Pieris brassicae*, *Pieris rapae*, and *Plutella xylostella* that are specialist herbivores on Brassicaceous plants) form ion channels in artificial membranes. They have been suggested to contain compounds that are directly involved in the induction of membrane depolarization, Ca\(^{2+}\) signalling, and subsequently in the initiation of defence responses in caterpillar-infested plants (Maischak et al., 2007). Since membrane depolarization depends on ion fluxes and subsequent intracellular signalling, peptides that produce ion channels within biological membranes can be used to study their potential effect on insect–plant interactions (Engelberth et al., 2000; Maffei et al., 2007). Here, the effects of an ion channel-forming peptide from a fungus on the induction of indirect plant defence have been investigated. The use of such a peptide allows for the manipulation of an early step in the signal transduction underlying induced plant defences. Thus, it allows investigation of the contribution of this early step to the induction of plant defence.

Alamethicin (ALA) is a voltage-gated ion channel-forming peptide mixture produced by the fungus *Trichoderma viride*. This mixture consists of at least 12 compounds each containing 20 amino acid residues (Brewer et al., 1987). In Lima bean, ALA treatment increases the levels of both JA and SA. Endogenous levels of JA peak early and transiently after treatment; SA levels rise more slowly, but remain at a high level for longer (Engelberth et al., 2000). Upon treatment with ALA, Lima bean leaves emit a less complex blend of volatiles than upon treatment with JA; this is probably due to increased levels of SA (Engelberth, 2000) inhibiting the JA response, occurring between 12-oxophytodienoic acid (OPDA; a precursor of JA in the octadecanoid pathway) and JA (Engelberth et al., 2001). ALA is also a potent inducer of methyl salicylate (MeSA) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) emission in *Arabidopsis thaliana* (Chen et al., 2003; Herde et al. 2008), and shares this with the effect of JA on this plant (Van Poecke et al., 2002). Although also a member of the Brassicaceae, it has not been studied whether the volatile release of Brussels sprouts is affected by ALA treatment and whether this affects the behaviour of carnivorous arthropods such as predators and parasitoids.

JA is a key compound in the octadecanoid pathway, involved not only in induced direct defence against herbivorous insects in plants, but also in induced indirect defence (Karban and Baldwin, 1997; Dicke et al., 1999; Thaler, 1999; Dicke and Van Poecke, 2002; Bruinsma et al., 2007, 2008), as well as resistance against abiotic stresses and pathogens (Creelman and Mullet, 1997; Wasternack, 2007). Treatment with JA or its volatile ester methyl jasmonate (MeJA) induces a late step in the signal transduction of the defence response and renders plants more attractive to carnivorous arthropods in many plant species, including Brussels sprouts (Bruinsma et al., 2009), Lima bean (Dicke et al., 1999; Heil, 2004), gherba (Gols et al., 1999), tomato (Thaler, 1999), *Arabidopsis thaliana* (Van Poecke and Dicke, 2002), tobacco (Kessler and Baldwin, 2001), maize (Ozawa et al., 2004), and rice (Lou et al., 2005). JA-induced plant volatile blends usually contain so-called green leaf volatiles and terpenoids (Boland et al., 1995; Dicke et al., 1999; Van Poecke and Dicke, 2002; Bruinsma et al., 2009). Chemical analysis has demonstrated that herbivory and JA treatment have similar, but not identical, effects on volatile induction in the plant studied here, Brussels sprouts (Bruinsma et al., 2009), as well as in Lima bean (Dicke et al., 1999; Koch et al., 1999) and *A. thaliana* (Van Poecke and Dicke, 2002). This difference may contribute to the phenomenon whereby although the predators or parasitoids prefer JA-treated plants to untreated plants, they are more attracted to herbivore-infested plants (Dicke et al., 1999; Van Poecke and Dicke, 2002; Ozawa et al., 2004; Bruinsma et al., 2009).

In this study, the effect of induction by ALA application, activating an early step in plant defence signal transduction, is addressed, and this is compared with the induction by JA application, affecting a late step, by measuring volatile emission and parasitoid behaviour. The tritrophic interactions between *Brassica oleracea*, *P. brassicae*, and *C. glomerata* as a model system were used to investigate (i) which volatiles are released from Brussels sprouts plants treated by ALA; (ii) whether induction by ALA can attract parasitoids; and (iii) whether there are interactions between induction by ALA and JA that affect volatile emission and parasitoid attraction.

**Materials and methods**

**Plant and insect material**

Brussels sprouts plants, *B. oleracea* var. *geminifera* cultivar *Cyirus* (Brassicaceae), were grown from seeds in a greenhouse in plastic pots (11×11 cm) at 24±4 °C, 60±20% relative humidity (RH), and a 16 h light:8 h dark photoperiod, with >200 μmol m\(^{-2}\) s\(^{-1}\) PAR during the photophase. All experiments were conducted with 5- to 6-week-old plants. The larval parasitoid, *C. glomerata* L. (Hymenoptera: Braconidae), was reared in a greenhouse at 23±1 °C, 60±10% RH, and 16 h light:8 h dark photoperiod on their preferred host, the large cabbage white butterfly, *P. brassicae* L. (Lepidoptera: Pieridae). Stock colonies of *P. brassicae* were maintained on Brussels sprouts plants in a climate room at 21±1 °C, 60±10% RH, and a 16 h light:8 h dark photoperiod.
**Plant treatments**

ALA (A&E Scientific, Marcq, Belgium) was dissolved in methanol at a concentration of 5 mg ml\(^{-1}\). From this stock solution the test solutions were prepared by adding water and 0.05% Tween-20 (Sigma-Aldrich, St Louis, MO, USA), resulting in final concentrations of 1, 5, 20, and 50 µg ml\(^{-1}\) ALA in the test solution (corresponding to 0.51, 2.55, 10.2, and 25.5 µM, respectively). For the JA treatment, 0.05 mM or 0.5 mM JA (Sigma-Aldrich) aqueous solutions containing 0.05% Tween-20 and 0.1% methanol were prepared. Concentrations were chosen based on ALA concentrations used previously for *A. thaliana* induction (Dicke and Van Poecke, 2002; Chen et al., 2003) and JA concentrations used for *B. oleracea* by Bruinsma et al. (2009). For the ALA+JA treatment, the plants were sprayed with an aqueous solution containing six (i.e. 3 ALA×2 JA) dosage combinations of ALA (5, 20, and 50 µg ml\(^{-1}\)) and JA (0.05 mM and 0.5 mM corresponding to 10.515 µg JA ml\(^{-1}\) and 105.15 µg JA ml\(^{-1}\), respectively), all containing 0.05% Tween-20.

The upper surface of all leaves with a main vein longer than 4 cm were rubbed with carborundum powder on a moist cotton pad. Subsequently, the plants were immediately sprayed with 10 ml of a test solution. Control plants were likewise rubbed with carborundum powder, after which the plants were sprayed with 10 ml of an aqueous solution containing 0.05% Tween-20 and 0.1% methanol. The caterpillar treatment consisted of plants infested with five second-instar larvae of *P. brassicae*. Plants were treated for 24±2 h before use in the experiments.

**Preference behaviour of parasitoids**

Parasitoid wasp odour preference bioassays were conducted to compare the attractiveness of differentially induced plants in dual-choice experiments. The behaviour of *C. glomerata* was tested in a windtunnel as described by Geervliet et al. (1994). Three- to six-day-old female wasps were used for all experiments and were assumed to have mated. Female wasps were separated from male wasps on the day before the experiment. Before the experiment the wasps were provided with water and honey, but had no experience with plants or caterpillars. The wasps were released individually at ~60 cm distance downwind from the two plants. They were released on a small piece of herbivore-damaged leaf from which caterpillars and faeces had been removed; this served to increase the responsiveness of the wasps but does not affect their choice. After release, the parasitoid was observed in the windtunnel until it landed on one of the plants (choice). If the wasp did not land on either plant within 10 min it was recorded as not having made a choice (no choice) and was discarded from the statistical analysis. The position of plants in the windtunnel was alternated after a maximum of five tested wasps to exclude possible directional bias of the set-up. All two-choice combinations were tested on at least 5 d, with new sets of plants on each day, and each wasp was used only once. The windtunnel conditions were set at 27±1 °C, 65±15% RH, a light intensity of 24±2 µmol m\(^{-2}\) s\(^{-1}\) PAR (Quantum meter QMSW-SS, Apogee Instruments Inc., Logan, UT, USA), and a wind speed of 20 cm s\(^{-1}\) (Thermisches Anemometer, Wilh. Lambrecht GmbH, Göttingen, Germany). The choices of the parasitoids between two odour sources were statistically analysed using the binomial test.

**ALA treatment compared with mechanical damage and herbivore infestation:** The behavioural preference of parasitoids for all combinations of control plants, plants treated with ALA (20 µg ml\(^{-1}\)), and herbivore-infested plants was tested using the dual-choice windtunnel assay.

**Dose–response relationship:** The effect of different concentrations of ALA on the response of the parasitoids was tested. Plants treated with 10 ml of a solution containing 1, 5, 20, or 50 µg ml\(^{-1}\) ALA were tested against control plants in the windtunnel. The experimental set-up was the same as described above.

**Treatment with combinations of alamethicin and jasmonic acid:** To test the effect of combinations of ALA with JA, the preference of the wasps for ALA-, JA-, or ALA+JA-treated plants was compared. Six ALA/JA dosage combinations were tested against ALA only and against JA only. Furthermore, ALA treatment and JA treatment were tested against each other at the dosages in which they were mixed. All combinations were tested against each other in the dual-choice windtunnel assay.

**Collection of headspace volatiles**

For the chemical analysis of volatiles emitted by mechanically damaged Brussels sprouts plants treated with either 0.05 mM JA, 20 µg ml\(^{-1}\) ALA, 20 µg/ml ALA+0.05 mM JA, or control solution, a dynamic headspace collection system was used. A plant of one treatment was placed in a 30.0 l glass jar. The plastic pot was removed from the plant and replaced by aluminium foil just before the plant was placed in the jar. The jar was closed air-tight with a glass lid that was pressed on the jar with a metal clamp with a Viton® O-ring in between. The lid had an air inlet and an air outlet. Air was filtered over silica gel, molecular sieve 4 Å, and activated charcoal, and sucked into the jar using a vacuum pump. Teflon tubing was used for all connections. Before the experiments the jars were cleaned with water and ethanol and were then purged with filtered air overnight with a constant flow rate of 100 ml min\(^{-1}\). The flow through the jars was controlled by flow meters (Brooks Instruments, Veenendaal, The Netherlands). The system containing the plants was purged for 1 h with filtered air before the volatiles were collected. Air was sucked out of the jar at 40 ml min\(^{-1}\) by passing it through a glass tube filled with 100 mg of Tenax-TA (Grace-Alltech) connected to the air outlet of the jar. Headspace collections were made in a climate chamber at 23±1 °C, 60±10% RH, 95±5 µmol m\(^{-2}\) s\(^{-1}\) PAR. This light intensity was used to...
be similar to the one used in the windtunnel bioassays with the parasitoids, in order to keep the emission rates of the plants comparable between the two experiments. Plant volatiles were collected for 4 h. Volatiles of two plants were collected simultaneously, and six replicates per treatment were collected. Six blank controls were taken to determine which compounds were present in the background. To correct the volatile emission for plant biomass, fresh weights of all plants were determined immediately after the experiments.

Chemical analysis of headspace volatiles: Headspace samples were analysed with a Thermo TraceGC Ultra connected to a Thermo TraceDSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Before thermodesorption, traps were flushed with helium at 30 ml min⁻¹ for 3 min to remove moisture and oxygen. After flushing, the collected volatiles were desorbed from the Tenax traps at 250 °C (Ultra; Markes, Llantrisant, UK) for 5 min with a helium flow of 30 ml min⁻¹. The released compounds were focused on an electrically cooled sorbent trap (Unity; Markes) at a temperature of 0 °C. Volatiles were injected on the analytical column (Rtx-5ms, 30 m×0.25 mm ID, 1.0 μm film thickness, Restek, Bellefonte, PA, USA) in splitless mode by ballistit heating of the cold trap to 250 °C for 5 min. The temperature program started at 40 °C (4 min hold) and rose 4 °C min⁻¹ to 250 °C (4 min hold). The column effluent was ionized by electron impact (EI) ionization at 70 eV. Mass scanning was done from 33 to 300 m/z with a scan time of three scans per second. The eluted compounds were identified using Xcalibur software (Thermo Fisher Scientific) by comparing the mass spectra with those of authentic reference standards or with NIST 05 and Wiley library spectra. Linear retention indices were calculated for each compound according to van den Dool and Kratz (1963) and were compared with those published in the literature.

Statistical analysis

The quantitative composition of the volatile mixtures of differently treated Brussels sprouts plants was evaluated by principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) using the software program SIMCA-P 10.5 (Umetrics AB, Umeå, Sweden) (Wold et al., 1989; Eriksson et al., 2001). In PCA, so-called scores are obtained by projecting data observations onto model planes, which are defined by the extracted principal components (PCs). Raw data (integrated peak areas corrected for the fresh weight of the plants) were normalized, i.e. peak areas of all analysed compounds (X variables) were summed and the relative amount of each variable was calculated. The normalized data were transformed to log (X+0.00001). The constant 0.00001 was added to provide non-detectable components with a small non-zero value (Sjödin et al., 1989). Transformed variables were then mean-centred, scaled to unit variance, and represented as a matrix X. The ellipse shown in the score plot defines the Hotelling’s T² confidence region (95%). The number of significant PCs was determined by cross-validation (Wold et al., 1989; Eriksson et al., 2001).

The objective of PLS-DA is to find a model that discriminates the X data according to the plant treatments in the best possible way (Eriksson et al., 2001). PLS-DA is a supervised technique, so class memberships of the observations need to be pre-defined. Therefore, an additional Y matrix was made with G columns containing the values 1 and 0 as dummy variables for each of the plant treatments, respectively. The number of significant PLS components was determined by cross-validation (Wold et al., 1989; Eriksson et al., 2001). In addition, the variable importance in the projection (VIP) was calculated, which is a numerical value describing the importance of the X variables, for both the X and the Y parts (Wold et al., 1993, 2001). Variables with VIP-values >1 are considered most influential for the model (Eriksson et al., 2001; Paolucci et al., 2004).

Results

Parasitoid preference

*Cotesia glomerata* females significantly preferred the volatiles from ALA-treated plants to those from control plants (binomial test, n=37, P <0.001; Fig. 1). However, the females were significantly more attracted to caterpillar-infested plants when given a choice between caterpillar-infested plants and ALA-treated or control plants (binomial test: n=35, P <0.001; and n=31, P <0.001, respectively; Fig. 1).

Dose–response relationship: The wasps significantly preferred volatiles from plants treated with the three higher concentrations of ALA, i.e. 5 μg ml⁻¹ (P=0.008), 20 μg ml⁻¹ (P=0.001), and 50 μg ml⁻¹ (P <0.001), to the control plants. Only for the lowest concentration tested, 1 μg ml⁻¹,

![Fig. 1. Response of *Cotesia glomerata* females in dual-choice tests in the windtunnel to control plants, plants sprayed with 10 ml of a 20 μg ml⁻¹ alamethicin (ALA) solution, and plants infested with five *Pieris brassicae* caterpillars. The numbers to the right of each bar represent the number of parasitoids making a choice, and the total number of parasitoids used in the windtunnel tests is indicated in parentheses (**P <0.001)).](image-url)
did the wasps not display a preference ($P=0.37$; Fig. 2). The percentage of wasps attracted by the ALA-treated plants increased with concentration (binomial test, $P >0.05$; Fig. 3). At the low and intermediate concentrations of ALA ($5\,\text{mg}\,\text{ml}^{-1}$ and $20\,\text{mg}\,\text{ml}^{-1}$) in combination with the low JA dose (0.05 mM), the ALA+JA-treated plants attracted significantly more wasps than the ALA-treated plants ($P <0.05$), but not more than the JA-treated plants ($P >0.05$; Fig. 3A, B). In combination with a high JA dose (0.5 mM), the wasps did not prefer the combination to the single treatments (Fig. 3D, E). However, at the high concentration of ALA ($50\,\text{mg}\,\text{ml}^{-1}$), the combination with 0.5 mM JA attracted significantly more wasps than JA alone ($P <0.05$; Fig. 3F). For the other combinations of JA and the highest concentration of ALA against single compound treatments, a tendency for attraction towards the combination of ALA and JA was observed (Fig. 3C, F).

Volatile emission

Thirty-four compounds were detected in the volatile blends of the four plant treatments (Table 1). The blends contained terpenoids, esters, alcohols, an aldehyde, and ketones. Regardless of the treatment, four monoterpenes represented the major components of the volatile blends: limonene (21–31% of total blend), 1,8-cineole (14–16%), sabine (13–15%), and $\alpha$-thujene (8–21%). The blends from the differently treated plants show quantitative rather than qualitative differences.

A PCA based on the relative amounts of 33 compounds (excluding hexanal, because of co-elution with octane) resulted in a model with three significant PCs, explaining 69% of the variation of the data. A plot of the scores of the first two PCs indicates that treating plants with JA or with a combination of JA+ALA induces volatile blends dissimilar from plants sprayed with ALA or control solution (Fig. 4). Volatile blends emitted by plants sprayed with JA are similar to those emitted by JA+ALA-treated plants (Fig. 4). Volatiles emitted by plants sprayed with ALA showed the largest variation (Fig. 4).

Further analysis of the data by PLS-DA was used to determine whether any two treatments differ from each other. Differences in the composition of the volatile blends were significant for all tested combinations, as at least one significant PLS component was extracted by cross-validation; except for the comparison JA versus JA+ALA which could not be separated (Table 2). For two well-separated groups ($G=2$) one would expect $G–1$ significant PLS components (Eriksson et al., 2004). More PLS components can indicate subclustering of the volatile blends. The volatile blends of JA and ALA treatments differed significantly in total emission; compounds such as: 2-pentenyl acetate, $\alpha$-pinene, $\alpha$-phellandrene, 1,8-cineole, $\gamma$-terpinene, $\alpha$-terpinolene, alloocimene, and (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) were emitted in higher amounts by JA-treated plants compared with ALA-treated plants (VIP $>1$). Compounds with the least influence on the separation of the groups (VIP $<0.5$) were: TMTT, 3-pentanone, MeSA, and (Z)-3-hexen-1-ol (PLS-DA JA versus ALA).

Discussion

So far, only a few studies have shown that ion channel-forming peptides of fungal origin may act as inducers of an early step in plant defence signal transduction resulting in defence responses of plants against different attackers. For example, treatment of *Nicotiana tabacum* with chrysospermin (produced by *Apiocrea* sp.) resulted in increased resistance against tomato mosaic virus infection (Kim et al., 2000), and two peptides from *Trichoderma virens* induced systemic protection against leaf bacteria in cucumber (Viterbo et al., 2007). ALA induces volatile emission in Lima bean (*Phaseolus lunatus*) and *A. thaliana*, as well as an increase in endogenous levels of plant hormones such as JA and SA (Engelberth et al., 2001; Chen et al., 2003). So far, only one study addressed the effect of ALA treatment on arthropod behaviour (M Dicke and H Dijkman, described in Dicke and Van Poecke, 2002). They showed that predatory mites (*Phytoseiulus persimilis*) prefer ALA-treated over control Lima bean plants. The induced volatiles in ALA-treated Lima bean plants: TMTT, DMNT, MeSA, and a trace amount of linalool (Engelberth et al., 2001), have been shown to be important for prey-searching behaviour of predatory mites (Dicke et al., 1990; De Boer et al., 2004). However, another compound attractive to predatory mites, the monoterpe (E)-$\beta$-ocimene, and
transcript levels of a (E)-β-ocimene synthase are not induced by ALA in the leguminous species Lima bean and Lotus japonicus (Arimura et al. 2004, 2008).

The parasitoid C. glomerata responds to herbivore-induced plant volatiles from Brassicaceous plants (Blaakmeer et al., 1994; Geervliet et al., 1996) and is also attracted to B. oleracea plants that are artificially induced with JA (Bruinsma et al., 2009). In this study, treatment of Brussels sprouts plants with ALA induces the emission of volatiles that attract parasitoid wasps. The parasitoid wasp C. glomerata preferred ALA-treated Brussels sprouts plants over control plants in three out of four concentrations tested. ALA treatment of Brussels sprouts plants did not result in higher emission rates of TMTT, DMNT, and MeSA as it did in Lima bean (Table 1; Engelberth et al., 2001). Because of the large variation in volatile emission after ALA treatment of Brussels sprouts plants recorded here, it is difficult to determine which compounds are responsible for the difference in preference of the parasitoids. It is not known whether the parasitoids respond to specific attractive compounds, or to ratios of attractive and repellent compounds, and whether responses increase with concentration above a certain threshold. Several studies suggest that green leaf volatiles, such as (Z)-3-hexen-1-ol, (E)-2-hexenal, and (Z)-3-hexenyl acetate, are important for the attraction of C. glomerata, but also other compounds such as terpenes have been suggested as attractants, and sulphur compounds as repellents (Smid et al., 2002; Fatouros et al., 2005; Scascighini et al., 2005; Shiojiri et al., 2006a, b; Soler et al., 2007). The total volatile emission of JA- and JA+ALA-treated plants was larger than that of control and ALA-treated plants. Possibly, the higher volatile emission rate of JA+ALA-treated plants is responsible for the preference of the parasitoids for these plants over ALA-treated plants. Yet, a higher volatile emission rate cannot explain the observed parasitoid choices in all tests. An unexpected result relative to the composition of the volatile blends is the similar response of the parasitoids to ALA- and JA-treated plants, especially so in view of the fact that ALA was applied at a molar dose

Fig. 3. Effect of combinations of alamethicin and jasmonic acid compared with the effects of either inducer alone on behavioural responses of Cotesia glomerata parasitoids in the windtunnel. The numbers to the right of each bar represent the number of parasitoids making a choice, and the total number of parasitoids used in the windtunnel tests is indicated in parentheses (*P <0.05).
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#### Table 1. Volatile compounds detected in the headspace of mechanically damaged Brussels sprouts plants sprayed with Tween-20 (control), or with a 10 ml solution of 20 μg ml⁻¹ alamethicin (ALA), 0.05 mM jasmonic acid (JA), or a mixture of 20 μg ml⁻¹ ALA and 0.05 mM JA, all three solutions also containing Tween-20 (n=6 per treatment)

Mean ±SE of GC peak area (1000 units g FW).

| Compound | Control | ALA | JA | ALA+JA |
|----------|---------|-----|----|-------|
| 1 (Z)-3-Hexen-1-ol | 5.7±3.8 | 15.2±3.9 | 31.0±9.9 | 42.6±4.6 |
| 2 1-Hexanol | 8.7±0.7 | 11.7±1.7 | 11.1±2.0 | 12.9±1.4 |
| 3 Hexanal* | 58.9±6.5 | 61.0±4.4 | 59.3±7.5 | 53.3±5.4 |
| 4 n-Butyl acetate | 22.0±2.5 | 22.1±3.9 | 21.3±4.6 | 18.7±2.2 |
| 5 2-Pentenyl acetate | 2.5±2.5 | 9.6±4.4 | 33.1±7.6 | 28.3±3.6 |
| 6 (Z)-3-Hexen-1-yl acetate | 40.8±16.0 | 56.9±22.2 | 390.2±138.0 | 331.5±85.0 |
| 7 Hexyl acetate | 1.9±1.9 | 7.0±1.9 | 22.1±4.4 | 25.2±4.6 |
| 8 Methyl salicylate | 27.5±8.4 | 23.3±3.5 | 21.2±3.4 | 30.7±6.6 |
| 9 3-Pentanone | 16.0±2.3 | 22.8±2.4 | 34.7±11.0 | 42.6±7.5 |
| 10 3-Methyl-2-pentanone | 2.9±1.2 | 2.3±0.9 | 6.9±1.0 | 6.9±1.3 |
| 11 2-Hexanone | 14.8±2.3 | 10.8±1.1 | 11.0±1.9 | 9.0±2.2 |
| 12 3-Heptanone | 12.5±2.7 | 9.8±2.5 | 7.3±1.9 | 8.4±0.8 |
| 13 2-Heptanone | 6.1±1.1 | 3.5±0.7 | 5.0±1.1 | 4.2±0.3 |
| 14 α-Thujene | 256.1±20.0 | 214.9±35.2 | 1390.5±1000.8 | 457.4±48.1 |
| 15 α-Piene | 52.0±2.4 | 50.9±4.0 | 81.8±8.6 | 78.5±6.2 |
| 16 Thuja-2,4(10)-diene | 4.3±0.4 | 3.0±1.0 | 5.1±0.7 | 3.9±1.0 |
| 17 Sabinene | 460.6±49.2 | 404.4±78.6 | 855.1±118.3 | 834.9±72.8 |
| 18 β-Piene | 72.3±9.9 | 59.9±9.1 | 97.7±10.9 | 100.7±6.8 |
| 19 α-Mycene | 107.7±18.0 | 99.2±25.7 | 179.0±39.4 | 215.5±32.9 |
| 20 α-Phellandrene | 27.8±3.0 | 21.8±4.9 | 60.7±9.9 | 52.4±3.4 |
| 21 3-Carene | 9.3±0.6 | 8.6±0.8 | 9.9±2.0 | 9.7±0.8 |
| 22 α-Terpine | 320.7±203.2 | 91.7±15.7 | 206.5±48.7 | 227.5±15.4 |
| 23 Limonene | 810.6±118.0 | 885.2±229.7 | 1369.5±267.4 | 1379.9±141.8 |
| 24 1,8-Cineole | 485.6±40.6 | 404.8±71.1 | 911.0±124.0 | 906.9±76.2 |
| 25 γ-Terpine | 114.0±11.2 | 93.3±20.2 | 237.1±38.3 | 226.3±16.8 |
| 26 α-Terpineol | 108.7±6.7 | 83.3±11.9 | 167.3±25.3 | 165.9±11.0 |
| 27 p-Mentha-1,8-dien-6-ol, l-carveol | 22.8±1.3 | 22.9±2.4 | 29.7±3.6 | 29.1±1.4 |
| 28 Alloocimene | 16.4±0.8 | 13.0±0.6 | 22.5±2.5 | 25.6±2.4 |
| 29 (β,4,8-Dimethyl-1,3,7-triene | 15.0±4.5 | 13.6±3.8 | 31.0±3.8 | 27.3±3.7 |
| 30 p-Carveone | 9.0±1.6 | 10.1±2.6 | 16.7±3.4 | 14.6±2.2 |
| 31 p-Cymen-9ol | 4.3±1.3 | 4.7±1.5 | 10.1±1.7 | 9.0±1.0 |
| 32 Thymol | 9.1±1.1 | 7.8±2.3 | 14.4±1.9 | 12.7±1.8 |
| 33 Isolongifolene/aromadendrene | 14.9±1.2 | 14.6±1.8 | 14.3±3.3 | 13.4±1.2 |
| 34 (E,E)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene | 14.2±5.5 | 19.9±5.2 | 12.8±3.9 | 31.4±9.1 |

| Total | 3248.9±313.0 | 2884.2±546.3 | 6473.8±159.8 | 5543.5±452.3 |

* Peak area estimated due to co-elution with octane.

† DMNT.

‡ TMTT.

20 times lower than that of JA. The total volatile emission and composition differed significantly between these two treatments; a range of compounds were emitted at higher rates by JA-treated plants compared with ALA-treated plants (Table 1). However, several compounds were emitted at similar rates in the two treatments; these compounds might be sufficient for the attraction of the parasitoids to the plants. Compounds that occurred in similar amounts had the least influence on the statistical separation of the groups are TMTT, 3-pentanone, MeSA, and (Z)-3-hexen-1-ol.

In Lima bean, both JA and SA are induced by ALA treatment (Engelberth et al., 2000). There is growing evidence that the JA and SA pathways can negatively interact with each other, e.g. in tomato (Peña-Cortes et al., 1993; Doares et al., 1995; Thaler et al., 2002b), tobacco (Niki et al., 1998; Felton et al., 1999; Rayapuram and Baldwin, 2007), and *A. thaliana* (Gupta et al., 2000; Traw et al., 2003; Cipollini et al., 2004). However, other studies show that the interactions between signalling pathways are not always negative, depending on the dose and timing of
application of the inducing compound, and the response measured (Niki et al., 1998; Schenk et al., 2000; Thaler et al., 2002a, b). For the Brassicaceous plant A. thaliana it was shown that both JA and SA are involved in the induced attraction of the parasitoid C. rubecula to P. rapae-infested plants (Van Poecke and Dicke, 2002).

An increase in SA due to ALA treatment inhibits the octadecanoid pathway between OPDA and JA in Lima bean plants; however, due to the slow increase in SA, inhibition occurs only after several hours, and thus after the typically transient JA burst (Engelberth et al., 2001). If ALA treatment would have a similar effect on Brussels sprouts plants, addition of JA to ALA-treated plants could compensate for the inhibition of the octadecanoid pathway by ALA. Indeed, in the present experiments, addition of JA to ALA treatment of plants generally increased the attractiveness of plants to the parasitoids compared with ALA-treated plants, significantly so at two ALA concentrations (5 μg ml\(^{-1}\) and 20 μg ml\(^{-1}\) ALA) in combination with 0.05 mM JA (Fig. 3) and marginally significantly (0.058 < P <0.088) in the other four combinations. Comparison of the behavioural responses in dual-choice tests with JA+ALA-treated plants versus JA-treated plants, however, did not yield such a clear-cut result. Only in the combination of JA and the highest concentration of ALA (50 μg ml\(^{-1}\)), did ALA increase attractiveness, although less strongly at the lower JA concentration (0.05 mM) than at the higher JA concentration (0.5 mM) (Fig. 3C, F). These data indicate that ALA increased parasitoid attraction at a molar dose 20 times lower than the JA dose to which it was added.

Phenotypic manipulation through the use of fungal inducers as well as phytohormones can increase our understanding of the signal transduction of plant defence responses and can provide more insight into the use of volatile cues in host searching by carnivorous arthropods. This is clear for the use of ALA in the studies described earlier with Lima bean, in which ALA induces a qualitatively different volatile blend from control plants, and the induced compounds were shown to be attractive to predatory mites (Dicke et al., 1990; Engelberth et al., 2001; Dicke and Van Poecke, 2002; De Boer et al., 2004). For Brussels sprouts plants, the regulatory network seems to differ from that of Lima bean as well as from that of A. thaliana, and results in quantitative rather than qualitative differences. In this study, ALA treatment induced a volatile blend in Brussels sprouts plants different from that induced by mechanical damage alone. The parasitoids were attracted

| Comparison  | No. of significant PLS components | \(R^2_X\) (cum) | \(R^2_Y\) (cum) | \(Q^2\) (cum) |
|-------------|-----------------------------------|----------------|----------------|----------------|
| ALA versus Ct | 4                                 | 0.767          | 0.992          | 0.821          |
| ALA versus JA | 2                                 | 0.688          | 0.9            | 0.722          |
| ALA versus ALA+JA | 4                                 | 0.83           | 0.99           | 0.882          |
| JA versus Ct | 2                                 | 0.667          | 0.864          | 0.612          |
| JA versus ALA+JA | 0                                 | 0.608          | 0.64           | -0.112         |
| ALA+JA versus Ct | 1                                 | 0.597          | 0.936          | 0.767          |
to the ALA-treated plants, demonstrating that ALA, as an elicitor of an early step in induced plant defence, induces a volatile blend that is attractive to parasitoids. Although JA treatment induced higher volatile emissions than ALA treatment, this resulted in equal attractiveness to parasitoids; yet JA was applied at a substantially higher dose. A combination of ALA and JA further increased the attractiveness of the plants to parasitoids at 20 μg ml⁻¹ or 5 μg ml⁻¹ ALA and 0.05 mM JA. Combining different treatments as presented here allows comparisons of the relative importance of specific steps of the signal transduction pathways for both plant volatile emission and indirect induced defence provided by parasitoid attraction.

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