Insect egg deposition induces Pinus sylvestris to attract egg parasitoids

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Summary

Plant volatiles released in response to feeding insects are known to attract enemies of the feeding herbivores. In this study, egg deposition by a herbivorous insect was shown to induce a gymnosperm plant to emit volatiles that attract egg parasitoids. Odour from twigs of Pinus sylvestris laden with egg masses of the pine sawfly Diprion pini attracts the eulophid egg parasitoid Chrysonotomyia ruforum. Volatiles released from pine twigs without diprionid eggs are not attractive. Oviposition by the sawfly onto pine needles induces not only a local response in pine needles laden with eggs but also a systemic reaction. Needles without eggs but adjacent to those bearing diprionid eggs also release the volatiles that attract the egg parasitoid. The eliciton of the attractive volatiles was shown to be present in the oviduct secretion coating the eggs of D. pini. When pine twigs are treated with jasmonic acid, a well-known plant wound signal, they emit volatiles that attract the egg parasitoid. These results show, for the first time, that a gymnosperm plant is able to attract parasitoids as soon as a herbivore has deposited its eggs on it. Thus, the plant appears to defend itself against herbivores prior to being damaged by feeding larvae.

Key words: induced plant response, induced defence, oviposition, insect/plant interaction, herbivore, sawfly, Diprionidae, Diprion pini, parasitoid, Eulophidae, Chrysonotomyia ruforum, Pinus sylvestris.

Introduction

Numerous studies have shown that damage caused by feeding herbivorous arthropods may induce defensive responses in plants (for reviews, see Karban and Baldwin, 1997; Tollrian and Harvell, 1998; Agrawal et al., 1999). On the one hand, these defensive responses may act directly against the herbivores by feeding deterrence, by impairing digestion or by producing toxins with targets that are not directly involved in food uptake and digestion (Duffey and Stout, 1996; Turlings and Benrey, 1998; Baldwin and Preston, 1999; Sabelis et al., 1999). On the other hand, the induced defensive responses of plants may act indirectly by inducing the release of plant volatiles that attract arthropod enemies of herbivorous insects and spider mites (Turlings et al., 1990; Tumlinson et al., 1993; De Moraes et al., 1998; Dicke and Vet, 1999).

The enemies of herbivorous insects have evolved the ability to respond to plant volatiles induced by herbivore attack. From the plant’s point of view, the emission of such volatiles may be of benefit if attraction of predators or parasitoids reduces the damage inflicted on the plant by the herbivores (Chattopadhayay et al., 2001). A few studies have shown that plants attracting larval parasitoids by the feeding-induced release of volatiles experience the advantage of a significantly higher seed production compared with plants fed upon by unparasitized herbivorous larvae (van Loon et al., 2000; Fritzsche-Hoballah and Turlings, 2001). Thus, the emission of plant volatiles has also been interpreted as a ‘call for help’ (Dicke and Sabelis, 1992). Nordlund and Lewis (1976) used the term synomones for chemicals that are favourable for both the emitter (here the plant) and the receiver (here the enemies of herbivores).

Studies on the mechanisms of induction of the emission of plant synomones have revealed that feeding induces plant reactions locally at the site of damage and systemically in leaves adjacent to those being attacked (Turlings and Tumlinson, 1992; Dicke, 1994; Röse et al., 1996). The herbivore’s oral secretion released during feeding contains the elicitor (Mattiacci et al., 1995; Turlings et al., 1995). Two classes of compound have been isolated from larval oral secretion and shown to elicit the emission of plant synomones when applied to mechanically damaged plant tissue: β-glucosidase from larvae of Pieris brassicae (Mattiacci et al., 1995) and fatty acid–amino acid conjugates from larvae of Spodoptera exigua (Alborn et al., 1997, 2000; Turlings et al., 2000) and Manduca sexta (Baldwin et al., 2001; Halitschke et al., 2001). The enzyme β-glucosidase is thought to act both by hydrolysing constitutive glycosides and by inducing de novo production of volatiles (Hopke et al., 1994; Felton and Eichenseer, 1999). Volicitin isolated from S. exigua larvae is a conjugate of plant-derived 17-hydroxylated linolenic acid and glutamine, which is of insect origin (Paré et al., 1998). The quantity and quality of volatiles emitted in response to insect
feeding have been shown to be specific for the plant and herbivore species and the age of the plant and the herbivore (Takabayashi et al., 1994; Takabayashi and Dicke, 1996; De Moraes et al., 1998).

Jasmonic acid and its precursors are known to act as wound signals in plant responses induced by herbivore feeding (Karban and Baldwin, 1997; Koch et al., 1999) (see also references therein). The herbivore-induced emission of volatiles can be mimicked by the application of jasmonic acid (Boland et al., 1992, 1995; Hopke et al., 1994). However, the pattern of volatiles induced by jasmonic acid is not identical to that induced by feeding (Dicke et al., 1999).

In addition to feeding by herbivorous arthropods, egg deposition may also induce plant responses. In a previous study, we demonstrated that egg deposition by the elm leaf beetle (Xanthogaleruca luteola) on elm (Ulmus minor) leaves induces the plant to emit volatiles that attract the eulophid egg parasitoid Oomyzus gallaeciae, which specializes on the eggs of the elm leaf beetle (Meiners and Hilker, 1997, 2000). No further studies have been published that demonstrate the induction of plant synomones by herbivore egg deposition.

The present study investigated whether a gymnosperm is able to respond to oviposition by a herbivorous insect by emitting synomones that attract egg parasitoids. The tritrophic system studied was the pine Pinus sylvestris, the diprionid sawfly Diprion pini and the eulophid parasitic wasp Chrysonotomyia ruforum, which specializes on diprionid eggs (Psborn-Walcher and Eichhorn, 1973; Eichhorn and Psborn-Walcher, 1976; Juutinen and Varama, 1986). We investigated this system from the perspective of each of the three trophic levels.

(i) From the plant’s point of view, we investigated (a) whether the plant responds to the sawfly’s egg deposition by emitting volatiles from the site of oviposition (local response), (b) whether plant parts without eggs, but adjacent to those bearing host eggs, also emit synomones (systemic response and proof of induction), and (c) whether jasmonic acid, the well-known wound signal in feeding-induced plant responses (see above), plays a role in the induction of synomones by egg deposition in pine.

(ii) Further experiments on the mechanism of induction of synomones by oviposition in this system focused on the herbivore’s oviposition behaviour and on how the herbivore’s oviposition activity induces the plant’s response. The sawfly female slits the pine needle tangentially using her sclerotized ovipositor valves prior to egg deposition (see Fig. 1A); we tested whether mechanical damage caused by slitting releases attractive volatiles. The oviduct secretion (see Fig. 1B), which coats the eggs of D. pini, and another greenish secretion of meringue-like consistency that covers the egg row within a needle (approximately 10–20 eggs per needle) were studied for the presence of an elicitor of the plant’s emission of volatiles in response to egg deposition.

(iii) From the parasitoid’s point of view, we wanted to know whether C. ruforum is able to respond to volatiles from a pine twig bearing eggs of D. pini and, thus, may be able to use them to find a host. Such oviposition-induced plant volatiles could provide highly reliable chemical information for egg parasitoids to find their host eggs. Host-associated chemicals such as sex pheromones have been considered as reliable cues enabling egg parasitoids to locate their host (Vet and Dicke, 1992; Godfray, 1994; Quickie, 1997; Vinson, 1998). The egg parasitoid C. ruforum is known to respond positively to the sex pheromones of the host sawflies D. pini and Neodiprion sertifer (Hilker et al., 2000). Such host pheromones might attract the egg parasitoid to the (micro)habitat of its host. Orientation in response to oviposition-induced plant volatiles might allow the eggs to be located within the habitat. Most insect parasitoids perform the later steps of host-finding behaviour within a habitat by walking (Vinson, 1984; Wajnberg, 1994). We therefore investigated the response of walking egg parasitoid females of C. ruforum to volatiles released from pine twigs bearing host eggs.

**Materials and methods**

**Plants**

Twigs of Pinus sylvestris L. were detached from 30- to 40-year-old trees in the forests near Berlin several days prior to the experiments. They were kept in tap water at 10 °C, 18h:6h L:D and 2000 lx. All tested twigs were approximately 20cm long and had approximately 160–190 needles, with the exception of the systemically induced twig and its control (see below). After treatment, pine twigs were transferred to an olfactometer (see below) to test the parasitoid’s response to pine volatiles.

**Insects**

The parasitoids Chrysonotomyia ruforum (Krauss) and sawflies Diprion pini (L.) were collected in the field and kept in the laboratory as described by Hilker et al. (2000) and Bombsch and Ramakers (1976). Only female parasitoids that had had prior contact with sawfly eggs on a pine twig for a period of 24 h were tested. After this exposure period, the parasitoids were kept isolated from host eggs on a pine twig in a Petri dish for a further 24 h. They were then used in bioassays.

**Olfactometer bioassays – general procedure and data evaluation**

A four-arm olfactometer was used for all bioassays, as described by Vet et al. (1983) and modified by Meiners and Hilker (1997). The air blown into the olfactometer by a pump was cleaned with a charcoal filter and humidified by passage through a glass cylinder filled with water. Before entering the olfactometer, the odorless, humidified air was separated into four flows (156 ml min⁻¹), each of which was directed to one of the four olfactometer fields. The air flowing into the test field of the olfactometer passed through a glass cylinder (250 ml) into which a pine twig had been placed. The cut end of the twig was wrapped in Parafilm to prevent the emission of odour from resin emerging from the cut end. The three control fields were provided with air that had been passed through glass cylinders.
containing a piece of Parafilm (the same size as that used to wrap the twigs). The air left the olfactometer through a small opening in the centre of the chamber.

At the start of each bioassay, a female parasitoid was placed into the exposure chamber of the olfactometer. We recorded how long the parasitoid spent walking within each of the four odour fields over a period of 600 s. Data for females that walked for less than 50% of the observation time were discarded. The odour source was changed after 5–9 females had been tested. In total, the responses of 22–30 females to volatiles from each pine twig treatment were tested.

The observations were recorded with help of the Noldus Observer program 3.0 (Wageningen, The Netherlands) (Noldus, 1991). The durations of walking within each of the four fields were compared statistically using the Friedman analysis of variance (ANOVA) and the Wilcoxon–Wilcoxon test for multiple comparisons (Sachs, 1992). A parasitoid that prefers to walk within the test field may be attracted to and/or arrested there by the test odour. Nevertheless, significantly longer walking periods in the test olfactometer field than in the three control fields is usually interpreted as a response of the actively walking parasitoid to an attractive odour (Vet et al., 1983; Meiners and Hilker, 1997, 2000). Thus, we term an odour ‘attractive’ when the parasitoid prefers walking in the olfactometer field provided with this odour.

**Pine twig treatments**

**Pine twig with host eggs**

A twig provided with tap water was placed into a small plastic box (20 cm × 20 cm × 8 cm) covered by a gauze lid. Two female and two male *D. pini* were added. After a treatment period of 72 h at 25 °C, 18 h:6 h L:D and 2000 lx, the twig carried 8–12 egg masses. To obtain an egg-free pine twig as a control, a twig was kept at the same abiotic conditions, but in the absence of sawflies.

**Systemically induced pine twig**

For this treatment, a pine twig was exposed to two male and two female *D. pini* as described above. The upper half of the twig was covered with polyethylene terephthalate (PET) foil to prevent egg deposition and adsorption of volatiles released from the lower half of the twig or from the sawflies (Hilker et al., 2000). Two small tubes (diameter 5 mm) were placed into the foil bag. Air that had passed through a charcoal filter was blown into the foil bag by a small pump (110 ml min⁻¹) via one of these tubes, while the other one served as a port for continuous efflux of air. This procedure ensured the presence of normally ventilated air around the foil-covered part of the twig. After 72 h, the lower half of the twig carried approximately 8–11 egg masses and was cut off. The egg parasitoid’s response to the volatiles released from the upper half of the test twig (approximately 10 cm long) was recorded after removal of the foil. As a control, the upper half of a pine twig was covered with PET foil while no sawflies were present in the box and no eggs were present on the lower half of the twig. The PET bag of this control twig was ventilated as described above. Three days after covering the upper half of the twig with foil, this half was cut and tested after removal of the foil as described above.

**Slit pine needles**

Eight to 10 needles of a pine twig were slit using a scalpel. The depth and length of the slit mimicked those made by an ovipositing female *D. pini* with her ovipositor valves. Prior to egg deposition, the female usually slits the needle tangentially, thereby wounding the epidermis, the parenchymatous tissue and the endodermis as well as one of the vascular bundles (Fig. 1B). After slitting the needles for this experiment with a scalpel, the twig was kept in tap water for a treatment period of 72 h under the same conditions as described above for the oviposition-induced needles.

**Slit pine needles plus covering secretion**

The needles of a pine twig were artificially slit 72 h prior to the experiment, as described above, and treated with the egg-covering secretion of *D. pini*. The abdominal gland producing and storing the covering secretion has been described in detail by Eliescu (1923). We dissected the gland from a gravid female of *D. pini* and applied this secretion to the artificial slits of two pine needles using a dissection needle. In total, 8–10 needles per twig were treated in this way. The treated twig was kept in tap water prior to the bioassay, as described above.

**Slit pine needles plus oviduct secretion**

Instead of the covering secretion, the secretion from the oviduct of *D. pini* was applied to the artificial slits in the needles. The oviduct secretion was scraped out of the oviduct of a gravid female. Again, the secretion of one female was applied to the slits of two needles.

**Treatment of pine twig with jasmonic acid**

Pine twigs were supplied for a period of 72 h with an aqueous Tween solution (0.05 %) containing 0.3 μmol ml⁻¹ racemic jasmonic acid (Sigma, Germany) through the cut stem of the test twig. Volatile plant emissions are known to be inducible without obvious induction of senescence by jasmonic acid at concentrations of 0.1–2 μmol ml⁻¹ (Hopke et al., 1994; Boland et al., 1995; Gols et al., 1999) when supplied in the water taken up by the plant. Control twigs were supplied with the aqueous Tween solution only.

**Results**

The egg parasitoid *C. ruforum* was attracted by volatiles emitted from pine needles laden with eggs of the sawfly *D. pini*. In contrast, odour from a twig without host eggs was not attractive (Fig. 2A,B). The attractiveness of a pine twig bearing eggs of *D. pini* was not restricted to needles laden with eggs. Pine needles without eggs but adjacent to those laden with eggs also emitted the volatiles that attract the egg parasitoid (Fig. 2C). This result clearly indicates systemic induction of pine synomones in response to egg deposition by *D. pini*. Since
the systemically induced pine twig had been wrapped in PET foil, a control experiment was conducted to determine whether wrapping in foil had any effect: no such effect was found (Fig. 2D).

The remaining experiments were intended to elucidate the mechanism of the inductive process. Artificial slitting of pine needles mimicking that performed by a female *D. pini* when inserting her eggs did not result in the release of attractive volatiles (Fig. 3A). The bioassay for the effect of the egg-covering secretion in synomone induction revealed that artificially slit pine needles with covering secretion applied to the slits did not emit volatiles attractive to the egg parasitoid (Fig. 3B). However, the egg parasitoids were attracted to volatiles released from small pine twigs with slit needles into which oviduct secretion had been applied (Fig. 3C). These results indicate that the oviduct secretion coating the eggs of *D. pini* contains an elicitor that induces the emission of synomones from pine needles.

Treatment of pine twigs with jasmonic acid revealed that a concentration of 0.3 μmol ml⁻¹ in aqueous Tween solution mediates the release of volatiles that attract the egg parasitoid (Fig. 4A). The control pine twig supplied with Tween solution only did not attract the parasitoids (Fig. 4B).

**Discussion**

Our findings show that egg deposition by the sawfly *D. pini* induces the pine locally and systemically to emit volatiles that attract the egg parasitoid *Chrysonotomyia ruforum*. These results parallel the findings in another tritrophic system studied by us. The elm responds systemically to egg deposition by a herbivorous insect, the elm leaf beetle, and emits volatiles that attract an egg parasitoid of the elm leaf beetle (Meiners and Hilker, 1997, 2000). The mechanisms of induction are similar in elm and pine. The oviduct secretion of both the elm leaf beetle and the pine sawfly elicit the emission of plant volatiles. Mechanical

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**Fig. 1.** (A) A female *Diprion pini* laying eggs on a pine needle. Eggs are laid sequentially in a row (mass) of approximately 10–20 eggs and are covered by a greenish secretion. (B) Cross section through a needle of *Pinus sylvestris* bearing an egg deposition of the sawfly *D. pini*. The ovipositing female slits the needle with her ovipositor valves starting at the epidermis (E) of one edge of the needle (arrow) and then cutting the parenchyma (Pa), the endodermis (En), the transfusion tissue (Tr) and one of the vascular bundles (V). After removal of the plant tissue, an egg is placed inside the needle. Ch, egg chorion with oviduct secretion; Y, egg yolk. Finally, the eggs are covered by a secretion (C) from an abdominal gland. Staining with Astrablau (Aldrich, Germany) and Safranin T (Fluka, Germany). Light micrograph. Scale bar, 0.43 mm.

**Fig. 2.** Induction of plant synomones by egg deposition. Responses of egg parasitoid female *Chrysonotomyia ruforum* to volatiles from differently treated (A–D) twigs of *Pinus sylvestris* offered in a test field (T) consisting of a four-arm olfactometer with three control fields supplied with clean air (1, 2 and 3). Mean values for the time the parasitoid females spent walking in the test and control fields are given over an observation period of 600 s. Values are means ± S.D. The dashed line is the mean time if the duration of walking had been equal in the test and control fields. *** indicates a significant (*P*<0.001) and NS a non-significant (*P*>0.05) difference evaluated by a Friedman ANOVA. Different letters indicate significant (*P*<0.05) differences evaluated by the Wilcoxon–Wilcox test.
wounding of elm leaves does not induce the release of these attractive volatiles, as we have shown here for pine needles.

The application of jasmonic acid to both elm leaves and pine needles mediates the release of synomones that attract their respective egg parasitoids (Meiners and Hilker, 2000). This result indicates that jasmonic acid may be involved in the plant signalling process elicited by egg deposition. Jasmonic acid has been identified in numerous angiosperms and gymnosperms (Sembdner and Parthier, 1993). We do not know whether endogenous levels of jasmonic acid increase after egg deposition, as has been shown for other mechanically and feeding-damaged plants (Karban and Baldwin, 1997; Staswick and Lehman, 1999).

Oviduct secretion was shown to elicit the release of pine synomones when applied to slit pine needles. This is similar to the effect of the oral secretion of herbivorous larvae in the induction of plant responses to damage. When artificially damaged leaves are treated with oral secretion, they release synomones that attract larval parasitoids (Turlings et al., 1990).

Fig. 3. Mechanism of synomone induction. Responses of egg parasitoid female Chrysonotomyia ruforum to volatiles released from differently treated (A–C) twigs of Pinus sylvestris offered in a test field (T) consisting of a four-arm olfactometer with three control fields supplied with clean air (1, 2 and 3). Mean values for the time the parasitoid females spent walking in the test and control fields are equal in the test and control fields. *** indicates a significant (P<0.01) and NS a non-significant (P>0.05) difference evaluated by Friedman ANOVA. Different letters indicate significant (P<0.05) differences evaluated by the Wilcoxon–Wilcox test.

Not only does the oral secretion elicit the release of synomones when applied directly onto wounded leaves, but emission of synomones was also observed when the cut stem of a plant with undamaged leaves was placed into water containing the oral secretion (Turlings et al., 1993). Wounding of the leaves prior to oviposition has also been observed in the elm leaf beetle (Meiners and Hilker, 2000). In contrast to the pine sawfly, the elm leaf beetle does not use its ovipositor valves to slit the leaf, but uses its mouthparts to remove the epidermis of elm leaves prior to oviposition. The transfer of elm leaf beetle eggs contaminated with oviduct secretion to undamaged elm leaves did not induce the emission of synomones attractive to the egg parasitoid (Meiners and Hilker, 2000). The role of the slitting process in the induction of pine synomones by egg deposition by D. pini needs further examination. The identification of the elicitor in the oviduct secretion of both the pine sawfly D. pini and the elm leaf beetle and the elucidation of its mode of action will be the subject of future studies.

The identity of the pine volatiles induced by egg deposition by the sawfly is currently under investigation. Induction of terpenoid synthesis in woody tissue of conifers is known to be triggered by the feeding activity of herbivores such as bark beetles (Gershenzon and Croteau, 1991). In conifer needles, the induction of monoterpene synthesis by herbivory was documented for the first time by Litvak and Monson (1998). Chemical analyses of oviposition-induced elm leaves revealed a very complex change in the pattern of volatiles. When comparing the headspace of egg-bearing elm leaves and those without eggs, the oviposition-induced leaves changed their...
pattern of emitted volatiles both qualitatively, by emitting components exclusively present in the headspace of induced plants, and quantitatively, by changing the relative amounts of specific volatiles and, therefore, their ratios within the blend (Wegener et al., 2001).

From the results presented here and previous studies (Meiners and Hilker, 1997, 2000), we can conclude that the induction of plant synomones by egg deposition by a herbivorous insect occurs in both angiosperms and gymnosperms. This production of synomones in response to egg deposition may be considered as a preventive plant defence strategy that acts prior to (further) damage by feeding of herbivorous larvae. However, no studies have examined whether the ability of a plant to respond to insect egg deposition by releasing synomones benefits the plants. Only perennial species have been shown to produce oviposition-induced synomones. However, we expect annual plants also to show this ability. These short-living plants may benefit even more from the release of oviposition-induced synomones to prevent feeding damage by larvae because of the limited period in which they can compensate for loss of fitness caused by feeding damage.

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References
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