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Insect egg deposition induces defence responses in *Pinus sylvestris*: characterisation of the elicitor

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Summary

Egg deposition by the phytophagous sawfly *Diprion pini* L. (Hymenoptera, Diprionidae) is known to induce locally and systemically the emission of volatiles in Scots pine (*Pinus sylvestris* L.) that attract the egg parasitoid *Chrysonotomyia ruforum* Krausse (Hymenoptera, Eulophidae). The egg parasitoids kill the eggs and thus prevent damage to the plant from feeding sawfly larvae. The elicitor inducing the pine's response is known to be located in the oviduct secretion which the female sawfly applies to the eggs when inserting them into a slit in the pine needle using the sclerotized ovipositor valves. In this study we have characterized the elicitor. The elicitor was still active when isolated from the oviduct and applied directly to slits made in the pine needles. However, as soon as the oviduct secretion was dissolved in Aqua dest. and stored for 3 h at room temperature or kept frozen at –80°C, its activity was lost. In contrast, oviduct secretion kept its eliciting activity, when dissolved in Ringer solution (pH 7.2) both after storage at room temperature and after freezing. The activity of the elicitor vanished after treatment of the oviduct secretion with proteinase K, which destroyed all proteins. This suggests that the elicitor in the oviduct secretion is a peptide or protein, or a component bound to these. SDS-PAGE revealed a similar, but not identical protein pattern from hemolymph and oviduct secretion. Hemolymph itself has no eliciting effect. The elicitor in the oviduct secretion is only active when transferred to slit pine needles, since its application on undamaged needles did not induce the emission of attractive volatiles.

Key words: elicitor, egg deposition, egg parasitoid, induction, oviduct secretion, sawfly, *Diprion pini*, volatiles.

Introduction

Both insect egg deposition and feeding by herbivorous arthropods is well-known to induce plant defensive responses. The plant's response may have direct detrimental effects on the eggs, the ovipositing female or the feeding herbivore (Hilker et al., 2002a). Furthermore, the plant is able to respond to egg deposition and feeding by recruiting predators and parasitoids of the herbivores (Baldwin and Preston, 1999; Dicke and van Loon, 2000; Hilker and Meiners, 2002; Turlings et al., 2002). In particular, many plants produce volatile substances in response to egg deposition or feeding that have been shown to attract enemies of the assailant (Boland et al., 1999; Colazza et al., 2004a,b; Dicke and Hilker, 2003; Hilker and Meiners, 2002; Mumm et al., 2003, 2005). While the plant responding to feeding herbivores acts in response to the damage, the plant responding to insect egg deposition acts prior to being damaged by feeding larvae. Thus, Hilker and Meiners (2002) interpreted the plant's defensive response to insect egg deposition as a preventive induced defense mechanism.

Egg deposition by the phytophagous sawfly *Diprion pini* has been shown to induce the plant to release volatiles attracting *Chrysonotomyia ruforum*, an egg parasitoid of *D. pini* (Hilker et al., 2002b). This induction of volatiles is not restricted to the oviposition site, but also occurs in surrounding tissue that is not damaged (systemic effect). During egg deposition, *D. pini* females make a tangential slit in the pine needles with their sclerotized ovipositor valves, and insert the eggs into the wounds in the needles. Finally, eggs are covered on the top by a mixture of a frothy secretion and needle tissue that hardens within a few hours (Eliescu, 1932). The elicitor inducing the pine's response was found to be located in the oviduct secretion coating the eggs, since application of the oviduct secretion into artificially wounded pine needles also resulted in the induction of volatiles attractive to the egg parasitoid, whereas artificial wounding alone did not (Hilker et al., 2002b). For application of the oviduct secretion to artificially wounded pine needles, oviducts were dissected from sawfly females and secretion was

obtained by washing the oviducts in distilled water. The freshly isolated secretion was directly transferred into the wound of a pine needle (Hilker et al., 2002b).

Prior to the study presented here, nothing was known about the chemistry and stability of the elicitor located in the oviduct secretion. Thus, we tested whether the elicitor could be isolated by distilled water or other solvents and we examined how to store it. Furthermore, we did not know whether wounding of the pine needle is necessary for the eliciting process or whether the elicitor is also active when being applied onto an intact, non-wounded pine needle. Additionally, since our method of obtaining oviduct secretion from sawfly females did not avoid contamination with hemolymph, the role of hemolymph in the eliciting activity was unclear. Since oviducts and accessory reproductive structures are known to be rich in proteins (Gillot, 2002; Hinton, 1981), we examined whether a proteinase destroys the eliciting activity of the oviduct secretion.

Materials and methods

Plants and insects

Branches of *Pinus sylvestris* L. used for experiments and rearing of sawflies were detached from crowns of 15- to 35-year-old trees near Berlin. All stems were cleaned and sterilized according to the method of Moore and Clark (1968). *Diprion pini* L. (Hymenoptera, Diprionidae) was reared in the laboratory on pine twigs, as described by Bombosch and Ramakers (1976) and Eichhorn (1976) at 25±1°C, 18 h:6 h, L:D and 70% relative humidity. The egg parasitoid *Chrysonotomyia ruforum* Krausse (Hymenoptera, Eulophidae) was collected in the field in France (near Fontainebleau), and in southern and central Finland. Parasitized eggs were kept in Petri dishes (i.d. 9 cm) in a climate chamber at 10°C, 18 h:6 h L:D photoperiod and 70% relative humidity. To induce parasitoid emergence, needles with parasitized eggs were placed in a climate chamber at 25°C, 18 h:6 h L:D photoperiod and 70% relative humidity. Emerging adults were collected daily and transferred in small Perspex tubes (75 mm long, 15 mm i.d.) covered with gauze at one end. A cotton-wool ball moistened with an aqueous honey solution closed the other end. The parasitoids were kept at 10°C, 18 h:6 h L:D until they were used for bioassays. Female parasitoids used in the bioassays were experienced, i.e. they previously had contact with pine twigs carrying eggs of *D. pini* (Mumm et al., 2005). Therefore, 48 h prior to the experiment, female and male parasitoids were brought into contact with a plant–host complex, consisting of a pine twig on which eggs of *D. pini* had been deposited, adult sawflies, and a cotton-wool pad with aqueous honey solution. Twenty-four hours later, the parasitoids were removed from the plants and kept without any contact with the plant–host complex for another 24 h prior to the experiments.

Olfactometer bioassay – general procedures and data collection

All bioassays were conducted with a four-arm olfactometer as described in detail by Hilker et al. (2002b). The airflow was adjusted to 155 ml min^{-1}. We recorded how long the parasitoid was present within each of the four odor fields over a period of 600 s using the software program The Observer 3.0 (Noldus, Wageningen, The Netherlands). Only data obtained from active parasitoids walking for at least 300 s of the observation period were used for statistical analyses (see below). Parasitoids preferentially walking in the olfactometer field provided with the test odor were defined as being 'attracted' since significantly longer walking periods in the odor field is usually interpreted as a response of the parasitoid to an attractive odor (Hilker et al., 2002b). The number of parasitoids used for each bioassay was 22 to 36 (see Table 1). The odor source was changed after five to nine parasitoids had been tested. One to two odor sources were tested per day.

Plant treatments

Small pine twigs of about 20 cm length with approximately 90–120 needle pairs were cut, placed into water, and treated for a period of 72 h at 25°C and 18 h:6 h L:D. In all but one bioassay (see Table 1) artificially wounded pine twigs were used. For artificial wounding, pine needles were slit tangentially with a scalpel prior to treatment, as described by Hilker et al. (2002b). If not mentioned otherwise, the differently treated oviduct secretion samples or respective control samples were applied into artificially wounded pine needles (see below). Eight needles of each twig were treated. All pine twigs were tested systemically as described in detail by Mumm et al. (2003), i.e. the lower half of a pine twig was treated, while the upper part was left untreated but was wrapped in a polyethylene terephthalate (PET) foil to avoid adsorption of volatiles from other parts of the twig. This ensured that any volatiles emitted by the lower, treated half of the twig could be adsorbed by the upper, untreated half. After 72 h, the upper half of the twig was cut off, the PET-foil was removed, the cut end of the twig was tightly wrapped with Parafilm®, and the twig was transferred into the olfactometer to test for systemic induction. If the twig had been systemically induced, the parasitoids were expected to be attracted to its volatiles (Hilker et al., 2002b).

Bioassay I: solution and storage of the oviduct secretion

(a) In a first experiment, the oviduct secretion was obtained by dissecting the oviducts (oviductus lateralis and o. communis) of four *D. pini* females. Oviducts were transferred to 8 µl of ice-cold Aqua dest (=distilled water). To remove oviduct cell fragments, samples were centrifuged (10 min at 12,700 ***g***) and the supernatant containing the oviduct secretion was immediately applied, in 1 µl portions, to wounded pine needles (8 needles per twig). After 72 h, the untreated upper part of the pine twig was tested in the olfactometer.

(b) We next examined whether oviduct secretion remains active after 3 h of storage at room temperature (ca. 20°C). This storage period was chosen because proteinase K treatment (see below) needs an incubation time of 3 h at room temperature. Oviduct secretion was obtained, diluted in Aqua dest. and treated as described for bioassay a. However, prior to

application to the wounded pine needles, the oviduct secretion was left in the dark at room temperature for a period of 3 h.

(c) To test whether oviduct secretion diluted in Aqua dest. keeps its activity when frozen directly after dissection, it was stored in a freezer for at least 2 days at –80°C. Samples defrosted at room temperature within a few minutes and were then directly applied to wounded pine needles. Other conditions were the same as described for bioassay (a). The retention of activity of the oviduct secretion after freezing would be very useful to yield larger amounts that are necessary for further analyses.

(d) As a control, slit pine needles were treated with Aqua dest. Eight needles were treated and 1 µl was applied per wounded needle.

(e) Oviduct secretion was obtained as described above (bioassay a), but transferred into Ringer solution (pH 7.2, Merck, Darmstadt, Germany) and then stored in the dark for 3 h at room temperature (ca. 20°C). Treatment of pine needles was the same as described for bioassay a.

(f) To test whether oviduct secretion diluted in Ringer solution can be stored frozen without loosing activity, we kept it frozen as described for bioassay c. For the bioassay, defrosted oviduct secretion in Ringer solution was directly applied to wounded pine needles.

(g) As a control, eight wounded pine needles were treated with Ringer solution (1 µl per needle) only.

Bioassay II: determination of whether damage of needles is necessary for the plant to respond to oviduct secretion

(h) Oviduct secretion was obtained as described for bioassay a and transferred into Ringer solution (pH 7.2, Merck, Darmstadt, Germany). However, pine needles were not wounded prior to application of the secretion. Instead, 1 µl secretion was slowly applied to an intact needle.

Bioassay III: the effect of hemolymph in eliciting activity

(i) Legs of sawfly females were cut and 1 µl of the emerging hemolymph was obtained with a glass capillary and transferred into Ringer solution. After 3 h storage in the dark at room temperature, slit pine needles were treated with these samples (compare bioassay e).

Bioassay IV: the effect of proteinase K on the activity of the elicitor

(j) Oviduct secretion was obtained and stored in Ringer solution as described for bioassay e. 10 vol.% proteinase K (pH 7.2; Merck, Darmstadt, Germany) was added to the sample. During an incubation period of 3 h, samples were stored in the dark at room temperature (ca. 20°C). The proteinase K was covalently bound to small latex bead so that it could be separated from the secretion after incubation, by centrifuging the sample for 10 min at 8800 ***g***. The supernatant was then applied to artificially wounded needles as described above.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

In order to examine the digestion of proteins in the oviduct secretion by proteinase K treatment, and to check that all proteinase K had been removed prior to the treatment of needles, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of digested and undigested oviduct secretion was performed. Samples were prepared as described for bioassays e and j. Digested samples were analyzed after removal of proteinase K by centrifugation. For comparison, hemolymph samples were also analyzed by SDS-PAGE. Electrophoresis was performed on a gradient gel (T=5–17.5%; C=4%) according to the procedure of Laemmli (1970). Protein molecular mass markers (Precision Plus Protein, BioRad, München, Germany) were used. The starting voltage for electrophoresis was 100 V until a uniform front occurred. Then, the voltage was raised to 200 V. The gels were stained with Coomassie Brilliant Blue R-250 (Roth, Germany). To obtain a better visualization of the bands, the gels were analyzed using the software program Scion Image (Scion Corp., Frederick, MY, USA).

Statistics

Bioassay data were statistically analyzed using the Friedman analysis of variance (ANOVA) for comparing residence time within each of the four olfactometer fields using the software program SPSS 11.0. (SPSS Inc., USA). The Wilcoxon–Wilcox test was used for post-hoc comparisons (Köhler et al., 1995).

Results

Bioassays

The egg parasitoid *C. ruforum* was significantly attracted to volatiles from a pine twig that was treated with oviduct secretion of *D. pini* when the freshly obtained oviduct secretion had been directly applied to the needles without storage (Table 1, bioassay a). Volatiles attractive to the parasitoid were released by the upper part of the twigs adjacent to the lower parts that had been treated. Thus, application of oviduct secretion into wounded pine needles is not only able to induce a local response, but also a systemic one, confirming the results by Hilker et al. (2002b). The activity of the elicitor within the oviduct secretion was not stable when the secretion was diluted in Aqua dest. and then stored for 3 h or kept frozen at –80°C (Table 1, bioassays b and c). However, when oviduct secretion was diluted in Ringer solution, its activity was stable both after storage at room temperature for 3 h and after freezing at –80°C (Table 1, bioassays e and f). The control experiments revealed that neither Aqua dest. nor Ringer solution themselves have any eliciting effect (Table 1, bioassays d and g).

Wounding of pine needles was found to be essential for the induction process. Volatiles from undamaged pine twigs treated only topically with oviduct secretion did not attract *C. ruforum* (Table 1, bioassay h). Therefore, the elicitor is only effective when transferred into damaged needle tissue.

Hemolymph of *D. pini* females did not induce the emission of pine volatiles attractive for the egg parasitoids (Table 1, bioassay i). Since the oviduct secretion does not normally come into contact with hemolymph during oviposition, free

Table 1. *Response of female egg parasitoids* C. ruforum *to volatiles released systemically from differently treated* Pinus sylvestris *twigs*

Bio-assay	Oviduct secretion	Solvent	Treatment	Application into	No. plants	Residence time (s) Test	Contr. 1	Contr. 2	Contr. 3	*N*	Statistics
I. How to dissolve and store the elicitor?											
a	+	Aqua dest.	Freshly obtained	Slit pine needles	7	221[a] (43–447)	97[a,b] (4–181)	53[b] (1–135)	49[a,b] (5–208)	36	* (*P*=0.02)
b	+	Aqua dest.	Kept at room temperature (3 h)	Slit pine needles	6	181 (56–292)	125 (52–250)	83 (18–209)	90 (46–135)	32	n.s. (*P*=0.15)
c	+	Aqua dest.	After freezing at –80°C	Slit pine needles	5	120 (64–248)	146 (48–208)	124 (61–197)	143 (46–204)	31	n.s. (*P*=0.91)
d	None	Aqua dest.	–	Slit pine needles	4	83 (19–168)	69 (27–188)	194 (49–410)	94 (8–239)	22	n.s. (*P*=0.16)
e	+	Ringer solution	Kept at room temperature (3 h)	Slit pine needles	5	184[a] (91–351)	99[a,b] (58–187)	58[b] (4–160)	115[a,b] (24–210)	26	* (*P*=0.02)
f	+	Ringer solution	After freezing at –80°C	Slit pine needles	4	198[a] (131–307)	95[a,b] (21–157)	55[b] (17–160)	128[a,b] (55–248)	25	* (*P*=0.02)
g	None	Ringer solution	–	Slit pine needles	4	92 (28–201)	96 (14–200)	145 (63–309)	125 (55–183)	27	n.s. (*P*=0.06)
II. Is damage of needles necessary for the eliciting effect?											
h	+	Ringer solution	Freshly obtained	Undamaged needles	4	74 (39–150)	141 (67–232)	166 (95–249)	127 (67–214)	28	n.s. (*P*=0.21)
III. Does hemolymph have eliciting activity?											
i	None	Ringer solution	Hemolymph	Slit pine needles	5	158 (48–283)	123 (52–193)	97 (29–227)	104 (63–202)	31	n.s. (*P*=0.27)
IV. Does a proteinase K destroy the activity of the elicitor?											
j	+	Ringer solution	Proteinase K treatment (3 h)	Slit pine needles	4	74 (23–143)	62 (17–223)	128 (59–248)	107 (38–315)	27	n.s. (*P*=0.89)

The time parasitoid females spent in test and control fields (Contr. 1–3) of a four-arm-olfactometer are given. Test field with odour from differently treated pine; Contr. 1, 2, 3=three fields with control (clean) air. Control field 2 is opposite to the test field, control fields 1 and 3 are adjacent to it.

+, needles treated with oviduct secretion; none ', not treated with oviduct secretion.

The columns 'solvent' and 'treatment' indicate how the oviduct secretion and hemolymph, respectively, have been treated prior to application to the pine twig.

Median and interquartile range (parentheses) are given. *Significant ($P<0.05$); n.s. 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.

hemolymph in the female's abdomen does obviously not play a role *per se* in the induction of the volatile compounds as a result of egg deposition.

The activity of the elicitor in the oviduct secretion was lost after treatment with proteinase K, because volatiles of pine twigs treated with proteinase K-digested oviduct secretion were not attractive to the parasitoids (Table 1, bioassay j).

SDS-PAGE

Electrophoresis of undigested oviduct secretion showed seven bands with molecular masses from ca. 10–250 kDa, visualized by seven peaks in the Scion image (Fig. 1). The band pattern of the hemolymph was similar, but one band (no. 1) was missing compared to the oviduct secretion. Band 2 was regularly visible in the hemolymph samples, although only a small peak is shown in Fig. 1. SDS gels of digested oviduct secretion showed no bands, confirming that all proteins were destroyed by proteinase K treatment. Furthermore, no proteinase K residues were detected, thus confirming that the enzyme was completely removed from the sample prior to its application to pine needles (Fig. 1).

Discussion

Our results clearly show that the oviduct secretion of *D. pini* contains an elicitor which induces a systemic release of pine volatiles attractive to the egg parasitoid *C. ruforum*. This confirms the results of Hilker et al. (2002b), giving further

support that oviposition and oviduct secretion induce similar response in *P. sylvestris*. The elicitor is unstable in Aqua dest., but can be stabilized by Ringer solution and its digestion by proteinase K indicates that the elicitor is a peptide or protein or a compound bound to a peptide or protein (Falbe and Regitz, 1990). SDS-PAGE revealed a protein fragment of ca. 12 kDa which is present only in oviduct secretion samples but not in the hemolymph (Fig. 1). In order to examine the bioactivity of this fragment, the oviduct secretion needs to be fractionated by gentle separation techniques that do not destroy the protein. The activity of this and further fractions needs to be tested in a bioassay. Active fractions will be subjected to further chemical analysis.

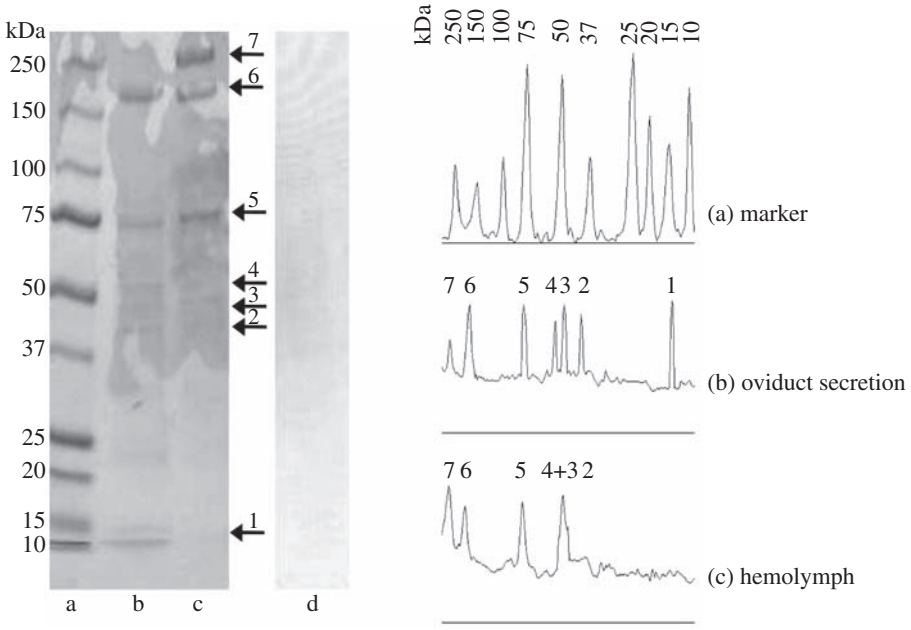

Fig. 1. (Left) SDS-PAGE after staining with Coomassie Brilliant Blue. Lanes: (a) marker, (b) oviduct secretion undigested, (c) hemolymph and (d) oviduct secretion digested by proteinase K. (Right) Scion image plot of the same SDS gel showing gel bands as peaks. Numbers 1–7 indicate bands detected on the gel.

To our knowledge, the biochemistry of only two elicitors inducing plant responses to egg depositions have been intensively investigated (Hilker et al., 2002a). (1) 'Bruchins' isolated from bruchid beetles, have been found to elicit a response in peas (*Pisum sativum*) that directly affects the herbivore. Egg deposition by the beetles induce growth of neoplasms at the site of egg attachment. The plant's response to egg deposition may protect the pea pod from larval feeding damage, because the egg on the neoplasm may easily be detached from the pod (Doss et al., 1995, 2000). When isolated bruchins are applied onto responsive pea pods, they also induce neoplastic growth. The bruchins are long-chain α,ω-monounsaturated C_{22}-diols and α,ω-mono- and diunsaturated C_{24}-diols (Oliver et al., 2000, 2002).

(2) In the tenthredinid sawfly *Pontania proxima***,** an elicitor of a plant reaction is known to be located in the secretion of accessory glands. When *P. proxima* lays an egg onto a *Salix fragilis* leaf, the egg deposition induces mitogenesis of plant tissue and growth of a gall is initiated. Chemical analyses revealed that the secretion of the accessory glands contains nucleic acids, protein (Hovanitz, 1959), uric acid, adenine derivatives, glutamic acid and possibly uridine (McCalla et al., 1962). Leitch (1994) suggested that precursors of cytokinins may be present in the ovipositional fluid and act as gall-initiators, however, Higton and Mabberley (1994) doubt that cytokinins *induce* the galls.

Several elicitors of plant defensive responses to feeding herbivores have been isolated from regurgitate of herbivorous larvae (Felton and Eichenseer, 1999). The components are known to be proteinous or to contain a peptide bond. β-Glucosidase has been isolated from the regurgitate of *Pieris brassicae* larvae (Mattiacci et al., 1995). Volicitin (*N*-[17-hydroxylinolenoyl]-L-glutamine) and other fatty acid–amino acid conjugates have been isolated from the regurgitate of several lepidopteran species (Alborn et al., 1997, 2000; Halitschke et al., 2001; Mori et al., 2001; Pohnert et al., 1999; Turlings et al., 2000). Musser et al. (2002) isolated a glucose oxidase from the saliva of *Heliothis zea.* Their results indicate that release of this glucose oxidase suppresses the plant's defensive response, and thus, has been interpreted as counteradaptation by the herbivore. Little is known about the mode of action of herbivore elicitors (Dicke and van Poecke, 2002). In contrast to elicitors isolated from plant pathogens, no herbivore defense-related plant perception mechanisms have been identified so far (Dicke and van Poecke, 2002; Felton and Eichenseer, 1999; Ham and Bent, 2002; Martin et al., 2003).

Our results revealed that the elicitor present in the oviduct secretion of *D. pini* needs to come into contact with wounded plant tissue. Similar results were found by Meiners and Hilker (2000) in another tritrophic system consisting of elm (*Ulmus minor*), the leaf beetle *Xanthogaleruca luteola*, and the egg parasitoid *Oomyzus gallerucae*. The elicitor present in the oviduct secretion of the elm leaf beetle also needs to contact wounded elm tissue to induce production of volatiles which attract the egg parasitoid of the elm leaf beetle. Elicitors inducing plant responses to feeding herbivores have also been shown to need disrupted plant tissue to become active (Mattiacci et al., 1995; Turlings et al., 1990). In contrast, Colazza et al. (2004a) could show that the pentatomid *Nezara viridula* lays its eggs on bean leaves without wounding the plant tissue. These egg depositions induce the bean leaves to release volatiles that attract the egg parasitoid. However, bean leaves carrying eggs of the bug only released volatiles attractive to the parasitoid, when the leaves had also been damaged by feeding.

Leaves with eggs, but without feeding damage did not emit the attractive volatiles. Thus, in this tritrophic system also, leaf damage seems to be necessary to induce the volatile blend attractive for the parasitoid. However, the elicitor associated with the egg deposition seems to be able to become active also when being applied to intact leaf surface.

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