

Insect oviposition induces volatile emission in herbaceous plants that attracts egg parasitoids

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Summary

The egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae) responded to synomones emitted by leguminous plants induced by feeding and oviposition activity of the bug *Nezara viridula* (L.) (Heteroptera: Pentatomidae). This was shown by laboratory bioassays using a Y-tube olfactometer. Broad bean leaves (*Vicia faba* L.) damaged by feeding activity of *N. viridula* and on which host egg mass had been laid produced synomones that attracted *T. basalis*. By contrast, undamaged leaves or feeding-damaged leaves without eggs did not attract wasp females. French bean plants (*Phaseolus vulgaris* L.) also emitted attractive synomones when they were damaged by host feeding and carrying egg masses. Thus, release of feeding- and oviposition-induced synomones does not seem to be plant-specific. Synomone production was shown to be a systemically induced plant physiological response to feeding damage and oviposition. Also, parts of the plant that were left undamaged and did not carry host eggs emitted attractive synomones when

other parts of the plant were damaged by feeding and carrying eggs. Furthermore, wasps were not attracted by *N. viridula* egg masses offered alone or combined with damaged broad bean leaves. Thus, the attractiveness of feeding-damaged leaves carrying eggs is due to induction by feeding and oviposition rather than due to a combined effect of attractive volatiles released from eggs and damaged leaves. The production of synomones was influenced by the age of the host egg mass, because feeding-damaged leaves bearing egg masses attracted the parasitoid until the eggs were ~72–96 h old but not once the larvae had hatched from the eggs (~120 h old). These results show that annual plants are able to produce synomones as a consequence of feeding and egg mass oviposition by a sucking insect.

Key words: Insecta, *Nezara viridula*, Heteroptera, Pentatomidae, *Trissolcus basalis*, Scelionidae, systemic induction, oviposition, insect/plant interaction, chemical ecology.

Introduction

The ability of parasitic arthropods to locate and attack hosts is a result of successive behavioural steps regulated by physical, chemical and biochemical factors (Vinson, 1985; Vet and Dicke, 1992; Godfray, 1994). In the host location and selection process, cues associated with the hosts have a hierarchical array, with volatile compounds emitted by plants as a consequence of herbivore activities playing a role in attracting parasitoids to patches infested with hosts (Vinson, 1985). When these volatiles are used to recruit natural enemies, they have been termed host-induced synomones, and they are regarded as an indirect chemical defence reaction by the plants (Dicke, 1999; Dicke and van Loon, 2000 and references therein).

In the case of hymenopteran parasitoids, studies of their ability to locate their hosts using host-induced synomones have focused primarily on parasitoids whose hosts are defoliators. These insects inflict substantial physical damage on the plant, which induces qualitative and quantitative changes in the

plant's profile of volatiles (Vinson, 1991; Turlings et al., 1995; Geervliet et al., 1994; van Alphen and Jervis, 1996; De Moraes et al., 1998, 2001; Turlings and Benrey, 1998; Guerrieri et al., 1999). These volatiles can be plant species specific and/or herbivore species specific (De Moraes et al., 1998) and are generally induced by elicitors in the herbivore saliva or oral secretions. To date, the main elicitors identified include an enzyme, β -glucosidase, isolated from the regurgitant of the large white butterfly caterpillar *Pieris brassicae* (L.) (Mattiacci et al., 1995), and volicitin, a 17-hydroxylated linolenic acid conjugated with glutamine, isolated from regurgitant of larvae of the beet armyworm *Spodoptera exigua* (Hübner) (Alborn et al., 1997, 2000). Other conjugates of fatty acids and amino acids that induce the release of volatiles by tobacco (*Nicotiana attenuata* Torrey ex Wilson) plants damaged by insect defoliation have also been detected in the regurgitant of larvae of the tobacco hornworm *Manduca sexta* (L.) (Baldwin et al., 2001; Halitschke et al., 2001). Furthermore, host-induced

synomones have now been demonstrated for those tritrophic systems in which the herbivores have phloem-feeding or stylet-sheath-feeding habits (Du et al., 1998; Guerrieri et al., 1999), although the elicitors have not yet been identified.

Plant synomones can also be induced by factors other than insect feeding activity. For example, insect egg deposition can induce a change in the profile of the plant volatiles, and these volatiles have been shown to attract egg parasitoids (for a review, see Hilker and Meiners, 2002; Hilker et al., 2002a). Plants that are able to attract egg parasitoids soon after herbivore eggs are laid benefit from increasing the parasitoid's effectiveness (Vinson, 1985) and from beginning to defend themselves against insect pests before any damage has occurred, i.e. before the larvae have hatched from the eggs (Hilker et al., 2002a). To date, detailed studies on oviposition-induced synomones have been carried out in two tritrophic systems, showing strong similarities. In both cases, the herbivores feed on perennial plants, the elicitor(s) is present in the oviduct secretions, the elicitor(s) must contact the wounded plant tissues, and the signal(s) is produced both locally and systemically (Hilker and Meiners, 2002; Hilker et al., 2002a). Recently, preliminary data suggesting the use of oviposition-induced synomones have been collected for another tritrophic system consisting of leguminous annual plants, the southern green stink bug *Nezara viridula* (L.) (Hemiptera: Pentatomidae) and its egg parasitoid *Trissolcus basalus* (Wollaston) (Hymenoptera: Scelionidae) (S. Colazza, A. Fucarino, E. Peri, G. Salerno, E. Conti and F. Bin, unpublished; Hilker and Meiners, 2002). Here, we present more complete details of this research.

Trissolcus basalus is a solitary egg parasitoid that attacks eggs of several phytophagous and predatory pentatomid bugs and it is now distributed worldwide as a result of extensive deliberate introductions for use as a biological control agent for *N. viridula* (Jones, 1988; Clarke, 1990). *N. viridula* is a highly polyphagous pest that attacks both perennial and annual plants. In Italy, it has been mainly recorded as a pest of annual plants (Colazza and Bin, 1995). *N. viridula* egg mass distribution is determined primarily by the degree of activity and movement patterns of gravid females (Todd, 1989). Data taken from a wide variety of annual crops indicate that there are primary sites for feeding and mating and then the gravid females disperse to other host plants for oviposition, so that egg masses are generally laid quite far from the adult feeding and mating sites (S.C., personal observation). Therefore, we predicted that *T. basalus* females would be unlikely to use cues from undamaged plants or plant cues induced by *N. viridula* feeding activity to locate hosts because host egg masses would not be nearby. Instead, we predicted that the wasps would be more likely to use cues originating from the host egg masses themselves or from interactions between the egg mass and the plant tissues as reliable host location cues.

In the present study, we examined whether volatile plant synomones are induced and released as a result of *N. viridula* oviposition, whether their activity is dependent on a synergistic effect between damaged plants and egg masses, whether the

plants respond systemically to the oviposition, and whether egg mass age has an effect on the oviposition-induced synomones.

Materials and methods

Plants

Seeds of broad bean plants (*Vicia faba* L., cv Aguadulce) and French bean plants (*Phaseolus vulgaris* L., cv Lingua di Fuoco) were individually planted in plastic pots filled with fertilized commercial soil and grown in greenhouse conditions ($20\pm 2^\circ\text{C}$, $60\pm 5\%$ RH). Plants 2–3 weeks old, with approximately six fully expanded leaves, were transferred into a climate controlled chamber ($24\pm 2^\circ\text{C}$, $70\pm 5\%$ RH, 16 h:8 h L:D) and used for the experiments.

Insect cultures

Adults of *Nezara viridula* (L.) were reared under laboratory conditions ($24\pm 2^\circ\text{C}$, $70\pm 5\%$ R.H., 16 h:8 h L:D) in plastic containers (30 cm \times 20 cm \times 15 cm) on a diet of sunflower seeds, seasonal fresh vegetables and water. *N. viridula* egg masses were collected daily and used to maintain cultures of both *N. viridula* and *Trissolcus basalus* (Wollaston). Cultures were augmented regularly with field-collected bugs. The *T. basalus* colony was established from wasps emerging from sentinel *N. viridula* egg masses placed in both cultivated and uncultivated fields located around Palermo, Italy. Wasps were kept in the laboratory at an 18-h photophase, a temperature of $24\pm 2^\circ\text{C}$ and an RH of $70\pm 5\%$ and were regularly exposed to *N. viridula* eggs for 24 h in glass vials. Parasitized egg masses were removed and stored in clean glass vials for development. At emergence, adults were fed with a honey–water solution. Parasitoid females used in bioassays were from a laboratory culture that had been in culture for no more than 3–5 generations. Each female was mated, naive, 2–3 days old (at which age they respond well to odours; S.C., personal observation) and isolated individually for ~24 h before the assay in a small vial with a drop of honey–water solution.

Y-tube olfactometer and general bioassay procedure

Wasps' responses to volatile chemicals were investigated with a dual choice Y-tube olfactometer (stem 90 mm; arms 80 mm at 130° angle; internal section 15 mm \times 15 mm) sandwiched between two glass sheaths as described by Colazza et al. (1997). An air stream (medical air, USP, 79% nitrogen and 21% oxygen by volume) was humidified by bubbling through a water jar and was then drawn through each arm of the olfactometer (30 ml min $^{-1}$ per arm). Before entering the olfactometer arms, each air stream passed through a 600 ml glass jar containing odour sources described below. The odour sources were randomly assigned at the beginning of the bioassays and were reversed after testing approximately five parasitoid females. The responses of 16–38 parasitoid females were tested for the various treatments and combinations. Tests were conducted from ~09:00 h to 16:00 h. After each trial, the whole system was rinsed with acetone and baked overnight at 200°C . Female wasps were tested one at a time, introducing

individuals into the olfactometer at the entrance of the stem and observing the behaviour for 10 min. Behaviours were recorded on video (monitor Sony® Trinitron; monochrome CCD camera Sony® SPT-M328CE, zoom lens 12.5–75 mm/F1.8), and a video frame grabber (Studio PCTV; Pinnacle Systems, Mountain View, CA, USA) was used to digitize the analogue video signals. Xbug, a video tracking and motion analysis software, was used to process data (S. Colazza, D. Peri, G. Salerno, E. Peri, M. Lo Pinto and G. Liotta, unpublished). For each recording, the total time spent in each olfactometer arm and the linear speed in the whole arena (mm s^{-1} ; sample rate, $\sim 6 \text{ images s}^{-1}$) were calculated. The temperature in the bioassay room was $\sim 26^\circ\text{C}$ at all times.

Experiments

The first experiments were designed to investigate female wasps' responses to undamaged plants *versus* plants damaged by adult bug feeding and *versus* plants damaged by feeding onto which an egg mass had also been laid. The possible effects of using different plant species on the oviposition-induced synomones were also tested. Broad bean plants and French bean plants were singly transferred into a wood-framed, nylon mesh cage (40 cm×40 cm×50 cm) and exposed to ~ 7 –10 *N. viridula* gravid females for ~ 1 –2 days, time enough for the adults to be conditioned to the new diet and for the females to lay at least one egg mass. About one hour prior to the bioassays, the bugs were removed and a set of two leaves was cut from the medium nodes of the treated plants. The cut petioles were wrapped in wet cotton and inserted in a 1-ml vial filled with distilled water and sealed with Parafilm®, and the leaves were then transferred to the glass holding chamber of the bioassay apparatus as described above. Leaves cut from undamaged plants held in the same conditions were used as controls.

The second experiment was carried out to evaluate whether the wasps' attraction to oviposition-induced synomones was the result of synergistic effects between the volatiles from both damaged leaves and the egg mass. The odour sources consisted of 24-h-old *N. viridula* egg masses (~ 60 –80 eggs each), laid on nylon screen and collected from rearing boxes containing 5–8 gravid *N. viridula* females, and feeding-damaged broad bean leaves prepared as follows. Volatiles from egg masses alone were tested *versus* odours from undamaged broad bean leaves and *versus* a treatment consisting of egg masses and feeding-damaged broad bean leaves held in the same glass jar.

A third experiment was carried out to investigate whether the volatiles that attract the wasps are released only locally from the damaged leaves carrying the egg mass or whether the volatiles are produced systemically throughout the plant. For this purpose, broad bean plants, reared under the same conditions described for the first experiment, were singly exposed to 3–4 mated *N. viridula* females in a cage longitudinally divided by a plastic sheet (40 cm×40 cm×50 cm). Some apical lateral leaves were carefully pushed through holes in the plastic sheet, and the holes were sealed at node level with cotton to reduce volatile exchange between the two halves of the cage. In this way, the test insects

were allowed to contact only ~ 4 –5 basal lateral leaves of the plant. A few hours after an egg mass had been laid (less than 24 h), a set of two leaves that had not been in contact with the bug were cut and prepared as described for the first experiments and tested *versus* undamaged broad bean leaves.

A fourth experiment was carried out to study the effect of time since oviposition of *N. viridula* egg mass on attraction of the wasps. *N. viridula* egg masses became less acceptable to the wasps once they were 72–96 h old (the eggs become pinkish-yellow due to the embryo development and hatch at ~ 120 h; Bin et al., 1993). Broad bean plants were exposed to 5–7 mated *N. viridula* females as described in the first experiment. Once an egg mass had been laid, the bugs were removed and the plant was held in the environment chamber until the egg mass was either 72–96 h old or 120 h old and then tested *versus* undamaged broad bean leaves.

Statistical analysis

Values for the resident times of parasitoids in each arm of the Y-tube olfactometer and their linear speeds in the whole arena were normally distributed (Kolmogorov–Smirnov test) and homoscedastic and were analyzed with parametric tests (*t*-test for paired samples; one-way analysis of variance followed by the Tukey HSD test for multiple comparisons between means). All the data were analyzed using the Statistica 5.1 Statistical Package (StatSoft, Inc., Vigonza, Italy).

Results

Volatiles from broad bean leaves damaged by feeding activity of adult *N. viridula* were no more attractive to *T. basalis* females than those from undamaged leaves ($t=0.81$, $P=0.43$; Fig. 1A). However, wasps were significantly more attracted to volatiles from feeding-damaged leaves carrying an egg mass than to leaves damaged only by feeding ($t=5.93$, $P=0.00002$; Fig. 1B). Feeding-damaged French bean leaves carrying an egg mass were also more attractive to wasps than leaves damaged only by feeding ($t=4.26$, $P=0.0001$; Fig. 1C).

Volatiles from bug egg masses laid on nylon screen did not attract the parasitoid ($t=1.66$, $P=0.11$; Fig. 2A). Furthermore, when combining volatiles from egg masses on nylon screen with those from feeding-damaged leaves that had never carried host eggs, this combination was no more attractive than volatiles from feeding-damaged leaves alone ($t=-0.77$, $P=0.44$; Fig. 2B). Therefore, the attractiveness of feeding-damaged leaves carrying egg masses appears to be due to volatiles produced by bug eggs in contact with the leaf and not from a combination of the odours emitted from the egg mass and the leaf held separately.

The odours of leaves without egg masses but belonging to a plant with other leaves damaged by feeding and carrying an egg mass emitted volatiles that attracted the parasitoid ($t=2.83$, $P=0.01$; Fig. 3). Thus, the attractiveness induced by feeding and oviposition is not confined to the leaves onto which an egg mass has been laid but is systemically induced throughout the plant.

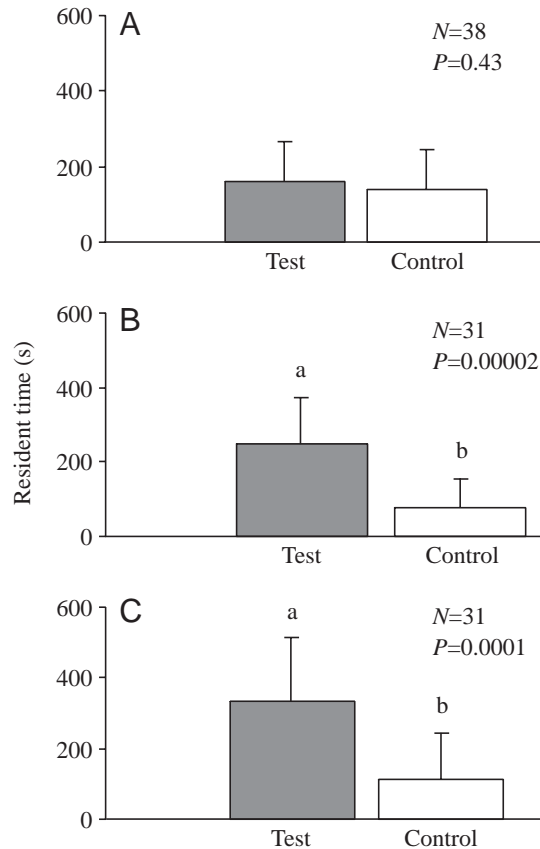


Fig. 1. Response of *T. basalis* females in a Y-tube olfactometer to volatiles from differently treated plants (test) of *V. faba* (A,B) or *P. vulgaris* (C) versus undamaged plants (control): (A) volatiles from feeding damaged leaves; (B,C) volatiles from feeding damaged leaves with egg masses (24 h old). N = number of replicates. Bars represent means \pm s.d. for the time spent by the wasp females in both the arms over an observation period of 600 s. Different letters indicate significant differences determined by *t*-tests for paired samples.

The age of the egg mass also influenced the attraction of wasps to leaves bearing egg masses. Thus, feeding-damaged leaves bearing egg mass were attractive to the parasitoid until the eggs were 72–96 h old ($t=2.96$, $P=0.005$; Fig. 4A) but not once the eggs had hatched (>120 h; $t=-0.77$, $P=0.44$; Fig. 4B).

Inside the olfactometer, the wasp's linear speed was significantly affected by the volatiles from different treatments. In the presence of feeding-damaged plant volatiles, wasps walked faster than when in the presence of volatiles from feeding-damaged plants with host egg masses or systemically induced leaves (d.f.=2, $F=12.34$, $P<0.001$; one-way ANOVA; Fig. 5).

Discussion

Our results suggest that egg deposition by the pentatomid bug *N. viridula* stimulates production of host-induced synomones that attract the egg parasitoid *T. basalis*. Oviposition-induced synomones have been described in only

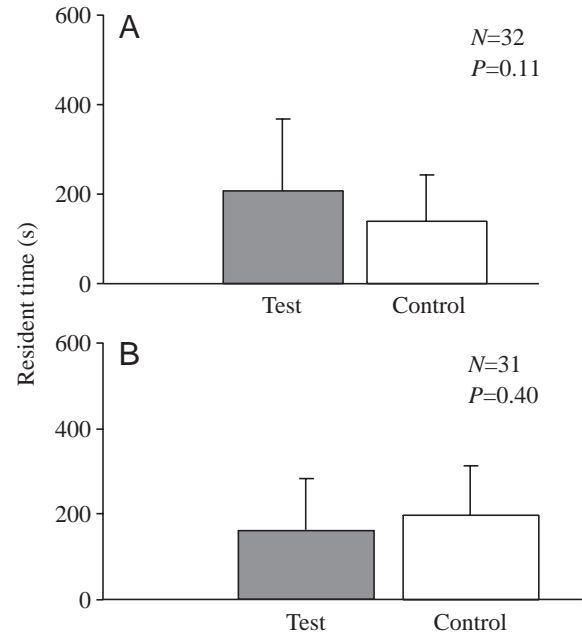


Fig. 2. Response of *T. basalis* females in a Y-tube olfactometer to volatiles from *N. viridula* egg masses offered (A) alone or (B) combined with *V. faba* leaves damaged by feeding activity (test) versus undamaged leaves offered in the control arm. N = number of replicates. Bars represent means \pm s.d. for the time spent by the wasp females in both the arms over an observation period of 600 s. Different letters indicate significant differences determined by *t*-tests for paired samples.

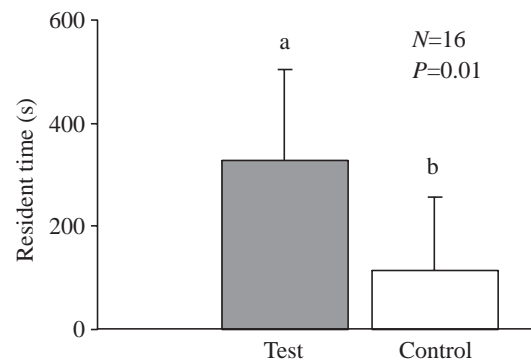


Fig. 3. Response of *T. basalis* females in a Y-tube olfactometer to volatiles from systemically induced *V. faba* leaves (test) versus undamaged leaves (control). N = number of replicates. Bars represent means \pm s.d. for the time spent by the wasp females in both the arms over an observation period of 600 s. Different letters indicate significant differences evaluated by *t*-test for paired samples.

two other tritrophic systems. First, oviposition by the elm leaf beetle *Xanthogaleruca luteola* Muller stimulates elm leaves (*Ulmus minor* Miller) to produce volatiles that attract the eulophid wasp *Oomyzus gallerucae* (Fonscolombe) (Meiners and Hilker, 1997). Second, oviposition by the pine sawfly *Diprion pini* (L.) induces odour production in needles of *Pinus sylvestris* L. to attract the eulophid wasp *Chrysonotomyia ruforum* (Krausse) (Hilker et al., 2002b). Although our results

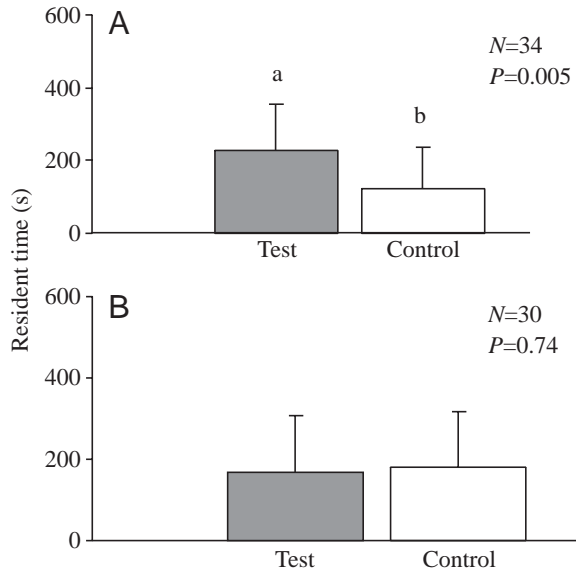


Fig. 4. Response of *T. basalis* females in a Y-tube olfactometer to volatiles from *V. faba* leaves damaged by feeding activity and with (A) a 72–96-h-old egg mass and (B) a hatched egg mass (120 h old) (test) versus undamaged leaves offered in the control arm. N = number of replicates. Bars represent means \pm S.D. for the time spent by the wasp females in both the arms over an observation period of 600 s. Different letters indicate significant differences determined by t -tests for paired samples.

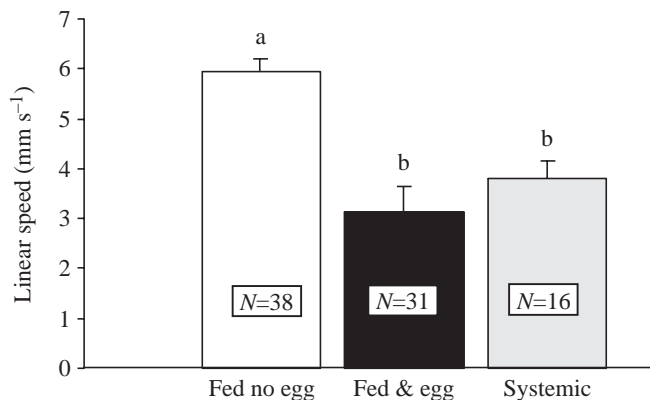


Fig. 5. Mean linear speed of *T. basalis* females in a Y-tube olfactometer in the presence of volatiles from differently treated leaves of *V. faba*. 'Fed no egg' = leaves damaged by feeding activity (see Fig. 1A); 'fed & egg' = leaves damaged by feeding activity with one egg mass less than 24 h old (see Fig. 1B); 'systemic' = systemically induced leaves (see Fig. 3). N = number of replicates. Bars represent means \pm S.E.M. Different letters indicate significant differences at $P < 0.05$ (ANOVA followed by Tukey HSD test).

are not yet as extensive and detailed as in these two systems, several similarities and differences are already apparent.

Results on our tritrophic system show, for the first time, that annual plants can produce oviposition-induced synomones. As was suggested by Hilker et al. (2002a), annual plants, having a short life cycle and a relatively small biomass, may benefit

more than larger perennial plants from egg parasitoid activity, which indirectly reduces the number of feeding larvae.

Synomones induced by oviposition by elm leaf beetle and pine sawfly, which have restricted host ranges, are characterized by a high specificity of response in their egg parasitoids (Hilker et al., 2002a). By contrast, *N. viridula* is highly polyphagous, developing on more than 150 species within ~30 plant families, although it has a distinct preference for leguminous plants (Todd, 1989; Panizzi et al., 2000). We demonstrated that *N. viridula* oviposition apparently induced synomones in two different leguminous annual plants. Because of *N. viridula*'s broad host range, we predict that synomones induced by *N. viridula* oviposition will be found in other annual and perennial host plants.

Unlike elm leaf beetle and pine sawfly, *N. viridula* females do not cut or otherwise physically damage the host substrate during oviposition. Instead, eggs are laid on the leaf surface in clusters that adhere to each other and to the plant by a sticky oviduct secretion. This secretion accumulates at the base of the egg while it descends the ovariole, and, once in contact with the air, the secretion rapidly oxidizes to a light brown film that extends over the egg mass border (Bin et al., 1993). Females of elm leaf beetle and pine sawfly also coat their eggs with oviduct secretion, with two resulting effects. First, the secretion induces production of synomones in the host plant when in contact with host tissues wounded during oviposition (Meiners and Hilker, 1997; Hilker et al., 2002b); elm leaf beetle females remove leaf lower surface prior to oviposition with their mouthparts, and pine sawfly females incise pine needles to insert the eggs (Meiners and Hilker, 2000; Hilker et al., 2002b). Second, the secretion is used as a contact kairomone that induces host acceptance behaviour in their egg parasitoids (Bin et al., 1993; Meiners and Hilker, 1997).

To date, our experiments have not yet characterized the elicitor associated with *N. viridula* oviposition nor its specific mechanism of action. Because of the absence of any apparent plant injury at the time of oviposition, elicitors other than those associated with oviduct secretions may be possible, such as those associated with the surface chemistry of the eggs, as is the case with hydrocarbons present in the surface wax of Colorado potato beetle (*Leptinotarsa decemlineata* Say) eggs (Nelson et al., 2003), or even the presence of egg-associated microorganisms (Städler, 2002). Moreover, in the oviposition-induced synomone cases studied by Hilker et al., the influence of adult feeding activity in inducing the synomones has been excluded (e.g. *D. pini* adult females do not feed on plants; Hilker et al., 2002a). In our experiments, volatiles released by leaves damaged only by *N. viridula* feeding activity were no more attractive than volatiles from undamaged leaves, but the volatiles produced by the combination of feeding damage and oviposition appeared to act synergistically. This synergistic activity between feeding and oviposition seems confirmed by the results of chemical and behavioural experiments currently in progress on the odours of bean plants induced by *N. viridula* adults as a result of their feeding activity, oviposition activity

and feeding and oviposition activity combined (S. Colazza, J. S. McElfresh and J. G. Millar, personal observation).

Synomones released as a response to attack by phytophagous insects could be produced or released at the site of the attack, as well as systemically by other parts of the plant, or the compounds could be produced at the site of the attack, transported to other sites and released far from the site of the attack (Dicke, 1999; Turlings and Benrey, 1998). All the oviposition-induced synomones investigated to date are emitted from both the leaves carrying the eggs and from insect- and egg-free parts of the same plant (Hilker et al., 2002a; present study). A systemically induced response may benefit the plant under attack by increasing the amount of synomones produced and increasing the surface area from which the synomones are released, thus creating a more apparent signal that could increase parasitoid attraction (Dicke and van Loon, 2000 and references therein).

It has been shown that the release of host-induced synomones is timed as a consequence of several factors such as the cost of defence and/or synchronization with the wasp's activity (Turlings and Benrey, 1998). Host egg resources are ephemeral, because host egg quality rapidly decreases with time as the host develops (Vinson, 1998). In our system, the age of host egg mass influences the acceptance behaviour of *T. basalis* (Bin et al., 1993). Therefore, it is expected that the production and/or activity of oviposition-induced synomones should be influenced by the age of the egg mass. Consistent with this hypothesis, leaves bearing eggs that are 72–96 h old still attract the parasitoid, while leaves bearing hatched eggs do not.

In conclusion, our knowledge of synomones induced in plants as a result of insect oviposition is still rudimentary, with the specific elicitors, synomones and mechanisms by which they work remaining to be identified. Work currently in progress aims to identify synomones induced by *N. viridula* oviposition and/or feeding and to examine the time course of their production as first steps in gaining a better understanding of the cues and signals mediating tritrophic interactions in this system.

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