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A NOVEL BIOASSAY FOR *Yponomeuta cagnagellus* OVIPOSITION IN RESPONSE TO EXTRACTS OF HOST AND NONHOST PLANT SURFACE COMPOUNDS

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Abstract—*Yponomeuta cagnagellus* is a phytophagous moth species specialized on *Euonymus europaeus*. Host discrimination by the adult female is an important aspect of host specialization and is based mainly on the distinctive secondary chemistry of host and nonhosts. This paper describes a bioassay that was developed to study the effect of isolated plant surface compounds on *Yponomeuta* oviposition. Adult moths recognize their hosts through chemical stimuli on the leaf or twig surface. Relatively apolar compounds extracted from the host twig surface by washing in dichloromethane do not stimulate oviposition. More polar, methanol-soluble compounds do, and this stimulation is dose dependent. Moths are able to recognize hosts solely by their surface compounds: females show a strong preference for artificial twigs treated with methanolic extracts of their hosts compared to those treated with methanolic extracts of nonhosts *Crataegus monogyna* and *Prunus spinosa* (both of which are hosts for closely related *Y. padellus*). Shape and surface characteristics of the oviposition substrate also influence oviposition. The substrate needs to resemble the basic form of a twig (i.e., cylindrical), and females prefer a coarse surface with irregularities over a smooth one.

Key Words—*Yponomeuta cagnagellus*, ermine moth, Lepidoptera, speciation, specialization, plant surface compounds, oviposition, host discrimination, *Euonymus europaeus*.

INTRODUCTION

The modification of host acceptance behavior and oviposition in particular can be viewed as the key to evolutionary modifications of host range in phytophagous

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insects (Dethier, 1982; Berenbaum, 1990; Bush, 1994; Menken and Roessingh, 1998). In lepidopteran species specialized on one or a few host plants, the correct choice by the adult female is critical, as the larvae generally will not readily feed on other plant species. Moreover, considering the small size of most first-instar larvae, they are not likely to find an acceptable food source without large fitness costs in the event of oviposition mistakes. However, the female is not able to assess the nutritional quality of her offspring's food plant directly, as she is unable to feed on it and is, thus, dependent on indirect cues present on the stem and leaf surface for host recognition.

Yponomeuta species (Lepidoptera: Yponomeutidae) form a genus of highly specialized phytophagous moths. Food plant associations are known in 38 of 70 species within this genus (exclusive of the American species): 31 species are monophagous on host plants belonging to one genus. Of these, 23 are found on plants in *Euonymus* (Celastraceae). Of the nine European *Yponomeuta* species, only three occur on a celastraceous host (*Euonymus europaeus* L.), the other six are specialized on plants belonging to the Rosaceae, Salicaceae, or Crassulaceae (Gershenson and Ulenberg, 1998). Their phylogeny indicates that shifts from an ancestral celastraceous host to other hosts—mainly rosaceous—may have been responsible for the evolution of the European species (Menken et al., 1992; Menken and Roessingh, 1998).

Currently, the potential for a change in the discriminative behavior of the adult *Yponomeuta* species and its possible contribution to the occurrence of host shifts and subsequent host race formation and speciation is being studied. As a starting point for understanding this behavior, identification of the specific plant compounds involved in adult host recognition is necessary. Therefore, in this study, we focus on the response of gravid females to plant surface extracts, asking whether *Yponomeuta cagnagellus* (Hbn.) is capable of recognizing the host from compounds available on the plant surface. An efficient tool for the assessment of *Yponomeuta* oviposition in response to chemical compounds was developed and used to evaluate stimulatory activity of extracts of plant surfaces washed with organic solvents.

METHODS AND MATERIALS

Insects. *Yponomeuta cagnagellus* moths were reared from larvae fed leaves of the host plant *Euonymus europaeus* in a climate room of 18°C or 23°C, 17L:7D. Caterpillars were collected in the field from host plants in June–July as fourth or fifth instars (Amsterdam), or in February as diapausing first instars by collecting *E. europaeus* twigs with *Y. cagnagellus* hibernacula (The Hague, Meyendel). The latter were stored at 5°C in complete darkness until use. After pupation, the pupae were placed individually in glass vials. Newly emerged

moths were fed with a solution of 10 g honey in 1 liter of 1% water–agar that was provided in 0.5 ml Eppendorf tubes every three to four days. *Yponomeuta cagnagellus* females take up to 14 days to become sexually mature (Hendrikse, 1979). To obtain gravid insects, individual females were provided with a mate and kept at 18°C, 17L : 7D for two weeks after emergence before using them in the oviposition experiments.

Bioassay. Artificial twigs (ATs) (Figure 1) were constructed from glass Pasteur capillary pipets (WU, Mainz, Germany). The diameter of these (70 mm) is similar to *E. europaeus* twigs with egg masses in the field. *Yponomeuta cagnagellus* preferably position their egg masses near buds, nodes, and other irregularities on the twig surface (Bremner et al., 1997). To simulate these structures, two orthodontic rubber bands (64 mm diameter) were placed on the pipets. For some experiments, the glass pipet was covered with paper adhesive tape (TESA, Beiersdorf BDF).

The ATs were sprayed with various plant extracts and solvents by using a compressed air reagents diffuser. To obtain a homogenous distribution of the

FIG. 1. Model of the cage and artificial twigs used for oviposition preference experiments.

extracts on the ATs, 5–15 pipets were fixed side-by-side on a cardboard strip, thus presenting a more-or-less two-dimensional surface. Since amounts of extract were limited, only the lower 5 cm were treated, excluding the first 1 cm by which the ATs were fixed in their holders. The ATs were mounted in PVC or polystyrene holders and placed in cages. In two-choice bioassays, one female moth was added to the Perspex cage (4 cm diam., height 15 cm; Figure 1) accompanied by her mate. For bioassays presenting a choice among more than two treatments, 5–10 pairs of moths were introduced in large cages of transparent plastic (70 cm high, 30 cm diam.) to obtain adequate amounts of egg masses for statistical analysis.

Yponomeuta cagnagellus deposits an egg mass once every two to three days, resulting usually in one to four and maximally 10 egg masses. Therefore, the moths were allowed to oviposit for their remaining life-spans (three to four weeks). During this period, they were fed with a solution of 10 g honey in 1 liter of 1% water-agar supplied in 0.5 ml Eppendorf tubes. The egg masses laid in the bioassays were counted every two to four days. Behavioral observations on *Y. cagnagellus* have shown that the final choice of the oviposition site is made before the actual egg deposition. During the deposition of the egg mass, the host is no longer examined (Hora and Roessingh, 1999). Therefore, preference was determined from the number of egg masses laid on each alternative rather than on the total number of eggs.

Plant Extracts. ATs were treated with phytochemicals extracted from the twig or leaf surface of host plant *E. europaeus*, or of non-hosts *C. monogyna* (Jacq.) and *P. spinosa* L. The latter two are hosts for the closely related oligophagous *Y. padellus* L. but are not accepted for oviposition by *Y. cagnagellus* (Bremner et al., 1997).

Surface extracts were prepared as described by Städler and Roessingh (1991). Approximately 8 m of leafless twigs, cut into 10-cm pieces, or intact leaves were dipped for 5 sec in 0.5 l CH_2Cl_2 followed by two 5 sec dips in two portions of 1 l MeOH. To enable calculation of the ensuing concentration of extract, the diameter (d) and length (l) of the twigs were measured, and the total surface of the extracted twigs was calculated as $\Sigma(l * \pi * d)$. For the leaf surface extract, the total leaf area was measured by using a photoplanimeter. The concentration of the extracts was expressed in twig (or leaf) surface equivalents (TSE or LSE), with units of square centimeters per milliliter of solvent. Concentration of the extract on the AT was controlled by application of volumes of the extract in proportion to the surface treated. Thus, 1 TSE on the AT equaled the concentration of compounds extracted from a similar twig surface.

Fractions were concentrated in a rotary evaporator under reduced pressure of approximately 190 mbar at a temperature of 40°C to a volume of approximately 40 ml. Methanolic extracts were pooled before evaporation. Thus, two fractions were obtained: the methanolic fraction containing the polar phyto-

chemicals: and the dichloromethane (CH_2Cl_2) fraction containing the more apolar substances.

Experiments with Surface Extracts of E. europaeus. A first series of experiments served to examine the stimulatory effect of surface extracts of the host *E. europaeus*. The ATs in these experiments consisted of plain glass pipets. The complete extract (i.e., a mixture of the methanol and CH_2Cl_2 fraction) was applied. The methanol fraction was applied first and allowed to dry, whereupon the CH_2Cl_2 fraction was added. The treated ATs were offered in two-choice experiments. The alternative choices were either a host plant twig (of equal length and diameter to the AT and kept fresh in 1% water agar) or a clean glass control (consisting of an AT treated with the solvents only). Two repetitions of 10 and eight individual females each in both choice situations were carried out. The concentration of the twig surface extracts was 1 TSE for the first trial and 0.1 TSE for the second. The results of the repetitions were pooled since no significant differences were found (Mann-Whitney, $P > 0.05$).

To determine which fraction contained compounds stimulating oviposition, the methanol and the CH_2Cl_2 fractions were applied separately on the artificial twigs. ATs treated with the two separate fractions were offered to individual females in two-choice tests ($N = 28$). Additionally, ATs treated with the methanolic extract were compared with either an *E. europaeus* twig ($N = 23$) or a control pipet treated with methanol ($N = 29$). Two repetitions were carried out with concentrations of 1 TSE.

The methanolic extract of host plant twigs was tested against the methanolic extract of leaves to determine whether the moths could discriminate between leaves and twigs when only phytochemical information is present. Both extracts were applied on ATs in a concentration of 1 TSE for the twig surface extract and 1 LSE for the leaf surface extract. Both were offered in a two-choice situation to 26 individual female moths.

The response of females to different concentrations of the methanolic twig surface fraction was determined by simultaneously offering six ATs treated with increasing concentrations of the extract to groups of 10 pairs of moths. A first series of experiments was carried out comparing concentrations of 0.0, 1×10^{-3} , 1×10^{-2} , 1×10^{-1} , 5×10^{-1} , and 1 TSE. This comparison was repeated 12 times with a total of 120 females. In the second series, a higher concentration of 5 TSE was added to the exclusion of the 5×10^{-1} TSE concentration. This was repeated 9 times with 90 female moths. The various concentrations were prepared by diluting increasing volumes of the original extract with MeOH to 1.5 ml, which was then sprayed on 9–12 ATs.

Experiments with Twig Surface Extract of Host and Nonhosts. ATs covered with paper tape and treated with 1 TSE of methanolic *E. europaeus* or *C. monogyna* extract were offered in a two-choice situation to 80 female moths in eight cages with 10 females per cage. The same experiment was carried out with

P. spinosa extract as the alternative choice to extract of *E. europaeus* (25 females in five cages with five females per cage).

Form and Structure of Oviposition Site. This experiment was carried out to determine the effect of the basic cylindrical form of a twig. Sixteen gravid females were offered 1 TSE of methanolic *E. europaeus* extract on a piece of filter paper (Whatman, 4.2 cm diameter) in a no-choice situation. The filter paper was treated with an amount of extract corresponding to the surface area. A ridge was folded in the center to present an irregularity in the surface. The papers were offered in round plastic Petri dishes (10 cm diameter, 1.5 cm high). The amounts and location of egg masses laid by these females were compared to those of 50 females from the same rearing unit that had been preconditioned under the same circumstances and were presented with cylindrical ATs treated with 1 TSE of the same methanolic extract. The ATs were covered with paper adhesive tape to present a similar surface texture.

Surface texture was tested by comparing oviposition on glass or tape ATs. Fifty females were tested in five cages containing a choice between glass ATs and ATs covered with paper adhesive tape, both treated with 1 TSE of methanolic *E. europaeus* extract.

RESULTS

Surface Extracts of *E. europaeus*. The complete twig surface extract of *E. europaeus* was preferred over the clean glass control (Wilcoxon, $P < 0.001$; Figure 2). However, it was not as attractive as the natural host twig (Wilcoxon, $P < 0.05$; Figure 2). Moths preferred the MeOH fraction to the CH_2Cl_2 frac-

FIG. 2. Number of egg masses deposited by *Yponomeuta cagnagellus* in two-choice situations with artificial twigs treated with the complete twig surface extract of *Euonymus europaeus* and either cut twigs of *E. europaeus* ($N = 18$ females) or artificial twigs treated with solvents ($N = 18$ females) only. *Significantly higher number of egg masses, Wilcoxon, $P < 0.05$; ***Wilcoxon, $P < 0.001$.

FIG. 3. Number of egg masses deposited by *Yponomeuta cagnagellus* in two-choice situations comparing artificial twigs treated with 1 TSE (twig surface equivalent) of the methanolic fraction of twig surface extract of *Euonymus europaeus* with cut twigs of *E. europaeus* ($N = 23$ females), with artificial twigs treated with solvent only ($N = 29$ females) or with 1 TSE of the CH_2Cl_2 fraction ($N = 28$ females). **Significantly higher number of egg masses, Wilcoxon, $P < 0.01$.

tion and the clean glass control (Wilcoxon, $P < 0.01$; Figure 3). Analogous to the complete extract, the MeOH fraction was not as attractive as the host twig (Wilcoxon, $P < 0.01$; Figure 3). We attempted to test the CH_2Cl_2 fraction against a negative control as well, but did not obtain an adequate level of oviposition; only one female of five deposited egg masses on the CH_2Cl_2 fraction, others did not oviposit at all.

The activity of the methanolic leaf surface extract did not differ from that of the twig surface extract (Wilcoxon, $P > 0.05$; Figure 4). The stimulatory activity of the twig surface extract was dose dependent (Figure 5). The highest number of egg masses was laid on the highest concentration of the extract in both repetitions. The two dose-response curves did not differ significantly (Kolmogorov-Smirnov, $P > 0.05$).

FIG. 4. Number of egg masses deposited by *Yponomeuta cagnagellus* in a two-choice situation with artificial twigs treated with either 1 LSE methanolic leaf surface extract or 1 TSE methanolic twig surface extract ($N = 26$ females). No significant difference was found (Wilcoxon, $P > 0.05$).

FIG. 5. Percentage of egg masses deposited by *Yponomeuta cagnagellus* in a six-choice situation with artificial twigs treated with increasing concentrations of methanolic twig surface extract. First series: 0.0, 1×10^{-3} , 1×10^{-2} , 1×10^{-1} , 5×10^{-1} , and 1 TSE ($N = 120$ females); Second series: 0.0, 1×10^{-3} , 1×10^{-2} , 1×10^{-1} , 1 TSE, and 5 TSE ($N = 90$ females).

Specificity of Twig Surface Extract. *Yponomeuta cagnagellus* preferred ATs treated with the twig surface extract of *E. europaeus* over ATs treated with either *C. monogyna* or *P. spinosa* extract (Wilcoxon, $P < 0.001$; Figure 6A and B).

Form and Structure of Oviposition Site. Of the 16 females, only four oviposited on the filter paper treated with the methanolic twig surface extract (on average 0.6 ± 0.3 egg mass/female). The others did not oviposit at all during the remainder of their life-spans. Of these four females, two also deposited

FIG. 6. Number of egg masses deposited by *Yponomeuta cagnagellus* in a two-choice situation with artificial twigs treated with 1 TSE of either methanolic twig surface extract of the host *Euonymus europaeus* or 1 TSE methanolic twig surface extract of non-host *Crataegus monogyna* (A; $N = 80$ females) or non-host *Prunus spinosa* (B; $N = 25$ females). **Significantly higher number of egg masses (Wilcoxon, $P < 0.01$).

FIG. 7. Number of egg masses deposited by *Yponomeuta cagnagellus* in a two-choice situation with either bare glass artificial twigs or artificial twigs covered with paper tape, both treated with 1 TSE methanolic twig surface extract of *Euonymus europaeus* ($N = 50$ females). *Significantly higher number of egg masses (Wilcoxon, $P < 0.05$).

egg masses beside the filter papers on the floor of their Petri dishes (40% of all egg masses). The average number of egg masses laid by the control group on ATs was higher (1.5 ± 0.2 egg mass/female) (Mann/Whitney, $P < 0.05$). Moreover, deposition of egg masses on the floor of the cage, away from the AT in the control group, occurred only incidentally (9% of all egg masses). As in the preceding experiments, egg masses on the ATs were preferably deposited against the orthodontic elastics.

Tape-covered ATs were preferred over bare glass ATs (Wilcoxon, $P < 0.05$; Figure 7). The slight ridge that was formed by the paper tape where the ends meet presented an additional preferred oviposition site.

DISCUSSION

Surface Extracts. The results show that oviposition of *Y. cagnagellus* is stimulated by polar, MeOH-soluble compounds that can be washed from the surface of *E. europaeus* twigs (Figure 3). The stimulatory activity of this extract is dose dependent: higher concentrations increase oviposition (Figure 5). Plant surface extracts have been described to be effective oviposition stimulants in a number of insects (e.g., Städler and Roessingh, 1991). In butterflies, it seems to be a general characteristic that stimulant surface compounds are quite polar (Renwick and Chew, 1994). The compounds in the *E. europaeus* extract stimulating oviposition in *Y. cagnagellus* conform to this pattern.

In moths, the role of surface compounds has not been as well studied as for butterflies, although reports of the effectiveness of host plant homogenates or compounds distilled from these homogenates are available for some moths (references in Renwick and Chew, 1994; Honda, 1995). However, homogenates are not necessarily representative of the chemical composition on the actual plant surface. The relative concentration of compounds in leaf tissue is not always the same as on the surface, and some secondary compounds may not penetrate

to the surface (Soldaat et al., 1996). Therefore, the reaction of moths to plant homogenates might not represent their natural behavior.

We believe a better approach for testing behavioral responses is the use of extracts of the plant surface as described in this paper. However, there are only a few reports of oviposition of moths in response to plant surface extracts [e.g., *Acrolepiopsis assectella* (Thibout and Auger, 1996), *Keiferia lycopersicella* (Burton and Schuster, 1981), *Manduca sexta* (Bordner et al., 1983), *Ostrinia nubilalis* (Derridj et al., 1992)]. The extracts eliciting positive responses in these moths were obtained by using water, ethanol, or methanol solvents. *Acrolepiopsis assectella* and *K. lycopersicella*, both specialist moth species, showed oviposition responses similar to *Y. cagnagellus*, i.e., substrates treated with host plant extracts were preferred strongly over controls.

Although *Y. cagnagellus* showed a strong response to the surface extract of its host, the artificial twig treated with host surface extract was not as attractive as the real host plant twig (Figures 2 and 3). This might be a result of a combination of deficiencies of the artificial substrate. First, the extraction method might not extract all relevant surface components, and their concentrations might not be similar to those on the twig surface. An indication that the latter might be involved was present in the dose-response test, where a concentration of 5 TSE was preferred over 1 TSE (Figure 5). However, gravid *Y. cagnagellus* did not behave differently on natural *E. europaeus* twigs or artificial twigs treated with 1 TSE methanolic twig surface extract (Hora and Roessingh, 1999), implying that all relevant chemical cues are present in the extract. Second, the host twig may emit general, possibly stimulatory cues—such as moisture—which can enhance attractivity of oviposition substrates (Renwick and Chew, 1994). Visual and tactile cues may also modify oviposition behavior in moths (Renwick and Chew, 1994) and contribute to the preference for real twigs.

Specificity of Twig Surface Extracts. In bioassays that use natural twigs, *Y. cagnagellus* shows an absolute oviposition preference for its host plant *E. europaeus* (Bremner et al., 1997). Our data show that females are able to choose the correct host only from the methanolic surface extract (Figure 6A and B). This implies that the compounds in the extract are mainly responsible for the choice of the host plant by the adult females. The stimuli are probably perceived by the insect with sensilla located on their antennae or tarsi (Hora and Roessingh, 1999). Nonhost extracts either do not contain these stimuli or possibly contain compounds deterring females from oviposition.

The compounds remain to be identified. Plant chemicals influencing oviposition in moths have been studied extensively, but only a few examples where specific compounds are identified are known (Honda, 1995). Moreover, even when active compounds are known, interpretation is complicated by fact that some Lepidoptera depend on synergistic action of various chemicals (Honda, 1990). Proportions of primary compounds (e.g., sugars or proteins) on the plant

surface can be species specific and may affect the reaction to the secondary compounds in the mixture (Soldaat et al., 1996).

Form and Surface Structure. *Yponomeuta cagnagellus* females seem to be well adapted to select the appropriate food plant for their offspring. One would expect the discrimination behavior of a specialist to reflect the suitability of the host plant for larval development (Wiklund, 1975). Specialist insects may be presumed to distinguish qualitative differences among host plants (Janz and Nylin, 1997). Thus, it is probable that various other cues, besides specific secondary compounds, will influence choice of the oviposition site. These may be chemical, visual, or tactile.

Although plant surface compounds seem to be a key stimulant, form and surface structure of the substrate also affect oviposition in *Y. cagnagellus*. The substrate needs to resemble the basic form of a twig (i.e., cylindrical), and flat filter paper is barely accepted, even if it is treated with twig surface extract. This seems adaptive behavior to distinguish leaves from twigs: leaves are an unsuitable oviposition site since they do not survive winter. This distinction is apparently not based on the surface compounds, as extracts of both leaves and twigs are acceptable for oviposition (Figure 4).

Surface texture affects the oviposition of many moths. They prefer smooth, hairy, or rough surfaces, depending on the peculiarities of their natural host plant(s) or relating to the species-dependent optimal condition for survival of the eggs (Ramaswamy, 1988; Renwick and Chew, 1994). The preference of *Y. cagnagellus* for paper tape (a rough surface) over glass (smooth) can be due to the rough surface of twigs in general. Alternatively, the tape-covered AT simply provides a better grip for the moths, making it more accessible.

CONCLUSION

Yponomeuta cagnagellus females appear to depend on a combination of specific chemical and tactile cues for host plant discrimination. If these are lacking, the moths will not oviposit. This is in contrast with the behavior of some (polyphagous) moth species that are reported to deposit eggs on blank controls and cage walls, even if generally better preferred substrates are available (e.g., Ramaswamy, 1988; Udayagiri and Mason, 1995). The fastidious discrimination behavior of *Y. cagnagellus* can be related to their specialization and tendency to deposit eggs in a few (1–10) large masses. This suggests that the moths are not time-limited in their search and discrimination behavior (Singer, 1984). It also implies there is strong selection pressure on females to be selective, as misplacement of an egg mass can lead to the loss of 10–100% offspring.

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