Microorganisms on *Aesculus hippocastanum* – olfactory perspective of *Cameraria ohridella* (Deschka & Dimic)

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Abstract: Mikroorganismen an *Aesculus hippocastanum* – olfaktorische Perspektive von *Cameraria ohridella* (DESCHKA & DIMIC).

Seit den 80er Jahren wird die Gemeine Rosskastanie *Aesculus hippocastanum* durch den Minierer *Cameraria ohridella* (Lepidoptera, Gracillariidae) befallen. Außerdem wird die Rosskastanie durch den Blattbräunepilz *Guignardia aesculi* und den Mehltau *Erysiphe flexuosa* attackiert. Oft treten alle drei Organismen parallel am gleichen Blatt auf. Weiterhin konnten endophytische Pilze aus dem Blattgewebe isoliert werden. Im vorliegenden Beitrag wird die volatile Interaktion zwischen Pflanze, Mikroorganismen und Insekt diskutiert.
Mit Hilfe der Gaschromatographie und gekoppelter Massenspektroskopie (GC-MS) wurden Duftproben gesunder und mit den pathogenen Pilzen *G. aesculi* und *E. flexuosa* gleichzeitig infizierter Blätter der Rosskastanie analysiert. Identifizierte Komponenten wurden elektrophysiologisch (EAG) an der Insektenantenne und in Verhaltensversuchen getestet. Mit den pathogenen Pilzen befallene Rosskastanienblätter geben 1-Octen-3-ol, 3-Octanon, ein Derivat von 2(5H)-Furanon, Nonanal und Decanal ab. *C. ohridella* war in der Lage, diese Substanzen zu detektieren. In Zweifachwahltests mit gesunden *A. hippocastanum* Zweigen reagierten Weibchen mit reduzierter Eiablage auf die Applikation von 1-Octen-3-ol, 3-Octanon, 2(5H)-Furanon und Decanal im Vergleich zur unbehandelten Kontrolle. Es ist bekannt, dass 1-Octen-3-ol und 3-Octanon von Pilzen selber emittiert werden. Nonanal und Decanal werden von Zellen, die nach Penetration durch Pilzhyphen unter oxidativem Stress stehen, produziert. Die Derivate von 2(5H)-Furanon wirken antimikrobiell und können auf einen Schutzmechanismus der Pflanze oder auf einen Konkurrenzmechanismus von Mikroorganismen um denselben Lebensraum hinweisen. Eine mögliche Erklärung wäre, dass diese Substanz von Endophyten zur Verteidigung des sie umgebenden Blattgewebes gegen die pathogenen Pilze produziert wird.

Key words: *Aesculus hippocastanum*, *Cameraria ohridella*, pathogenic fungi, endophytic fungi, GC, MS, EAG, volatiles, bioassay, oviposition

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Since the 80s, the popular park- and city tree *Aesculus hippocastanum* has been infested with the leaf miner *Cameraria ohridella* (DESCHKA & DIMIC 1986). Additionally, the pathogenic fungi *Guignardia aesculi* causes leaf blotch disease and *Erysiphe flexuosa* causes powdery mildew on horse chestnuts. Often, all three diseases occur in parallel at the same leaves leading to a situation of competition. Moreover, recently some endophytic fungi were isolated from the leaf tissue of *A. hippocastanum*. In the present study, the volatile interaction between three trophic levels plant, insect, and fungi are discussed.

Methods and Materials

Healthy leaves and leaves co-infected by the pathogenic fungi *Guignardia aesculi* and *Erysiphe flexuosa* were collected from trees of *A. hippocastanum*. The fifteen odour samples from healthy and co-infected leaves were obtained using the CLSA-method (closed-loop-stripping-analysis) (BOLAND et al. 1984). For each sample five leaves were put into 250 ml glass flasks from which air was circulated through a charcoal

filter with a flow of 1 l/min for a sampling time of 45 min. Volatiles were eluted with 75 µl of a mixture of methylene chloride and methanol (2:1). Furthermore, the pathogenic fungus *G. aesculi* (pure culture from Centraalbureau voor Schimmelcultures CBS) was cultivated (22°C, UV) on malt agar (malt extract 2%, International Diagnostics Group; agar 1%, Roth; distilled water). The odours were sampled from *G. aesculi* cultures and pure malt agar (control) using thermodesorption (TDS) tubes OD 6.0 mm, filled with Tenax TA 60/80 (Gerstel). For sampling open Petri dishes were enveloped in plastic roasting bags free of plasticizer. The sampling time was 30 min; 1l/min. Odour samples were analysed with a gas chromatograph (model 6890N, Agilent, Palo Alto, USA) equipped with a HP-5MS column (length 30m, ID 0.25 mm, film thickness 0.25 µm, Agilent) and coupled with a mass spectrometer (model 5973N, Agilent). Helium was used as carrier gas at a constant flow of 1 ml/min. The GC employed the following temperature programs: for CLSA samples start 50°C, hold for 1.5 min, ramp 6°C/min to 200°C, hold for 5 min and for TDS start 40°C, hold for 3 min, ramp 7,5°C/min to 200°C, hold for 5 min. The NIST mass spectral library (National Institute of Standards and Technology) was used for identification of compounds.

For electrophysiological investigations insect antenna were fixed in an antenna holder (FÄRBERT et al. 1997). Dose-response series (amplified 100x) of insect antennae were measured by manually puffing (puls duration 0.5s) of 5 ml air from glass syringes, which contained pieces of filter paper drenched with standard dilutions of stimulus compounds in paraffin oil into a constant stream of clean air (flow rate 400 ml/min). A minimum of three insect antennae originated from different individuals were tested per compound. Each antenna was stimulated for three times to get a reproducible result. The following compounds were tested: 1-octen-3-ol (Merck-Schuchardt), 3-octanone (Acros), 2(5H)-furanone (Sigma-Aldrich), nonanal (Merck-Schuchardt) and decanal (Acros).

In "dual choice tests" individual olfactory active compounds were tested regarding effects on oviposition. For the assays a lighted (1 klx) cage (180×75×75 cm) was used. On leaflets of two green twigs of *A. hippocastanum* filter papers covering 30-50 % of the leaf surfaces were fixed with a needle. The filter papers of one twig were soaked with paraffin oil solutions of a single odorant compound ($1 \cdot 10^{-3}$) whereas the filter papers of the second twig were treated with paraffin oil only (control). In this way, the volatile pattern of host tree leaves was overlaid with an additional compound typical for fungal infection. Fresh odour solution was added every 6 hours (aldehydes) or 12 hours (control-bioassay, other compounds). Per assay 250 adults were captured in the field one day before the experiment and chilled overnight in a dark chamber (5°C). In the beginning of the experiments they were released in the middle of the cage. Eggs on leaves were counted after 24 hours (aldehydes) or 48 hours (other bioassays). The statistical analyses for bioassays were carried out with Chi-square Test ($\alpha = 0.05$); Statistica 6.1.

Results

Co-infection by the fungi *G. aesculi* and *E. flexuosa* induced the release of additional volatiles on *A. hippocastanum* leaves (Fig. 1) Volatiles from *G. aesculi* cultivated on malt agar extract were not detected. The leafminer *C. ohridella* was able to detect fungi induced volatiles (Fig. 2). In bioassays the influence of single fungi induced volatile on oviposition of *C. ohridella* was tested. The total number of deposited eggs varied widely. However, on leaves treated with odorant compounds typical for fungi infection fewer eggs were deposited than on control leaves. Only nonanal had no significant impact on oviposition (Fig 3).

Discussion

Pathogenic fungi infection by *G. aesculi* and *E. flexuosa* induced the release of additional volatiles in leaves of *A. hippocastanum*. 1-Octen-3-ol and 3-octanone are known to be produced by fungi (KAMINSKI et al. 1974). On the malt agar *G. aesculi* grew very slowly and without detectable volatile emissions. The release of volatiles and characteristics of fungi can depend on cultural conditions and composition of the growth media (KAMINSKI et al. 1974). In order to examine the production of volatiles by *G. aesculi*, different cultural conditions and media will be tested in further experiments. To our knowledge, it is not possible to cultivate *E. flexuosa* on artificial media.

Volatiles related to fungal infections were detected by *C. ohridella* antennae. Except for nonanal, the compounds led to a decreased oviposition on leaves of *A. hippocastanum*. The variation in total numbers of eggs deposited in each bioassay is most probably caused by the oviposition activity of generations in the

field. Additionally, individual compounds offered in the bioassay cages may have stimulating/inhibitory effects on oviposition. Further studies concerning this topic are planned.

Nonanal and decanal indicate oxidative stress in cells (SCHÜTZ 2001) and may be released by penetration of plant cells by fungi. The pathogenic fungi and the leafminer use the same food resource. The leaf blotch disease causes necroses on the leaf tissue. One larva of *C. ohridella* needs a mining space of about 4 cm² (PSCHORN-WALCHER 1994). In order to ensure an uninhibited growth of larvae, an avoidance of leaf tissue infected by pathogenic fungi for oviposition might be beneficial. So, most probably, the females use 1-octen-3-ol, 3-octanone and decanal as volatile markers for detection of fungal infection on leaves of *A. hippocastanum*.

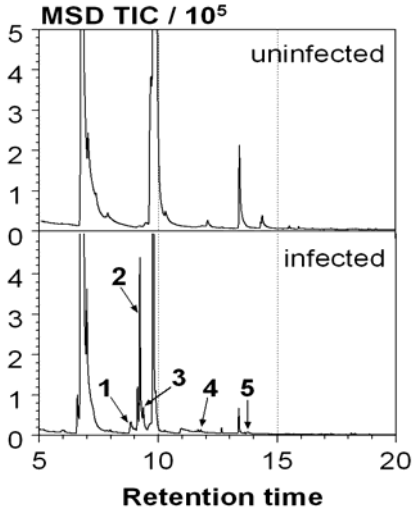

Fig. 1: Chromatograms of *A. hippocastanum* leaf volatiles. Compounds related to co-infection of *G. aesculi* and *E. flexuosa*. **1**: 5-ethyl-2(5H)-furanone; **2**: 1-octen-3-ol; **3**: 3-octanone; **4**: nonanal; **5**: decanal.

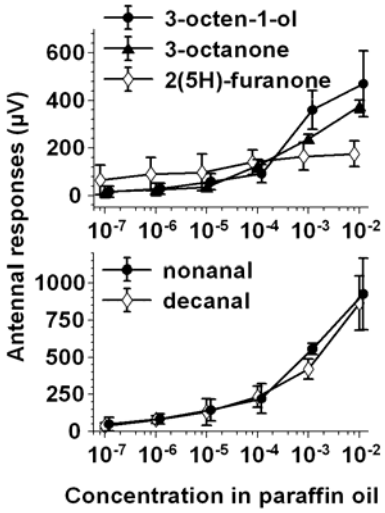

Fig. 2: Dose-dependent responses of *C. ohridella* antennae to compounds emitted by fungal infected leaves of *A. hippocastanum*.

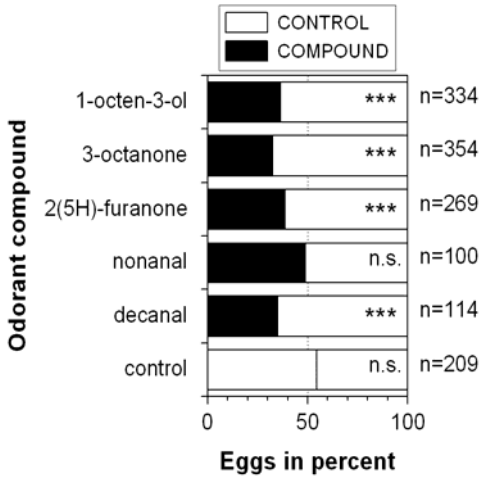

Fig. 3: Oviposition of *C. ohridella* on leaves of *A. hippocastanum* treated with fungus odour compounds solved in paraffin oil ($1 \cdot 10^{-3}$, COMPOUND) and paraffin oil only (CONTROL) in dual-choice-tests; chi²-test: n.s. not significant, *** $p \leq 0.001$; n=total number of eggs.

Moreover, 5-ethyl-2(5H)-furanone was released by leaves because of fungal infection. Derivatives of 2(5H)-furanone are known to have antifungal as well as antibacterial activity (PAULITZ et al. 2000). The derivatives were found to be produced by higher plants (*Ranuculus* ssp.; MIRSA & DIXIT 1980), bacteria *Pseudomonas aureofaciens* with antifungal activity (PAULITZ et al. 2000) and fungi (*Trichoderma harzianum*; ORDENTLICH et al. 1992). 5-ethyl-2(5H)-furanone may be produced by *A. hippocastanum* to protect itself againstpathogenic fungal infection, or by microorganisms living on leaves of the tree. Recently, endophytic fungi have been isolated from leaf tissue of *A. hippocastanum* (PASTIRČÁKOVÁ 2004). On leaves infected by *G. aesculi* and *E. flexuosa* the pathogenic and endophytic fungi compete for the same habitat. Therefore it is also conceivable that endophytic or pathogenic fungi release the 2(5H)-furanone derivatives to protect the surrounding leaf tissue. Endophytic fungi are known to produce antimicrobial volatiles that inhibit or kill other fungi or bacteria (STROBEL et al. 2001). Whether the release of this compound indicates a response of the plant against pathogenic fungi or the competition between microorganisms on the same host remains to be revealed. *C. ohridella* is able to detect the antimicrobial compound 2(5H)-furanone and responds with reduced egg deposition. The compound 2(5H)-furanone was tested as a substitute for its antifungal derivative 5-ethyl-2(5H)-furanone identified in leaf samples of *A. hippocastanum.* Therefore, this result has to be interpreted with care. The presence of microorganisms like endophytes may have a negative effect on larvae. In leaf mines on oak trees injected with endophytic fungi larvae of *Cameraria* ssp. developed slower than larvae in mines without fungi (FAETH & HAMMON 1997). Further on, it was shown for *Cameraria* ssp. on *Quercus emoryi* that the presence of microorganisms causes a reduction in oviposition (WILSON & FAETH 2001).

The present study demonstrated that *C. ohridella* detect odorant compounds emitted by or induced by microorganisms colonizing leaves of *A. hippocastanum*. In response to the majority of these compounds, *C. ohridella* females reduce the number of eggs deposited on treated leaves if compared to untreated control leaves of the horse chestnut.

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