Pollinator-attracting semiochemicals of the wasp-flower *Epipactis helleborine*

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Abstract: Die Orchideenart *Epipactis helleborine* gilt als typische Wespenblume. Die Blüten weisen Anpassungen an den Besuch und die Bestäubung durch soziale Faltenwespen (Hymenoptera: Vespidae) auf und werden häufig durch *Vespula vulgaris* und *V. germanica* bestäubt. In früheren Untersuchungen konnte gezeigt werden, dass olfaktorische Reize bei der Bestäuberanlockung eine übergeordnete Bedeutung vor optischen Reizen haben (Hölzler 2003). Die Frage, warum *E. helleborine* fast ausschließlich ihren optimalen Bestäuber, die soziale Faltenwespe, zur Bestäubung anlockt, und nicht auch auf andere Blütenbesucher attraktiv wirkt, ist noch unbeantwortet. Wir untersuchten die Hypothese, dass *E. helleborine* Blüten GLVs, die von Herbivoren befallenen Pflanzen abgegeben werden, nachahmen, um Beute jagende Wespen zur Bestäubung anzulocken. Dazu sammelten und analysierten wir Duftstoffe von *Epipactis* Blüten und mit *Pieris*-Raupen befallenen Kohl und identifizierten vier gemeinsam vorkommende GLVs. In Y-Rohrtests konnte die wespenanlockende Wirkung dieser Verbindungen nachgewiesen werden.

Key words: *E. helleborine*, social wasps, mimicry, infested cabbage, GLVs, floral scent, pollination

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The orchid genus *Epipactis* is represented by 25 species in Europe (Richards 1982). *Epipactis helleborine* (L.) Crantz is the most common and widely distributed species of the genus (Wiefelspütz 1970), and is a prime example for wasp-flowers, because it is mainly pollinated by social wasps (Hymenoptera: Vespidae), like *Vespula vulgaris* and *V. germanica* (Müller 1873). Darwin (1888) already noticed that *E. helleborine* is almost exclusively ignored by bees and bumblebees, an observation that was confirmed in recent investigations (Keppert 2001). The flowers of *E. helleborine* show morphological, physiological and phenological adaptations to the visit and the pollination by Vespidae (Keppert 2001). They possess a reddish-brown or dirty purple coloration of the inflorescence (Keppert 2001), have relatively small, mostly bulbous blossoms with a broad entrance and bulbous widened, nectar-rich juice holders (Müller 1873, 1881; Schremmer 1962).

Although there is much reported about wasp-pollinated flowers there is little known about the signals that are responsible for the attraction of wasps. Wiefelspütz (1970) proclaimed the statement that only the visual stimulus is responsible for the wasp attraction. Recently studies, however, assumed that odour is involved in the wasp attraction (Keppert 2001). Hölzler (2003) showed that the main attraction of the wasp-flower *Epipactis* for pollinators is its olfactory stimulus. It remains an unanswered question why *E. helleborine* flowers almost exclusively attracts social wasps, as opposed to bees and bumblebees.

In this study we analysed the role of floral volatiles which are responsible for the specific attraction of social wasps. We supposed a mimicry-system in *E. helleborine* for the specific attraction of pollinators for the following reasons. So-called "green leaf volatiles" (GLVs) are emitted by plants while herbivorous insects, for example caterpillars, feed on them. GLVs thereby attract predators or parasitoids of the herbivorous insects (Dicke & Sabelis 1988; Turlings & al. 1990, 1995; Dicke & Vet 1999). Among the GLVs so far identified in former studies there are aldehydes, compounds that were also found in flower extracts of *E. helleborine* (Hölzler 2003). Therefore, we postulated that *E. helleborine* flowers produce GLVs in order to attract prey hunting social wasps for pollination. We performed bioassays and analysed flower odour gained to headspace-sampling using gas chromatography (GC), mass spectrometry (GC-MS) and gas chromatography coupled

with electrophysiological analysis (GC-EAD) to investigate the hypothesis that *E. helleborine* flowers mimic "green leaf volatiles" (GLVs) to attract their pollinators.

Materials & Methods

Plant Material and Volatile Collection

Floral scent emitted from *E. helleborine* flowers and cabbage infested by *Pieris brassicae* caterpillars was collected using headspace-sampling. Plants were enclosed in polyester oven bags (Toppits®, Germany), and volatiles were trapped in an absorbent tube (CLSA, 1.5mg, Gränicher & Quartero and Super Q, 5mg, Poropak Q, Waters Devision of Millipore) by using a membrane pump, adjusted to a flow rate of 500ml/min for app. 9 h. The inflowing air stream was cleaned by a charcoal filter (activated charcoal, Supelco, Orbo 32 large). The trapped volatiles in the absorbent tube were desorpt with dichloromethane (Sigma-Aldrich, HPLC grade).

Chemical analyses

The headspace samples were analyzed on a HP 6890 gas chromatograph (Hewlett-Packard, Agilent Technologies) equipped with a non-polar DB-5ms and a polar DB-Wax capillary column (J&W, 30m x 0.25mm). The gas chromatograph was operated splitless at 50°C for 1min. Thereafter the splitter was opened and the oven temperature increased with a rate of 10°C/min to 240°C when using a DB-Wax column and to 310°C when using a DB-5ms column. Structure elucidation of individual compounds of the samples was based on gas chromatography-mass spectrometry (GC-MS), performed on a VG70/ 250 SE instrument, Vacuum Generators, Manchester, England, linked to a HP 5890 gas chromatograph (FFAP column, 50m) at the Institute of Organic Chemistry, University of Hamburg.

Electrophysiology

Electrophysiological analyses of the headspace samples from *E. helleborine* flowers and cabbage infested by *Pieris brassicae* caterpillars were performed with a GC-EAD system. Workers of *V. germanica* and *V. vulgaris* females were caught from two nests in the surrounding of the campus of the University of Ulm. The GC-EAD system consisted of a gas chromatograph (HP 6890, Hewlett-Packard, Agilent Technologies) equipped with a flame ionization detector (FID) and an EAD setup provided by Syntech (Hilversum, Netherlands). After performing gas chromatographic analyses (see above) the gas stream was splitted (split ratio 2:1), 30ml/min of make-up gas (Nitrogen) was added and the gas stream was simultaneously directed to the detector of the GC (FID) and the wasp antennae, mounted between two glas-electrodes (EAD). As carrier gas we used hydrogen (2ml/min). The gas chromatograph was operated splitless at 50°C for 1min, following by opening the split and programming to 240°C/310°C at 10°C/min. GC-EAD active compounds were identified by GC-MS and co-injection technique. To identify GLVs in the *E. helleborine* samples we compared the odour samples from *E. helleborine* with cabbage infested by *Pieris brassicae* caterpillars, which emitted GLVs.

Bioassays

A Y-tube experiment was performed with the intention to compare the attractiveness of individual odour resources for *V. vulgaris* and *V. germanica* workers. The olfactometer consisted of a Y-shaped glass tube (length 22cm, Ø 0.8cm) horizontally fixed in a polystyrene box (18 x18 x 16cm). To avoid light irritations the only light resource was a cold light lamp (Schott KL 1500 LCD, 2950K) placed above the centre of the Y-tube. The test plants were put in glass cylinders, which were connected with teflon or silicon tubes to the Y-tube. Using a motor pump (Volcraft, Laboratory Power Supply, PS-302A) the air stream (air flow 100ml/min) was sucked through an activated charcoal filter (activated charcoal, Supelco, Orbo 32 large) before it passed the two glass cylinders where the air was enriched with the odour of the test plants. The air stream was directed in every leg of the Y-tube. We also tested a synthetic mixture of GLVs. Therefore filter paper was impregnated with 5µl of the mixture and placed in each end of the shorter Y-tube arms.
A wasp was put into the long arm of the Y–tube and its choice behaviour was noticed. For the statistical analyses of the Y-tube experiments we used the "Sign test".

Results

Electrophysiology and chemical analyses

Using GC-EAD analyses we found in headspace samples collected from cabbage infested by *Pieris brassicae* caterpillars and *E. helleborine* flowers four electrophysiologically active compounds in common on which *V. germanica* as well as *V. vulgaris* female antenna responded. Using the non-polar DB-5ms capillary column we found three EAD active substances that are produced in infested cabbage and by *E. helleborine* flowers. Substance x1 was identified as a common GLV (Fig.1). In GC-EAD analyses with the polar DB-Wax capillary column we found a further GLV and two aldehydes also known as GLVs. All the compounds were analyzed and synthesized.

Bioassays

The result of the Y-tube experiments showed that the wasps significantly preferred the odour of cabbage and infested cabbage compared to the empty control (Sign test, n=20/24, p=0.01). Furthermore, they preferred infested cabbage compared to non-infested cabbage (Sign test, n=43, p=0.001). A synthetic mixture consisting of two GLVs, that are produced by infested cabbage and by *E. helleborine* flowers was significantly more often preferred in comparison to the control (Sign test, n=28, p=0.02) (Fig.2).

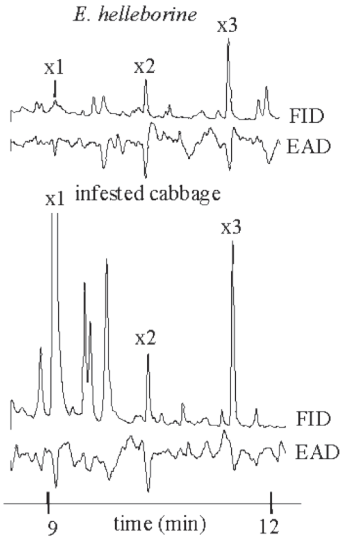

Fig.1: Comparison of GC-EAD chromatograms from *E. helleborine* and infested cabbage using a non-polar DB-5 ms capillary column. The substance x1 was identified as a common GLV.

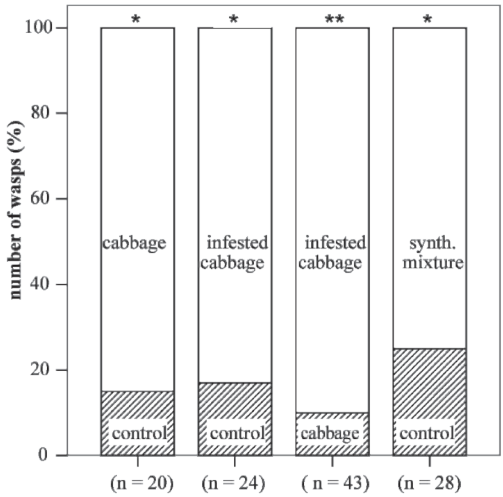

Fig.2: Comparison of the attractiveness of the odour of cabbage, infested cabbage and a synthetic mixture of GLVs for social wasps in a Y-tube olfactometer.
* p≤ 0.05, ** p≤ 0.01, Sign test

Discussion

In electrophysiological studies and chemical analyses we could prove that *E. helleborine* flowers emit four common GLVs that can also be found in cabbage invested by *Pieris* caterpillars. The assumption that the GLVs are used to attract pollinating wasps was confirmed by the Y-tube experiments we performed. *Vespula* workers preferred cabbage invested by *Pieris* caterpillars over non-infested plants and were attracted by a synthetic mixture of GLVs in common. This supports our hypothesis that *E. helleborine* flowers mimic GLVs in order to attract insect hunting wasps for pollination.

However, why should food rewarding flowers perform mimicry? We assume that wasps that are visiting *Epipactis* flowers for a first time are attracted by the volatile GLVs that will increase the probability that *Epipactis* plants can be located by the pollinators. While feeding on nectar they associate the odour with the reward and afterwards use this information to visit further *Epipactis* flowers. This will minimize pollen loss and ensure *E. helleborine* an effective pollination.

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