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Evolution of 'pollinator'-attracting signals in fungi

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Fungi produce a plethora of secondary metabolites yet their biological significance is often little understood. Some compounds show well-known antibiotic properties, others may serve as volatile signals for the attraction of insects that act as vectors of spores or gametes. Our investigations in an outcrossing, self-incompatible fungus show that a fungus-produced volatile compound with fungitoxic activities is also responsible for the attraction of specific insects that transfer gametes. We argue that insect attraction using this compound is likely to have evolved from its primary function of defence—as has been suggested for floral scent in the angiosperms. We, thus, propose that similar yet convergent evolutionary pathways have lead to interspecific communication signals in both fungi and plants.

Keywords: *Epichloë*; volatiles; scent; fly pollination

1. INTRODUCTION

Many fungi depend on insects for the dispersal of spores, which serve either as propagules or gametes for fertilization. The latter function is analogous to pollination in plants, and for 'pollinator' attraction, some fungi produce showy flower-mimics with sugar rewards, that are visited by a range of pollinator insects (Roy 1993; Roy & Raguso 1997). Other fungi attract only a few, specific pollinators, primarily by olfactory signals. Such a specialized relationship is found in endophytic fungi of *Epichloë* (Clavicipitaceae, Ascomycota), which systemically infect pooid grasses where they develop an external fruiting structure, the stroma, for sexual reproduction (Schardl *et al.* 2004). *Epichloë* spp. are self-incompatible, and sexual ascospores are only formed if gametes (conidia) of one mating type are transferred to stromata of the opposite mating type. Fungal stromata specifically attract female flies of the genus *Botanophila* (Anthomyiidae) that actively cross-fertilize and then oviposit on the fungus (Bultman *et al.* 1998). Hatching larvae consume part of the fungal tissues, which produce ascospores, but enough is left for reproduction as excessive exploitation of the fungus is prevented by greater larval mortality with increasing egg load (Bultman *et al.* 2000). Since fly larvae depend on fertilized stroma as food source, both fly and fungus clearly profit from this mutualistic association. The grass host, in turn, benefits from the fungus-produced secondary metabolites that may provide increased resistance to herbivores or pathogens (Shimanuki 1987; Breen 1994; Brem & Leuchtmann 2001).

We investigated the chemical communication in the specialized *Epichloë–Botanophila* relationship and provide an intriguing link between the antimicrobial function of a secondary metabolite and the attraction of gamete vectors in this fungus. We collected volatiles from *Epichloë* fungal stromata of two species and analysed the samples by gas chromatography coupled with electroantennographic detection (GC–EAD) to detect compounds that are physiologically active in the flies' olfactory neurons, and conducted bioassays to investigate the behavioural activity of the EAD-active compounds.

2. MATERIAL AND METHODS

(a) *Fungi, plants and insects*

For volatile collection *Epichloë typhina* infecting *Anthoxanthum odoratum* (two genotypes), and *Epichloë sylvatica* infecting *Brachypodium sylvaticum* (six genotypes) were used. Infected plants were grown in pots maintained outdoors at the Botanical Garden Zürich. Female *Botanophila* flies used for EAD were collected with hand nets while visiting *Epichloë* stromata of infected *A. odoratum* plants. Females of these flies are virtually impossible to identify by morphological traits. Thus, we are as yet unable to assign species names to the specimen caught. Flies, however, were identified by molecular markers based on sequence analysis of the mitochondrial protein coding gene cytochrome oxidase (*COII*), and comparison of sequences with those from previously identified *Epichloë*-associated *Botanophila* (Leuchtmann 2005).

(b) *Chemical and electrophysiological analysis*

Volatiles were collected from freshly emerged, unfertilized fungal stromata by headspace sorption (Huber *et al.* 2005); samples for structure elucidation were collected by rinsing 30×10 stromata in 2 ml dichloromethane for two minutes and pooling the samples. Until use, all samples were stored in a freezer at −20 °C. For GC–EAD the GC was equipped with a HP5 column (30 m× 0.32 mm internal diameter (i.d.)×0.25 μm film thickness) and a flame ionization detector (FID); helium was used as carrier gas. One microlitre of each sample was injected splitless at 50 °C (1 min) into a GC (Agilent 6890N) followed by opening the split valve and programming to 300 °C at a rate of 10 °C min^{-1}. For EAD recording, heads of individual flies were cut off and mounted on a grounded glass electrode filled with insect ringer solution and mounted on a micromanipulator. A second glass electrode (recording electrode) was connected to the tip of the funiculus of one of the flies' antenna; the arista of this antenna was cut off at its base.

(c) *Structural assignment*

Analysis by coupled GC/mass spectrometry (MS) (double focusing instrument VG 70-250 S, Vacuum Generators, Manchester, UK, separation conditions as above) indicated the active compound to have a molecular weight of $M=222$. Chemical ionization (isobutane) as well as high-resolution MS revealed a molecular formula of $C_{15}H_{26}O$ (three double bond equivalents). Loss of water (m/z 204) and a most abundant signal at m/z 69 suggested a sesquiterpene alcohol with a β,β-dimethylallyl group as a substructure. Mass spectra and retention times of the active compound were identical in *E. typhina* and *E. sylvatica*. Published data on the mass spectrum of the sesquiterpene alcohol chokol K, identified from *E. typhina* (Tanimori *et al.* 1994) were in good accordance with our mass spectrum.

From the dichloromethane-extract of 350 stromata of *E. sylvaticum*, the biologically active compound could be isolated by preparative GC. The device consisted of a HP 5890 GC, an autosampler HP 7673 (both Hewlett Packard, Palo Alto CA, USA)

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and a fraction collector (Gerstel, Mülheim, Germany). The isolation was carried out by using a 30 m, 0.53 mm i.d. fused silica capillary Optima 5, film thickness 1 µm (Macherey & Nagel, Düren, Germany). Hydrogen served as the carrier gas. For NMR-investigations of the isolated compound, dissolved in $CDCl_3$, a DRX-500 instrument (Bruker, Karlsruhe, Germany) was used. The sets of data obtained with ^{1}H-NMR-, HMBC-, HMQC- and NOE-experiments were in full accord with those published for chokol K (Trost & Phan 1993; Tanimori *et al.* 1994). In hexane, the natural product showed a negative rotation value, as in an ethanolic solution of natural chokol K (Koshino *et al.* 1989; Trost & Phan 1993). Upon enantioselective GC (25 m, 0.25 mm i.d. fused capillary column coated with a 1 : 1 mixture of OV1701 and heptakis (2,3-di-*o*-acetyl-6-*o*-*tert*-butyldimethylsilyl)-β-cyclodextrin, isothermal at 110 °C), racemic chokol K could be well resolved (rt (−)=42.2 min, α=rt (−) : r (+)=1.0374). GC investigations on natural extracts revealed chokol K from *E. sylvaticum* and *E. typhina* to be the (1*R*,2*S*,3*R*)-stereoisomer (Trost & Phan 1993), showing an enantiomeric purity of at least 98% enantiomeric excess.

(**d**) ***Synthesis of chokol K***

The synthesis of racemic chokol K (1*R**,2*S**,3*R**)-1,2-dimethyl-3-(6-methylhepta-1,5-dien-2-yl) cyclopentanol was carried out by employing a modification of the procedure of Tanimori *et al.* (1994; see electronic supplementary material). The synthetic product, a colourless oil, was purified by chromatography (silica gel, pentane/Et_2O 1 : 1) and bulb-to-bulb distillation (55 °C, 10^{-2} Torr). Spectroscopic data (IR, NMR and MS) of the product were found to be in complete accordance with published data (Tanimori *et al.* 1994).

(**e**) ***Bioassays***

To test the electrophysiologically active compound for behavioural activity, attraction experiments with sticky traps were carried out at the Botanical Garden Zürich (Huber *et al.* 2005). The traps consisted of a white plastic disc 8 cm in diameter to which insect glue was applied (commercial insect exclusion adhesive; Temmen Insektenleim, Hattersheim) and covered with a plastic bowl. Synthetic, racemic chokol K (0.1; 1; 10 mg) was applied on a small rubber GC septum placed in the middle of a plastic disc. Release rates of volatiles from the septa were controlled for and found to be in the range of equal to three times as much as one intact stroma (data not shown). Traps were set up in grassland near the Botanical Garden where no *Epichloë*-infected grasses occurred within a radius of 100 m. A chi-square test was used to compare the frequency of flies attracted to 'chokol'- and control traps.

3. RESULTS

In all GC–EAD recordings, one substance proved to be active (figure 1), which was identified as the sesquiterpene alcohol chokol K, previously known as a fungitoxic compound from *Epichloë* (Koshino *et al.* 1989). *Epichloë typhina* emitted chokol K from fungal stromata on infected grass culms (table 1), as well as from pure, cultured mycelium on agar medium (Steinebrunner 2005, unpublished data), confirming the fungal origin of the compound (Koshino *et al.* 1989).

The pollinator attracting function of chokol K was demonstrated in field bioassays where 11 *Botanophila* flies were caught on traps emitting synthetic chokol K, whereas no *Botanophila* were caught on control traps treated with solvent only during five subsequent days (χ^2=8.33; p=0.004). On both types of traps, however, several unrelated insects (mostly dipteran) were also present. *Botanophila* flies were identified by molecular markers and 10 individuals matched with those previously known to visit and fertilize stromata of *E. typhina* and *E. sylvatica*. One individual belonged to a *Botanophila* taxon not found previously on these hosts. The attractiveness of the traps corresponded well to natural stromata, as not more than three *Botanophila* individuals per hour may typically visit stromata of an infected grass clump with warm weather under field conditions (Leuchtmann 2004, personal observation) indicating that fly visitation frequency is rather low. These results suggest that chokol K is a key compound in the attraction of *Botanophila* flies to *Epichloë* stromata. In addition to its pollinator attracting function, chokol K, as well as several other chokols produced by *Epichloë* species, has fungitoxic properties (Koshino *et al.* 1989; Tanimori *et al.* 1994).

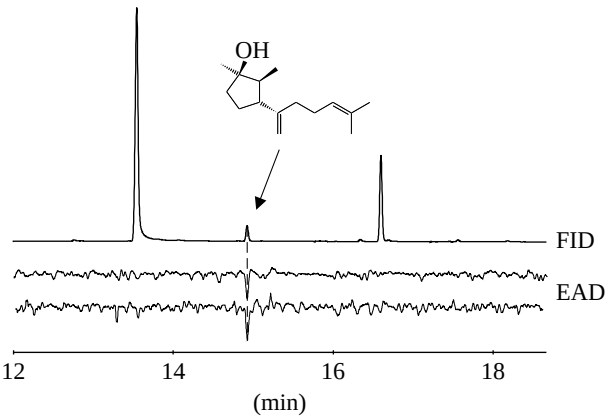

Figure 1. Gas chromatographic analysis with flame ionization detector (FID) of *Epichloë typhina* stromata-extract and simultaneous electroantennographic detection (EAD) using heads of *Botanophila* flies caught from *E. typhina* stromata. Chokol K triggered consistent EAD responses in analyses with three fly individuals. The major, non-active components eluting before and after chokol K are coumarin and methyl 2,3-dihydrofarnesenoate, respectively.

Table 1. Amounts of chokol K emitted from *Epichloë* stromata on plant host.

Epichloë species	headspace (ng l^{-1})	pooled extract (ng stroma^{-1})
E. typhina	82.37±58.86	18.1
E. sylvatica	50.72±42.05	240.5

4. DISCUSSION

Antagonistic interactions among fungi are common, and fungal secondary metabolites with antimicrobial functions are known since long, the most famous example being the powerful antibiotics produced by *Penicillium chrysogenum* (Fleming 1929). Recently, volatile compounds with inhibitory effects on a range of micro-organisms were discovered in endophytic species of *Muscodor* and *Gliocladium* (Strobel *et al.* 2001; Stinson *et al.* 2003). Generally, volatile compounds are better known as signal transmitters in intra- and interspecific communication, and are important for pollinator attraction in many higher plants (Raguso 2001). In fungi, dependent upon insects for gamete transfer, odour signals can mediate the attraction of insects. For example, in some endophytic rust fungi, pollinator attracting volatile compounds emitted from infected plant parts have

been identified (Connick & French 1991; Raguso & Roy 1998; Naef *et al*. 2002). It is, however, often not clear whether the fungi produce the volatiles *de novo*, or use plant precursors, or induce emission of volatiles upon infection.

Our data provide evidence that a single fungal volatile is attractive to a specific pollinator insect. Interestingly, this compound has a dual function by also inhibiting other fungi that may secondarily infect the fungal stromata, posing the question of which role evolved first. Although phylogenetic analyses, useful to answer this question, are not yet available, we assume that antibiotic compounds are evolutionarily ancient among fungi, because resistance to competitors and parasites is a basic need for most soil and plant inhabiting fungi. In contrast, sexual reproduction involving insect vectors for the exchange of gametes is known from only few groups of fungi, such as *Epichloë*, and, thus, is likely to be of more recent origin. Therefore, we suggest that the function of chokol K as a pollinator attractive has evolved secondarily in addition to its antimicrobial properties. Similar arguments have been raised for angiosperms, where gamete transfer by animals has become common and was probably linked to the spectacular diversification of this plant group (Lunau 2004). The important role of floral volatiles for pollinator attraction in many angiosperms is thought to have evolved from the primary function of warding off herbivores and microbes from the reproductive structures of the plant (Pellmyr & Thien 1986). Our finding of a fungus derived antimicrobial compound that also attracts insects suggests that in fungi and plants the same parsimonious pathway was employed to evolve signals for the attraction of pollinators, a solution for gamete transfer in sessile, outcrossing organisms.

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ENDNOTE

[1]IR (*Perkin–Elmer* FT-IR spectrophotometer, model *Spectrum ONE*; film) ν_{max} [cm^{-1}]: 3422*m*, 3079*w*, 2964*s*, 2930*s*, 2874*s*, 1640*m*, 1452*s*, 1376*s*, 1287*w*, 1194*m*, 1152*m*, 1103*m*, 1022*m*, 917*s*, 886*s*, 826*w*, 639*w*, 543*w*, 447*w*. ^{1}H-NMR (*Bruker DRX-500*, 500 MHz, $CDCl_3$) δ [p.p.m.]: 5.13 (*tsept*. $^3J = 6.9$, $^4J = 1.4$ Hz, H–C (5′)); 4.78, 4.76 (2 m_c, H_2C (1′)); 2.39 (*dt*, $^3J = 11.3$, 9.0 Hz, H–C (3)); 2.14 (br. *q*, $^3J \approx 7.4$ Hz, H_2C (4′)); 2.01–1.91 (*m*, H_2C (3′), H–C (4)); 1.75 (*t*, $^3J = 7.9$ Hz, H_2C (5)); 1.69 (*d*, $^4J = 0.9$ Hz, H_3C–C (6′)); 1.62 (br. *s*, H_3C–C (6′)); 1.55 (*dq*, $^3J = 11.3$, 6.8 Hz, H–C (2)); 1.43 (*dq*, $^2J = 13.0$ Hz, $^3J = 8.1$ Hz, H–C (4)); 1.28 (*s*, H_3C–C (1)); 1.14 (br. *s*, HO–C (1)); 0.87 (*d*, $^3J = 6.8$ Hz, H_3C–C (2)). ^{13}C-NMR (*Bruker DRX-500*, 125.8 MHz, $CDCl_3$) δ [p.p.m.]: 151.59 (*s*, C (2′)); 131.53 (*s*, C (6′)); 124.36 (*d*, C (5′)); 108.09 (*t*, C (1′)); 80.30 (*s*, C (1)); 51.98 (*d*, C (3)); 47.55 (*d*, C (2)); 39.99 (*t*, C (5)); 33.77 (*t*, C (3′)); 28.63 (*t*, C (4)); 26.82 (*t*, C (4′)); 26.60 (*q*, H_3C–C (1)); 25.69, 17.73 (2 *q*, $(H_3C)_2C$ (6′)); 10.66 (*q*, H_3C–C (2)); assignments *via* ^{1}H,^{13}C-correlation spectra. EI-MS (electron impact ionization, *MAT 95* spectrometer at 70 eV) *m*/*z* (%): 222 (5, $M^{+\cdot}$), 207 (4, $[M-CH_3]^+$), 204 (13, $[M-H_2O]^{+\cdot}$), 189 (7), 179 (7), 164 (12), 161 (52), 149 (6), 135 (25), 121 (28), 109 (48), 108 (17), 107 (16), 95 (25), 93 (17), 91 (10), 82 (10), 81 (12), 79 (14), 71 (12), 69 (100), 67 (20), 55 (15), 43 (30), 41 (45).

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