

Ecological role of volatiles produced by *Epichloë*: differences in antifungal toxicity

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Abstract

Species of *Epichloë* (Ascomycota, Clavicipitaceae) are endophytic symbionts of pooid grasses. Sexual reproduction of the fungus depends on gamete-transferring *Botanophila* flies, which in earlier studies were shown to be specifically attracted by the fungal volatiles chokol K and methyl (*Z*)-3-methyldodec-2-enoate. As several *Epichloë* volatiles are known to have antimicrobial properties, it was hypothesised that the original function of insect-attracting volatiles is microbial deterrence. However, the origin of volatile compounds and their toxicity within an ecological context has not yet been clarified. We examined the inhibitory effect of chokol K and methyl (*Z*)-3-methyldodec-2-enoate on mycoparasites, plant pathogenic fungi and on *Epichloë* itself at ecologically relevant concentrations, and assessed volatile production in pure cultures of *Epichloë* on complex and defined media supplemented with inorganic sources of carbon and nitrogen. Chokol K reduced the spore germination of all tested fungi, whereas methyl (*Z*)-3-methyldodec-2-enoate had no inhibitory effect. Moreover, only chokol K was produced in culture, confirming its fungal origin. Our findings are consistent with the proposed scenario that fungal volatile substances have followed an evolutionary pathway from defence to attraction.

Introduction

Fungal endophytes are defined as fungi that inhabit plants without causing visible disease symptoms (Schulz & Boyle, 2005). It is thought that virtually all higher plants are hosts of one to many endophytes (Stone *et al.*, 2000; Strobel, 2003). These symbiotic interactions can be variable in nature, ranging from parasitic to mutualistic.

Among the best-investigated endophytic systems are the fungi of genus *Epichloë* (Ascomycota, Clavicipitaceae). *Epichloë* endophytes are wide spread in pooid grasses forming systemic infections (Schardl, 1996; Schardl *et al.*, 2004). Following intercellular, asymptomatic growth inside the host, they form an external fruiting structure (stroma) preventing the emergence of inflorescences, known as choke disease. Typically, the grass host is infected with one *Epichloë* strain only, which is self-incompatible and has to be fertilized by the opposite mating type for sexual reproduction. For fertilization, *Epichloë* depends on flies of the genus *Botanophila* (Diptera, Anthomyiidae) that transfer gametic

spores endochorously from stroma to stroma (Bultman *et al.*, 1998; Bultman & Leuchtman, 2003). Upon fertilization through deposition of faeces containing gametic spores, female *Botanophila* lay eggs on the stroma surface and hatched fly larvae feed and develop on the stoma tissue until they drop and pupate in the soil. It has been demonstrated that *Botanophila* acting as gamete vectors are attracted by a volatile sesquiterpenoid alcohol, named chokol K (Schiestl *et al.*, 2006), and a methyl ester, methyl (*Z*)-3-methyldodec-2-enoate (Steinebrunner *et al.*, 2008), emitted by the fungus.

For successful fungal reproduction, it is crucial that the exposed stroma remains free from contaminating parasites before fertilization, and as long as possible thereafter. Stromata are rarely found to be infected by hyperparasites in nature, and there are only a few reports on mycoparasitic fungi found on *Epichloë* (Schroers, 2001). Mycoparasitism, defined as the interrelationship between a fungus parasite and a fungus host (Barnett, 1963), is a common phenomenon in fungi (Barnett, 1963; Jeffries, 1997), and the rareness of mycoparasites in *Epichloë* may indicate that some

protection mechanisms exist, perhaps mediated by secondary compounds produced by the fungal stroma.

In flowering plants, certain substances involved in pollinator attraction are suggested to have had a primary role in the plant defence mechanism (Pellmyr & Thien, 1986; Pichersky & Gershenzon, 2002; Knudsen *et al.*, 2006). Possibly, such substances were produced as general deterrents, but became identification cues for insects that could bypass the negative effects (Pellmyr & Thien, 1986). Likewise, in the *Epichloë* system it was hypothesized that stroma volatiles involved in *Botanophila* attraction have functionally evolved from a mere defence compound to a 'pollinator' attractant (Schiestl *et al.*, 2006). This hypothesis was based on an earlier finding that reported a chokol-induced toxic effect on *Cladosporium herbarum*, an opportunistic grass parasite (Koshino *et al.*, 1989a, b), and on the notion that antimicrobial compounds are a common feature in endophytic fungi (Tan & Zou, 2001; Strobel, 2003). Fungitoxic methyl esters have been reported from *Epichloë typhina* (Pers.: Fr.) Tul. (Koshino *et al.*, 1987), whereas methyl (*Z*)-3-methyldodec-2-enoate (Steinebrunner *et al.*, 2008) is a newly discovered natural compound and, therefore, its fungitoxic properties are not known. Moreover, defensive qualities of the two *Botanophila* attractants have not been tested in an ecological context using mycoparasitic target organisms, and for both volatiles, chokol K and methyl (*Z*)-3-methyldodec-2-enoate, it is unknown whether they are of pure *Epichloë* origin or are the product of host plant and fungus biosynthetic interaction.

In this paper, we test the toxic effects of chokol K and methyl (*Z*)-3-methyldodec-2-enoate on a number of different fungi, including natural mycoparasites (e.g. *Clonostachys* sp.), plant pathogenic fungi and *Epichloë* isolates. By keeping the concentrations within the same range as actually present on fungal stromata, we applied an ecologically meaningful setting for our toxicity bioassays. In addition, we measured volatile production in pure cultures of *Epichloë* to test whether or not the volatiles are of fungal origin.

Materials and methods

Sampling of *Epichloë* volatiles *in vivo*

Extracts

Unfertilized stromata were collected shortly after emergence from potted plants that were maintained outside on an open terrace. Infected plants originated from various locations in Switzerland and France, and thus are considered distinct genotypes. In total, 44 stromata from nine *Epichloë sylvatica* Leuchtman & Schardl genotypes infecting *Brachypodium sylvaticum* (Huds.) P.B. (9701, 9702, 9703, 9704, 9705, bsD2, bsS2, bsS5, bsS6), six stromata from one *Epichloë clarkii*

White genotype infecting *Holcus lanatus* L. (9401) and five stromata from one *Epichloë festucae* Leuchtman *et al.* genotype infecting *Festuca rubra* L. (9267) were extracted each in 100 µL dichlormethane for 1 min. After extraction, samples were stored in amber glass vials at -20°C .

Headspace

Potted plants with unfertilized, freshly emerged stromata, when volatile production is considered at its peak, were taken to the lab for sampling. Tillers bearing stromata from *B. sylvaticum* with *E. sylvatica* (genotype bsS5), *H. lanatus* with *E. clarkii* (9401), *Bromus erectus* Huds. with *Epichloë bromicola* Leuchtman & Schardl (9630) and *F. rubra* with *E. festucae* (9267) were covered with polyethylene terephthalate oven bags (Nalo[®], Kalle GmbH, Germany) and air was pulled out of the bags by a vacuum pump (SCK Inc.) at a rate of *c.* 100 mL min⁻¹ (Schiestl & Marion-Poll, 2002). Volatiles were trapped on custom-made micropipette filters containing 5 mg of Porapak Q (Salzmann *et al.*, 2006). Before sampling, the Porapak Q adsorbent was cleaned with 100 µL of dichlormethane and 100 µL of hexane. Blank air samples were collected to control for background contaminants. After a sampling period of 24 h, adsorbed volatiles were eluted from Porapak Q with 50 µL of a mixture of hexane:acetone (9:1) (Merck, Uvasol).

Sampling of volatiles from *Epichloë* cultures

Sampling

Epichloë isolates used were obtained in the endophytic stage from infected grasses as described by Leuchtman & Clay (1988): *E. clarkii* from *H. lanatus*, La Rippe, Ct. VD (isolate no. 9401), *E. festucae* from *F. rubra*, Vesancy, France (9267), *E. bromicola* from *B. erectus*, Vesancy, France (9632) and *E. sylvatica* from *B. sylvaticum*, Sihlwald, Ct. ZH (bsS2). Volatiles were sampled from actively growing isolates on potato dextrose agar (PDA) petri dishes using the headspace sampling method (described above). To further evaluate variation in chokol K production, we selected isolate 9401 (chokol produced on PDA, Difco) and isolate 9267 (no chokol produced on PDA) and sampled them on seven defined and two complex media in Petri dishes using the solid-phase micro-extraction method (SPME). The basal medium was prepared as described in Blankenship *et al.* (2001) and supplemented with either ornithine as the N-source and one of the following C-sources each: D-fructose, D-glucose, D-mannose and D-sucrose; or D-glucose as the C-source and one of the following N-sources each: arginine, asparagine and urea. The amount of N-source was adjusted to give a final concentration of 40 mM of N-atoms, and the C-source to give a final concentration of 150 mM of

C-atoms. The two complex media tested were PDA and yeast sucrose agar (20 g L⁻¹ yeast extract, 10 g L⁻¹ D-sucrose, 15 g L⁻¹ agar).

SPME

For this more sensitive system, the fibre material 75 Carbox-enTM on polydimethylsiloxane mounted on a stable flex fibre (Supelco) was used. Sampling was made with a syringe that was placed through a small hole drilled at the side of the Petri dish and exposed to the headspace for 1 h. Before each sampling, the fibre was conditioned at 250 °C for 15 min under a flow of hydrogen.

Chemical analysis and identification of volatiles

Before GC analysis, 100 ng of *n*-octadecane was added to either extracts or headspace samplings to calculate absolute amounts using the internal standard method (Schomburg, 1990). One microlitre of each sample was injected splitless into a gas chromatograph (Agilent 6890N) equipped with an HP5 column (5% phenyl-methylpolysiloxane, 30 m × 0.32 mm Ø × 0.25 µm film thickness) and a flame ionization detector (FID). The oven was temperature programmed as follows: 50 °C for 1 min, then increasing to 300 °C at 10 °C min⁻¹ with a final hold of 10 min at 300 °C. The carrier gas was hydrogen, and the GC was operated at constant flow (2 mL min⁻¹). Data acquisition and data analysis were performed using the AGILENT CHEMSTATION software package (Agilent Technologies). For SPME, the syringe was inserted into the gas chromatograph for 1 min, with the settings as described above. For quantitative analysis of the SPME samples, 200 ng of synthetic chokol K was injected into the GC and this peak area was compared with the peak area after desorption from the SPME fibre.

Compounds were identified by retention time comparison with authentic standards and confirmed by comparison of spectra obtained by GC-MS (HP G 1800 A, Hewlett Packard Inc., Palo Alto) with the same operating conditions as described above.

Fungal isolates used for toxicity tests

Mycoparasites were collected at natural sites from older *Epichloë* stromata (at least 3 weeks after emergence), where they formed easy-to-detect greenish spore masses. Strain PEc0501 was isolated from one of several infected stromata of *E. clarkii* on *H. lanatus* near the Zürich Botanical Garden (Switzerland), and PEt0502 from a single infected stroma of *E. typhina* on *Anthoxanthum odoratum* L. Strain PEb0601 was found on stromata of *E. bromicola* on *B. erectus* near Merishausen (Switzerland). Small amounts of mycoparasitic conidia were transferred to PDA Petri dishes containing oxytetracycline (50 mg L⁻¹). Colonies grown from conidia were streaked out twice to obtain single spore colonies. Pure

cultures were stored on malt agar tubes (20 g L⁻¹ malt extract, 15 g L⁻¹ agar) at 4 °C.

Two wheat pathogens, *Stagonospora nodorum* (Berk.) E. Castell. & Germano (*Pleosporales*) and *Mycosphaerella graminicola* (Fuckel) J. Schröt. (*Mycosphaerellales*), were obtained from the culture collection of the Plant Pathology group at ETH Zürich. *Mycosphaerella graminicola* was grown on liquid medium as described in Zhan *et al.* (2005). The *S. nodorum* isolate was grown on 2% sucrose agar medium for 2 months until pycnidia formation.

Isolates of *E. bromicola* and *E. clarkii* were obtained as described above. Pure cultures were stored on malt agar tubes (20 g L⁻¹ malt extract, 15 g L⁻¹ agar) at 4 °C. The species used for the bioassay were selected based on their difference in chokol K production *in vivo*. *Epichloë bromicola* produces low amounts of chokol K not exceeding 1 ng h⁻¹ stroma⁻¹, whereas *E. clarkii* emits a mean amount of c. 260 ng h⁻¹ stroma⁻¹ (this study, Steinebrunner *et al.*, 2008).

DNA extraction and sequencing of mycoparasitic fungi

To support morphological identification of mycoparasites, part of the internal transcribed spacer (ITS) region delimited by the primers TW81 and AB28 (Curran *et al.*, 1994) was sequenced. Mycoparasites were grown for 14 days on PDA Petri dishes, and then mycelium was harvested and lyophilized. Total fungal DNA extraction and PCR conditions were essentially as described in Schardl *et al.* (1994) and Moon *et al.* (2002). Sequencing reactions were performed in 10 µL volumes using the Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems). Sequencing products were analysed using a capillary 3100-Avant Genetic Analyzer (PE Applied Biosystems) and analysed with Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI). Sequences of the hyperparasites were submitted to GenBank (National Center for Biotechnology Information, Bethesda, MD; <http://www.ncbi.nlm.nih.gov/>) under accession numbers EF600031, EF600032 and EF600033.

Identification of mycoparasitic fungi

Sequences of the nuclear ribosomal ITS region of mycoparasitic fungi were compared using BLASTN searches with other sequences available from GeneBank. The growth characteristics and morphology of isolates were observed in cultures grown on PDA at room temperature.

Spore preparation for bioassays

For the sporulation of mycoparasites, small amounts of mycelium were transferred from malt agar tubes to PDA Petri dishes and grown at room temperature for 1–2 months. Conidiospores of *M. graminicola* were obtained

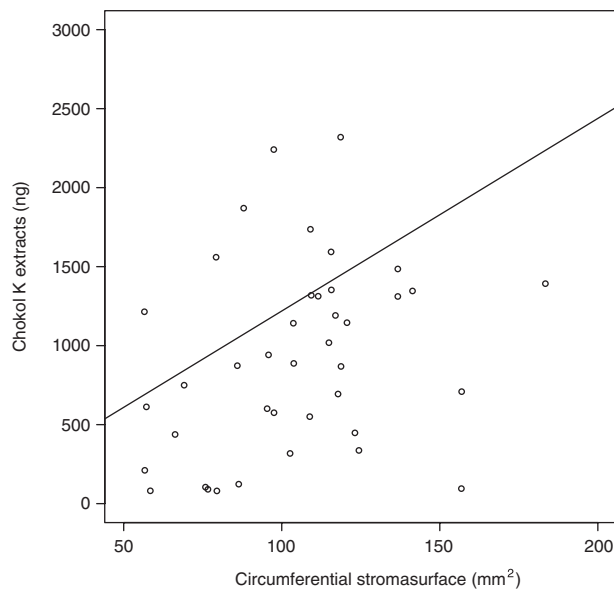


Fig. 1. Relationship between circumferential stroma surface (mm^2) of stomata of *Epichloë sylvatica* and chokol K amount (ng) as determined by dichloromethane extraction. The solid line indicates a significant regression ($y = 12.191 * x$; $r = 0.59$, $n = 43$, $P < 0.001$).

from liquid culture as described in Zhan *et al.* (2005), and spores of *S. nodorum* were collected from pycnidia squeezed in Eppendorf tubes using a spatula. Conidia of *Epichloë* isolates were collected from cultures grown on PDA Petri dishes for 2 weeks at room temperature. Spores of all isolates were taken up in autoclaved water and transferred to sterile 1.5 mL Eppendorf tubes, and suspensions were adjusted to 1500 spores mL^{-1} using a Thoma haemocytometer.

Toxicity tests

Synthetic chokol K and methyl (Z)-3-methyldodec-2-enoate were applied to PDA in Petri dishes at concentrations of 60, 100 and 200 μg to test for antifungal activities against the two mycoparasites *M. graminicola* and *S. nodorum* and two *Epichloë* isolates. The minimal concentration of 60 μg approximately corresponds to the amount of chokol K present in natural stomata of *E. sylvatica* and was determined as follows: the extractable amount of chokol K was regressed against the circumferential stroma surface (Fig. 1), with 1 cm^2 approximately corresponding to 1.29 μg chokol K. This amount, when extrapolated on the surface of an 8 cm Petri dish equals the threshold concentration of *c.* 60 μg chokol K used in our study. We also tested the effect of 100 and 200 μg chokol K per Petri dish to reflect the probably much higher effective concentrations resulting from continuous *de novo* production of compounds by stomata in nature. For methyl (Z)-3-methyldodec-2-enoate the same concentrations were used, although the actual concentration

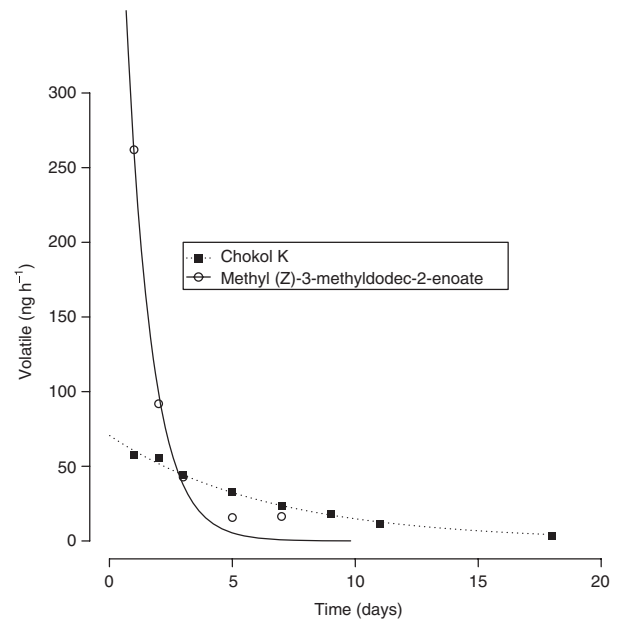


Fig. 2. Volatile evaporation from 100 μg synthetic chokol K (dotted line) and methyl (Z)-3-methyldodec-2-enoate (solid line) added to a PDA Petri dish. Evaporation closely followed a negative exponential growth curve (chokol K: $y = 70.69e^{-0.156 * x}$; methyl (Z)-3-methyldodec-2-enoate: $y = 685e^{-0.968 * x}$). Measurements are based on repeated SPME of Petri dish headspace.

in stomata is unknown. Approximate molarities of the applied concentrations calculated for 20 mL of agar were 0.014, 0.023 and 0.045 mM.

The effectiveness of volatile evaporation in our agar *in vitro* system was validated by measuring concentrations of synthetic chokol K at days 1, 2, 3, 5, 7, 9, 11 and 18, or of methyl (Z)-3-methyldodec-2-enoate at days 1, 2, 3, 5 and 7 after application of 100 μg of each volatile in a Petri dish (Fig. 2). Concentrations of chokol K as determined by SPME in the headspace only dropped gradually over the time span of the experiment following a negative exponential growth curve, while concentrations of methyl (Z)-3-methyldodec-2-enoate rapidly decreased until day 5.

Each concentration was repeated four times by adding the synthetic compound in 100 μL dichloromethane in the middle of the dish and streaking out evenly over the agar surface with a sterilized glass rod. Dishes applied with 100 μL dichloromethane served as control. Before applying the test spore solutions, dishes were incubated for 1 h at room temperature to allow the solvent to evaporate. Subsequently, 100 μL of a 1500 spores mL^{-1} spore solution of test fungi was applied and streaked out as described above. The dishes were then sealed with two layers of parafilm and incubated at room temperature. Toxicity was assessed by eye counting of germinated conidiospores after 2–3 days (depending on the isolate). After 6 days, we reassessed a subsample to test for

Table 1. Mean values (\pm SEM) of chokol K and methyl (Z)-3-methyldodec-2-enoate (methyl) emitted from stromata and from isolates in culture, measured by headspace and solvent extraction technique

	<i>In vivo</i>				In culture	
	Extracts*		Headspace†		Headspace	
	Chokol K Mean \pm SEM (ng stroma ⁻¹)	Methyl Mean \pm SEM (ng stroma ⁻¹)	Chokol K Mean \pm SEM (ng h ⁻¹)	Methyl Mean \pm SEM (ng h ⁻¹)	Chokol K Mean \pm SEM (ng h ⁻¹)	Methyl Mean \pm SEM (ng h ⁻¹)
<i>E. sylvatica</i>	1255.0 \pm 188.4	0.0 \pm 0.0	261.5 \pm 54.23	1.00 \pm 0.25	0.15 \pm 0.02	0.0 \pm 0.0
<i>E. clarkii</i>	619.1 \pm 154.4	0.0 \pm 0.0	194.2 \pm 43.01	7.61 \pm 1.38	6.64 \pm 1.39	0.0 \pm 0.0
<i>E. bromicola</i>	NA	NA	0.06 \pm 0.03	0.03 \pm 0.02	0.0 \pm 0.0	0.0 \pm 0.0
<i>E. festucae</i>	0.0 \pm 0.0	0.0 \pm 0.0	0.08 \pm 0.05	45.41 \pm 13.35	0.0 \pm 0.0	0.0 \pm 0.0

Due to host plant differences in flowering time and tiller formation *Epichloë* species could not be sampled at the same time in one experiment. Results from extracts are based on one to seven genotypes per species and three to seven stromata replicates per genotype. Results from headspace sampling are based on one genotype per species and four to seven stromata replicates per genotype. For *Epichloë bromicola*, no stroma extracts were available.

*Measured using dichloromethane solvent extraction.

†Measured using headspace, porapak[®] filters.

NA, not available.

possible delayed germination and to confirm the quality of the colony counting. The size of colonies was determined with the image analysis software *ASSESS* (Lamari, 2002) from 10 randomly chosen colonies per Petri dish. As germination rates fluctuated among the isolates tested, we calculated the normalized response, expressed as germinated conidia relative to germination of the respective fungal strains on control agar Petri dishes containing only dichloromethane.

Statistical analyses

Homogeneity of variances of the data was tested using Levene's test. To approximate normal distribution of residuals, data were transformed. If no appropriate transformation could be found, data were analysed using nonparametric tests (Kruskal–Wallis test, Wilcoxon rank sum test). Linear regression was applied to assess the relationship of extractable chokol K and the circumferential stroma surface. The effects of chokol K and methyl (Z)-3-methyldodec-2-enoate on spore germination of isolates were tested using a two-way nested ANOVA with fungus species, fungal group (mycoparasites, pathogens and *Epichloë* isolates) and concentration of compounds as factors. Fungus species was treated as random and was nested within a fungal group. All statistical analyses were performed using R (R Development Core Team, 2005).

Results

Production of the volatiles *in vivo*

For quantification of chokol K and methyl (Z)-3-methyldodec-2-enoate, four *Epichloë* species differing in the production of these compounds under natural conditions were

chosen. *Epichloë sylvatica* infecting *B. sylvaticum* and *E. clarkii* infecting *H. lanatus* belong to a group of strong chokol K producing genotypes, whereas *E. bromicola* on *B. erectus* shows low production. *Epichloë festucae* infecting *F. rubra* mainly emits methyl (Z)-3-methyldodec-2-enoate (Steinebrunner *et al.*, 2008).

There was considerable variation in chokol K production among stromata of *E. sylvatica* ($n = 44$) and *E. clarkii* ($n = 6$), determined by dichloromethane extraction (Table 1). However, variation could not be related to the nine different genotypes of *E. sylvatica* used in the study originating from different locations. Clearly, the mean amount of chokol K was larger for *E. sylvatica* than for *E. clarkii* (t -test, $t = 2.594$, $df = 25.062$, $P = 0.016$), although stroma circumferential surface was smaller in *E. sylvatica* compared with *E. clarkii* (t -test, $t = -4.84$, $df = 5.60$, $P = 0.0035$). For *E. sylvatica*, a significant linear relationship could be established between extractable chokol K per stroma and circumferential stroma surface (Fig. 1). No chokol K was detected in the solvent extracts of *E. festucae* and *E. bromicola* stromata, and no methyl (Z)-3-methyldodec-2-enoate was detected in the extracts of any of the four *Epichloë* species tested (Table 1).

The mean chokol K production per stroma as determined in the headspace was significantly different between the *Epichloë* species (Kruskal–Wallis, $\chi^2 = 13.10$, $df = 3$, $P = 0.004$). The highest production was again measured for *E. sylvatica*, followed by *E. clarkii* (Table 1). Only traces of chokol K were detected from *E. bromicola* and *E. festucae*. For *E. sylvatica* and *E. clarkii*, the amount of chokol K in the headspace was about five times lower than the solvent extractable amount. Therefore, approximately every 3–5 h the solvent-extractable amount may be renewed. A converse pattern was observed for methyl (Z)-3-methyldodec-2-enoate production. The concentrations were significantly

higher in the headspace from *E. festucae* stromata than from *E. sylvatica*, *E. clarkii* and *E. bromicola* (Table 1) (Kruskal–Wallis, $\chi^2 = 16.58$, $df = 3$, $P = 0.0008$).

Production of the volatiles in culture

Volatile production of four *Epichloë* isolates was measured in pure cultures using the headspace from the filter elution method. Chokol K was detected in the headspace of *E. clarkii* and *E. sylvatica*, while no chokol K could be measured in the respective headspace of *E. festucae* and *E. bromicola* (Table 1). The more sensitive SPME measurements of *E. clarkii* and *E. festucae* headspace on two complex media and seven defined media confirmed these results, with chokol K being present only in the headspace of *E. clarkii*. With *E. clarkii*, ANCOVA revealed a significant effect of the medium on chokol K production; the covariable colony area had no significant effect on the production (one-way ANCOVA, $F_{8,3} = 13.57$, $P < 0.0001$, data not shown). Highest production was measured on PDA (22.45 ng h^{-1} , $SEM = 4.57 \text{ ng h}^{-1}$, $n = 3$) and lowest amounts were emitted from the ornithine–mannose defined medium (2.21 ng h^{-1} , $SEM = 0.25 \text{ ng h}^{-1}$, $n = 3$). No methyl (Z)-3-methyldodec-2-enoate was detected in all four tested isolates. Again, results from *E. clarkii* and *E. festucae* were consistent with the two different sampling techniques.

Identification of mycoparasitic fungi

The two isolates of the first mycoparasite obtained from *E. clarkii* and *E. bromicola* formed a relatively slow-growing colony with scanty, white aerial mycelium and greenish spore masses in coalescing pustules (sporodochia) on PDA. Penicillate, sparsely branched conidiophores produced phialidic conidia ($4.0\text{--}7.0 \mu\text{m} \times 2.0\text{--}3.0 \mu\text{m}$) in short chains. No teleomorphic state was observed either in culture or on the natural host. From GeneBank searches, the closest match (98% identity) of sequences of the nuclear ribosomal ITS region was *Bionectria epichloë* (AF210675), a mycoparasite described from *Epichloë* with *Clonostachys* as an anamorphic state that was similar to our isolates (Schroers, 2001). Thus,

both morphological and molecular identification indicate that the mycoparasite infecting *E. clarkii* (PEc0501) and *E. bromicola* (PEb0601) is correctly referred to as the genus *Clonostachys*.

The second mycoparasite obtained from *E. typhina* (PEt0502) formed greenish, velvety mycelium on PDA and readily formed hyphomycetous conidia ($3 \times 3 \mu\text{m}$) of *Nodulisporium* type (Ellis, 1971, 1976). The closest match (84% identity) of ITS sequences was *Nodulisporium* sp. (AF280629; Polishook et al., 1996).

Toxicity tests

Synthetic chokol K significantly reduced the spore germination of all fungi tested relative to the control when added in the highest concentration ($200 \mu\text{g}$) to agar Petri dishes (Table 2, Fig. 3a). At lower concentrations, reaction differed among tested fungal isolates, including mycoparasites, plant pathogenic fungi and *Epichloë* isolates. *Nodulisporium* sp. was the most sensitive as concentrations of $60 \mu\text{g}$ reduced the germination of conidiospores significantly. Spore germination of *Clonostachys* sp., *E. clarkii* and *E. bromicola* was slightly increased at 60 and $100 \mu\text{g}$ compared with the control. The other isolates were not affected at lower concentrations of chokol K. In an ANOVA, significant differences between the tested fungal isolates were found (Table 2).

The average size of colonies grown from germinating spores was generally smaller on chokol K amended media compared with the control (data not shown). Particularly for the mycoparasitic *Clonostachys* sp., colony sizes at $100 \mu\text{g}$ chokol K were significantly reduced (one-way ANOVA, $F_{2,4} = 7.76$, $P = 0.011$, Table 3), although spore germination at this concentration was not affected.

Methyl (Z)-3-methyldodec-2-enoate had no negative effect on spore germination. However, significant interactions between fungal isolate and methyl (Z)-3-methyldodec-2-enoate treatment were found (Table 2). They can be attributed to the considerable variation in spore germination of *M. graminicola* on the different concentration treatments (Fig. 3b).

Table 2. Summary of ANOVA for the effects of compound concentration (chokol K and methyl (Z)-3-methyldodec-2-enoate), fungal species (*Clonostachys* sp., *Nodulisporium* sp., *Stagonospora nodorum*, *Mycosphaerella graminicola*, *Epichloë clarkii* and *Epichloë bromicola*) and fungal group (hyperparasites, pathogens and *Epichloë* isolates) on conidiospore germination

Source	df	Chokol K			Methyl (Z)-3-methyldodec-2-enoate		
		MS	F	P	MS	F	P
Compound concentration	3	11 476	53.34	< 0.0001	187	1.07	0.36
Fungal group	2	80	0.06	0.94	1767	2.53	0.23
Fungal species	3	1383	6.43	0.0006	696	4.01	0.010
Compound × fungal group	6	316	1.68	0.14	580	3.34	0.006
Error	80	215			173		

Significant values are in bold type.

Fig. 3. Normalized response of mycoparasites (*Clonostachys* sp. and *Nodulisporium* sp.), plant pathogenic fungi (*Stagonospora nodorum* and *Mycosphaerella graminicola*) and *Epichloë* (*E. bromicola* and *E. clarkii*) isolates to increasing concentrations of chokol K (a) and methyl (Z)-3-methyldodec-2-enoate (b), respectively (bars at measuring points indicate range of response for individual Petri dishes).

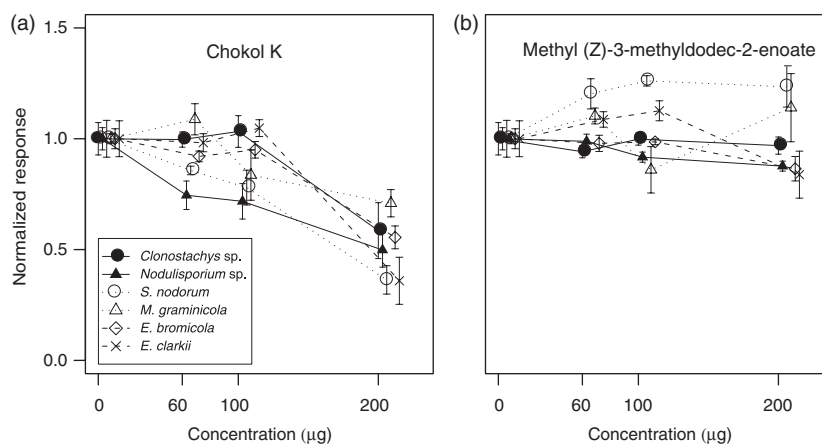


Table 3. Mean colony size (cm²) of germinated conidiospores of *Clonostachys* sp. 3 days after plating on chokol K amended medium at 60 and 100 µg per Petri dish

Concentration of chokol K	Mean (cm ²) ± SEM
60 µg	0.025 ab ± 0.002
100 µg	0.008 b ± 0.002
Control	0.037 a ± 0.009

Measurements are based on 10 colonies per replicate ($n=4$). Levels followed by the same letter are not significantly different based on pairwise Bonferroni correction.

Discussion

Bioassays of this study showed that chokol K can reduce the spore germination and growth of tested fungi, at least at the highest, ecologically relevant concentration. In contrast, methyl (Z)-3-methyldodec-2-enoate did not negatively affect spore germination. Moreover, chokol K was detected in the headspace of *Epichloë* cultures *in vitro* on complex and defined media confirming the fungal origin of this volatile, whereas methyl (Z)-3-methyldodec-2-enoate was not emitted from the cultures.

Concentrations of volatiles used in our toxicity tests were converted from stroma measurements of four *Epichloë* species based on headspace and solvent extraction techniques, and thus are believed to conform to actual, ecologically relevant concentrations *in vivo*. For chokol K, a concentration of 200 µg per agar Petri dish, and in some cases even 60 µg, was sufficient to diminish spore germination of various fungi including two *Epichloë* species, two mycoparasites and two plant pathogens. Previous studies have also investigated the toxic effects of chokol K and other secondary metabolites of *Epichloë*, but used different fungi and unrealistically high concentrations (Koshino *et al.*, 1987, 1989a; Yue *et al.*, 2000). Koshino *et al.* (1989b) assessed the toxic effect of chokol K on *Cladosporium herbarum*, an

opportunistic grass pathogen, by applying a spore suspension directly onto the resolved fraction of chokol K on a TLC plate. Although this technique is suitable for screening purposes in the search for new bioactive substances, the results are difficult to interpret biologically as concentrations of active compounds are arbitrary. Nevertheless, our findings are consistent with those of Koshino *et al.* (1989b), but show that chokol K has a much wider antimicrobial activity against distantly related fungi.

Methyl (Z)-3-methyldodec-2-enoate, a newly discovered natural compound (Steinebrunner *et al.*, 2008), did not have an antimicrobial effect on the fungi tested in this study. On the contrary, at a concentration of 200 µg per Petri dish, spore germination of the two plant pathogenic fungi was even stimulated compared with the control. Possibly, methyl (Z)-3-methyldodec-2-enoate was used as a food resource, or shares characteristics with substances stimulating spore germination of plant pathogens.

The two fungi isolated from *Epichloë* stromata were considered to be mycoparasites, because they apparently live and sporulate on stroma tissue. The one referred to as *Clonostachys* sp. appears to be a specialized mycoparasite of *Epichloë* and has previously been reported from *E. typhina* in Japan and America (Schroers, 2001), whereas *Nodulisporium* sp. may be opportunistic and its occurrence on *Epichloë* accidental. The genus *Nodulisporium* comprises saprophytic and endophytic species, and to our knowledge, no association of *Nodulisporium* with *Epichloë* or other fungi is reported in the literature. As mycoparasites were only found on older, often unfertilized stromata, it is unlikely that *Botanophila* flies are involved in spore dispersal; we rather think that wind or rain splashes are the main vectors of mycoparasites. Differences in sensitivity of the two species to chokol K may be explained by their different incidence and degree of specificity. The more tolerant *Clonostachys* sp. may have experienced selection pressure for increased tolerance through repeated exposure to *Epichloë* defence compounds.

An unexpected finding of our study was the self-toxicity in bioassays with *Epichloë* isolates. Gametic spores, at least those of *E. clarkii* which produce high amounts of chokol K, should have evolved to be tolerant to chokol K. Canals *et al.* (2005) reported self-toxicity in plants of the annual grass *Lolium rigidum*, where plant extracts adversely affected the germination and seedling growth of conspecific individuals. Moreover, germination was increased at high plant densities compared than at low densities, which was interpreted as dilution of the negative effect through sharing of the toxic compounds among germinating plantlets. In our toxicity bioassay, we kept the number of applied spores low to facilitate the scoring. However, the number of spores deposited by *Botanophila* flies can be considerably larger, with up to 80 000 spores mL⁻¹ present in fly faeces (F. Steinebrunner, pers. obs.). Although our study did not investigate density-dependent spore germination, it would be interesting to test whether similar effects, as those seen in plant studies, are also valid for *Epichloë* germination on stromata. If so, self-toxic effects might be smaller under field conditions when large amounts of *Epichloë* spores are deposited.

An alternative explanation is that the addition of 200 µg chokol K per Petri dish might have been unrealistically high and, therefore, self-toxicity was observed. Our results indicate that at a chokol K concentration of 100 µg per Petri dish, spores of *Epichloë* isolates and the specialized mycoparasite *Clonostachys* sp. germinated slightly better than most of the other isolates that may be less adapted. Even though differences in germination are small, they could be decisive to gain a competitive advantage when spreading over the stroma surface.

Numerous other fungitoxic substances of different chemical classes have been reported from *Epichloë* (Koshino *et al.*, 1992; Yue *et al.*, 2000; Seto *et al.*, 2005); therefore, chokol K might only be one element of the defence system. This may particularly apply to the low chokol-producing species such as *E. bromicola*. In studies with *Muscodor albus*, an endophyte from *Cinnamomum zeylanicum*, the toxicity against test organisms was greatly increased when a high number of volatiles, stimulated by rich growth media, were present (Ezra & Strobel, 2003). By contrast, the suppressive capacity was reduced under nutrient-poor conditions, where the complexity of the volatile blend was depleted suggesting that synergism between individual compounds may be important. Although we have not investigated synergistic effects, we suggest that a similar scenario could apply to *Epichloë*, and that protection against contaminating parasites is far greater under field conditions than results of our bioassays imply using the single compound chokol K.

Epichloë isolates were grown in pure culture on defined and complex media to assess whether volatiles are of fungal origin and based on genes encoded in the genome of

Epichloë. Half of the tested *Epichloë* cultures produced detectable amounts of chokol K on complex media. This indicates that at least some *Epichloë* species possess the biosynthetic capacity to produce chokol K without precursor substances from the host plant. However, for chokol K-producing species the amount of chokol K was considerably larger in the *in vivo* system. This might be attributed to either missing stimuli from the host plant *in vitro* or the presence of precursory substances of plant origin *in vivo* promoting volatile production. This observation would merit further investigation.

In contrast, we did not detect methyl (*Z*)-3-methyldodec-2-enoate emission with either of the sampling methods or on any media tested. Although our data cannot definitively reject the possibility of fungal origin of this substance, we think it is likely that for the production of methyl (*Z*)-3-methyldodec-2-enoate host stimuli or precursor metabolites are needed.

By analogy with volatile evolution scenarios of angiosperm–pollinator relationships, we have hypothesized that the primary role of chokol K might have been antimicrobial, and that this compound secondarily became involved in *Botanophila* ‘pollinator’ attraction (Schiestl *et al.*, 2006). The present study supports this hypothesis. We demonstrated that chokol K has a fungitoxic effect at ecologically relevant concentrations and that chokol K production is intrinsic to *Epichloë*. In fact, cyclonerodiol, a chemically related sesquiterpene that likely originates from the same biochemical pathway (Yue *et al.*, 2000), is found to be widespread in fungi (Nozoe *et al.*, 1970; Cross *et al.*, 1971; Hanson *et al.*, 1975; Ghisalberti & Rowland, 1993). This may suggest that at least part of the chokol K producing pathway is ancestral to fungi. In a second step of the evolutionary scenario, *Botanophila* spp., closely related to flower visiting species (Vitou *et al.*, 2001) or other grass feeding *Anthomyiidae* (Väre & Itäemies, 1995), also started to exploit the fungal tissue and may have then been selected for detecting *Epichloë* volatiles. Interestingly, a *Botanophila* sp. whose larvae develop inside the grass stem, and thus may represent the transitional stage from a purely plant-associated to fungus-associated species, was most basal in the phylogenetic tree of *Epichloë* visiting *Botanophila* species (Leuchtmann, 2007).

Conclusions

The two volatile metabolites, chokol K and methyl (*Z*)-3-methyldodec-2-enoate, emitted from *Epichloë* stromata differ in their fungitoxic effect at ecologically meaningful concentrations. Measurements from pure cultures suggest that chokol K but not the methyl ester is encoded by *Epichloë* genes. For chokol K, constraints in the production may exist as evidenced by self-toxicity or incomplete protection. Further research is needed to evaluate the significance of

endogenous (host plant) and exogenous (*Botanophila*, parasites) factors for *Epichloë* volatile evolution, and possible synergism between the two antimicrobial compounds from *Epichloë* reported here. In addition, the local mycoparasite abundance, their distribution within natural *Epichloë* populations and the possible role of *Botanophila* flies in inoculum transmission warrant further investigation.

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