biology letters

Biol. Lett. (2007) **3**, 523–525
doi:10.1098/rsbl.2007.0338
Published online 7 August 2007

Evolutionary biology

Secondary chemicals protect mould from fungivory

Marko Rohlfs[1,*], Martin Albert[1], Nancy P. Keller[2] and Frank Kempken[3]

[1]*Zoological Institute, Department of Animal Ecology, and*
[3]*Botanical Institute, Department of Plant Genetics and Molecular Biology, Christian-Albrechts-University of Kiel, Am Botanischen Garten 1-9, 24098 Kiel, Germany*
[2]*Department of Plant Pathology, University of Wisconsin, 1630 Linden Drive, Madison, WI 53706, USA*
**Author for correspondence (rohlfs@zoologie.uni-kiel.de).*

The vast repertoire of toxic fungal secondary metabolites has long been assumed to have an evolved protective role against fungivory. It still remains elusive, however, whether fungi contain these compounds as an anti-predator adaptation. We demonstrate that loss of secondary metabolites in the soil mould *Aspergillus nidulans* causes, under the attack of the fungivorous springtail *Folsomia candida*, a disadvantage to the fungus. Springtails exhibited a distinct preference for feeding on a mutant deleted for LaeA, a global regulator of *Aspergillus* secondary metabolites. Consumption of the mutant yielded a reproductive advantage to the arthropod but detrimental effects on fungal biomass compared with a wild-type fungus capable of producing the entire arsenal of secondary metabolites. Our results demonstrate that fungal secondary metabolites shape food choice behaviour, can affect population dynamics of fungivores, and suggest that fungivores may provide a selective force favouring secondary metabolites synthesis in fungi.

Keywords: chemical defence; filamentous fungi; food choice; LaeA; secondary metabolites; soil arthropods

1. INTRODUCTION

The fungal kingdom encompasses some of the most important organisms in the world, not only owing to their critical position as main decomposers in various ecosystems (Dighton 2003) but also because of their impact on humans and human-related activities (Deacon 2006). Often this impact has been attributed to the fungi's ability to produce a prodigious diversity of secondary metabolites (SMs) that affect humans in beneficial (e.g. pharmaceuticals; Demain 1999) and detrimental (e.g. mycotoxins; Nesbitt *et al.* 1962) ways. Despite the wealth of knowledge on the manifold influences of SMs on humans, the function of these compounds in interaction with fungi's natural environment is still an unresolved problem (Sherratt *et al.* 2005; Deacon 2006; Schiestl *et al.* 2006). Since fungi constitute an important food source for many invertebrates, one recurring hypothesis suggests fungal SMs evolved, in part, as antifeedants (Demain & Fang 2000; Scheu & Simmerling 2004) and provide a type of chemical protection analogous to SM protection of plants against herbivory (Schoonhoven *et al.* 2006). In contrast to this latter case, there is so far no experimental proof of a role for fungal SMs as chemical antifeedants.

To address this deficiency, we examined the effect of genetic silencing of the global SM transcription regulator LaeA in the common soil mould *Aspergillus nidulans* on predatory interactions with the fungivorous springtail *Folsomia candida*. The nuclear protein LaeA has recently been discovered to control expression of many SMs, including mycotoxins, in several *Aspergillus* species (Bok & Keller 2004; Bok *et al.* 2006) and appears to be conserved in various filamentous fungi. Deletion of the *laeA* gene (Δ*laeA*) results in fungal strains with vigorous vegetative growth but reduced or eliminated SM gene transcripts (Bok & Keller 2004; Perrin *et al.* 2007). In a series of food preference experiments presented to *Folsomia*, we demonstrate a role for SMs in shaping animal food choice behaviour, where loss of SM production results in disadvantage in fungal growth but an advantage to arthropod reproductive success. To our knowledge, this provides the first genetic support for the shield hypothesis of secondary chemicals in fungi.

2. MATERIAL AND METHODS

In all experimental set-ups 25–35 days-old mature *F. candida* were used. Springtails were reared on wet plaster of Paris and fed with soya bean flour. Prior to the experiments, the springtails had not received any food for 7 days. The wild-type *A. nidulans* strain RDIT2.3 and *laeA* mutant strain RJW46.4 (Bok & Keller 2004) were used in this study. Both strains were maintained on standard malt extract agar at 28°C. Isolated spores were applied to inoculate experimental malt extract agar patches (see below). All experiments were conducted at 25°C and in constant darkness.

(a) *Preference and springtail fitness*

Preference tests were performed in Petri dishes (9 cm diameter, 1.5 cm height) containing two smaller dishes (2.5 cm diameter, 0.7 cm height) that were glued to the bottom of the larger dish. Aliquots of 2 ml autoclaved malt extract agar were filled into the small dishes and inoculated with approximately 1000 spores. The remaining area of the large dish was filled with agar, containing the anti-fungal agent nipagin. At a fungal colony age of 5 days, 20 springtails were released into the centre of each arena equidistant and the two *Aspergillus* containing dishes. In total, 17 replicates were run simultaneously. After 24 hours, the number of springtails on each fungal colony was counted to determine their feeding preference. A *t*-test for dependent samples was used to evaluate statistical differences in feeding preference. Prior to analysis, data were arcsine-square-root transformed in order to meet assumption of normality and equality of variances.

The effect of fungal secondary metabolites on springtail reproduction was studied in experimental arenas containing only a single 5 days old fungal colony (WT or Δ*laeA*). Again 20 springtails were released into each arena (10 replicates per treatment). The number of eggs in each arena was counted at 1, 2, 4, 6, 8 and 13 days. At the same time, we recorded the number of dead springtails. Following log-transformation, we performed a repeated measurement analysis of variance (ANOVA) to test for different effects of fungi on the springtails' egg-laying activity. Survival of adult springtails as a function of fungal diet was analysed by means of a Cox regression (SPSS 13.0).

(b) *Fungal biomass loss*

Prior to inoculation with spores, a sterile piece of circular gauze (mesh width, 100 μm; approx. 2.8 mm in diameter) was placed on each malt extract agar patch (one patch per experimental arena, see above). Individual pieces of gauze were weighed beforehand by using a microbalance. Fungal spores (approx. 1000) were applied onto the gauze that had direct contact with the entire malt agar area. At the end of the experiments, the pieces of gauze including the fungal colonies, now growing on the gauze, were taken off the

Electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2007.0338 or via http://www.journals.royalsoc.ac.uk.

Received 26 June 2007
Accepted 9 July 2007

agar surface. Fungal tissue plus gauze were dried at 60°C for one day, and were immediately weighed to the nearest 0.0001 mg. The previously determined individual gauze weight was subtracted from total object weight to obtain pure fungal dry weight. To assess the impact of fungivory on mould biomass loss, 20 springtails were released into each arena containing either one wild-type (WT) or one Δ*laeA A. nidulans* colony (5 days old). Springtails were allowed to feed for 5 days on the colonies; subsequently, fungal dry weight was determined. To obtain replicated data ($n=22$ for the WT treatment and $n=24$ for the Δ*laeA* treatment) on fungal biomass loss, single control arenas (undisturbed fungal growth) were randomly assigned to single treatment arenas (fungivory) before substrates were inoculated with spores. Although WT colonies were generally heavier than Δ*laeA* colonies, biomass loss was calculated by subtracting dry fungal biomass in springtail treatments from dry biomass in the corresponding control treatments for the specific strain. We used linear regression analysis to test if the intercept significantly differed from zero (equals to no changes through fungivory); negative values indicate fungal biomass loss. According to Kolmogorov-Smirnov and Levene's test, data met criteria of normality and variance homogeneity, respectively.

3. RESULTS

When given the choice between WT and Δ*laeA A. nidulans*, 88.52%±2.87 s.e. of the springtails were found feeding on Δ*laeA* ($t=-9.349$, d.f.=16, $p<0.0001$). This was paralleled by springtail preference for Δ*laeA* chemical extracts over WT extracts (see electronic supplementary material) where a series of mixed extract feeding choices neatly demonstrated the preference for Δ*laeA* as one of avoidance of WT metabolites (see electronic supplementary material).

Springtails started egg-laying at the same time on both fungi, but significantly more eggs were laid on Δ*laeA* during the course of our observation, and the increase in the number of deposited eggs was greater on Δ*laeA* than WT (figure 1; repeated measurement ANOVA; between subject effect (fungal strain), $F=16.09$, d.f.=1, $p=0.0009$; within subject effects (day), $F=41.92$, d.f.=5, $p<0.0001$; day×fungus, $F=16.22$, d.f.=5, $p<0.0001$). Mortality rates in springtails were low and did not differ between the two fungal diets (Cox regression, *Wald*=0.793, d.f.=1, $p=0.373$; figure 1).

Changes in fungal biomass loss through fungivory are given in figure 2. Variation in biomass changes was attributable to initial differences (e.g. size of inoculum) between control and treated colonies. On average, changes in biomass were expected not to be different from zero (intercept=0) if there were no fungivory or if springtail feeding does not cause any biomass loss. Although we observed springtails feeding on WT during a period of 5 days, on average no biomass loss was detected (intercept=0.1250±0.3638, $\chi^2_1=0.12$, $p=0.7313$; figure 2). If springtails consume significant amounts of hyphal tissue, mean deviation from zero was expected to be negative (intercept<0). In strong contrast to the effect on WT, Δ*laeA* indeed suffered from a significant loss in dry biomass through fungivory (on average 30%; intercept=−1.7808±0.2264, $\chi^2_1=61.89$, $p<0.0001$; figure 2).

4. DISCUSSION

In the food choice experiments *F. candida* displayed a strong preference for the SM deficient *A. nidulans* strain (Δ*laeA*), which demonstrates that the springtails can discriminate between the two fungal strains.

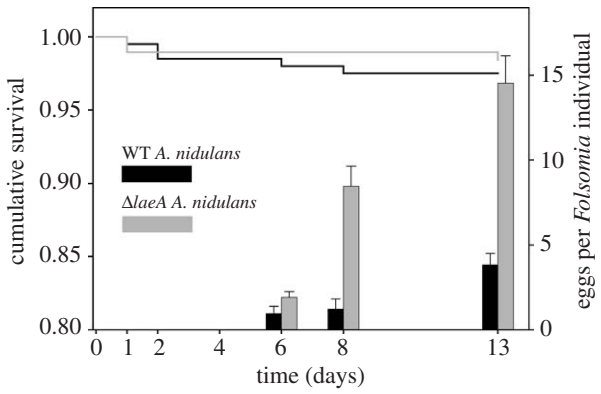

Figure 1. Egg-laying activity and survival of *F. candida* on WT and Δ*laeA A. nidulans*. The curves illustrate springtail survival. Bars indicate mean number of eggs per *F. candida* individual (+s.e.).

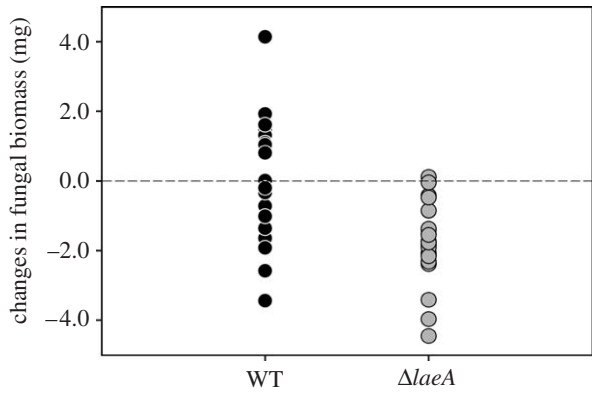

Figure 2. Effect of fungivory on changes in biomass of WT and Δ*laeA A. nidulans*. Data were obtained by subtracting the weight of single undisturbed control colonies from randomly assigned single colonies treated with springtails (see §2).

Folsomia candida is a blind soil microarthropod that should base its preference on chemical information, whether repulsion or attraction. Mixed chemical feeding experiments (see electronic supplementary material) strongly suggest that the lack of one or more SMs in Δ*laeA* makes this strain more attractive to the fungivores. Springtails are thus likely to directly respond to the presence/absence of SMs although one cannot discount a role for SM independent signals genetically co-regulated with SM expression.

Independent of the exact mechanism driving the springtails' food choice decisions, fungus-borne and SM-related information evidently exists and is exploited by the animals to increase their evolutionary fitness. *Folsomia candida* indeed derives a distinct benefit from choosing the chemical deficient *A. nidulans*, because the animals' reproduction was positively affected when feeding on Δ*laeA* in contrast to WT. Since mortality rates of adult springtails on WT were as negligible as on Δ*laeA*, food preference for the mutated strain was adaptive in terms of increasing reproductive output. Interestingly, a recent study could not demonstrate preference for a melanin (hyphal pigment) deficient mutant of *Aspergillus fumigatus* in springtails although feeding on the mutant yielded a reproductive advantage, compared with the WT fungus (Scheu & Simmerling 2004). Possibly, not all SMs can be chemically perceived or springtails respond only to a

limited set of SMs, e.g. mycotoxins. Being choosy with respect to fungal SM expression may explain why springtails can be highly selective when feeding on fungi (Jørgensen *et al.* 2005), which might in turn critically affect spatial distributions of fungivores in soils and hence decomposition processes.

In addition to being more attractive to *F. candida*, Δ*laeA* suffered from significant biomass loss under springtail feeding attack. Therefore, the reproductive benefit that accrues from feeding on Δ*laeA* seems to be attributable to a more successful exploitation of the fungal tissue, implicating that the SM deficient fungus is more vulnerable to fungivory since its proposed chemical shield has been removed. In combination with the SM-related influences on springtail behaviour and reproduction, the negative effect of fungivory on fungal growth strongly suggests that fungivores can be a selective force favouring the evolution of SM biosynthesis in fungi.

In relation to fungivory, crucial SMs appear to be regulated by LaeA, however, we still do not know which substances operate as antifeedants, if and how they are induced by fungivores, and what kind of chemical signals springtails perceive and use to optimize food choice. Fortunately, critical advances have been made in sequencing the genome of several filamentous fungi (Galagan *et al.* 2005), so that microarray technology should permit rapid screening of a wide range of fungal (SM) genes, as recently demonstrated in the aspergilli (Bok *et al.* 2006), to determine the identity and interactions of those involved in deterring fungivores and improving chemical protection. Coupled with analytical chemistry, this genomic approach will help us to address the pivotal question whether secondary metabolism in the fungal kingdom has been subject to similar evolutionary forces as chemical defence in plants (Schoonhoven *et al.* 2006).

This work was supported by NIH 1 R01 Al065728-01 to N.P.K. Two anonymous reviewers gave valuable comments on an earlier draft of this paper.

Bok, J.-W. & Keller, N. P. 2004 LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukar. Cell* **3**, 527–535. (doi:10.1128/EC.3.2.527-535.2004)

Bok, J.-W., Hoffmeister, D., Maggio-Hall, L. A., Murillo, R., Glasner, J. D. & Keller, N. P. 2006 Genomic mining for *Aspergillus* natural products. *Chem. Biol.* **13**, 1–7. (doi:10.1016/j.chembiol.2005.10.008)

Deacon, J. 2006 *Fungal biology*. Oxford, UK: Blackwell Publishing.

Demain, A. L. 1999 Pharmaceutically active secondary metabolites of microorganisms. *Appl. Microbiol. Biotechnol.* **52**, 455–463. (doi:10.1007/s002530051546)

Demain, A. L. & Fang, A. 2000 The natural function of secondary metabolites. In *Advances in biochemical engineering/biotechnology*, vol. 69 (ed. T. Sheper), pp. 1–39. Berlin, Germany: Springer.

Dighton, J. 2003 *Fungi in ecosystem processes*. New York, NY: Marcel Dekker, Inc.

Galagan, J. E. *et al.* 2005 Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438**, 1105–1115. (doi:10.1038/nature04341)

Jørgensen, H. B., Johansson, T., Canbäck, B., Hedlund, K. & Tunlid, A. 2005 Selective foraging of fungi by collembolans in soil. *Biol. Lett.* **1**, 243–246. (doi:10.1098/rsbl.2004.0286)

Nesbitt, B. F., O'Kelly, J., Sargeant, K. & Sheridan, A. 1962 *Aspergillus flavus* and turkey X disease: toxic metabolites of *Aspergillus flavus*. *Nature* **195**, 1062–1063. (doi:10.1038/1951062a0)

Perrin, R. M., Fedorova, N. D., Bok, J.-W., Cramer, R. A., Wortman, J. R., Kim, H. S., Nierman, W. C. & Keller, N. P. 2007 Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. *PLoS Pathog.* **3**, e50. (doi:10.1371/journal.ppat.0030050)

Scheu, S. & Simmerling, F. 2004 Growth and reproduction of fungal feeding Collembola as affected by fungal species, melanin and mixed diets. *Oecologia* **139**, 347–353. (doi:10.1007/s00442-004-1513-7)

Schiestl, F. P., Steinebrunner, F., Schulz, C., von Reuß, S., Francke, W., Weymuth, C. & Leuchtmann, A. 2006 Evolution of 'pollinator'-attracting signals in fungi. *Biol. Lett.* **2**, 401–404. (doi:10.1098/rsbl.2006.0479)

Schoonhoven, L. M., van Loon, J. J. A. & Dicke, M. 2006 *Insect–plant biology*. Oxford, UK: Oxford University Press.

Sherratt, T. N., Wilkinson, D. M. & Bain, R. S. 2005 Explaining dioscorides' "double difference": why are some mushrooms poisonous, and do they signal their unprofitability? *Am. Nat.* **166**, 767–775. (doi:10.1086/497399)