Chapter 1
Secondary Metabolites in Soil Ecology

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1.1 Introduction: Chemical Interactions in Soil

Interactions among organisms are central to understanding any ecosystem, perhaps with the exception of a short period when a newly created niche is colonized by its first inhabitants. Soil environment is not an exception, but biotic interactions dominating soil biology differ from those in other systems because of the dominating role of sessile organisms and the lack of autotrophy in soil (chemolithoautotrophs being an interesting but not significant exception). When chemical processes in soil are discussed, the traditional concept of food webs comes first to mind as a framework for the exchange of organic substances and flow of energy. Feeding, predation, degradation of macromolecular substrates and absorption of nutrients have dominated thinking about biogenic chemical processes in soil. The food web approach proved extremely fruitful in generating hypotheses and inspiring experimental approaches concerning the bulk transformation of organic matter, but it did not address phenomena related to chemical interactions which are more specific both on the chemical and on the taxonomical level and which cannot be adequately described in terms of energy flow and biomass transformation. These interactions involve compounds named secondary metabolites, which are not strictly needed for the survival and reproduction of their producers. Secondary metabolites are structurally highly diverse and each of them is produced only by a small number of species. They exert various biological effects, often at very low concentrations, and can be regarded as carriers of chemical communication among soil inhabitants.

The high complexity and heterogeneity of soil makes this matrix recalcitrant to chemical analysis. Methods for the determination of pesticides, polychlorinated biphenyls and other xenobiotics in soil have existed for a long time to monitor pollution of the environment, but it is only recently that dedicated analytical methods for natural metabolites in soil have been available (Mortensen et al. 2003). Apart

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from the complexity of soil matrix, analytical methods for secondary metabolites in the soil have to cope with the enormous diversity of the analyte itself. The resolution of current metabolomics approaches is far from adequate even for the metabolome of a single organism, let alone for systems orders of magnitude more complex. Adsorption phenomena, large differences in concentrations among metabolites and their heterogeneous distribution further complicate profiling of secondary metabolites in soil by current metabolomics techniques. We may need to focus on dominant metabolites and major effects first, gradually zooming into the system as the progress of analytical techniques allows us.

Most secondary metabolites produced by soil microbes appear to be secreted, an observation which corroborates their role if controlling biotic interactions. The research field addressing the role of secreted metabolites in an ecosystem is ecological chemistry. Concerning soil microorganisms, the antibiotics paradigm has dominated experimental approaches to the ecological role of secondary metabolites so far, followed by pathogenic interactions between microorganisms and plants. Other roles of secondary metabolites, such as facilitating symbiosis with insects, plants and higher animals, are documented but have rarely been addressed (Demain and Fang 2000; Sect. 1.6.3). For instance, it has been known since ancient times that fungal products may poison animals, but the idea that microbes produce toxins to protect their substrates from ingestion by animals did not surface until Janzen's pioneering paper was published in *The American Naturalist* (Janzen 1977). Even then, attempts to test this hypothesis experimentally have rarely been reported. The role of secondary metabolites in interactions among soil microorganisms or between a microorganism and a plant might appear to be easier to address, but rigorous testing of a working hypothesis in this area is tricky (see Sects. 1.6.2, 1.6.3, 1.7). Without the capability of manipulating secondary metabolite synthesis or their targets genetically, conclusive results are difficult if not impossible to obtain.

1.2 Should the Term "Secondary Metabolites" Be Abandoned?

More than a century ago, Kossel (1891) defined secondary metabolites by exclusion (compounds that do not belong to primary metabolites), provoking criticism which has never ceased. The current, generally accepted concept in line with Kossel's view is that primary metabolites are chemical components of living organisms that are vital for their normal functioning, while secondary metabolites are compounds which are dispensable. A distinguishing feature of secondary metabolites is that their production is limited to a group of species or genera and is rarely conserved over a wide taxonomical range, while primary metabolism is conserved among phyla and across kingdoms.

The specificity of secondary metabolism encouraged botanists and mycologists to use secondary metabolite production as a taxonomical characteristic in plants (Smith 1976) and fungi (Frisvad et al. 1998). Chemotaxonomy harbors risks, because on

the one hand a single-point mutation might block a whole biosynthetic pathway, and on the other hand there are indications that some gene clusters involved in secondary metabolite biosynthesis have been transmitted among species by a horizontal gene transfer. The use of chemotaxonomy for elucidating phylogenetic relationships was therefore limited, and it became obsolete with ready access to DNA sequences. However, chemotaxonomy has not lost its appeal as a rapid and inexpensive support for taxonomical classification of microbial isolates.

Many scientists studying secondary metabolites dislike the term, because it appears to imply an auxiliary importance of secondary metabolites compared with the importance of primary metabolites. Numerous attempts to replace "secondary metabolites" by other labels were undertaken without gaining wide acceptance. Several initiatives emphasized the biological role of these compounds. For example, the designation "ecological metabolites" stresses the role of secreted metabolites in interactions of their producers with other organisms. Similarly, Frisvad's creation of "extrolites" (an outwardly producers directed chemically differentiated product of a living organism) is based on the notion that the function of many secondary metabolites is to control or modulate interactions with the environment. In the meantime the author has been using his term as a synonym for all secondary metabolites (Frisvad et al. 2004). The problem is that not all secondary metabolites fit his definition of extrolites, and for the majority of secondary metabolites we do not know whether they are "outwardly directed" or not. I suppose this is the reason why the term "extrolites" has not been embraced by the scientific community. In their recent review of fungal metabolomics, Frisvad's colleagues abandoned the term "extrolites" completely, consistently using "secondary metabolites" (Smedsgaard and Nielsen 2005). Substitutes like the term "extrolites" will unlikely replace the established term "secondary metabolites" because their definitions do not cover the full range of natural products known as secondary metabolites, and because their applicability relies on information which is seldom available. Let us look at a couple of examples. Leaf-movement factors in nyctinastic plants are clearly secondary metabolites, but one would not call them ecological metabolites or extrolites. Sometimes both secondary and primary metabolites serve the same purpose, defeating any classification based on function. For instance, pyochelin is secreted by *Pseudomonas* sp. and citric acid is secreted by plant roots, both facilitating the uptake of mineral nutrients by their producers. A functional classification would blur the distinction between secondary metabolites (pyochelin as a nonribosomal peptide) and primary metabolites (citric acid as a member of the Krebs cycle), the preservation of which is desirable. From a practical point of view, the main problem with functional classifications is that for most newly described natural products we do not know anything beyond their structure and taxonomical affiliation of the producer, the latter information often being limited to a genus.

The traditional distinction between primary and secondary metabolism is straightforward and knowledge of the structure is usually sufficient for the assignment of a compound to primary or secondary metabolism. As useful as some of the suggested substitutes are in emphasizing functional aspects, terms like "extrolites," "special metabolites" (Gottlieb 1990), "idiolites" (Demain 1986), "ecological

metabolites" (Sirenko et al. 1979) and so on will unlikely replace the term "secondary metabolites." We do not need to search for a substitute as long as we do not associate "secondary" with unimportant or uninteresting.

1.3 Overcoming the Phytochemist's Approach to Secondary Metabolites

Secondary metabolites are the study object of natural product chemistry. The amazing structural variability of these compounds has attracted the curiosity of chemists and the biological activities possessed by natural products have inspired the pharmaceutical industry to search for lead structures in microbial cultures and plant extracts. This strategy proved highly successful: until the advent of molecular genetics, natural product chemistry was the main source of innovation in drug development. An impressive number of compounds have been purified and their structures elucidated in the past four decades. Neither computer-aided drug design nor combinatorial chemistry has surpassed nature as a source of structural variability.

Paradoxically, the success of natural product chemistry in applied research and product development steered the field towards a dead end in basic research. While commercial interests generated pressure to purify and run though bioassays more and more compounds each year, little effort has been devoted to questions of primary scientific interest—namely, for what reasons plants and microbes make them and what happens to them in nature. The vast majority of publications on secondary metabolites have been limited to structure elucidation, at best accompanied by arbitrarily selected bioassays. Any randomly selected issue of the *Journal of Natural Products* will illustrate this practice. This situation is reminiscent of old-time entomology, when scholars were collecting and meticulously describing insects but devoted little effort to the physiology, genetics, ecology or ethology of their subjects.

Apart from searching for new structures and commercially exploitable biological activities, a natural product chemistry field progressing well in the past few decades was the elucidation of biosynthetic pathways. Feeding isotopically labeled precursors proved an efficient strategy to this end even before the implementation of spectroscopic techniques, when stepwise chemical degradation and elementary analysis dominated the tedious process of structure elucidation. Labeling with heavy isotopes remained a major tool of pathway elucidation after the coupling of nuclear magnetic resonance with mass spectrometry became the workhorse of natural product chemistry because both techniques can distinguish isotopes. A practical reason for the interest in biosynthetic pathways was that feeding different precursors provides access to new derivatives with potentially improved properties. The elucidation of biosynthetic pathways by natural product chemists was limited to establishing sequences of intermediates, and it usually failed short of experimentally addressing the enzymatic reactions involved. Enzymes were a domain of biochemistry, which was well isolated from organic chemistry at traditional universities, being affiliated with the faculty of biology rather than that of chemistry. Biochemists was

still busy investigating the intricacies of primary metabolism, while natural product chemists were publishing hundreds of weird and beautiful structures each year as on the assembly line.

Chemical ecology has formally existed for more than a century (Mitchell-Olds et al. 1998), but compared with the proliferation of natural product chemistry its achievements have been modest. It is difficult to understand why so few people seriously addressed the question why those fancy structures published by phytochemists each year actually existed in nature. It seems that the voluminous literature on natural products remained largely unnoticed by biologists, and those who were aware of the growing need for a scientific inquiry did not possess the expertise and tools needed. For natural product chemists, describing new structures was what describing new species was for a traditional taxonomist. Bioassays were used to assess the potential commercial value of new metabolites rather than a means of addressing their function in nature. Describing and cataloging items is a necessary first step towards understanding, but it is not more than a first step. Resources available for research are limited and it is my view that rather than following a convenient routine purify–elucidate–publish–abandon (and purify–elucidate–patent–license in rare lucky cases), natural product chemistry needs to attach more meaning to its results. For example, chemists occasionally experimented with growing conditions in order to maximize the yield or to generate new products. The inventor of the one strain, many compounds (OSMAC) concept, A. Zeeck, explicitly suggested that varying cultivation parameters could provide insights into the role of secondary metabolites in microbial communities (Bode et al. 2002), but the approach has never been used systematically to this end. Another rarely used option is to select bioassays applied to new metabolites according to the natural environment of the producer. The narrow traditional concept of natural product chemistry and its isolation from microbiology and biochemistry contributed to the discrepancy between the volume of descriptive work and the scarcity of functional approaches.

Only in the 1990s did research on secondary metabolites began to overcome its limits. On one hand, biologists installed gas chromatography and high-performance liquid chromatography systems in their laboratories and learned how to purify secondary metabolites from plant extracts and microbial cultures. On the other hand, chemists learned that apart from growing producing strains in fermenters, they can genetically manipulate biosynthetic pathways and use cell-free extracts or purified enzymes to perform biosynthetic reactions in test tubes. The transition was all but smooth because questions arising in biology traditionally caused little excitement in chemistry. Natural product chemists retiring these days remember how difficult it was at the beginning of their careers to compete for chemistry grants with projects proposals on natural products. As it took time for them to establish the same reputation as physical and synthetic chemists had, concepts like metabolomics face difficulties now to be accepted within the realm of chemistry. But the new paradigm has been set. Not only research on natural products became interdisciplinary, involving fields as diverse as molecular genetics and entomology, but the boundary between disciplines has started to dissolve as laboratory members are compelled to learn techniques adequate for their research

subjects, rather than picking topics amenable to techniques which they have mastered for years. My laboratory in the Department of Crop Sciences uses mass spectrometry to elucidate biochemical transformations of secondary metabolites and my colleagues in the Institute of Botany study biosynthetic pathways. Our colleagues in the Faculty of Chemistry investigate the biophysics of biological membranes and perform transposon mutagenesis in *Actinomyces*. This development was a necessary prerequisite for natural product chemistry to overcome its descriptive tradition.

1.4 Chemical Ecology of Microorgansims Has Been Neglected

Ecological chemistry of soil is dominated by microbes. Most research activities labeled as chemical ecology worldwide have so far been concerned with interaction between insects and plants. The selection of papers published in the *Journal of Chemical Ecology* provide a good example. According to its mission statement, the journal is devoted to "promoting an ecological understanding of the origin, function, and significance of natural chemicals that mediate interactions within and between organisms," but the majority of its articles deal with insect–plant interactions. This is just another manifestation of a phenomenon known from systematic biology: the smaller the dimensions of members of a taxonomical group are, the more species the group possesses and the fewer the taxonomists that deal with it. While whole institutes are devoted to ecological studies of insect–plant interactions, only a handful of laboratories seriously investigate chemical communication among microbes in nature. Three systems with a high potential for practical applications are prominent exceptions: quorum sensing in bacteria, biological control of plant diseases, and interaction of plant pathogens with their hosts. A review of advances in ecological chemistry written by the late Jeffrey B. Harborne (1999), one of the most influential doyens of phytochemistry, nicely documents this bias. The review is divided into four sections according to interacting organisms: animal–animal, plant–animal, plant–plant and plant–microbe. A section on microbe–microbe interactions, which would arguably be concerned with chemical interactions more substantial for the survival of their participants than any of the four combinations listed above, just did not occur. Similarly Bell (2001) claims in his review on ecological biochemistry to have selected "examples … of different types of biochemical relationships," but he presents merely the following sections (apart from the introduction and conclusions): beetles and seeds, caterpillars and leaves, biochemical polymorphism in plants, biochemical polymorphism in herbivores and, finally, induced response to herbivory. Sections on microbes such as "bacteria and plants" or "induced response to fungi" are missing, though the title of the review "Ecological biochemistry and its development" did not indicate that it is limited to plant–insect interactions. Overcoming a bias towards creatures that can be seen by the naked eye and collected by hand is the first prerequisite for maintaining progress in chemical ecology in a broader sense.

1.5 The Origin of Chemical Diversity in Soil

Secondary metabolism continues to be a rich source of new and often surprising structures. The number of secondary metabolites discovered so far, which is estimated to be at most 50,000 (Demain and Fang 2000), appears to represent only a fraction of the chemical diversity possessed by extant plants and cultivable fungi, bacteria and protists. Even worse is the fact that the vast majority of microbes inhabiting natural biota cannot be cultivated under laboratory conditions. The metagenome approach pioneered by Diversa Corporation is unlikely to recover intact and functional biosynthetic pathways involving several enzymes, nonubiquitous cofactors or specific precursors. The consequence is that most of the chemical diversity on Earth is not accessible for humans and it is likely to remain out of our reach in the foreseeable future.

An intuitive concept that the force driving the diversification of secondary metabolites produced by soil-borne or soil-inhabiting microorganisms is competition is widespread. In terms of interference competition, an organism which acquires the ability to produce a new antibiotic will experience a gain in fitness. The efficiency of the antibiotic declines as resistance mechanisms arise and spread, in analogy to the race between the pharmaceutical industry and human-pathogenic bacteria. Intuitively, this situation appears to favor diversity in antimicrobial metabolites. This view has recently been corroborated by the outstanding work by Czaran et al. (2002). The authors simulated an evolutionary arms race which takes place in a spatially structured environment. The basic idea was that the production of a secondary metabolite which blocks competitors either increases or decreases the net fitness of the producer, depending on the presence of the competitor and its resistance towards this particular toxin. The crucial point that led to the generation of diversity was the introduction of costs of resistance. In a spatially segmented, two-dimensional substrate, several strains survived at a stable total density but with periodically fluctuating abundance at local regions. The final version of the model consisted of 14 systems, each containing an immune producer, a resistant nonproducer and a sensitive nonproducer. It is significant that this groundbreaking result was achieved by a computer simulation. Because of the enormous complexity of soil ecosystems and the inherent limits of our experimental tools, numerical simulations are likely to play an important role in research into chemical interactions in soil in the future.

The most valuable outcome of computer modeling is an experimentally testable hypothesis. Davelos et al. (2004) recently documented spatial fragmentation of interference competition in soil experimentally. The authors showed that in *Streptomyces* from prairie soil, antibiotic production is highly variable in space, implying that the fitness benefit resulting from antibiotic production varies among locations. Resistance patterns were consistent across locations, indicating that the costs of resistance were low. This contradicts the results of Czaran et al. (2002), because selection against resistance was a crucial factor promoting chemical diversity in their model. The apparent discrepancy shows that we are still at the beginning of understanding chemical diversity in ecosystems. In addition to variation in space, variation in time needs to be addressed experimentally. Maintenance of chemical

diversity by selection in a fragmented environment is one of the most promising areas of current secondary metabolite research.

A factor not considered in the model of Czaran et al. (2002) is that secondary metabolites may act additively, synergistically or antagonistically. Challis and Hopwood (2003), again focusing on *Streptomyces*, investigated antibiotic effects regarding synergy and contingency, which they defined as the production of several metabolites targeting the same competitor. Their work took advantage of rich data on the production of antibiotics by *Actinomyces* and the complete genome sequence of two *Streptomyces* species. The coproduction of clavulanic acid and cephamycin C, the common regulation of both pathways (both are controlled by *ccaR* protein) and the location of the gene clusters in the genome, as well as the comparison of clavulanic acid and cephamycin C production by different strains, supported a view that clavulanic acid synthesis developed as a response to the acquisition of β-lactamase by one of the organisms targeted by cephamycin C. Similar arguments are presented for siderophores (iron chelators), streptogramins and further secondary metabolites, showing that the synergistic and contingent effect of secondary metabolites against the same competitor was one of the reasons for the development of multiple pathways for antimicrobial secondary metabolites.

How do microorganisms generate and maintain chemical diversity on a biochemical level? Firn and Jones (2000, 2003) suggested that a small set of enzymes with relaxed specificities may generate a large set of different but structurally related metabolites. Only some among these products exert effects which enhance the fitness of their producer under current conditions. The other metabolites serve merely as a supply of diversity for future needs. Apart from postulating how relaxed enzyme specificities generate structural diversity, which can easily be accommodated by the current framework of evolutionary theory, a novel and controversial aspect of their metabolic grid concept is the notion that evolution optimized retention of chemical diversity at minimum metabolic cost, including the production of metabolites which do not exert any beneficial effect on their producers. If such "useless" metabolites exist, one might suggest an alternative explanation by considering them to be side products of biosynthetic pathways which have not been optimized yet for specificity. Structurally related metabolites usually exert similar effects, while the efficiencies of individual metabolites differ. This is well known not only for antibiotics, but also for all groups of mycotoxins (e.g., fumonisins, trichothecenes, aflatoxins, enniatins and zearalenone derivatives). Apart from the hard-to-swallow idea of evolution maintaining chemical diversity for future needs, a problem with the hypothesis is that it is impossible to prove for any secondary metabolite that it does not enhance the fitness of its producer under certain conditions. The concept was derived from the so-called screening hypothesis, which sought to reconcile the diversity of natural products with the observation that the majority of these compounds are not active in bioassays used in screening programs developed by the pharmaceutical industry. Even if the assertion that "potent biological activity is a rare property for any one molecule to possess" is true, it may not be relevant for ecosystems with complex interorganismal interactions, because activity does not need to be strong in order to positively affect the fitness of its producer. Moreover,

even potent activity may remain unnoticed in bioassays unless adequate target organisms are used. Because most natural targets of metabolites secreted in soil are unknown and possibly uncultivable, the value of in vitro bioassays for explaining the biological role of secondary metabolites in soil is inherently limited.

1.6 Secondary Metabolites and Fitness: Evolution Meets Ecology

1.6.1 Chemical Interactions and Coevolution of Soil Species

Metabolites involved in interorganismal interactions affect the relative fitness of interacting partners in a distinctive way. The simplest scenario is that the biological activity of an ecological metabolite has been optimized by evolution to affect a target organism in a way benefiting the producer. This idea is the basis of many concepts of metabolite-mediated interaction, including interference competition among fungi, attraction of pray by carnivorous plants and protection of plants from herbivores by repellant volatiles and antifeedants. These ideas are straightforward and as long as the production of the metabolite in question is amenable to control by genetic engineering or by induction/suppression of its synthesis, it is relatively easy to design experiments for testing working hypotheses in natural environments. Elementary evolutionary considerations require us to assume that the selection pressure exerted by a secreted secondary metabolite on the population of the target organism will affect allele frequencies, speed up the elimination of genotypes responding in unfavorable ways and facilitate fixation of mutations enhancing the fitness of the target under the effect of the metabolite. Eventually, an evolutionary change will occur which will overcome the fitness depression of the target organism and eliminated fitness gain, benefiting the metabolite producer. In reality, both interacting partners are subjected to selection pressures at the same time, leading to reciprocal adaptation in a process called coevolution.

Coevolution became the basic explanatory framework in research on plant–insect interactions, which is a field in which ecological chemistry has been developed most extensively. In spite of relentless criticism by Jermy (1988, 1998), the coevolutionary theory proliferated and ramified into its most recent incarnation known as geographic mosaic theory of coevolution (Thompson 2005). Unfortunately, this development has little benefited ecological chemistry of soil. Belowground research has always played a poor cousin's role in ecology, possibly because field trips, insects and flowering plants are more attractive for most students than soil microcosms, complex instrumentation and methods requiring considerable training time. But even when we compare applications of the same technique to aboveground and belowground space, soil biology gets the short end of the stick. Studies of volatiles provide a revealing example. Volatile compounds in soil are likely to be more important for the orientation of invertebrates than in aboveground environments because visual orientation in soil is impaired. Furthermore, concentration gradients

of volatiles in soil air are more stable than gradients in aboveground space because of limited air convection. In spite of this, students of plant volatiles rarely turn their headspace gas chromatography (Tholl et al. 2006) and insect-antenna-derived sensors (Weissbecker et al. 2004) to rhizosphere air. Although experimental data are largely lacking, volatile-mediated relationships similar to those known from aboveground ecosystems (Harrewijn et al. 2005) are likely to have been established by the coevolution of herbivorous invertebrates and plants in soil. Volatiles generated by soil microorganisms, plant roots and germinated seeds are well known to affect soil fungi and stimulate plant growth (Schenck and Stotzky 1975; Ryu et al. 2003; Kai et al. 2007). Coevolutionary relationships based on chemical communication via nonvolatile components of soil solutions, including olfactory cues evaluated by soil invertebrates, are likely to play an even more significant role, but available experimental data are equally scarce.

1.6.2 Cost of Biosynthesis

Let us look at the metabolic costs of secondary metabolite synthesis, which can be easily investigated in simple systems. In plant–insect interactions this issue has been extensively addressed (Gershenzon 1994). Determining the cost of biosynthesis of a particular metabolite by a microorganism appears to be a straightforward issue, providing suitable mutants are available. Wilkinson et al. (2004) recently determined the effect of a stepwise deactivation of the sterigmatocystin biosynthesis pathway in *Aspergillus nidulans* on the fitness of the fungus. Their result was surprising: the number of conidia produced in axenic cultures increased with the progression of sterigmatocystin synthesis. The lowest number of conidia was found in cultures of a mutant in which the complete pathway had been shut off via a regulatory gene *afl*R; the highest number of conidia was found in the wild-type strain. Because the strains were isogenic, hidden effects of additional mutations can be excluded. The authors showed that the effect cannot be explained by protection against light.

The result of Wilkinson et al. (2004) is counterintuitive: the synthesis of sterigmatocystin is thought to provide ecological benefits to its producer called indirect effects (Strauss et al. 2002), but the direct effect of the biosynthesis on the fitness of its producer is expected to be negative because it consumes energy and metabolic precursors, which could otherwise be used to build up biomass and reproductive structures. Because the experiments were performed in axenic cultures, observed positive effects of sterigmatocystin synthesis on conidia formation did not involve interactions of *A. nidulans* with other organisms. Sterigmatocystin is known as a carcinogenic mycotoxin (it serves as a precursor of aflatoxin synthesis in other *Aspergillus* species) and although its ecological role is not known, it is a common belief that its function is to inhibit organisms which compete for resources with sterigmatocystin producers. An alternative explanation to direct benefits to the fungus as postulated by the authors is that the observed effect could have resulted from regulatory phenomena. This hypothesis is corroborated by the fact that both conidia

development and sterigmatocystin synthesis are derepressed by a common activator FluG, which counteracts the affect of the repressor SfgA (Seo et al. 2006).

The work of Wilkinson et al. (2004) was the first one addressing the effect of a stepwise deactivation of a biosynthetic of a secondary metabolite on fungal fitness, but the observation of a negative rather than a positive effect of the loss of a dispensable pathway on fitness under axenic conditions is not unique. For example, Gaffoor et al. (2005) disrupted all polyketide synthase (PKS) genes of *Fusarium graminearum* and observed inhibition of mycelial growth in mutants that lost two out of 15 PKS genes. Similarly, Zhou et al. (2000) observed growth inhibition in *A. parasiticus* after disruption of PKS FLUP. The mechanisms of these effects are unknown. Regulatory phenomena may be responsible for apparent benefits caused by the synthesis of these metabolites in axenic cultures. To test this hypothesis, one would need to isolate regulatory mutants which reverse the effect of the disruption of the biosynthesis on fitness. In axenic cultures, the fitness of double mutants should be even higher than the fitness of the wild-type, nondisrupted strain.

The work of Wilkinson et al. (2004) makes clear that the effect of the synthesis of a secondary metabolite presumed to have ecological roles in the fitness of its producer needs to be assessed experimentally on a case-to-case basis. Knockout mutants are now available for many secondary metabolite pathways in fungi, but most of them are not ideal for experiments involving fitness estimation because they contain genes conferring resistance against hygromycin, phleomycin or other antibiotics used for selection of transformants. These resistance genes are expressed constitutively and are likely to have a negative impact on fitness. The best strategy for experiments involving fitness estimation appears to be the use of clean gene deletions, which can be achieved with the help of site-specific excision by recombinases such as Cre or ϕC31. However, this procedure is much more laborious than gene disruption. Alternatively, ectopic insertions can be used as controls instead of wild-type strains. Because the insertion of the resistance cassettes into the genome may cause unpredictable effects, several independently generated ectopic transformants have to be used.

Well-designed experiments with carefully engineered strains in axenic and mixed culture will allow us to assess the affect of selected metabolites on the fitness of their producer and on other organisms in the system. The interpretation of the results may be complicated by regulatory effects (see later), synergy or contingency effects (Challis and Hopwood 2003) or detoxification (Karlovsky 1999). In spite of this complexity, carefully engineered mutants in systems imitating natural conditions open the only window currently available for unbiased direct observation of biological functions of secondary metabolites in soil.

1.6.3 Complexity of Chemical Interactions in Soil

Microbial populations in soil are complex and their total population density is high. One-to-one correspondence between a producer of a metabolite and its target, as

known from insect–plant interactions, will rarely be encountered. In interactions among soil microorganisms, all partners are producers and many if not all are targets of ecological metabolites. In terms of fitness, the outcome of chemical interactions of a particular microorganism will be determined by how well the blend of its own secondary metabolites is adapted to the current environment and how efficiently its countermeasures (resistance, detoxification, export, etc.) prevent the harmful effects of metabolites produced by other inhabitants of the niche. As already mentioned in the context of fitness, this inherent complexity needs to be taken into account when studying effects of perturbations of chemical interaction (e.g., by gene knockouts) in natural systems.

Growth inhibition or toxicity in general are not the only effects exerted by metabolites involved in chemical warfare in soil. Microorganisms may avoid harmful effects of antimicrobial compounds produced by their competitors by suppressing their synthesis. The interpretation of such effects from an ecological point of view is straightforward. For example, fusaric acid is a mycotoxin and presumably a virulence factor of *F. oxysporum*. Plant infection by *F. oxysporum* can be suppressed by certain strains of *Pseudomonas fluorescens* which produce the antifungal metabolite 2,4-diacetylphloroglucinol (see Chap. 5). Notz et al. (2002) showed that fusaric acid suppresses the production of 2,4-diacetylphloroglucinol by *P. fluorescens*. Importantly, this effect was demonstrated not only in vitro, but strains carrying reporter fusions for 2,4-diacetylphloroglucinol synthesis were investigated in the rhizosphere and the effects of *F. oxysporum* strains producing different amounts of fusaric acids were compared.

Secondary metabolites involved in antagonistic interaction may affect other functions and activities of competitors to benefit their producers. For instance, mycotoxin deoxynivalenol produced by *F. graminearum* appears to inhibit the expression of a chitinase gene in *Trichoderma atroviride* (Lutz et al. 2003). Because chitinase activity is a decisive factor determining the efficiency of the biocontrol agent *T. atroviride* against *F. graminearum*, the repression of chitinase production by deoxynivalenol may be regarded as a defense mechanism. This results revealed a new ecological role for mycotoxin deoxynivalenol, which was known to act as a virulence factor of *F. graminearum* in wheat. Deoxynivalenol obviously plays at least two different and unrelated ecological roles. (Because of the induction of vomiting and food refusal by deoxynivalenol in mammals, the mycotoxin might also be involved in interference competition between *Fusarium* and grain- or seed-consuming animals.)

Detoxification is a widespread mechanism of defense of target organisms against harmful secondary metabolites (Karlovsky 1999). Antimicrobial plant metabolites are often detoxified by a phytopathogenic microorganism (Pedras and Suchy 2005; Pedras and Hossain 2006; Morrissey and Osbourn 1999; Glenn et al. 2003). These processes have been studied with plant metabolites extracted from leaves and stems, but plant phytoalexins and phytoanticipins also reach soil with root exudates (see Chap. 11) and with plant debris (see Chap. 10). Detoxification of plant defense chemicals is therefore as important in the rhizosphere as it is in aboveground plant organs.

The effects of secondary metabolites on the biology of soil inhabitants are too numerous to list here exhaustively. Metabolites of plant origin induce germination of fungal spores and microsclerotia, attract and repel nematodes, mediate allelopathy among plants and induce chemotaxis in zoospores and protozoans. Strigolactones (Humphrey and Beale 2006) belong to the most interesting compounds not discussed in this volume. These plant secondary metabolites, which are secreted by roots in extremely low quantities challenging our most sensitive analytical techniques, stimulate the germination of parasitic weeds and mycorrhiza fungi. Siderophores are another group of secreted metabolites involved in complex interactions. They are synthesized to facilitate the uptake of iron by their producers, but many microorganisms hijack foreign siderophores to lower their costs of iron extraction, or even use them decadently as a cheap nutrient. Similarly as in marine ecosystems (Engel et al. 2002), nontoxic concentrations of antimicrobial compounds involved in interference competition may effect microbial behavior, corroborating the view that chemical communication is the primary factor controlling interorganismal interactions in soil.

1.6.4 Regulation of Biosynthesis as a Key to Function

Producers of metabolites with ecological roles need to adapt to changing environments by controlling their biosynthesis pathways because the mobility of microbes is limited and the production of any ecological metabolite incurs metabolic costs. Therefore, the regulation of the production of secondary metabolites, regarding both their qualitative spectrum and their quantities, appears to be a crucial factor affecting the success of a microbe in a biotope. Apart from commercially relevant antibiotics, the most thoroughly investigated regulation of secondary metabolite synthesis includes mycotoxins. In line with the prediction that a well-tuned regulation is an important factor maximizing fitness, the regulation of the synthesis of mycotoxins by *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. appears to be very complex. The effects of many environmental factors on the synthesis of a number of mycotoxins have been experimentally determined and regulatory elements involved in the control of mycotoxin synthesis have been identified and cloned. Unfortunately, we have not been able to extract much biological meaning out of these data so far. For example, we know how nitrogen, phosphorus and starch affect fumonisin synthesis in *F. verticillioides*, that a very high sugar concentration is needed for zearalenone synthesis and that deoxynivalenol is produced in media with a high amount of yeast extract. The effects of water activity, temperature and substrate on mycotoxin production have been mapped in detail in Naresh Magan's group. We know that the highest amounts of fumonisins and zearalenone accumulate when their producers are grown on rice, which is not their natural substrate. What does it all mean? We do not know yet, but it is reasonable to assume that mycotoxin synthesis is regulated in order to limit metabolic costs and/or self-poisoning. Deciphering regulatory patterns of mycotoxin biosynthesis should therefore provide us with clues about their

biological function. In general, it appears that we do not have data from relevant conditions yet, or we were unable to look at the data in the right way.

In the course of the characterization of PKS genes in *F. graminearum*, Gaffoor et al. (2005) investigated the expression of all 15 PKS genes of this fungus under 18 culture conditions and discerned seven expression patterns, some of which can be interpreted in ecological terms (e.g., plant infection-specific expression and grain-specific expression). On a different note, their work documents an immense gap in our understanding of fungal secondary metabolites: although *F. graminearum* is the most thoroughly studied *Fusarium* species, its whole genome has been sequenced and disruptions of all its 15 PKS genes are available, the chemical products of nine of its PKS genes are still unknown!

The induction of the synthesis of metabolites putatively involved in interference competition by cultivation of their producers in the presence of competitors provides information which may be more valuable than the results of bioassays. This strategy was used successfully for *Heterobasidion annosum* (Sonnenbichler et al. 1989) at times when gene disruptions in fungi were not readily available. Apart from corroborating the role of certain secondary metabolites with antifungal activity in the interaction of this tree pathogen with antagonistic fungi, these experiments revealed that the antifungal metabolites produced by *H. annosum* can be detoxified by putative target organisms (Sonnenbichler et al. 1993).

1.7 Pitfalls in Search for Function

Interference competition dominated thinking about chemical interactions in soil, inspired by the potent effects of antibiotics isolated from soil *Actinomyces*. Competition among coprophilous fungi in dung was a popular experimental system for these studies because of easy experimental access and a well-described, predictable sequel of colonizing organisms. However, most of the investigations were performed on isolated organisms. For example, Gloer and Truckenbrod (1988) began their report by stating "Isoepoxydon has been established as the major causative agent of interference competition between *Poronia punctata*…," while, in fact, only in vitro effects have been established. The bioassay used by Gloer and Truckenbrod was based on a species competing with the producer of isoepoxydon, but too often the role of a secondary metabolite in interference competition is postulated on the basis of bioassays with human pathogens or other ecologically inappropriate organisms.

On the other hand, antibacterial or antifungal effects may be overlooked when a metabolite is well known in a different context. For instance, a strong toxic effect of mycotoxin zearalenone on filamentous fungi remained unnoticed for decades (Utermark and Karlovsky 2007). Zearalenone is known as a potent estrogen and the ingestion of contaminated food and feeds poses a health risk to humans and farm animals. This prominent biological activity and the label "mycotoxin" apparently prevented people working with zearalenone from subjecting it to a standard antifungal assay.

Zearalenone provides an instructive example of a wrong assignment of function too. The estrogenic activity of the metabolite inspired speculations about its role as a sex hormone and regulator of reproduction in *Gibberella zeae* (Nelson 1971). The hypothesis was seemingly corroborated by observations that zearalenone added to *G. zeae* cultures increased perithecia production (Wolf and Mirocha 1973) and that dichlorvos, an inhibitor of zearalenone biosynthesis, reduced perithecia production (Wolf et al. 1972). In spite of the facts that many chemicals, including commercial fungicides in sublethal doses, stimulate perithecia formation, that dichlorvos unspecifically inhibits many PKSs, and that *F. culmorum*, which does not possess a sexual stadium, produces large amounts of zearalenone, the sex hormone hypothesis survived for over three decades. Nelson's idea was so appealing that it persisted even after the exposure of zearalenone–perithecia correlation as a fallacy (Windels et al. 1989).

Neither isoepoxydon nor zearalenone has been shown to enhance the fitness of their producers in the presence of competing fungi in natural environments so far, but the role of zearalenone in interference competition is strongly supported by finding that mycoparasite *Gliocladium roseum*, which preys on *Fusarium* spp., developed an enzymatic detoxification mechanism for zearalenone (El-Sharkawy and Abul-Hajj 1988). *G. roseum* is resistant to zearalenone and the inactivation of its detoxification activity renders it susceptible (Utermark and Karlovsky 2007).

Research on secondary metabolites involved in interaction of microbial pathogens with plants suffered from serious setbacks. Gäumann (1954) and his disciples postulated half a century ago that phytotoxins are causally involved in all plant diseases. A generation of phytopathologists generated phytotoxicity data to support their hypothesis, but a convincing proof did not surface even for a single toxin at that time because of the lack of appropriate experimental tools. Referring to this era, Robert Scheffer and Steve Briggs once wrote: "The literature on toxins affecting plants is vast, but much of it is meaningless." Their harsh judgment was embraced by the next generation of phytopathologists, who went to the other extreme and abandoned research into secondary metabolites acting as virulence factors for nearly three decades. (Host-specific toxins were a noticeable exception.) As a consequence, opportunities to design novel resistance mechanisms for crops based on detoxification of fungal toxins were considerably delayed and our understanding of pathogen–plant relationships was deprived of one of its principal facets. A renewed interest of phytopathologists in non-host-specific toxins, as we experience it now, will likely benefit not only plant protection but also basic research on secondary metabolites in general.

1.8 Future of Secondary Metabolite Research

Thousands of secondary metabolite structures have been published, but educated guesses about biological function are possible only for a negligibly small fraction of them. Besides, they are seldom more than guesses: when a bioassay demonstrates

toxic effects upon a competitor, we still do not know whether the substance is produced under relevant conditions in nature, whether its local concentration is sufficient to exert the effects observed in vitro and how adsorption, degradation and interaction with other metabolites modulates its toxicity in situ. It is not possible to determine or control all these factors. The only reliable way to address the biological role of a particular metabolite is to manipulate its biosynthesis or degradation by genetically engineering interacting organisms and investigating the consequences of the perturbation under natural conditions. This strategy has been used extensively and successfully in interactions between plant pathogens and their hosts. In a few cases, the role of secondary metabolites in biological control of plant pathogens has also been studied with the help of genetically engineered microbes. It is time now to extend the concept to chemical ecology of soil in a broad sense.

How is secondary metabolite research advancing beyond its traditionally descriptive approach? Natural product chemistry is extending its scope and embracing techniques and concepts originating from biochemistry and genetics, while ecologists and environmental microbiologists recognize that chemical interactions mediated by secondary metabolites are crucial for our understanding of soil ecosystems. Empirical screening of natural products for biological activities, as well as high-throughput purification and structure elucidation of natural products from arbitrarily selected sources, should be left to the responsibility of the pharmaceutical industry and service laboratories, releasing capacity in academia and basic research to address fundamental questions. The following emerging approaches and technologies are likely to play a role in this transition:

- Application of genetic engineering in systematically controlling the production and/or degradation of secondary metabolites, followed by monitoring how these perturbations affect the system, allows us to assess the effect of secondary metabolites on the fitness of soil organisms.
- Analytical techniques for the quantification of many metabolites in matrices as complex as soil are needed to follow the dynamics of secondary metabolite production, transformation and degradation in soil. In situ detection and nondestructive analysis are needed in order to take into account the heterogeneous structure of soil ecosystems.
- Routine techniques available for monitoring microbial populations in soil are differential gradient gel electrophoresis (DGGE) of amplified ribosomal RNA genes or reverse-transcribed ribosomal RNA, terminal restriction fragment length polymorphism (T-RFLP) of ribosomal RNA genes and in situ hybridization of taxon-specific oligonucleotides labeled by fluorescent dyes (FISH). In future these techniques they will be extended by large-scale metagenome sequencing (Eisen 2007; Rusch et al. 2007).
- Modeling chemical interactions in microbial ecosystems and their evolutionary consequences will be increasingly important. The interplay of factors such as metabolic costs, competition, spatial heterogeneity, synergy of antibiotic effects of many metabolites, adsorption and detoxification can be investigated by computer modeling, while it is difficult to address more than one factor experimentally.

Soil is arguably the most complex and difficult system to chose for the study of ecological functions of secondary metabolites. However, soil is also the ecosystem in which chemical interactions play the most substantial role, and from where major insights into the evolution of chemical diversity are expected to come.

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