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REVIEW ARTICLE

Fungal Phytotoxins in Sustainable Weed Management

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Abstract: Fungal phytotoxins are natural secondary metabolites produced by plant pathogenic fungi during host–pathogen interactions. They have received considerable particular attention for elucidating disease etiology, and consequently to design strategies for disease control. Due to wide differences in their chemical structures, these toxic metabolites have different ecological and environmental roles and mechanisms of action. This review aims at summarizing the studies on the possible use of these metabolites as tools in biological and integrated weed management, *e.g.* as: novel and environmentally friendly herbicides; lead for novel compounds; sources of novel mechanisms of action. Moreover, the limiting factors for utilizing those metabolites in practice will also be briefly discussed.

Keywords: Phytotoxins, weed management, natural herbicides, mechanisms of action, fungal metabolites, plant pathogens.

1. INTRODUCTION

Weed management has been a major problem since the dawn of agriculture. Unmanaged weeds represent the greatest cause of crop yield reduction, more than any other agricultural bio-constraint [1]. For centuries, manual weeding represented the most time-consuming labor in farming practices, and it is still widely exploited in agricultural contexts where labor is available at low costs. The recent availability of highly effective synthetic herbicides was first considered as a "panacea" for managing weeds, increasing control effectiveness and reducing costs, since many of these compounds have very good selectivity toward crops and are relatively inexpensive. Later on, although most of the currently used synthetic herbicides have low impact on the environment and the wildlife, their use has become increasingly controversial in some quarters. Partly due to this, organic agriculture has received a recent surge in popularity, although its market share is globally still negligible [2].

Despite the progress in the use of novel and (questionably) environmentally friendly non-chemical tools for weed control (*e.g.* mechanical, physical, biological methods), herbicides continue to be a major tool in weed management. Today herbicides still account for more than half of the volume of all agricultural pesticides applied in the world.

Due to stricter regulations to ascertain safety and environmental impact, the procedures to register herbicides have become a longer and more accurate process that also involved the already registered herbicides [3]. This resulted in the withdrawal from the market of a number of dangerous compounds previously registered and commercialized, and negatively influenced the possible introduction of herbicides with novel mechanisms of action (MOAs). Indeed, no herbicide with new MOAs has been introduced in a commercial product in around the last 25 years. Before this, a new mode of action was discovered and introduced with quite constant intervals, leading to the current availability of less than 15 modes of action [4].

All the 137 "new" herbicide active ingredients introduced from 1980 to 2009 have the same modes of action of herbicides introduced before 1990 [5]. Some older herbicides or new herbicides with old modes of action have sometimes been exploited thanks to the use of additional compounds, the so-called "safeners". From a practical point of view, the reduction in number of the available herbicides has increased the difficulties

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in managing weeds, causing a faster appearance/selection of resistant weeds. The evolution of resistance to most of the currently available herbicides makes the discovery of new sites of action a particularly pressing issue for agrochemical companies and farmers.

After years of modest attractiveness, thanks to their enormous and still unexplored diversity, natural compounds from living organisms are now receiving a renewed interest in the field of herbicide research [6, 7]. Among the possible sources of natural herbicidal compounds, plant pathogenic fungi, with their toxins, deserve greater attentions.

The structural diversity and evolved biological activity of natural phytotoxins offer opportunities for the: (i) direct application as natural herbicides; (ii) use as lead compounds for chemical synthesis of novel herbicides; (iii) exploitation as starting material for subsequent chemical or microbiological modification; (iv) discovery of new modes of action.

The advent and the availability of novel technologies allow research approaches not possible just a few years ago. High-throughput bioassays allow faster and more accurate screening procedures; faster, cheaper and more environmentally friendly extraction procedures from the microbial cultures have enhanced the speed of the metabolites purification; analytical procedures and structure determination have been simplified by more sophisticated and automated equipment; 'omics' approaches allow an easier determination of the modes of action of phytotoxins; nanomaterials, nanotechnologies, precision agriculture can allow a better delivery to the target plant or increase effectiveness of the compounds.

Conversely, there are also disadvantages and limitations hampering the discovery and the use of fungal/microbial metabolites, *e.g.* difficulties in scaling-up the fermentation processes; metabolites having structures hardly reproducible through synthesis; reduced stability/persistence of the compounds; off-target effects.

All these different aspects have been the object of a number of excellent and focused reviews. The aim of the present review is to make a brief and more general overview of the use of fungal toxins as tools for herbicide development, underlining some novel opportunities and applications, including approaches to overcome constraints and limitations to their development and exploitation, referring to those reviews and making a few examples in each of the discussed topics.

2. FUNGAL DIVERSITY

A first intriguing argument encouraging the search for natural herbicides from microbial sources is the awareness that fungal biodiversity is far from being fully explored. Indeed, it has been postulated that fungal diversity encompasses over 5 million species, exceeding that of terrestrial plants by an order of magnitude [8]. Only a fraction of all fungal species has been described (about 100,000), and an even smaller fraction explored for the production of biologically active secondary metabolites (SMs).

Among the eukaryotic microscopic fungi, the SM producing capability of the "imperfect" (or "mitosporic") fungi, the ascomycetes and of several other filamentous and endophytic fungal species are the most significant. The basidiomycetes are also frequently reported to produce important SMs, while yeasts, phycomycetes, slime moulds rarely produce bioactive metabolites.

Among the many different ecological groups of fungi, the phytopathogenic ones could be particularly interesting for the discovery of novel herbicides. They can be classified as necrotrophs, hemi-biotrophs and biotrophs, depending on the tactic they use to colonize/attack the host. Fungal pathogens represent major infectious agents in plants, being able to cause severe damages during all the developmental stages including post-harvest, obtaining nutrients from the plants they invade. Conversely, they could also be very interesting sources of SMs for beneficial purposes.

Obligate biotrophs are entirely dependent on the living plant they colonize, being characterized by several sophisticated infection structures (*e.g.* appressoria, penetration and infection hyphae, secreted effectors), that allow the invader to suppress plant defense responses and to gain access to host nutrients, resulting in disease [9-11]. Conversely, necrotrophic fungal pathogens, usually have broader host ranges, thrive on dead host tissue that has become necrotized by phytotoxic SMs, secreted cell wall degrading enzymes, peptides, and reactive oxygen species [12, 13]. Hemi-biotrophic pathogens initially establish a biotrophic relationship with their host. They produce toxic metabolites only at later stages of the infection, in order to kill the host cells and complete its life cycle on the dead tissue [12]. Secreted phytotoxins contribute to virulence or pathogenicity by disrupting host cells and inducing the release of nutrients to facilitate colonization of host tissues [14].

Thus, fungal phytopathogens, especially the necrotrophic ones, very often produce SMs that are involved in the infection process and in the symptoms appearance. They are attractive sources of bioactive metabolites having herbicidal potential.

A number of phytopathogenic necrotrophic genera, such as *Cercospora*, *Ramularia*, *Rhynchosporium*, *Alternaria*, *Fusarium*, *Helminthosporium*, *Sclerotinia*, and *Verticillium*, just to cite a few examples, have been quite intensively studied to understand the plant-pathogen interactions and the infection processes, and then to find ways to protect the crops from the pathogen attack. Moreover, SMs of many of those genera have been also studied in relation to the risks posed to human and animal health when they accumulate in agricultural commodities and enter the food chain. Conversely, many other pathogens, *e.g.* those attacking weeds or wild plants, or weak pathogens, were merely isolated and described, but their metabolic potential has never been deeply explored. Moreover, a further source of interesting SMs could be symptomless endophytes that, in association with host plants, have the capacity to either develop as pathogens or saprophytes, and in either case can produce toxins [15]. Thus, this tremendous source of biodiversity has been only partially explored and deserves a greater attention.

For example, *Alternaria* species with lifestyles ranging from saprophytes to endophytes, to pathogens, cause diseases in nearly 400 plant species including a wide variety of economically important crops and cause severe economic problems. *Formae speciales* of *A. alternata* alone can infect more than 100 plant species [16], and over 100 species occurring world-wide have been described [17]. However, they still represent a mostly unexplored source of novel metabolites. Moreover, some species produce diverse host specific toxins (HSTs) considered as a key reason for the success of these pathogens [18].

The *Magnaporthe grisea* species complex comprises many phylogenetic species [19] that cause disease to some 50 grass and sedge species. They were mainly studied because of the diseases they cause to rice, wheat, and many cereals, but they attack numerous weed and ornamental grasses as well. The filamentous ascomycete genus *Cochliobolus* (anamorph *Bipolaris*/*Curvularia*) is composed of more than 40 closely related pathogenic species with particular specificity to their host plants [20]. Over 140 species have been described within the genus *Cercospora* [21].

These are only a few examples of the dimension of some common phytopathogenic fungal genera, only partially explored for the production of SMs.

3. METABOLIC DIVERSITY BY A CHEMICAL ECOLOGICAL APPROACH

The chemical interactions between organisms have had an important role as evolutionary forces driving the survival of species with positive functions in interactions such as mutualistic and symbiotic relationships and negative ones in competitive and parasitic relationships. These processes have led to the development of secondary metabolic pathways, which permit producing a vast array of structurally diverse and biologically active molecules [22]. Although they are usually considered not essential for life, it is widely accepted that they have important ecological roles for many organisms. The studies of these ecological phenomena have and can further lead to the discovery of novel natural products that have bioactivities of practical importance, including novel compounds with herbicidal potential. Fungi are not exempt from these phenomena, and thus they are sources of thousands of low-molecular weight SMs.

According to estimates made a decade ago, the total number of bioactive fungal products amounts to several thousands, representing over one third of all the known microbial products [23]. Moreover, around 30-35 out of the 100-150 metabolites of microbial origin that are currently and practically used (*e.g.* antibiotics for human therapy, or for agricultural and veterinary purposes) have fungal origin. However, the known SMs represent only a very limited fraction of the immense biodiversity available in nature. Exploration of natural sources for novel bioactive agents has been successfully exploited for drug discovery and development. Some of the most successful drugs and agrochemical fungicides on the market have been developed from fungal SMs based on random screenings. Those include, for example, antibiotics, anti-fungal agents and immunosuppressive drugs [24].

Conversely, the application of an "ecological rationale" to discover novel compounds is based on the evolutionary concept that each SM provides some environment advantage. Examples of this approach include compounds produced by plants that serve as chemical defenses against herbivores, or produced by marine invertebrates that deter attacks by predators [25]. Fungi commonly thrive in competitive environments, and thus some of their secondary metabolic capabilities may be influenced by the selection pressure exerted by

other organisms. In case of phytopathogenic fungi, secondary metabolites can be important determinants in the virulence of the pathogen, in symptoms appearance, or conversely in overcoming the resistance capability of a potential host plant. Thus, when searching for natural compounds having herbicidal effects, the "ecological" rationale would be to look for fungal pathogens able to cause symptoms such as necrosis and chlorosis, and try to identify the fungal SMs whose macroscopic effects resemble those due to the pathogen's attack. Application of rationale at the beginning of a screening process would limit the number of organisms needing to be investigated, reduce the costs of the screening procedures, and facilitate dereplications. The chemical ecological approach is complementary to random screening because they incorporate rationale into the process of selecting fungi for chemical studies. Moreover, it would be particularly well suited to academia or public research center laboratories that usually cannot afford high-throughput screening and bioassays approaches.

This approach must be integrated by fusing the expertise of groups having different scientific backgrounds. Indeed, mycologists and plant pathologists have to recognize plants, and above all weeds, having disease symptoms that could be associated to a fungal attack, and that could be co-caused by phytotoxins. After a fungal pathogen has been identified, and considered interesting for the production of bioactive metabolites (*e.g.* lack of literature on the subject), its real capability to produce phytotoxic compounds has to be ascertained. It must be grown in proper cultural conditions (*e.g.* a liquid defined medium). Then the phytotoxicy of the culture filtrates should be ascertained using suitable bioassays, that will also guide the successive chemical purification steps by identifying the toxic fractions. Once pure metabolites are obtained, their structures can be determined, and the biological characteristics can be evaluated by more suitable biological assays. Ascertaining structure-activity relationships, absolute configuration, mechanisms of action, synthetic production, optimization of microbial fermentation processes, analytical determination, dose-activity effects, and application methods can follow, with the involvement of different types of expertise [26].

Toxins of the same structural group are often produced by microorganisms that belong to different genera, suggesting that there had been horizontal gene flow of a clustered group of genes responsible for metabolite biosynthesis. It is the case of trichothecenes, a family of mammalian toxic tetracyclic sesquiterpenoid substances (more than 50) which are produced by different genera, including *Fusarium* (producing at least 25 different trichothecenes), *Myrothecium* (producing roridins and verrucarins), *Stachybotrys* (satratoxins) and *Trichoderma* (trichodermins). Ophiobolins, a group of sesterterpenoids that includes at least 23 analogues, are produced by phytopathogenic species of different species, such as *Bipolaris* or *Drechslera* spp. [27].

As a further element increasing biodiversity, it is noticeable that species belonging to the same genus often are able to produce a wide variety of metabolites, as in the case of the genera *Alternaria*, *Claviceps* or *Fusarium*, whose species can produce tens of different toxic metabolites. For example, *A. alternata* has the ability to produce more than 60 SMs. From about 20 HSTs that have been documented, at least seven are produced by *A. alternata* pathotypes. Several other phytotoxins are also produced by *Alternaria* such as brefeldin A, altertoxin, and tentoxin, and many other mycotoxins as well [28].

4. STRUCTURAL DIVERSITY

An analysis of the structural diversity of the organic compounds available in the CAS (Chemical Abstracts Service) Registry confirmed that most of the 24 million organic compounds in the database can be included in less than 150 basic structural groups [29], with a distribution responding to a power law. It seems that the frequency in the use of a structural framework for the synthesis of a compound influences the likely to be used in another compound. The cost of synthesis has an impact on that because a new derivative made from an already known framework is probably less costly than starting from a novel frame. Thus, despite the tremendous number of organic compounds available, their diversity is limited by a short of "steered" synthetic patterns.

Conversely, natural products are often complex compounds possessing a higher structural diversity, with more chiral centers, sp3-hybridized carbons, and rings than synthetic compounds, because they evolved to interact efficiently with their biological targets.

Accordingly, it is not surprising that 40% of the chemical scaffolds in the Dictionary of Natural Products occupy unprecedented chemical space that was not represented by synthetic compounds [30]. Moreover, few natural products contain halogens (Cl, F, and Br), but conversely they tend to be rich in oxygen and nitrogen, and may contain sulfate or phosphate groups. This diversity may make available new classes of natural compounds, not obtainable by classic synthesis ap-

proaches [31]. New cheminformatic and synthetic techniques have been developed to identify and design compounds with natural product-like properties (see above).

In general, fungal SMs can be divided into four main chemical classes: polyketides, terpenoids, shikimic acid derived compounds, and non-ribosomal peptides. Moreover, hybrid metabolites composed of moieties from different classes are common, as in the meroterpenoids, which are fusions between terpenes and polyketides [32]. However, within this simplification, fungal phytotoxins belong to many different groups of naturally occurring compounds. A number of reviews have already highlighted the different and unique structures of bioactive metabolites produced by plant pathogenic fungi [33-35]. The use of a chemical ecological approach (see above) has allowed our and other research groups to produce, purify and structurally and biologically elucidate a large number of phytotoxins having both original structures and promising herbicidal activities. A few examples include, among others: non proteinogenic amino acids (ascaulitoxin and ascosonchine) [36, 37], aromatic compounds (agropyrenol, agropyrenal, agropyrenone) [38], cytochalasans (cytochalasins, deoxaphomin) [39], nonenolides (putaminoxins and pinolidoxin) [40, 41], oxazatricycloalkenones and other complex carbon skeleton compounds (phyllostictines, phyllostoxin, drazepinone) [42, 43], terpenes (phomentrioloxins, ophiobolins) [44, 45], furopyrans (chenopodolans) [46]. Some of the structures are displayed in Figs. (**1** and **2**).

5. MECHANISMS OF ACTION

Because natural products offer an unparalleled source of structural diversity, with little overlap with synthetic compounds generated by traditional organic synthesis, this wider structural diversity may endow natural products with unique mechanisms of action (MOAs) [47]. Currently, there is a large number of registered herbicides on the market that are based on many different active ingredients belonging to several chemical families. However, they act only against a restricted number of target sites with a relatively few MOAs [4]. In the past, the frequent introduction into the market of novel herbicides allowed overcoming quite easily the appearance of weed populations resistant to a specific herbicide. Indeed, applying herbicides with different mechanisms of action would generally hamper the ap-

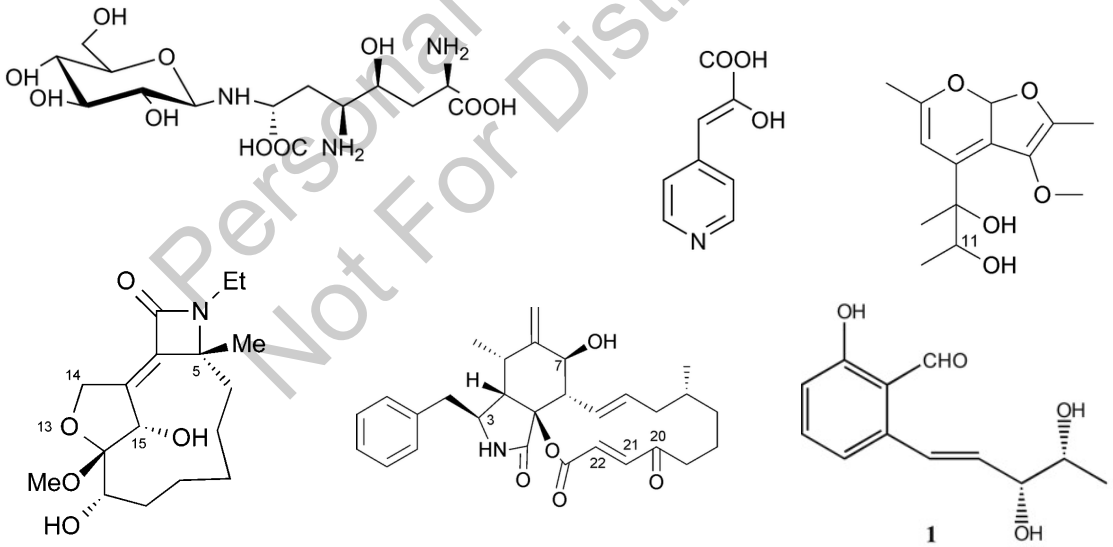

Fig. (1). Examples of phytotoxin structural diversity: (upper row, from left) ascaulitoxin and ascosonchine (non proteinogenic amino acids), chenopodolan A (furanopyrans); (lower row, from left) phyllostictine (oxazatricycloalkenones), cytochalasin A (cytochalasans), agropyrenol (aromatic compounds).

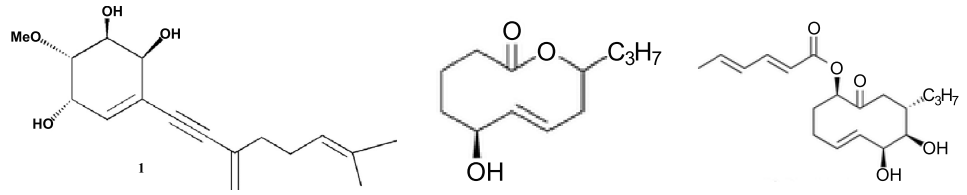

Fig. (2). (From left) phomentrioloxin (terpenes), putaminoxin and pinolidoxin (nonenolides).

pearance of herbicide-resistant weeds. The new herbicides registered for weed control in the last 25 years do not contain an active ingredient with a new MOA, but simply they are novel mixtures or novel formulations of existing herbicides.

A number of reasons have hampered or depleted the registration and the introduction into the market of herbicides with novel mechanisms of action, among which: (1) the reduced demand from the market of new compounds due the availability of cheap and effective herbicides; (2) the introduction of herbicide-resistant crops, that have strongly oriented the herbicide market in some of the major crops in the world to one major herbicide; (3) the increasingly higher costs for discovering and registering novel molecules; (4) the contractions of the number of companies involved in the synthesis of novel compounds; (5) the stricter regulatory criteria imposed by the authorities with an increased expectation of safer and more environmentally friendly compounds.

However, as recently analyzed, despite the potential of SMs for the discovery herbicides with novel MOAs, of the 19 classes of herbicides recognized by the Herbicide Resistance Action Committee [4] with defined sites of action, and although several natural products (NPs) have served as models, only one NP, bialophos is a herbicide of commercial significance [7].

6. NEW (EXPLOITABLE) MECHANISMS OF ACTION

From an ecological and evolutionary standpoint, knowing the modes of action of natural compounds allows to have decisive information of its function in nature. In case of a phytotoxins produced by a plant pathogen, a specific molecular target found only in plants provides evidences that the compound could have evolved as a virulence factor for the pathogens [48]. From a practical point of view, natural compounds can be sources of herbicides with new MOAs, and new MOAs are needed to combat the increasingly rapid evolution of weed resistance to the currently used herbicides (see above).

For a number of phytotoxins the mechanism of action has been more or less clearly determined (Table **1**), showing differences with the known herbicides, and thus increasing the interest for applicative uses of the compounds. Some of them were studied long time ago, with the main aim of understanding their function in the development of the disease caused by the producer.

Although not of fungal origin, the story of bialaphos deserves to be mentioned as an example of possible future developments in this research field. Bialaphos is produced from the fermentation broth of the actinomycete *Streptomyces hygroscopicus*. It is marketed as an herbicide in eastern Asia. It is a pro-herbicide, meaning that it is inactive by itself, and it is metabolized into phosphinothricin by plants before exerting its herbicidal action. It has a broad-spectrum post-emergence effect to control a wide range of weeds in agricultural settings. Glufosinate, the synthetic form of phosphinothricin, is produced as an herbicide in the rest of the world. Bialaphos is a unique inhibitor having an unusual P-methylated amino acid, that is a structural analogue of glutamate and acts as an inhibitor of glutamine synthetase [49].

Cornexistin is a fungal metabolite produced by *Paecilomyces variotii* [50] (Fig. **3**). It is patented as an herbicide, inhibiting aspartate amino transferase activity at high concentrations. Probably it is a pro-herbicide, too, that must be metabolized to an amino transferase inhibitor, because it acts only after incubation in a plant cellular extract [51].

T-toxins are trichothecene phytotoxins produced by different fungal pathogens, *e.g. Cochliobolus heterostrophus* and *Bipolaris maydis*. In sensitive plants they bind an inner mitochondrial membrane protein and inhibit mitochondrial respiration, resulting in pore formation, leakage of NAD+, and other ions, as well as subsequent mitochondrial swelling [52].

2,5-anhydro-D-glucitol, a fungal phytotoxin isolated from *Fusarium solani*, inhibited root growth. It is a natural analogue of fructose and thus it was supposed that plants utilize this fructose analogue as a substrate [53]. Indeed, 2,5-anhydro-d-glucitol was bisphosphorylated when incubated with a plant-cell-free extract, probably by enzymes as glycolytic glucosyltransferases, hexokinase and phosphorfructokinase, and the successive binding of the phosphorylated fungal toxin to aldolase interferes with the normal catalytic function of this enzyme.

Beticolins (Fig. **3**) are a family of nonpeptidic toxins (with 20 identified members to date) produced by *Cercospora beticola*, the fungal agent responsible for the leaf spot disease on sugar beet. In particular, they induce dramatic loss of solutes such as amino acids and β-cyanin from root tissues, and possess the capability to self assemble into multimeric ion channels that disrupt membrane function [54].

Table 1. Examples of phytotoxins with known MOAs (see the text for further details and references).

Toxin	Producer	MOA
2,5-anhydro-d-glucitol	*Fusarium solani*	Interferes with the normal catalytic function
AAL toxin	*Alternaria alternata*	Causes loss of plasma membrane integrity
Beticolins	*Cercospora beticola*	Disrupts membrane function
Cercosporin	*Cercospora* spp.	Induces rapid membrane peroxidation and cellular death
Cerulenin	*Cephalosporium cerulens*	Inhibits *de novo* fatty acid synthesis
Colletotrichin	*Colletotrichum* spp.	Disintegrate plasma membrane
Cornexistin	*Paecilomyces variotii*	Inhibits aspartate amino transferase
Cyperin	*Ascochyta cypericola*	Inhibits plant enoyl reductase
Cytochalasins	*Phoma exigua*	Hampers actin polymerization into filaments
Fumonisin	*Fusarium* spp.	Causes loss of plasma membrane integrity
Fusicoccin	*Fusicoccum amygdali*	Inhibits stomata closure with subsequent lethal wilting
HC-toxin	*Cochliobolus carbonum*	Inhibits growth and cell division
Moniliformin	*Fusarium moniliforme*	Arrests mitosis
Ophiobolins	*Bipolaris* spp.	Affects plasma membrane
Pyrenophorin	*Drechslera avenae*	Causes chlorophyll retention
Sirodesmin PL	*Leptosphaeria maculans*	Inactivates protein function
Tentoxin	*Alternaria alternata*	Inhibits the energy transfer
T-toxins	*Cochliobolus heterostrophus*	Inhibit mitochondrial respiration
Victorin	*Cochliobolus victoriae*	Causes collapse of the mitochondrial transmembrane potential

Tentoxin is a cyclic tetrapeptide produced by *Alternaria alternata.* It causes severe leaf chlorosis of sensitive species by inhibiting chloroplast development [55]. Tentoxin inhibits the energy transfer of the chloroplast-localized CF1 ATPase, and interferes with the transport of the nuclear-coded enzyme polyphenol oxidase into the plastid of sensitive plants, without effects on insensitive species.

A series of structurally related fungal metabolites specifically inhibit ceramide synthase (sphinganine-*N*-acyltransferase) in plants. These include several analogues of AAL toxin (Fig. **3**) and fumonisin [56, 57], produced by *Alternaria alternata* tomato pathovars and by *Fusarium* spp., respectively. Indeed, those compounds are analogues of the substrate for ceramide synthase. When plants are treated with these inhibitors, the sphingolipid precursors and precursor derivative levels are rapidly elevated to concentrations well higher than those found in untreated tissues [58], followed by the fast loss of plasma membrane integrity.

Cerulenin, a product of the fungus *Cephalosporium cerulens*, proved to inhibit *de novo* fatty acid synthesis in plastids [59].

Cyperin, a diphenyl ether compound produced, among others, by *Ascochyta cypericola* a pathogen of *Cyperus rotundus* [60], inhibits plant enoyl reductase, which results in light-independent disruption of membrane integrity [61].

Fusicoccin, one of the first well-studied fungal phytotoxins, is produced by *Fusicoccum (Phomopsis) amygdali*. It was shown to irreversibly activate the plant plasma membrane H+-ATPase, leading to inability of stomata to close and subsequent lethal wilting [62].

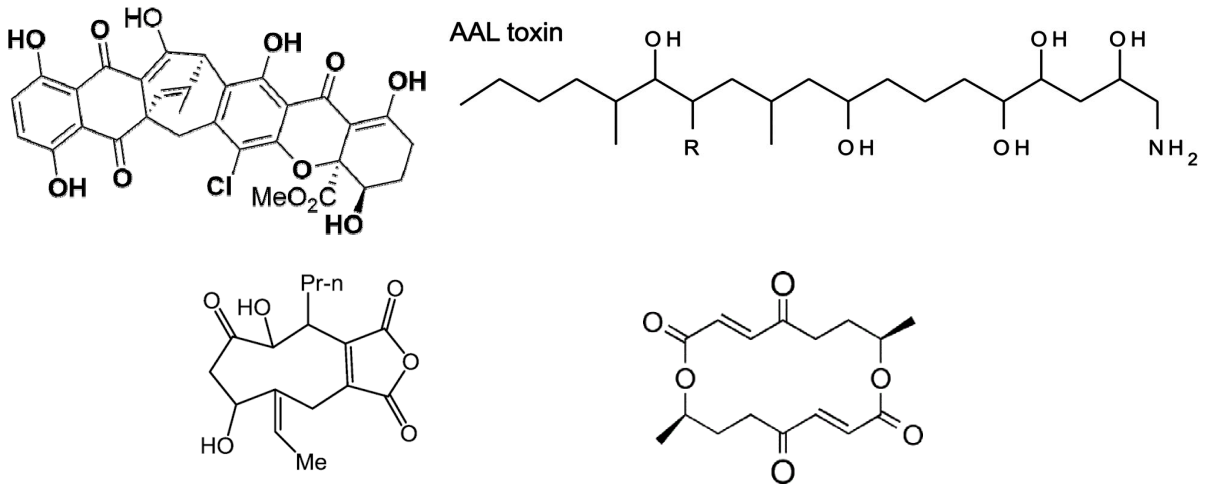

Fig. (3). Examples of toxins with interesting mechanisms of action: beticolin 0 (up-left); AAL-Toxin (up-right); cornexistin (down-left); pyrenophorin (down-right).

Victorin is a host-selective toxin produced by *Cochliobolus victoriae*, the causal agent of victoria blight of oats. Victorin is a toxin essential for the pathogenesis. It causes the collapse of the mitochondrial transmembrane potential, resulting in a mitochondrial membrane transition [63]. However, the retention of plasma membrane and tonoplast integrity supported the hypothesis that it induces an apoptotic-like cell death response. It may also act at the cell surface to cause a hypersensitive response *via* plasma membrane ion fluxes [64].

Colletotrichin is a highly phytotoxic compound from several *Colletotrichum* species, *e.g.*, *C. nicotianae*. It causes first the disintegration of the plasma membrane, accompanied by massive cellular leakage [65]. The many symptoms caused on plants by ophiobolins, tricyclic sesquiterpene phytotoxins produced by several fungal species, are largely due to effects on the plasma membrane [66]. Moniliformin, a bioactive metabolite produced by *Fusarium moniliforme*, is phytotoxic and arrests mitosis of maize root meristematic cells at the metaphase stage [67]. Cytochalasins (Fig. **1**) are metabolites of several fungal species, such as *Phoma exigua*, binding actin. This hampers actin polymerization into filaments, thus resulting into the inhibition of the processes that require actin filaments, such as mitosis and other plant processes [68]. HC-toxin is a specific cyclic tetrapeptide produced by the corn pathogen *Cochliobolus carbonum* that inhibits growth and cell division of target plants by aspecifically acting on histone deacetylase. HC-toxin may also significantly alter gene expression in ways that would be detrimental to the plant [69].

The trichothecenes, a large class of sesquiterpene mycotoxins produced by several plant pathogens exert most of their effects by inhibiting protein synthesis. They do this apparently by targeting the peptidyltransferase center of mitochondrial ribosomes [70].

Some fungal phytotoxins such as sirodesmin PL from *Leptosphaeria maculans* and gliotoxin have internal disulfide bridges that conjugate proteins inactivating the protein function [71]. Such compounds are generally broadly cytotoxic.

Cercosporin is a photodynamic photosensitizer pigment first isolated from species of the fungal genus *Cercospora* that in the presence of light and oxygen, generates singlet oxygen and superoxide ions that induce rapid membrane peroxidation and cellular death [72].

Pyrenophorin (Fig. **3**) is a non-selective phytotoxin which not only causes chlorophyll retention (known as "green islands") in leaves but affects root growth of graminaceous species. The presence of PSII inhibitor diuron did not reverse electrolyte leakage and bleaching caused by pyrenophorin, indicating that the oxidative damage triggered in *A. sterilis* by the phytotoxin does not depend on light and is exerted through a mechanism involving electron misdirection and generation of reactive oxygen species [73].

Some of the toxins mentioned above often possess, beside the interesting phytotoxic properties, other non-target effects (*e.g.* mammalian toxicity) or broad MOAs not specific for plants. It is the case of trichothecenes, moniliformin, or T-toxins, just to make a few examples, that are very potent mycotoxins and thus they were studied much more for the risks rather than the benefits of their use. In any case, understanding the MOA of a novel metabolite remains a priority in order to evaluate its potential use as an herbicide.

7. PHYTOTOXINS WITH UNDEFINED MECHANISMS OF ACTION

In addition to natural phytotoxins with known MOAs, there are many potent phytotoxins for which the MOA is still unknown [47] (Table **2**). In several cases, there are indications that the compounds could have unique MOAs.

Table 2. Examples of toxins with undefined MOAs (see the text for further details and references).

Toxin	Producer
Agropyrenol	*Ascochyta agropyrina* var. *nana*
Ascaulitoxin aglycone	*Ascochyta caulina*
Chenopodolin	*Phoma chenopodiicola*
Cinnacidin	*Nectria* sp.
Lentisone	*Ascochyta lentis*
Macrocidins	*Phoma macrostoma*
Mevalocidin	*Coniolariella* sp.
Phomentrioloxin	*Phomopsis* sp.

One example is ascaulitoxin aglycone, a non-specific phytotoxin isolated from the liquid culture of *Ascochyta caulina*, a fungus proposed for the biological control of *Chenopodium album* [36]. It is more effective as a growth inhibitor to duckweed (*Lemna paucicostata*) than many commercial herbicides. Supplementation of growth media with most amino acids reverses the effects of this potent phytotoxin [74]. Certain tricarboxylic cycle intermediates also reversed or reduced the effects. Metabolomic analysis of the toxin effects revealed both up and down regulation on pools of some amino acids.

Mevalocidin was discovered from static cultures of two fungal isolates originally identified as *Rosellinia* sp. and *Fusarium* sp. [75], and successively as *Coniolariella* sp. Mevalocidin has broad spectrum post-emergence activity on grasses and broadleaves with greater than 50 % injury to all of the species tested at 4 kg/ha after 16 days and lethality after 21 days. Due to the symptoms it produces, a novel mode of action has been supposed. Moreover, mevalocidin is rapidly absorbed and translocation occurs from the treated leaf to other plant parts including roots confirming phloem as well as xylem mobility.

Cinnacidin was isolated from the fermentation extract of a strain of *Nectria* sp., a fungal pathogen causing cankers on many tree species. It consists of a cyclopentalenone ring system linked to an isoleucine subunit *via* an amide bond. It showed promising herbicidal activity, as it caused stunting and chlorosis, which spread throughout the foliar tissues. It may act as a hormone-like herbicide by mimicking the role of jasmonic acid [76].

Macrocidins (Fig. **4**) are cyclic tetramic acids mostly responsible for the activity of *Phoma macrostoma*, a bioherbicide controling broadleaf weeds such as *Cirsium arvense* and *Taraxacum officinale* [77]. Post-emergence application of macrocidins caused on broadleaf weeds symptoms, as chlorosis and growth inhibition, similar to those caused by the pathogen in in the infected tissues. Although the observed symptoms were consistent with those typically observed for inhibitors of HPPD, *in vitro* tests against this enzyme resulted in no observable effects on enzyme activity. More recently, the carotenoid profiles and other physiological parameters were compared in several plants treated with macrocidin, withanother known carotenoid biosynthesis inhibitor, diflufenican, or untreated control plants. Only in susceptible plants, macrocidins induced photobleaching symptoms, reduced total chlorophyll content and photosynthetic gas exchange while increasing the percentage of the carotenoid precursor phytoene. These interesting results seem to prove that macrocidins act on the carotenoid biosynthesis by partially inhibiting the carotenoid biosynthetic enzyme phytoene desaturase (PDS) and on one or more other steps in carotenoid biogenesis [78].

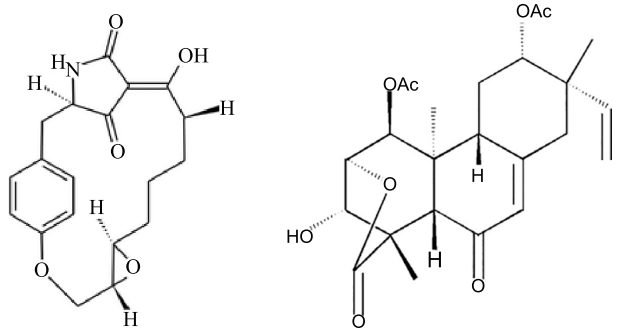

Fig. (4). Macrocidin A (left); chenopodolin (right).

A strain of the fungus *Ascochyta agropyrina* var. *nana*, a pathogen of the perennial weed *Elytrigia repens*, produced several toxins when grown in a liquid medium. Its main toxin, named agropyrenol (Fig. **1**), was characterized as a substituted salicylaldehyde, based on its chemical and spectroscopic properties.

When assayed on leaves of several weed plants, agropyrenol was phytotoxic, causing the appearance of necrotic lesions, interestingly not associated to antibiotic, fungicidal or zootoxic activities [38].

Chenopodolin (Fig. **4**), a new phytotoxic unrearranged ent-pimaradiene diterpene, was isolated from the liquid culture of *Phoma chenopodicola*, a fungal pathogen of *Chenopodium album*, a common worldwide weed of arable crops such as sugar beet and maize. At a concentration of 2 mg/mL, the toxin caused necrotic lesions on *Mercurialis annua*, *Cirsium arvense*, and *Setaria viride* [79]. The fungus produces also three tetrasubstituted furopyrans, named chenopodolans A–C (Fig. **1**) [46].

A new phytotoxic geranylcyclohexenetriol, named phomentrioloxin (Fig. **2**), was isolated from the culture of *Phomopsis* sp., a fungal pathogen proposed for the biological control of *Carthamus lanatus*, a widespread and troublesome thistle weed belonging to the Asteraceae family causing severe crop and pastures losses in Australia. The toxin was not specific, as at a concentration of 6.85 mM, it caused the appearance of necrotic spots on a number of plants. Its interesting phytotoxicity includes growth and chlorophyll content reduction of fronds of *Lemna minor* and inhibition of tomato rootlet elongation [44].

An aggressive isolate of *Ascochyta lentis* obtained from lentil *(Lens culinaris*) produced various metabolites *in vitro*. In particular, a new phytotoxic anthraquinone, named lentisone, was isolated and characterized. Although the mechanism of action is unknown, interestingly the toxicity of these compounds proved to be light-dependent [80].

These are only a few examples of potent phytotoxins that have interesting macroscopic phytotoxic effects with yet undefined MOAs. Interestingly, some of those compounds seem to have no off-target effects, and thus show potential as novel herbicides. Considering the already established wide range of MOAs not associated with commercial herbicides that are possessed by natural compounds, and the number of metabolites whose MOA is still unknown, one might expect that even more new MOAs have still to be discovered, and could be viable for a commercial herbicide MOA.

8. "OMICS" TOOLS

The study of biological entities at the systems level is becoming a very frequent approach in life sciences. For that, analytical tools that can identify the component parts of the system and measure their responses to a changing environment are necessary. A multitude of genomic, transcriptomic, proteomic, physionomics and metabolomic profiling technologies have been developed towards this end. The study of fungal toxins should be included into this trend. Omics methods could offer novel support in this respect. Considering that some of these technologies started to be developed and exploited only in recent years, they are far from being fully exploited to answer questions related to natural phytotoxins. Those methods can be applied, with different purposes, to both the producer fungus and to the target weed, allowing the discovery of the molecular targets of the phytotoxins, the understanding of their mechanisms of action, the identification of the pathway for their biosynthesis, or even the production of novel metabolites. Considering that excellent reviews on the possible approaches for understanding the phytotoxin MOAs have been recently published [81-83] and a rich literature is available in this and other research fields [84-86], only brief notes are made in the present section, whereas reference is made to those readings.

The increasing availability of fungal genome sequences demonstrates that their biosynthetic potential is still very far from being fully exploited. In fungi, the genes required for the biosynthesis of a secondary metabolite are clustered, and often they are silent under standard laboratory conditions (*e.g.* are activated only in the presence of the host). Consequently, many of these gene clusters can be discovered only bioinformatically, as no products can be found. Strategies have been successfully applied during the last years to activate such silent gene clusters in filamentous fungi and thus to allow the activation of unexpressed biosynthetic pathways and the production of novel metabolites [87]. Until now, the majority of successful approaches have been based on molecular biology like: (i) the generation of gene ''knock outs", as in the case of the discovery of emericellamides [88]; (ii) the promoter exchange, as for the production of asperfuranone [89]; (iii) overexpression of transcription factors or other pleiotropic regulators, as for the formation of the aspyridones A and B [90]. Moreover, epigenetic strategies opened new avenues for the elucidation of the regulation of secondary metabolite formation and will certainly continue to play a significant role in the elucidation of cryptic natural products [87].

Metabolomics can be used for the assessment of perturbations in biological systems. In the case of phytotoxins, this approach can allow the identification of metabolic changes in a treated weed compared to the

unperturbed ones [91]. During the last twenty years the development of robust, high-throughput analytical techniques, such as 1H nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) allows the simultaneous measurement of large numbers of metabolites from a single biological sample. This has catalyzed the development of metabolomics as a distinct and very active, multi-disciplinary research field. Even though modern instrumentation and software have reduced the cost and labor in omics studies, the amount and the complexity of data produced to be analyzed still represents a bottleneck for a wider application of the available tools [81].

9. STRUCTURE-ACTIVITY RELATIONSHIPS

Correlating the structure of a phytotoxic compound to its biological properties is of utmost importance in order to understand the active and essential part(s) of the moiety. This can represent the basis for: studying the possible synthesis of the active compound; use its structure as a lead for designing novel metabolites, having the same active sites associated to simplified and more easily synthesizable structures; hypothesizing possible chemical transformations, useful for increasing the bioactivity or the stability; predicting the fate and the stability in the environment; understanding biosynthetic pathways for their natural production.

Structure-activity relationship studies can be performed when some structurally closely-related metabolites are naturally available that are produced by one or more organisms, or by obtaining some laboratory derivatives, by chemically modifying one or more of the reactive sites of the compound.

For example, putaminoxin and pinolidoxin (Fig. **2**) have functional features at the nonenolide ring and the functional groups bonded to its skeleton as hydroxy, propyl and hexadienoyl residues. Therefore, a number of derivatives modified in these structural features were prepared, and a number of natural analogues of the two toxins were used to study structure-activity relationships.

Putaminoxin was the most toxic compound assayed on the host plant, annual fleabane (*Erigeron annuus*), as well as on some other weeds and crop plants by leaf-puncture assay, compared to putaminoxin analogues and derivatives. Any relatively small structural change in putaminoxin resulted in the complete loss of phytotoxicity, *e.g.* extension of the propyl side chain at C-9 to a pentyl side chain, saturation of the C-6/C-7 double bond, or esterification of the C-5-hydroxy group. On the basis of these results, the structural features of primary importance for the phytotoxic activity of putaminoxin were the presence and the stereochemistry of both an unchanged hydroxy group at C-5 and the propyl side chain at C-9. In addition, the functionalization and conformational freedom of the nonenolide ring plays an important role [92].

The primary important features of pinolidoxin were the presence of an unmodified diol system between C-7 and C-8, associated with the functionalization and the conformational freedom of the nonenolide ring. Conversely, the hexadienoyloxy residue at C-9 did not seem to affect activity.

Seven derivatives of phomentrioloxin were synthesized. This phytotoxin is produced by *Phomopsis* sp., a potential mycoherbicide proposed for the control of the annual weed *Carthamus lanatus*. They were assayed for phytotoxic, antimicrobial, and zootoxic activities, and the results provide insights into an investigation of the structural requirements for activity. The hydroxy groups at C-2 and C-4 were important features for the phytotoxicity, as well as an unchanged cyclohexentriol ring. Unsaturation of the geranyl side chain also contributed to the overall activity [93].

Weeds of the genera *Striga* (witchweeds) and *Orobanche*/*Phelipanche* (broomrapes) are unique parasitic weeds. The seeds of these parasitic weeds only germinate in response to specific compounds, *i.e.* germination stimulants, present in the rhizosphere of host plants and of some non-host plants as well. The germinated seed attaches itself to the host root, connects with its vascular system, extracts nutrients and water. This parasitic process has an enormous impact on the growth of the host plant, and the ultimate consequence is a substantially lower crop yield. After the identification of first naturally occurring germination stimulant [94], a number of other stimulants were discovered and, having some common chemical features, were collectively named strigolactones (Fig. **5**). Besides other interesting biological characteristics, these compounds were and are still intensively studied as natural herbicides, to induce the so-called "suicidal germination" (*i.e.* germination without the presence of a suitable host) in order to reduce the soil seed-bank of parasitic weeds [95].

The identification of the active part of the molecule responsible for the biological activity is essential for understanding the molecular interaction of stimulant molecules with the protein receptor in the seed of the weed. This bioactiphore was studied by systematically simplifying the strigolactone skeleton. Moreover, several A and A/B analogues of strigol lacking the D-ring

and the ABC enol ether were also prepared and were biologically inactive in inducing germination. From these structure–activity data, it was concluded that the bioactiphore of strigolactones resides in the CD part of the molecule. The methyl substituent in the D-ring at C-4 is essential for bioactivity, and thus the D-ring in strigolactones must be retained [96]. Indeed, all natural strigolactones isolated so far contain this key CD structure unit. These studies are thus essential also for the possible design of synthetic compounds, having simpler structures compared to natural strigolactones, but retaining their herbicidal properties [97].

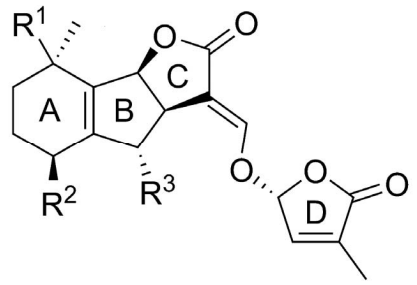

Fig. (5). General structure of strigolactones.

10. PRODUCTION/FERMENTATION PROCESSES

Despite the large number of fungal phytotoxins identified, there are several technological, biotechnological, and physiological factors that hamper further research or industrial development. Strategies to overcome these limiting factors and allow fungi to express their "real" metabolic potential, probably in some cases far superior to that of bacteria and plants, are currently being only partially exploited. Procedures for obtaining or producing compounds of botanic or bacterial origins are easier compared to fungal fermentation, as plants can be easily grown and harvested as crops, and bacteria can be grown more easily than fungi in bioreactors by liquid shaken fermentation. Thus, the amount of a metabolite produced by a fungus and its cost of production could represent real constraints. Indeed, promising metabolites are often produced by fungi in laboratory conditions in amounts sufficient only for the preliminary bioassays. For example, phomentrioloxin (Fig. **2**) was produced by *Phomopsis* sp. at only 10 mg/L of culture filtrate [44], whereas drazepinone, another original phytotoxin isolated from *Drechslera siccans* cultures, can be found in amounts as low as 2.4 mg/L [43].

Many fungal metabolites are obtained in the laboratory by using basic and traditional mycological methods, consisting in axenically growing the fungus on liquid media having a mineral defined composition, in order to more easily isolate and identify the metabolites produced by the fungi. In other cases, fungi are grown in static conditions, or even on solid substrates. However, many microorganisms are very difficult to grow *in vitro*, or are still considered uncultivable. The successful cultivation of a new fungal strain is often sufficient to identify new natural products, as in the case of the finding of deoxypodophyllotoxin, a pro-drug of podophyllotoxin, in the endophytic fungus *Aspergillus fumigatus* [98].

A limited attention has been given to the production aspects, and only a limited number of secondary metabolites have been really investigated for a possible scale-up in bioreactors [99]. The most used bioreactors are liquid shaken, unfortunately often not suitable for the highly branched fungal mycelia, that can cause problems due to the formation of mycelial clumps and pellets in the fermentor. This not only limits mass transfer, but also increases the broth viscosity, reduces oxygen transfer, and causes difficulty in mixing. In order to overcome these inconveniences, cell immobilization methods have been employed to manage cell morphology and achieve higher cell density and productivity in several filamentous fungal fermentations. Different bioreactor strategies have been developed to overcome those problems and increase metabolite production. One is the semi-solid fermentation, in which mycelia can grow on the surface and in the interior of a solid substrate in the absence of free water; another is the formation of a biofilm, obtained by the development of mycelium on an inert support, such as stainless steel, glass or Teflon material integrated in an adapted reactor. Considering that the excretion capacity is increased when fungal biomass is attached on a given support, these two latter strategies can be more suitable for the production of fungal metabolites of biotechnological interest in higher amounts[100].

Another critical element is the manipulation of nutritional and environmental factors promoting secondary metabolite biosynthesis. Indeed, little changes in the cultural conditions or in the nutrient source can have a strong impact on both the quantity and nature of fermentation products. The OSMAC (one strain, many compounds) approach is the strategy consisting in simple modifications of the cultural parameters in order to augment the metabolic diversity of a strain [101].

Thanks to the advent of new technologies, the availability of high-throughput bioassays and of novel analytical methodologies it is now possible to create un-

usual environmental and nutritional conditions and thus metabolite production can be modified in an almost unlimited and unpredictable manner. Changes in temperature regimes, light exposure, amount and quality of nutrients available, agitation conditions, atmosphere composition, can have a strong impact on the ability of fungi to quantitatively and qualitatively produce secondary metabolites [102].

11. BIOTRANSFORMATION

Considering that microorganisms co-inhabit a wide variety of environments and interact each other in their natural environments, and considering that metagenomes isolated from environments such as soil are rich sources for novel natural products, close interactions of microorganisms would constitute a driving force for the production of secondary metabolites. Thus, simulation of these habitats *in vitro*, *e.g.* by culturing two or more strains together, should be a rational way to exploit the metabolic potential of cultivatable microbes (mixed fermentation or biotransformation). The presence of neighboring microbes or the use of microbial metabolites as nutrients for other microbes can result in: (a) production of extracts with enhanced biological activity; (b) production of higher amounts of known or of novel metabolites; (c) biosynthesis of new analogues of known metabolites due to a combination of pathways; (d) novel products obtained by biotransformation and induction of previously unexpressed pathways for bioactive constituents [103, 104].

For example, *Heterobasidion* spp. produce many secondary metabolites having different activities, such as fomannosin, fomajorin S and fomannoxin. This latter is a phytotoxic dihydrobenzofuran metabolite that has been successfully isolated from naturally infected wood. When fomannoxin was added to cultures of rhizosphere-associated *Streptomyces*, the compound disappeared and three novel fomannoxin derivatives without phytotoxic activity were detected, together with two fomannoxin amide and fomannoxin acid, which conversely retained toxicity [104].

Fungal metabolism can also be used as a method of bioconversion of important natural metabolites which do not have a fungal origin [105]. As an example, enzymes from mushrooms are commercially developed for bioconversion of natural metabolites into bioactive products. Several studies have shown the possibility of producing optimised derivatives of plant-derived components obtained by fungal fermentation [106].

Another interesting biosynthetic process involving a fungus and a bacterium is that leading to the production of the phytotoxin rhizoxin. Indeed, in this case *Burkholderia rhizoxinica*, a bacterial endosymbiont of the rice pathogenic fungus *Rhizopus microsporus* produces the rhizoxin backbone that is then modified by an oxygenase produced by the fungus. The modified compound is more phytotoxic than the initial bacterial product [107].

Although trichothecenes are very stable mycotoxins, several types of microbial bioconversions have been reported, among which oxygenation, acetylation, deacetylation and oxidation. Some of these transformations cause complete loss (*e.g.* deepoxidation) or reduction (*e.g.* acetylation, oxidation) of toxicity [108]. Further supporting the possibility to modify the activity of fungal metabolites by microbial biotransformations.

12. SYNTHESIS

The inability of microorganisms to produce large amounts of a toxin or the high costs of purification represent potential constraints to their practical use as natural herbicides. Their chemical syntheses or the chemical synthesis of the active moiety could overcome those limitations. Unfortunately, the natural compounds often have very unusual or too complex structures, and sometimes only very partial structures can be achieved by synthesis. However, the identification of the active sites of a moiety could allow the synthesis of some structurally simpler compounds than the natural ones, but retaining their effectiveness.

For example, cinnacidin is a phytotoxin obtained by the fermentation extracts of the fungus *Nectria* sp. It contains a cyclopentalenone ring system having an isoleucine subunit linked through an amide bond. It proved to have promising herbicidal activity and, in order understand the possible action site, two synthetic analogues were prepared, comparing their herbicidal activities with that of cinnacidin. The synthetic compounds were highly phytotoxic on several weeds and it was hypothesized to have a mode of action similar to that of coronatine and jasmonic acid, on the basis of the symptom appearance. However, coronatine proved to be more active against warm-season grasses, whereas cinnacidin benzyl ester was more effective on cool-season grasses. Conversely, the activities of the two compounds were equivalent in a seedling growth bioassay conducted on bent grass [76].

The structural features of phomentrioloxin [44] (Fig. **2**), associated to the variations in biological activity as a function of structural modification [93], suggested that phomentrioloxin could be a useful lead for the development of novel and environmentally friendly

herbicides. For this purpose, the enantiomeric form of phomentrioloxin was synthesized in seven steps, using by starting material the homochiral *cis*-1,2- dihydrocatechol, a compound obtained in enantiomerically pure and stereochemically well-defined form. These studies also allowed to assign the correct stereostructure to phomentrioloxin [109].

The nonenolides herbarumin I and II were obtained from the culture broth of the fungus *Phoma herbarum,* thank to the bioassay guided fractionation. These lactones were significantly phytotoxic effects on germination and growth of *Amaranthus hypochondriacus* seedlings. These compounds are structurally related to pinolidoxin (Fig. **2**), a metabolite isolated from the phytopathogenic fungus *Ascochyta pinodes*, which proved to be a potent inhibitor of induced phenylalanine ammonia lyase (PAL) activity [110], an enzyme having a key role in the phenylpropanoid defense metabolism of higher plants. Compounds able to interfere with plant self-defense represent highly promising lead structures in the search for novel herbicidal agents. Considering that the stereochemistry of the three contiguous chiral centers of herbarumin I can be matched by the pattern displayed by d-ribose, the d-ribonolactone acetonide derivative was chosen as a readily accessible starting material which could be converted on a multigram scale into tosylate. The total synthesis of the potent phytotoxic 10-membered lactones (putaminoxin, herbarumin I and II) was achieved in number of successive steps. A sound basis was set out for further studies on the structure/activity profile of this promising family of lead compounds [111].

(+)-Phenguignardic acid, a phytotoxic metabolite of the grape black rot fungus *Guignardia bidwellii* and its enantiomer were synthesized in eight steps from (R)-phenyl- lactic acid and 3-phenylprop-2-yn-1-ol. The formation of the carboxylate at a late stage was crucial for the synthesis in order to prevent enolization of the precursor used in the central acetalization step. The assignment of its hitherto unknown absolute configuration was also obtained [112].

13. NOVEL TECHNOLOGIES

Novel delivery systems (*e.g.* the so-called drones), nanosensors, nanomaterials (*e.g.* nanoparticles), are receiving increasing attention for the possible applications in agriculture [113]. Such technologies could be used to facilitate the application and distribution of the herbicides, but could also be specifically developed to overpass some of the limiting aspects hampering the practical application of phytotoxins. A few examples are discussed.

Nanoformulations allow the controlled release of the metabolites, either regulated by time, location or triggered under certain circumstances, making this approach very attractive for future developments. This could be done using several nanodevices based on different basic frames such as nanoparticles, nanocapsules, nanoclays, liposomes [114]. The toxin could be attached to, or loaded inside a nanocarrier, which protect the first one against degradation. Additionally, specific ligands could be added to the nanocarrier aiming for specific targets and/or allowing release of the active compound under certain conditions. For example, most herbicide treatments against parasitic plants involve systemic application of the pesticide through the host crop, in order to reach the parasite using the vascular system [115]. A common problem in this case is phytotoxicity against the crop and detoxification of the active compound, damaging the crop in one side and reducing efficiency of the agrochemical on the other. The nanocarrier would protect the toxin against detoxification and avoid damage to the host crop. Once the nanocarrier reaches the parasitic plant through the haustorium, the chemical load would be magically released and the toxin could act specifically against the parasite, reducing the necessary effective dose.

For toxins acting as root inhibitors, nanoformulations could also improve soil applications. For instance, the metabolite could be applied encapsulated in nanoparticles providing a slow and constant release during the crop season [116]. This would allow good control with just a single application, reducing rates, costs, and environmental risks.

Additionally, nanoencapsulation could allow the joint application of several substances, preventing interactions between them until they are released. For example, different toxins/herbicides with different modes of action could be encapsulated separately and applied at the same time, thus improving the synergistic effects against the weed, or reducing the risk of selection of organisms resistant to the active compounds. Even more, the use of nanocarriers can improve the solubilisation of active compounds, enabling the use of metabolites that, due to their physico-chemical properties, would not be applicable in the fields, solving problems such as a too fast degradation, or a too low solubilisation.

14. ADVANTAGES AND DISADVANTAGES

Utilizing fungal metabolites for the discovery of new herbicides offers a number of advantages, but it is far from being a panacea. Conversely, there is a num-

ber of aspects that could limit using such compounds for large-scale weed management.

One of the indirect and important benefits of the chemical composition and structural characteristics of natural products (*e.g.* the absence of 'unnatural' ring structures and the low amount of 'heavy' atoms) is that most of these compounds are expected to be rapidly degraded after application, thus having a limited or no impact, including on weeds. From an "environmental" standpoint this can be an element favoring the use of SMs, with a better public acceptance. Conversely, from an industrial and practical standpoint this can be detrimental, because using an excessively fast-degrading natural herbicide several application could be necessary in order to assure a sufficient level of weed control.

It could be expected that SMs, being of natural origin, could be toxicologically safer. This is not always true, because some of the worst metabolites coming in contacts with the food chain are mycotoxins produced by some plant pathogenic fungi, and thus safety concerns would certainly arise in case of registration of a fungal metabolite for herbicidal purposes. Some of the phytotoxins studied for novel MOAs possess adverse effects toward not target organisms (*e.g.* mammalian toxicity), too, or are carcinogenic, and thus they can hardly afford the registration procedures.

Novel structures with uncommon molecular target sites could be found for SMs, and this would be certainly a positive aspect. However, the production of those compounds could be too low when obtained by natural processes (fermentation) and the structures could be too complex to be obtained by chemical synthesis, and thus be not suitable for an industrial production.

The availability of novel improved instrumentation makes screening, testing and identifying active compounds easier than the past, and requires smaller amounts of the compounds. This would allow the re-evaluation of metabolites and producers already considered in the past. If from a research point of view would be extremely encouraging, because the dereplication tools circumvent what was once the costly and time-consuming process of purifying and isolating previously known molecules, from a practical point of view it would be detrimental, because a patent protection of already known compounds could be very limited, and thus not attractive for companies.

Beside the lower environmental impact of natural herbicides compared to the synthetic ones, it could be expected a low-rate use of phytotoxins as herbicides in the field. This is not necessarily true, because those compounds could be very effective when produced *in planta*, but much less potent when applied exogenously. Indeed, SM structures may be optimized by nature for the biological activity but their physico-chemical properties may be less than ideal for uptake into and translocation in plants to produce an adequate effect at an economical dose. Thus, higher doses could be necessary for a phytotoxin in order to be effective.

Finally, the registration process of novel herbicides from fungal origin could be as long and expensive as the synthetic ones, and thus be not so attractive for companies. Indeed, from one side the registration process could be simplified and less expensive under the category of the biopesticides, as it could be possible for the USA. At the European level, the regulatory procedure for their inclusion in the legislation would be that defined by Regulation (EC) No. 1107/200949 concerning the placing on the market of Plant Protection Products (PPPs), which defines the rules for the assessment and the authorisation of active substances, safeners and synergists, adjuvants and coformulants. Safety of each active substance is evaluated before it can be placed in the market within a PPP. Indeed, lack of harmful effects on human and animal health, and of unacceptable effects on the environment and non-target species should be proved for active substances and their residues in food, besides general or specific beneficial effects against organisms harmful for plants (including other plants). Registration and authorization of PPPs use have long and laborious procedures, requiring the support of many experts; furthermore, they are also very expensive because many information must be provided by the producer to the competent authorities for the evaluation and the successive authorization of the product use. Depending on the type and complexity of the products, the whole procedure can cost from a few hundreds of thousands of euros to a few millions of euros.

CONCLUSION

The "World" of the phytotoxins produced by plant pathogens, and their possible use for herbicidal purposes is still far to be fully explored. The availability of novel research tools, combined with a renewed interest of the agro-chemical companies for the natural products, and an increased attention of the public opinion toward health and environmental protection, offer a unique opportunity to invest resources in this interesting research and applicative field. Combined supports from the private and public sectors would certainly

speed up the chances of success. The discovery and the introduction on the market of a fungal-produced herbicide, having a new MOA, and possessing low environmental impact, would definitively be a breath of fresh air for this research field.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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