Review

The past, present and future of secondary metabolite research in the Dothideomycetes

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SUMMARY

The Dothideomycetes represents a large and diverse array of fungi in which prominent plant pathogens are over-represented. Species within the *Cochliobolus*, *Alternaria*, *Pyrenophora* and *Mycosphaerella* (amongst others) all cause diseases that threaten food security in many parts of the world. Significant progress has been made over the past decade in understanding how some of these pathogens cause disease at a molecular level. It is reasonable to suggest that much of this progress can be attributed to the increased availability of genome sequences. However, together with revealing mechanisms of pathogenicity, these genome sequences have also highlighted the capacity of the Dothideomycetes to produce an extensive array of secondary metabolites, far greater than originally thought. Indeed, it is now clear that we appear to have only scratched the surface to date in terms of the identification of secondary metabolites produced by these fungi. In the first half of this review, we examine the current status of secondary metabolite research in the Dothideomycetes and highlight the diversity of the molecules discovered thus far, in terms of both structure and biological activity. In the second part of this review, we survey the emerging techniques and technologies that will be required to shed light on the vast array of secondary metabolite potential that is encoded within these genomes. Experimental design, analytical chemistry and synthetic biology are all discussed in the context of how they will contribute to this field.

Keywords: Dothideomycetes, plant pathogens, secondary metabolites.

INTRODUCTION

Plants and fungi have shared a long evolutionary history. There is evidence that plants first colonized the surface of the Earth by establishing symbiotic associations with fungi (Heckman *et al.*, 2001; Humphreys *et al.*, 2010). Over time, evolution has shaped and diversified the fungal–plant interaction into different forms:

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mycorrhizic, endophytic, saprophytic and pathogenic. Renowned by their plant-associated members, the Dothideomycetes is the largest class of fungi within the biggest phylum, the Ascomycota (Kirk *et al.*, 2008). The Dothideomycetes is a very heterogeneous class, occupying the most varied habitats and niches: from the Arctic to the tropics, over rocks facing the inclemency of weather (Ruibal *et al.*, 2009), colonizing dung (Kruys *et al.*, 2006), as lichenized symbionts (Nelsen *et al.*, 2011) or living under water as either marine or freshwater organisms (Shearer *et al.*, 2009; Suetrong *et al.*, 2009). One notable feature of the Dothideomycete class is the over-representation of devastating plant pathogens (Arnold, 2007; Schoch *et al.*, 2009).

Members of the Dothideomycetes cause disease in every major crop (Ohm *et al.*, 2012). Species from *Alternaria*, *Botryosphaeria*, *Cochliobolus*, *Mycosphaerella*, *Phaeosphaeria* and *Pyrenophora* affect the production of cereal crops, the base of human diet. *Didymella* is responsible for many major legume diseases, and *Leptosphaeria* is devastating in Brassicas. Consequently, members of the Dothidiomycetes represent a primary food biosecurity risk, through either crop yield losses or product quality issues caused by mycotoxin contaminations (Solomon, 2011).

Fungi infect plants using different strategies: biotrophs exploit plants' resources, whilst keeping the host alive; necrotrophs kill the host in order to thrive off dead or dying tissue; and hemibiotrophs have an asymptomatic phase followed by a necrotrophic stage (Horbach *et al.*, 2011). Despite their lifestyle, a common mechanism for many plant-infecting fungi is the production of effectors. Typically, these effectors interact with a specific host target, triggering a response that may lead to either the suppression or development of disease (Wolpert *et al.*, 2002). For example, ToxA, a small secreted protein from *Parastagonospora nodorum* and *Pyrenophora tritici-repentis*, is a classic example of an effector involved in an inverse gene-for-gene interaction (Ciuffetti *et al.*, 2010; Friesen *et al.*, 2006). When both *ToxA* and its host susceptibility gene *Tsn1* are dominant, disease ensues. However, if either of these genes is missing, no susceptibility is observed (Oliver and Solomon, 2010). Although, in the above example, the term 'effector' refers to small secreted proteins, some definitions of plant–microbe effectors have been expanded. These expanded definitions consider all substances secreted into the plant by microorganisms (e.g. proteins, small molecules, small

RNAs) which cause an effect on the host cell (Hogenhout *et al*., 2009). In consequence, this would therefore include some of the microbial secondary metabolites (SMs) (Collemare and Lebrun, 2011).

SMs, also known as natural products, have fascinated researchers for many decades. They are often small molecules that do not play an obvious role in the basic cellular functions, but have bio-ecological roles in helping the producing organism adapt to its environmental niche (O'Brien and Wright, 2011; Williams *et al*., 1989). Studies of fungal SMs have traditionally been focused on two main areas: pharmaceutical and agronomical (Hanson, 2008). From ancient times, fungi have been recognized for their medicinal and toxicological properties. When Fleming discovered penicillin, the interest in fungal SMs as a source of drugs increased significantly (Aly *et al*., 2011). Partly driven by the invigorated pharmaceutical interests, agricultural researchers began looking for SMs involved in pathogenicity and mycotoxicosis, as well as new sources of agrochemicals. These SM studies have not only demonstrated that fungal SMs possess remarkable biological activities which can be harnessed for the benefit of humankind, they have also driven important developments in analytical, synthetic and medicinal chemistry (Nakanishi, 1999). However, there remains much to be learnt, especially with regard to the biological and ecological roles of SMs in fungal adaptation and survival.

We are only beginning to understand how fungal SMs work in plant pathosystems. For example, some phytotoxic compounds produced by plant-pathogenic fungi can act as plant-damaging photosensitizers, target host proteins to disrupt their function, affect the membrane or trigger apoptosis in plant cells (Möbius and Hertweck, 2009). Some of these are host-specific toxins (HSTs), whereas others have activities against a broader range of organisms. Other roles of SMs from plant pathogens include mineral uptake (e.g. siderophores), protection against biotic and abiotic factors, mediation of interspecific interactions and even manipulation of the genetic regulation of the hosts (Scharf *et al*., 2014). However, regardless of the identified *in vivo* roles, SMs can exert physiological effects in different organisms. For example, SMs with a negative effect on humans and other higher animals, independent of their role in fungal biology, are called mycotoxins (Bennett and Klich, 2003; Reverberi *et al*., 2010).

Fungal SMs exhibit considerable structural diversity, but can be broadly grouped into four main categories based on their biosynthetic origin: polyketides, non-ribosomal peptides (NRPs), terpenoids and tryptophan derivatives. These are made by the core enzymes polyketide synthases (PKSs), non-ribosomal peptide synthases (NRPSs), prenyltransferases (PTs)/terpene synthases (TSs) and dimethylallyl tryptophan synthases (DMATSs) (Wiemann and Keller, 2014). The conserved sequence features, within the respective family of core enzymes, can be exploited to allow the rapid identification of genes encoding PKSs, NRPSs, PTs, TSs and DMATSs within fungal genomes. The fact that genes involved in fungal SM biosynthesis are often clustered together on the chromosomes, either to simplify regulation and horizontal gene transfer or to prevent the accumulation of toxic metabolic intermediaries, further facilitates the identification of these enzymes (Keller *et al*., 2005; McGary *et al*., 2013). From the number of fungal genomes that have been sequenced so far, it has been observed that the Dothideomycete fungi encode a remarkable number of SM gene clusters, suggesting their immense potential at producing vast chemical diversity. However, although the genetic template for this diversity is identifiable, the number of SM compounds known in each of the sequenced fungi remains limited. It is clear that what has been discovered to date is just a small portion of the fungal SM diversity.

The modern genomic era is transforming natural product research into an interdisciplinary area in which vastly different approaches are now being used to tackle the many facets of SMs from their evolutionary origins, genetic regulations and biosynthesis to their natural bio-ecological functions. As Stadler and Keller (2008) wrote: there are new paradigms in fungal SMs. This review is an opportunity to survey the path we have walked and to consider where we are heading. The first half of this article presents an overview of what is known about Dothideomycete SMs in terms of compound diversity, biosynthesis and bioactivities. The second part reviews some of the new technologies for the study of SMs, with a focus on their application in the characterization and understanding of SMs and their roles from an ecological perspective (e.g. their role in plant–microbe or microbe–microbe interactions)

PATHOGENIC DOTHIDEOMYCETES AND THEIR SMs

The Dothideomycetes is a class so vast that it is not the aim of this review to describe each genera or to list all the known SMs. In this section, we summarize some particular examples within the Dothideomycetes with the aim of not only presenting what we currently understand, but, more importantly, highlighting what we do not.

Cochliobolus

Cochliobolus (teleomorph of *Bipolaris* and *Curvularia*) is a genus that contains a large number of devastating necrotrophic plant pathogens which are reliant on SMs to cause disease. Figure 1 shows examples of these SMs. Some of these compounds have been identified as HSTs and their biosynthetic genes and mode of action have been investigated and reviewed (Panaccione, 1993; Stergiopoulos *et al*., 2013). The three iconic HSTs from *Cochliobolus* are T-toxin, HC-toxin and victorin.

The polyketide T-toxin is a virulence determinant from the maize pathogen *Cochliobolus heterostrophus* race T and is encoded by a
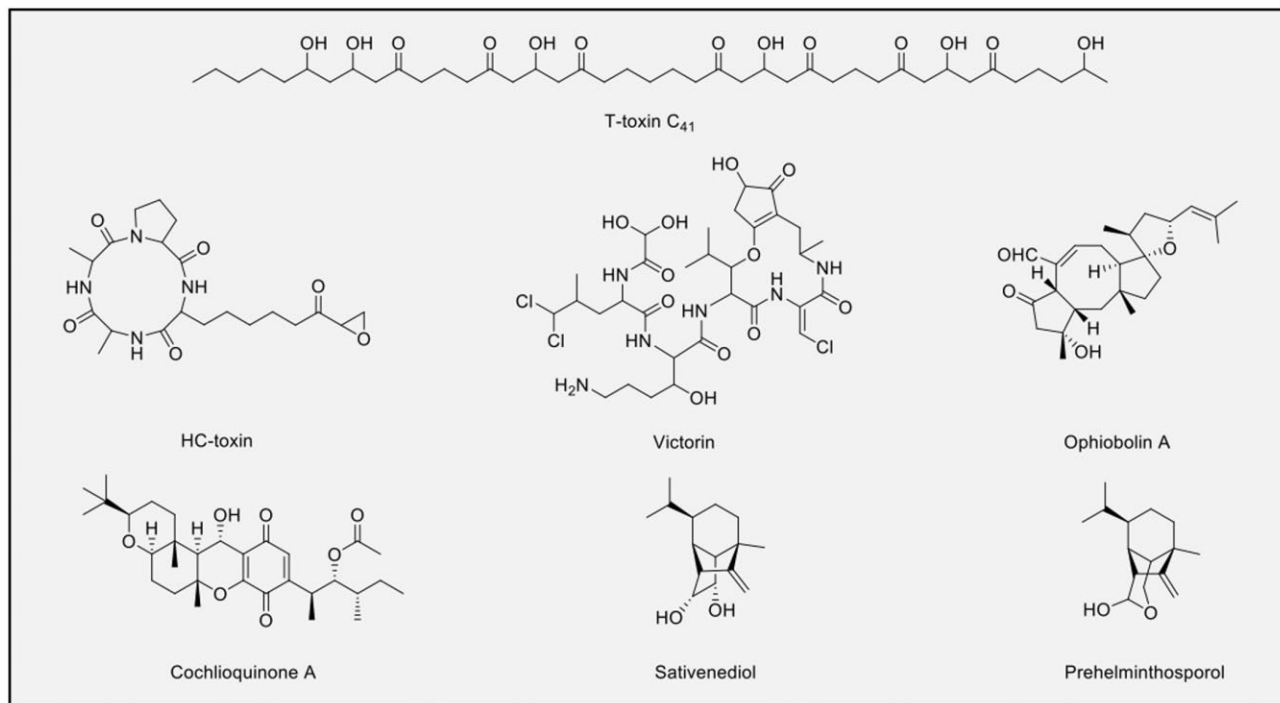
Fig. 1 Secondary metabolites isolated from *Cochliobolus*.

total of nine genes (Inderbitzin *et al*., 2010). Two of these are PKS genes located on two unlinked loci. T-toxin triggers apoptosis when it interacts with a receptor, URF13, which is located on the inner mitochondrial membrane, resulting in small-molecule leakage and necrotizing tissue (Inderbitzin *et al*., 2010). HC-toxin is a cyclic NRP from the maize pathogen, *Cochliobolus carbonarum* race 1. This toxin is produced by the core NRPS, HST1, and other enzymes encoded in the *TOX2* locus. HC-toxin is an inhibitor of histone deacetylase that causes disease in maize lines which lack the carbonyl reductase enzymes, *HM1/HM2*, required for the detoxification of this compound (Walton, 2006). Victorin is an NRP from *Cochliobolus victoriae*, the causal agent of Victoria blight in oats. Although the genome of *C. victoriae* has been sequenced, the NRPS gene that is responsible for the production of victorin remains to be identified (Condon *et al*., 2013). Victorin elicits plant cell death in oat lines containing the resistance gene Pc-2 against *Puccinia coronata*, allowing *C. victoriae* (a necrotroph) to proliferate (Lorang *et al*., 2007). Victorin also affects *Arabidopsis thaliana*, but the susceptibility of this host is caused by a different 'resistance' gene, LOV1, which encodes a protein containing a nucleotide-binding domain and leucine-rich repeat domain (NB-LRR). This NB-LRR protein is activated when victorin binds to a thioredoxin, TRX-h5. Using *Arabidopsis* and virus-induced gene silencing (VIGS), it was shown that the susceptibility response to this chemical toxin from *C. victoriae* is equivalent to the classical resistance response against proteinaceous effectors from biotrophs (Gilbert and Wolpert, 2013).

In addition, *Cochliobolus* produces non-HSTs, such as ophiobolins, a large family of more than 20 phytotoxic sesterterpenoids with a characteristic structure of a 5-8-5 ring. Originally isolated from *Cochliobolus miyabeanus* (Tsuda *et al*., 1967), ophiobolins have also been reported in other *Cochliobolus* species (Strobel *et al*., 1988), and also in *Aspergillus* and *Cephalosporium* (Cutler *et al*., 1984; Itai *et al*., 1967). Ophiobolins are active against plants, microorganisms and animals, affecting different cellular processes (Au *et al*., 2000). Ophiobolin A targets the calcium receptor, calmodulin, causing ion leakage in corn roots (Leung *et al*., 1984, 1985), and also inhibits β-1,3-glucan synthase, affecting oomycete cell wall formation (Fukushima *et al*., 1993). Recently, the gene involved in ophiobolin F biosynthesis in *Aspergillus clavatus* has been reported, *ACLA_076850*; it is the first discovered sesterterpene synthase (Chiba *et al*., 2013). The identification of this ophiobolin gene will facilitate the discovery of homologues in *Cochliobolus* and other organisms. In addition, this could influence not only basic research, but also drug discovery, as the ophiobolin structure has been proposed as a promising anticancer template (Bury *et al*., 2013).

Other noteworthy compounds from this genus are cochlioquinones from *C. miyabeanus*, *Bipolaris cynodontis*, *Bipolaris bicolor*, *Bipolaris brizae* and an endophytic *Cochliobolus* sp. These phytotoxins are hybrid compounds incorporating a *p*-benzoquinone ring and an isoprenoid moiety (Carruthers *et al*., 1971). Cochlioquinones have been shown to inhibit the root growth

of finger millet, rice and Italian rye grass, and also to affect complex I in electron transport in mitochondria by interfering with NADH-ubiquinone reductase (Lim *et al*., 1996, 1998; Miyagawa *et al*., 1994). In addition, these SMs exhibit activities with medical relevance against cancer (Machida *et al*., 1995), human immunodeficiency virus (HIV) (Yoganathan *et al*., 2004) and *Leishmania* (Campos *et al*., 2008). Other important SMs are the sativenediol- and helminthosporol-type compounds from the pathogenic *Cochliobolus sativus* (Nukina *et al*., 1975; Tamura *et al*., 1963). Unlike the cochlioquinones, these SMs promote seedling elongation in rice and their activity has been compared with the gibberellins (Kato *et al*., 1964). Structurally, the sativenediols, helminthosporins and gibberellins share a bicyclo(3.2.1)octane moiety with the same stereochemistry. Hence, it can be hypothesized that the growth-promoting activity of these compounds is conferred by the bicyclo group (Briggs, 1966; Coombe *et al*., 1974).

The *Cochliobolus* genus has one of the highest representations amongst the Dothideomycete pathogens that have publicly available genomic sequences, including *C. carbonarum*, *C. heterostrophus*, *Cochliobolus lunatus*, *C. miyabeanus*, *C. sativus*, *C. victoriae* and *Curvularia protuberata*. This therefore provides an excellent opportunity for comparative genomic studies. A recent *Cochliobolus* comparative genomic study has shown that around 50% of the NRPS genes and 30% of the PKS genes are highly conserved among the first six species aforementioned (Condon *et al*., 2013). However, some of these enzymes are unique at a species level, making them good candidates for specific virulence factors. As it stands, most of the products of these genes are yet to be characterized (Condon *et al*., 2013).

Alternaria

Alternaria is a wide group of anamorphic fungi, mainly from the *Lewia* genus. Many of these are described as saprobes, but plant pathogens are also well represented in *Alternaria* (Thomma, 2003). *Alternaria* species are known for their prolific production of chemical structures (Fig. 2). The production of mycotoxins by this genus has long been studied. For instance, alternariol (AOH) was isolated and characterized in 1953 (Raistrick *et al*., 1953) and its biosynthetic origin was determined to be derived from a heptaketide chain by feeding ^{14}C-labelled acetate to *Alternaria alternata* (Thomas, 1961). Recently, an *Al. alternata* genome-wide PKS expression analysis has shown that two PKS genes, *pksH* and *pksJ*, are expressed at the same time as AOH and alternariol methylether (AME) production. Down-regulation of *pksJ* reduced the amount of AOH and AME produced, suggesting that *pksJ* is responsible for the biosynthesis of these polyketides (Saha *et al*., 2012). However, as *pksJ* encodes a highly reducing PKS that usually produces non-aromatic compounds, it is surprising that it plays a role in AOH synthesis.
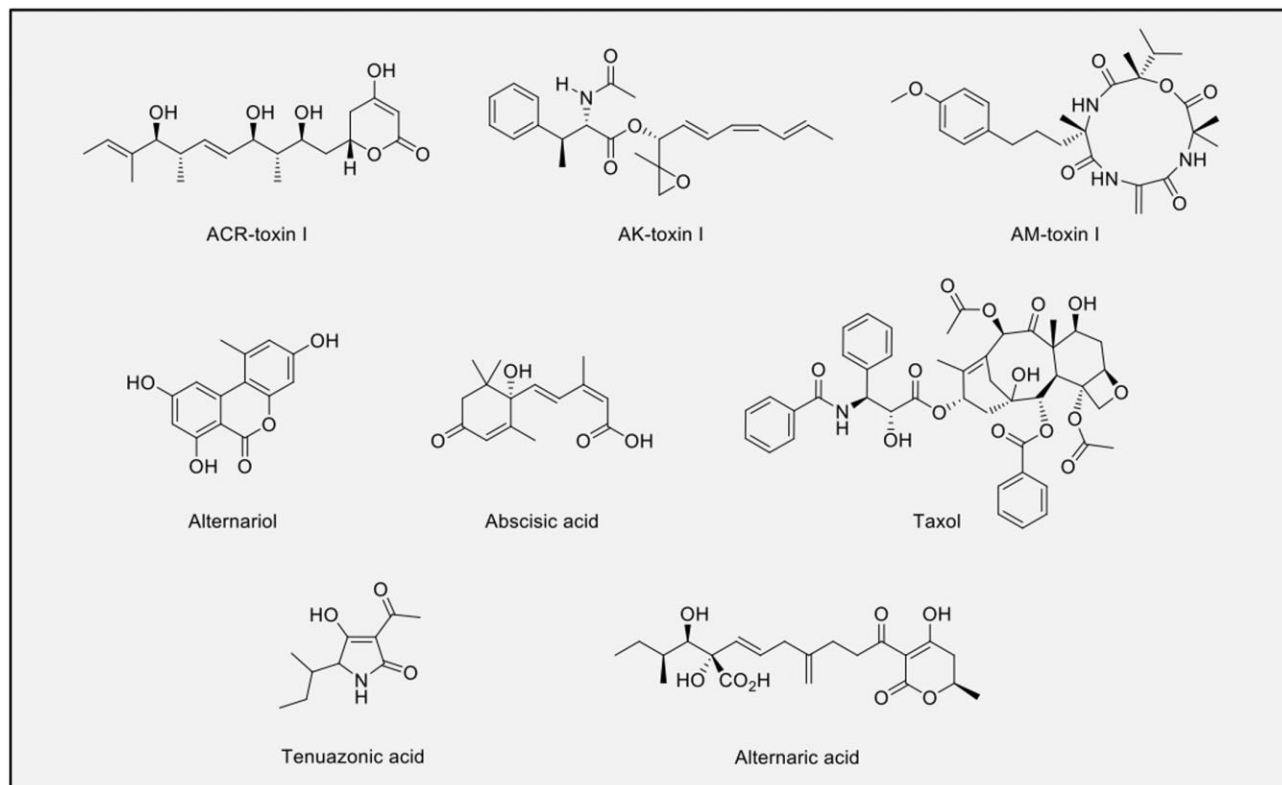

Fig. 2 Secondary metabolites isolated from *Alternaria*.

Table 1 Type and number of secondary metabolites (SMs) from *Alternaria* species reviewed by Lou *et al*. (2013).

Alternaria SMs by chemical groups	
Alkaloids	61
Cyclopeptides	22
Steroids	7
Terpenoids	49
Pyranones	56
Quinones	45
Phenolics	38
Miscellaneous	12
Total	**268**

As demonstrated for *Cochliobolus*, HSTs form part of the SM repertoire of *Alternaria* species. For example, *Al. alternata* pathotypes have specific toxins depending on their host, such as ACR toxins for citrus, AK toxins for Japanese pear, AM toxins for apples and pears and AT toxin for tobacco. For most of these toxins, the structures, effect, biological targets and biosynthetic genes have been described in two recent reviews (Akimitsu *et al*., 2013; Tsuge *et al*., 2013). However, the structure of some compounds, such as AT toxin, have not been elucidated.

Recently, Lou *et al*. (2013) have published a review with 268 SMs reported from *Alternaria* species (summarized in Table 1). Bioassays have identified phytotoxic and antimicrobial activities for some of these compounds. In addition, cytotoxic/antitumour effects have also been reported frequently, and specific bioactivities have arisen from targeted SM screenings (Lou *et al*., 2013). Included on this list are abscisic acid (a plant hormone involved in developmental processes), Taxol or Paclitaxel (the anticancer drug discovered in the bark of *Taxus brevifolia*) and tenuazonic acid (a mycotoxin with wide-ranging activities, including antibiotic, phytotoxic, antitumour and antiviral properties). However, despite the many reports on *Alternaria* SMs, the functions for most of these compounds remain unknown.

Mycosphaerella

Mycosphaerella contains several anamorphic genera known to be economically important plant pathogens, including *Septoria* and the well-known producer of SMs *Cercospora* (Crous, 2009). Some *Mycosphaerella* SMs are shown in Fig. 3. Probably the most renowned SM of this genus is the perylenequinone compound cercosporin, a non-specific light-activated phytotoxin first reported from *Cercospora kikuchii* (Kuyama and Tamura, 1957). A role for cercosporin in plant pathogenesis has been shown by using cercosporin-deficient mutants (Upchurch *et al*., 1991). However, non-pathogenic fungi also produce these perylenequinones which are likely to have other roles, including non-plant-associated interactions (e.g. microbe–microbe)(Daub and Chung, 2009; Daub and Ehrenshaft, 2000).

Another interesting class of SMs in the *Mycosphaerella* are the beticolins (cebetins). These are a family of 20 polyketide-non-specific toxins produced by *Cercospora beticola*, a pathogen of sugar beet. These compounds are chlorinated heterodimers of anthraquinone and xanthone derivatives (Milat *et al*., 1992). Beticolins have shown activity against plants and bacteria, they complex different metals, interfere with H^+ and K^+ transport, affect ATPases and depolarize membranes (Jalal *et al*., 1992). These activities have been linked to the ability of beticolins to form pores in the plasma membrane (Goudet *et al*., 2000).

Similar to the *Cercospora* perylenequinones, the rubellins from the barley pathogen *Ramularia collo-cygni* are also light-activated toxins (Miethbauer *et al*., 2006). Rubellins are anthraquinone derivatives of polyketide origin which have demonstrated cytotoxicity in tumorous cell lines and also have light-dependent antibiotic activity (Miethbauer *et al*., 2009). Although rubellins are not a determinant for the infection of the host (they are not host specific), their ability to induce necrosis *in planta* suggests that they are pathogenic factors, as *R. collo-cygni*, a necrotroph, thrives in necrotic tissue (Heiser *et al*., 2004; Walters *et al*., 2008).

Another example of SMs from the *Mycosphaerella* genus is dothistromin, isolated from the pathogen *Dothistroma septosporum* (anamorphic *M. pini*), the causal agent of Dothistroma needle blight of *Pinus* ssp. (Barnes *et al*., 2004). Dothistromin is an aflatoxin-related compound with a wide spectrum of toxicity; it exhibits activity against microorganisms and phytotoxicity against the host. Furthermore, it causes the characteristic red coloration of infected needles (Shain and Franich, 1981). However, it has been shown that dothistromin knockouts of *M. pini* maintain the ability to produce the disease, demonstrating that dothistromin is not needed for pathogenicity, but a possible role of this compound could be to compete against other *Pinus*-infecting microorganisms (Schwelm *et al*., 2009). However, it is interesting to note that the genes for dothistromin synthesis are not clustered as typically observed for SM synthetic genes, but appear to be scattered throughout the genome (Zhang *et al*., 2007). This has been proposed to be the product of a gene cluster fragmentation which could be a mechanism for retooling SM biosynthetic machinery and, consequently, for niche adaptation (Bradshaw *et al*., 2013).

Cladosporium fulvum is a biotrophic pathogen of tomato and, presumably, an anamorphic *Mycosphaerella* (Thomma *et al*., 2005). A genomic analysis has shown that it has 21 SM core enzymes, including those coding for the perylenequinone and dothistromin structures (Collemare *et al*., 2014). Using comparative genomics and transcriptomics data, Collemare *et al*. showed that, in contrast with other biothrophs, which have a reduced number of SM genes, *Cl. fulvum* has a comparable number of SM biosynthetic genes to that found in hemibiotrophs and necrotrophs. However, their expression during the infection phase is down-regulated, representing an alternative mechanism of biotrophic lifestyle (Collemare *et al*., 2014). The only major compound that can be detected and identified *in vitro* is a
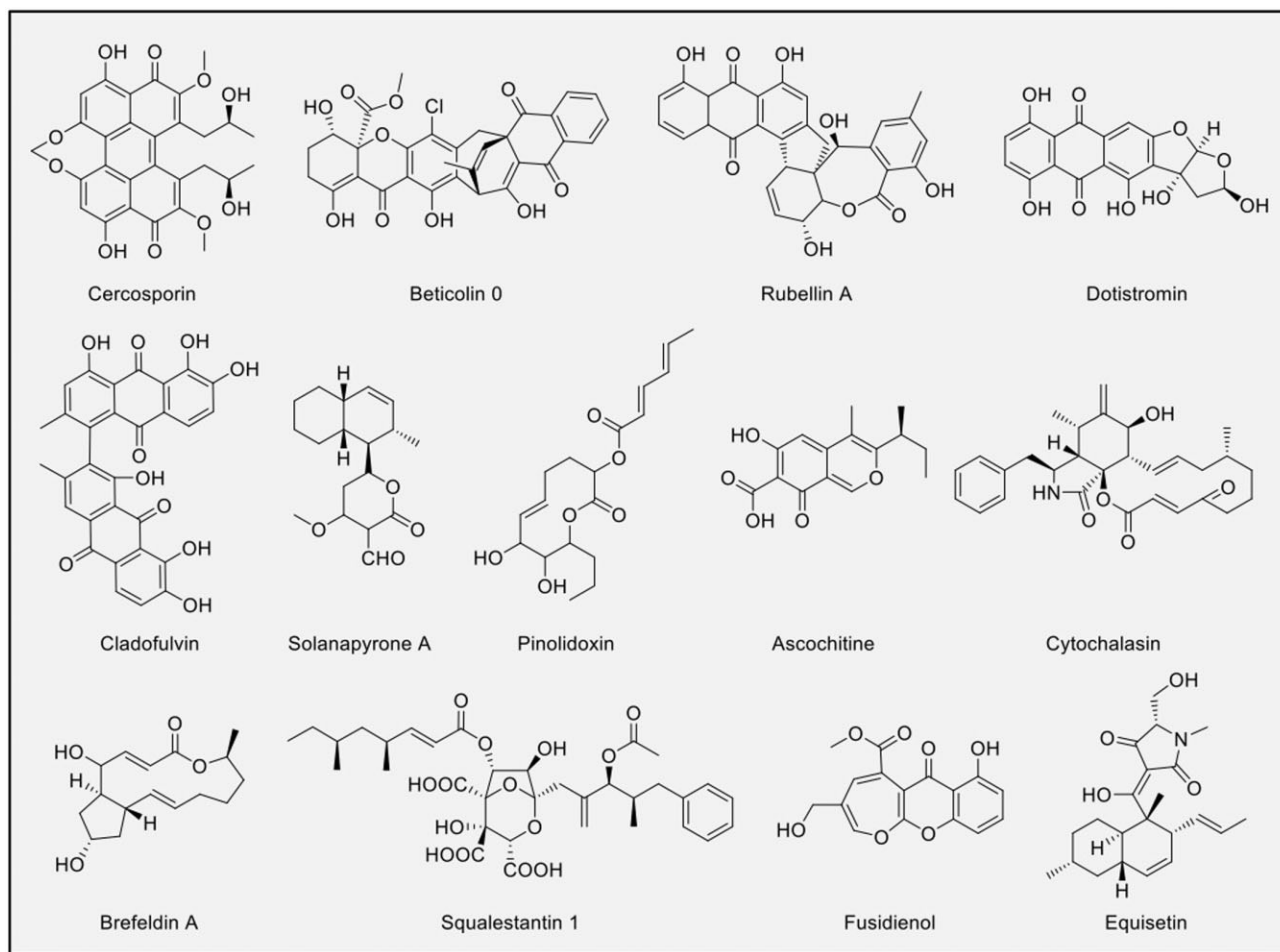
Fig. 3 Secondary metabolites isolated from *Mycosphaerella* and *Didymella*.

bianthraquinone, cladofulvin. *Cladosporium fulvum pks6*, whose expression correlates with the production of cladofulvin, has been proposed to be involved in the biosynthesis of this molecule. However, cladofulvin does not appear to play a role in causing necrosis of the plant host.

As a result of the polyphyly of *Mycosphaerella*, a new genus, *Zymoseptoria*, has been proposed for some of the species within this genus, most notably *Zymoseptoria tritici* (formerly known as *M. graminicola*) (Quaedvlieg *et al*., 2011). Interestingly, despite being one of the major wheat pathogens in Europe and considered by some as a model organism (Dean *et al*., 2012), there have been no reports describing toxins or SMs.

Didymella

A large number of *Ascochyta* and *Phoma* pathogens are *Didymella* anamorphs and have been reported to produce SMs; some of these compounds are presented in Fig. 3. Species within the *Aschochyta* represent the causal agents of many of the main diseases of legumes: *Ascochyta pisi* and *Ascochyta pinodes* infect peas, *Ascochyta lathyri* infects sweet peas, and chickpeas and beans are infected by *Ascochyta rabiei* and *Ascochyta fabae*, respectively.

The virulence of *A. rabiei* isolates has been correlated with their ability to produce the phytotoxic solanapyrones A, B and C (Kaur, 1995). However, when applying these compounds on chickpea shoots directly, they cannot be detected after symptoms develop, suggesting that these substances may be metabolized *in planta*. It has been suggested that solanapyrones react with glutathione-*S*-transferase, becoming covalently bonded to glutathione, and thereby reducing the toxicity (Hamid and Strange, 2000; Riaz *et al*., 2013). Pinodiloxines, five nonenolides from *A. pinodes* and ascosalitoxin, a precursor of ascochitine produced by *A. pisi* and *A. fabae*, have also been shown to exhibit phytotoxicity. These SMs produce similar symptoms to those observed during fungal infection. In addition, their effect on other microorganisms was tested and no inhibitory activity was found, suggesting that these compounds are plant-specific toxins (Evidente *et al*., 1993a, b). In contrast, compounds from the cytochalasin family

identified in *A. lathyri* (Vurro *et al*., 1992), *A. rabiei* (Latif *et al*., 1993) and *Phoma exigua* (Capasso *et al*., 1987, 1988; Evidente *et al*., 1992, 1996) displayed antimicrobial activities, but no visible effects on plants, when tested as pure compounds (Capasso *et al*., 1991). However, cytochalasins, which inhibit actin polymerization, have been shown to allow fungi to penetrate into non-host plant cells (Kobayashi *et al*., 1997a, b) by suppression of the plant defence response, involving cytoskeleton reorganization (Hardham *et al*., 2007).

Phoma species from *Didymella* are also known to produce diverse SMs. For example, *Phoma medicaginis* has been shown to produce brefeldin A, originally a protein transport inhibitor isolated from *Eupenicillium brefeldianum* (Härri *et al*., 1963). *Phoma medicaginis* only produces brefeldin A in dead pre-colonized plant tissue and *in vitro*, but not in living plants (Weber *et al*., 2004). As brefeldin A has been shown to suppress spore germination and the growth of other fungi, it has been suggested that it may play a role in substrate defence at the saprophytic stage (Weber *et al*., 2004).

Other *Phoma* SMs have been reviewed by Rai *et al*. (2009). *Phoma* SMs have shown activities that make them pharmaceutically attractive: squalestatins are cholesterol lowering (Dawson *et al*., 1992), fusidienol A has antitumour properties (Singh *et al*., 1997) and equisetin shows anti-HIV properties (Hazuda *et al*.,

1999). Unfortunately, many of the natural product screenings do not report on species identification and thus describe the fungi as *Phoma* sp., preventing their placement in the correct telemorphic group, as *Phoma* species could be either from the *Didymella* or *Leptosphaeria* genus.

Leptosphaeria

Leptosphaeria maculans, the causal agent of blackleg disease on canola, is the most prominent member of the *Leptosphaeria*. As such, several SMs from *L. maculans* have been identified with putative roles assigned (Howlett *et al*., 2001). Examples of these SMs can be found in Fig. 4. Sirodesmins are the hallmark compounds of *L. maculans*. The non-HST, siderodesmin PL, is an antibacterial and antiviral compound of the epipoly-thiodioxopiperazine family. Sirodesmin biosynthesis is encoded by 18 genes in a cluster, including an NRPS responsible for the formation of the diketopiperazine core from a tyrosine and a serine (Ferezou *et al*., 1980; Gardiner *et al*., 2004). The disulphide bond bridging the diketopiperazine ring that is characteristic of epipolythiodioxopiperazine compounds is presumably responsible for the bioactivity, as it can interact with cysteine residues of proteins (Mullbacher *et al*., 1986). Another known compound from *L. maculans* is the antimicrobial polyketide phomenoic acid,
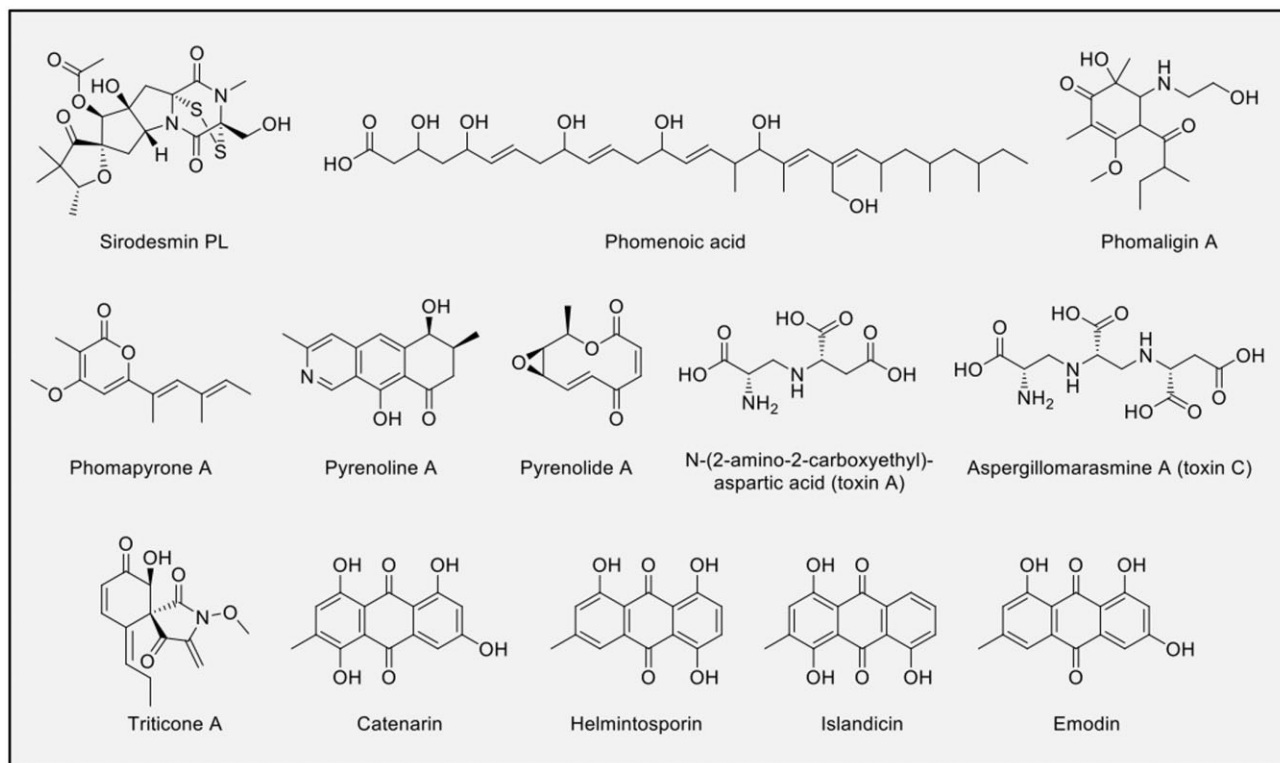

Fig. 4 Secondary metabolites isolated from *Leptosphaeria* and *Pyrenophora*.

which is found in stem and stubble. This long-chain polyketide was isolated during a traditional natural product screen looking for compounds toxic to fungi (Devys *et al*., 1984). Using gene silencing, the PKS gene, together with the gene cluster responsible for its biosynthesis, has been found, *PKS2* (Elliott *et al*., 2013). As phomenoic acid exhibits antifungal activity, but not phytotoxicity, it has been proposed that it plays a role in the suppression of competing fungi.

Initially considered to be the same species, *Leptosphaeria biglobosa* was separated from *L. maculans* (Shoemaker and Brun, 2001). Both fungi have distinct *in vitro* SM production (Pedras and Yu, 2009). Two groups of molecules, phomaligins and phomapyrones, have been isolated from *L. biglobosa*, but their biological activities and ecological roles have not been studied (Pedras *et al*., 1993; Soledade *et al*., 1994).

Pyrenophora

The *Pyrenophora* genus includes several significant cereal pathogens, such as the devastating *Pyrenophora tritici-repentis* (wheat) and *Pyrenophora teres* (barley) (Ellwood *et al*., 2010; Manning *et al*., 2013). As a result of their potential role in facilitating disease, the SMs of these pathogens have received considerable attention. Some of these SMs are shown in Fig. 4. *Pyrenophora teres* produces the phytotoxic SMs isoquinoline pyrenolines (Coval *et al*., 1990), the antifungal macrolide pyrenolides (Nukina *et al*., 1980a, b) and five phytotoxic peptide alkaloids. Amongst the five peptide alkaloids, *N*-(2-amino-2-carboxythyl)-aspartic acid (toxin A) and aspergillomarasmine A (toxin C) are the most phytotoxic, and their activity *in planta* correlates with the latter symptoms caused by the pathogen (Bach *et al*., 1979; Friis *et al*., 1991; Weiergang *et al*., 2002). *Pyrenophora teres* also produces proteinaceous toxins, and it has been reported that they work in a complementary way to the SMs of this pathogen. The symptoms observed at an early stage of *P. teres* infection correspond to those caused by the proteinaceous toxins, whereas the presence of small-molecule toxins correlates with the latter stages (Sarpeleh *et al*., 2007).

Phytotoxic pirocyclic lactam triticone SMs have also been reported from *P. tritici-repentis* (Hallock *et al*., 1993; Sugawara *et al*., 1988). The most active is triticone A, an intercellular toxin that causes necrosis, suppresses photosynthetic processes and has been reported to interfere with sulphydryl (SH)-containing enzymes (Kenfield *et al*., 1988). In addition, seven anthraquinonic mycotoxins have been reported from *P. tritici-repentis*: emodin, catenarin, helminthosporin, islandicin and the hydroxylated derivatives of the last three. These mycotoxins show antimicrobial activities, suggesting that they may play a role during the saprophytic stage of the interaction (Bouras and Strelkov, 2008; Kachlicki and Wakuliski, 2002).

Phaeosphaeria

The *Phaeosphaeria* genus contains pathogens of weeds and grasses; among them, *Phaeosphaeria nodorum* (anamorph *Parastagonospora nodorum*) is the causative agent of Stagonospora nodorum blotch (Shoemaker and Babcock, 1989). The genome sequence of *P. nodorum* reveals a large number of SM genes (Hane *et al*., 2007), some of which are expressed *in planta* during infection (Ipcho *et al*., 2012). To date, 11 SMs have been isolated and identified from *P. nodorum* (Fig. 5): mycophenolic acid, mellein and four of its derivatives (Devys *et al*., 1980, 1994), septorin and its two derivatives (Devys *et al*., 1982), AOH (Tan *et al*., 2009) and 4'-methoxy-2-methylbutyrophenone (Yang *et al*., 2013). The mellein and septorin compound families have reported phytotoxic activities when tested as pure compounds. However, the necrotic effect of the proteinaceous toxins in *Ph. nodorum* (ToxA, Tox1 and Tox3) is so strong that the possible activity of the SMs may be masked *in vivo*.

Other *Phaeosphaeria* species, including pathogens, endophytes, saprotrophs, endolichenic fungi as well as those found in aquatic environments, are the source of more than 75 reported natural products. These include the aforementioned perylenequinones (Li *et al*., 2012), the gibberellins (Sassa *et al*., 1989) and the phytotoxic stagonolides that could be involved in the bio-control of weeds (Evidente *et al*., 2008) (Fig. 5). This indicates the potential of this genus to be a rich source of SMs with a wide diversity of activities and structures.

Botryosphaeria

Similar to *Phaeosphaeria*, most of the compounds reported from *Botryosphaeria* (Fig. 5) do not come from plant pathogens, even though many species from this genus are plant-associated microorganisms. *Botryosphaeria* is mainly known for its forestry and agricultural pathogens. Amongst these, *Botryosphaeria obtusa*, a pathogen of apples and grapevines, has been studied chemically, leading to the commonly isolated mellein and its derivatives, as well as tyrosol and *p*-hydroxybenzaldehyde. All these showed phytotoxicity in apple, but there was no correlation between the amount of compound produced by the strain and its pathogenicity (Djoukeng *et al*., 2009; Venkatasubbaiah and Chilton, 1990; Venkatasubbaiah *et al*., 1991). It is hypothesized that most, if not all, *Botryosphaeria* have an endophytic stage (Slippers and Wingfield, 2007); thus, it is not surprising that the majority of SMs reported from this genus come from endophytes. In a recent study in an endophytic *Botryosphaeria dothidea* from *Melia azedarach*, 18 SMs were isolated with a range of activities (Xiao *et al*., 2014). For instance, the perylenequinone derivative, stemphyperylenol, presented cytotoxic activity, and also showed, together with AOH, AME and other metabolites, toxicity against *Alternaria solani*; however, altenusin, with an opened lactone AME structure,
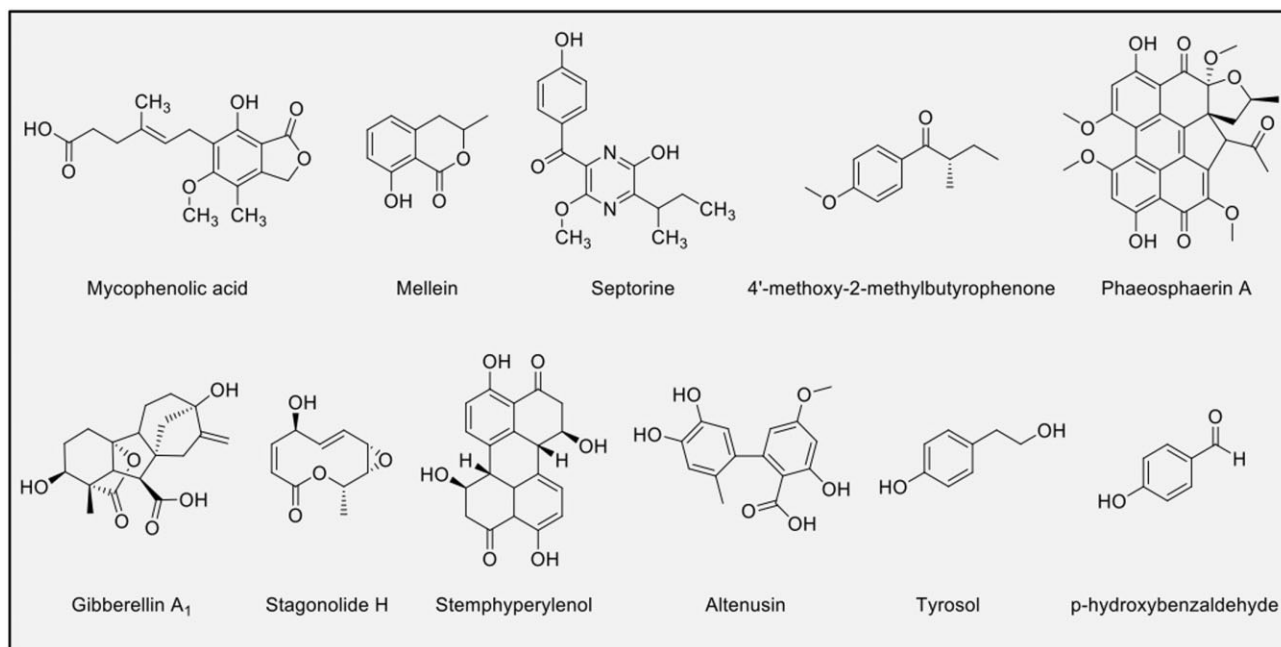
Fig. 5 Secondary metabolites isolated from *Phaeosphaeria* and *Botryosphaeria*.

showed an activity that its cyclic counterparts lacked: it scavenged free radicals (Xiao *et al.*, 2014). This activity could be an important mechanism in plant-associated microorganisms, as plant defence responses include the production of reactive oxygen species. It is interesting to consider, however, that many of the SMs reported from endophytic *Botryosphaeria* are phytotoxic. For example, *Botryosphaeria mamane* and *Botryosphaeria parva*, endophytes from *Garcinia mangostana* and *Eugenia jambolana*, respectively, produce mellein and other phytotoxic derivatives, raising the question as to what roles these compounds play during interaction (Araujo *et al.*, 2012; Pongcharoen *et al.*, 2007).

THE FUTURE OF SM RESEARCH IN THE DOTHIDEOMYCETES AND THE ROLE OF EMERGING TECHNOLOGIES

The above overview of Dothideomycete SMs provides a snapshot of their diversity, in terms of both their chemical structures and bioactivities. It suggests that members of the Dothideomycetes are a good source of phytotoxic compounds, which is not surprising given the numbers of plant-associated fungi in this class. Interestingly, several of these SMs have been isolated from endophytes, such as the cochlioquinones and the mellein family, highlighting the fact that phytotoxicity may be a coarse measure to explain their biological role. It is plausible that these 'phytotoxic' substances may be involved in tuning the interaction between plant and microorganism at subtoxic concentrations, thus supporting the argument against equating laboratory-observed biological activities to biological roles. In addition, there remains a lack of

knowledge of their biosynthesis and the genes that encode them for many of the SMs presented above. From our perspective, one of the aspects of SMs in which our understanding is still lacking is the natural functions of SMs. What are their biological functions?

However, it would also seem clear that we have really only touched the surface in terms of the identification of what SMs are produced by Dothideomycete fungi. A large number of fungal SMs have been discovered by traditional application-driven natural product screening programmes based on bioactivity-guided isolations, e.g. many Dothideomycete phytotoxins have been found with the aim of obtaining mycoherbicides. Although these studies have enriched our understanding of SMs, they are also limited, as the fungus is typically grown in artificial conditions and the assays focus on a specific desired activity. Rarely do these studies consider the natural environment of the organism. During *in vivo* growth, SMs would be produced by fungi only when required and under specific conditions in order to economize resources. Furthermore, if we compare the number of SM gene clusters in the sequenced Dothideomycete genomes with the number of identified molecules, it is clear that our knowledge of secondary metabolism in this broad class of fungi is limited at best.

Indeed, the availability of fungal genome sequences has revolutionized SM research in many ways. It has allowed us to obtain a complete inventory of the SM biosynthesis genes of a fungus and to systematically determine the SM product(s) of each SM gene cluster (see below: Identification of cryptic SMs). Apart from facilitating the identification and characterization of the genes involved in the biosynthesis of SMs, comparative genomics and transcriptomics have allowed the inference of the evolutionary

and functional role of SMs. For example, comparative genomics of 18 Dothideomycete fungi has revealed that the specialization of SMs and the diversification of their biosynthetic machinery play major roles in the adaptation of an organism to its lifestyle (Ohm *et al*., 2012). By comparing two closely related dothideomycete pathogens, *Cl. fulvum* and *D. septosporum*, with different hosts and lifestyles, it was shown that the evolution of SM biosynthesis genes, via gene gain, gene loss, changes in gene regulation and pseudogenization, plays an important role in the adaptation of the organisms to different environmental niches (de Wit *et al*., 2012). Nevertheless, to advance our understanding of the biological and ecological roles of SMs in fungi, it is important to: (i) study the SM molecules produced by the organisms in different natural environments and correlate with possible functions; (ii) identify these molecules; and (iii) elucidate or validate their biological functions.

In the next section, we focus on the current approaches and technologies available for the comparative study of the SM molecules produced by the organisms in different natural environments and the identification of these bio-ecologically relevant molecules.

Unveiling the roles of fungal SMs

An alternative to activity-based screenings could be ecological role-based screenings. Ecological role-based screenings look for SMs using the whole producer organism in different environmental conditions, rather than focusing on testing extracts, fractions and pure compounds. Broadly, these screenings compare SM profiles between a control state and a challenged state; SMs that are induced during the challenged state may have a role in organism adaptation to the challenge. A plant pathogen may be challenged with the presence of different hosts or tissues, by introducing an antagonistic microorganism, by changing the substrate in which it grows or by subjecting it to various abiotic stresses, such as water stress, heat stress, mineral deprivation, etc. This is followed by the analysis of the SM profiles of the organisms at different states. Similarly, instead of comparing SM production from challenged and unchallenged states, SM profiles from different developmental or life cycle stages can be compared.

New analytical techniques using mass spectrometry (MS) and nuclear magnetic resonance (NMR)

MS systems hyphenated with chromatographic devices are powerful tools for microbial SM profiling (Kersten *et al*., 2010). MS essentially gives the molecular mass of the compounds analysed. When high-resolution MS (HRMS) is employed, the molecular formula can be deduced. MS can provide some structural data if fragmentation occurs; for this purpose tandem MS (MS^n) is employed, which produces a characteristic fragmentation spectrum (Niessen, 2006). Extracts from the control and challenged samples are analysed, obtaining the retention time, exact mass

and, if possible, fragmentation spectra of the components in these extracts. The collected data for each treatment are SM profiles that can be compared to highlight the differences and to select the compounds to be identified.

Nonetheless, it is seldom that either HRMS or MS^n provides sufficient structural data to unequivocally identify a substance (Seger *et al*., 2013). Commonly, a purification process follows the screening and the purified compound is identified using NMR, the main technique to elucidate the structure of natural products (Halabalaki *et al*., 2014). Despite the advantages of MS (less expensive, greater sensitivity, shorter analysis times and easier data to interpret), NMR provides detailed structural information in a non-destructive way that allows the *de novo* determination of a compound's structure (Sarker and Nahar, 2012; Wolfender, 2009). The full capability of NMR, however, can be limited by the time required (relative to MS) to generate sufficient data at low analyte concentrations. Because of this, NMR is being replaced by stop flow or online solid phase extraction coupled with liquid chromatography (LC-SPE-NMR) (Seger *et al*., 2013). The field has recently further advanced with the multiplexing of a combination of MS, NMR and other analytical techniques, providing platforms which are now being used for de-replication, novel compound discovery and *de novo* identification (Sturm and Seger, 2012). Such analyses to date have been predominantly restricted to the plant natural product field and, to our knowledge, there are no reports in which these advanced analytical techniques have been applied to identify fungal SMs. However, these approaches offer immense potential for the deciphering of fungal SMs and their roles, and there is little doubt that, as they become more accessible, these techniques will improve the throughput of fungal SM discovery. For further information on these techniques, interested readers are directed to Li *et al*. (2013).

Imaging MS (IMS) is emerging as a powerful technique for the study of the ecological function of SMs in microorganisms by allowing the targeted or untargeted analysis of the spatial and temporal production of SMs in response to specific environmental stimuli. IMS overlaps spectrometric data of locally desorbed and ionized samples over the image of these samples, mapping the distribution of compounds over a two-dimensional render (So *et al*., 2013; Watrous and Dorrestein, 2011). For example, IMS has been used to analyse the growth of *Streptomyces coelicolor* when co-cultured with other actinomycetes. Several SMs were found to be selectively induced by the interaction, and some SMs were shown to be antagonist specific (Hopwood, 2013; Traxler *et al*., 2013). When such analytical systems are not available, targeted gas chromatography (GC-MS) or liquid chromatography (LC-MS) may be used to analyse extracts from different sections of a sample, allowing the localization of SM production. This approach identified *O*-methylmellein in the interaction zones from plates containing *Botryosphaeria obtusa* and *Eutypa lata* using LC-MS and NMR (Glauser *et al*., 2009). Both fungi are common elements

in the mix of microorganisms causing esca disease in grapevines. Although extracts obtained from pure cultures did not show any activity, those present in the interaction zones were phytotoxic and fungitoxic. Consistent with this observation, *O*-methylmellein showed germination inhibition of both plant and fungi. Researchers propose that the SMs produced by esca fungi in order to interact with each other could also drive the disease of the host (Glauser *et al*., 2009). It is clear that the emergence of these advanced analytical techniques is unveiling not just new SMs, but also novel biological and ecological interactions, highlighting their importance in the future of fungal SM research.

Identification of cryptic SMs

Even with the latest analytical technology described above for compound resolution and identification, at some point, all SM investigations require the isolation of the pure compound for structural elucidation and/or for the determination of the mechanism of action. However, these isolations may prove difficult if SMs are minor components in the extract or if they are not expressed in laboratory culture conditions. In the case of plant-associated fungi, some SMs may only express *in planta* at levels that are difficult to isolate from the complex plant metabolic background. However, techniques are emerging to facilitate the biosynthesis and purification of these SMs for subsequent study (Lim *et al*., 2012; Scherlach and Hertweck, 2009; Takahashi *et al*., 2013).

Modification of culture conditions

As mentioned above, the *in vitro* culture of Dothideomycete fungi is likely to limit the expression of SMs. As some SMs are known to be antimicrobial and to inhibit the growth of competitors, perhaps growth in a heterogeneous environment is necessary to trigger the production of SMs not normally observed in homogeneous cultures (O'Brien and Wright, 2011). For example, it has been shown that *Streptomyces rapamycinus* triggers chromatin remodelling through histone modification in *Aspergillus nidulans*, leading to the expression of silent SM genes in the fungus (Nützmann *et al*., 2011). Several investigations to date have proven the success of co-culturing studies, demonstrating their worth not just for scientific research, but also for SM production at industrial levels (Bader *et al*., 2010; Nützmann *et al*., 2012). Similarly, based on the fact that differences in the culture conditions can lead to different SM profiles, Bode *et al*. (2002) isolated more than 100 compounds from only six microbial strains, proposing 'One Strain MAny Compounds' (OSMAC), which refers to a systematic modification of the medium composition and culture conditions of the microbial fermentations.

Epigenetic regulation

Although the use of culture conditions to activate or promote cryptic and minor SMs has proven to be effective, perhaps most potential lies

in the use of genetic-based approaches to decrypt Dothideomycete secondary metabolism. For example, several global SM regulators have been identified and exploited for the activation of silent SM gene clusters in fungi (Brakhage and Schroeckh, 2011). It has been demonstrated that the overexpression of a global SM regulator, *laeA*, increases penicillin and lovastatin, but not sterigmatocystin, production in *As. nidulans* (Bok and Keller, 2004). Overexpression of *laeA* also leads to the production of terrequinone A, a cryptic metabolite not previously described from *As. nidulans*. LaeA works at a transcriptional level, but has no homology to any transcription factor; it has a methyltransferase domain and is involved in histone methylation, regulating SM production by restructuring the chromatin (Keller *et al*., 2005). LaeA, together with VeA and VelB, form the velvet complex, which is involved in sexual development and in the regulation of secondary metabolism at the global level (Bayram *et al*., 2008). Other chromatin-regulating genes, a histone deacetylase *hdaA* and a histone methylase *cclA* from *As. nidulans*, appear to have contrasting action to *laeA* in that they suppress the expression of several SMs (Bok *et al*., 2009; Shwab *et al*., 2007). In addition, a mechanism of chromatin regulation was found in *Fusarium graminearum* involving silencing by a trimethylated histone H3 lysine 27 (H3K27me3); transcriptomic analysis of mutants without H3K27 methyltransferase showed that 14% more genes were expressed (Connolly *et al*., 2013). *Epichloe festucae* also presents this type of mechanism, which is linked to its characteristic endophytic lifestyle (Chujo and Scott, 2014). However, H3K27 methylation has not been observed in all organisms; *Aspergillus*, a model for the study of SM regulation, lacks this mechanism, showing how there is much still to be learned about the different layers of gene regulation (Connolly *et al*., 2013). Chromatin regulation of SM gene clusters has also been manipulated through the use of epigenetic modifiers (Williams *et al*., 2008). For example, supplementation of potato dextrose with histone deacetylase chemical inhibitors led to the discovery of 4'-methoxy-(2*S*)-methylbutyrophenone in *Ph. nodorum* (Yang *et al*., 2013).

Molecular biology of SM pathway activation

The above-mentioned methods are not SM specific and approaches targeting particular SM biosynthetic genes may be needed. Traditionally, the linkages between gene clusters and SMs are identified by genetic knockouts and mutant complementation, but this will not be applicable if the gene cluster of interest is silent. Recent advances in our understanding of SM biosynthetic genes and the development of bioinformatics tools, such as SMURF (Khaldi *et al*., 2010) and AntiSMASH 2.0 (Medema *et al*., 2011), have accelerated the identification of SM gene clusters in sequenced fungal genomes. These resources expedite the identification of SM gene clusters within individual fungal genomes and allow a targeted approach to acquire SM products encoded by specific gene clusters. One direct approach is by exchanging the promoters of individual genes of an SM gene cluster in the native host; however, this could be cumbersome if the gene cluster of

interest contains many genes. Two main strategies can be exploited to characterize specific SMs or gene clusters of interest: (i) pathway-specific regulatory gene manipulation; and (ii) heterologous expression of SM clusters. These two strategies not only allow the isolation of cryptic or minor SMs, but also simultaneously link the genes or clusters to the SM products.

The basis of manipulating the regulation of specific pathways lies in the fact that some clusters contain genes encoding for transcription factors that activate the biosynthesis of the SMs coded within that cluster (Yin and Keller, 2011). Pioneered by Bergmann *et al*. (2007), it was found that a single ectopic insertion of *apdR*, a putative Zn_2Cys_6-type activator gene in a silent gene cluster, encodes for a hybrid PKS–NRPS pathway. The activation of *apdR* promotes the expression of the novel aspyridones and the cryptic products within the cluster. This strategy has been employed with success in several other laboratories, especially amongst the *Aspergillus* species (Chiang *et al*., 2009; Zabala *et al*., 2012). However, not all SM gene clusters are regulated by a pathway-specific regulator and other complementing strategies are required.

Heterologous expression

As an alternative to overcome the SM pathway activation difficulties, an emerging approach is to transfer the SM genes of interest into a well-characterized heterologous host, such as *Saccharomyces cerevisiae* or *Escherichia coli*. Successful attempts have been made in expressing SM genes in *S*. *cerevisiae* by incorporating a phosphopantetheinyl transferase gene, *npgA*, for post-translational modification of acyl carrier protein domain-containing enzymes, which include the PKSs and NRPSs (Ma *et al*., 2009; Tsunematsu *et al*., 2013). However the expression of fungal SMs in standard laboratory organisms is technically challenging because of the lack of appropriate intron splicing and post-translational modification machinery in these models (Schümann and Hertweck, 2006). Thus, there is an advantage of using filamentous fungi, such as the well-characterized *Aspergillus* spp., for the expression of SM gene clusters, as there is a high possibility that the fungal host has compatible intron splicing and post-translational machinery. However, heterologous expression of SM clusters in a filamentous fungal host is often complicated by the metabolic complexity of the host and the possibility that the host biosynthetic enzymes cross-talk with the heterologous pathway. For this reason, a strain of *As. nidulans* that lacks many of its SM clusters is being developed as a 'clean host' for the heterologous expression of fungal SMs (Chiang *et al*., 2013).

THE FUTURE OF DOTHIDEOMYCETE SM RESEARCH IS PROMISING

A clear theme that can be garnered from this review is that, although we have made significant progress towards the under-standing of secondary metabolism in Dothideomycete fungi, there remains much to learn. For example, cryptic SMs and orphan genes are a rule and not the exception, the biological role of individual SMs is unknown and their biosynthetic regulation is often poorly understood. However, the pieces of the puzzle are starting to come together. The next-generation sequencing revolution means that access to genome sequences is no longer limited to models, and the bioinformatics tools are now available to readily identify SM clusters within these genomes. The analytical chemistry tools needed to identify and characterize SMs are rapidly advancing in resolution and, importantly, are becoming more easily available to researchers in the field. The emergence of synthetic biology tools to enable the expression and analysis of SM clusters that typically appear silent will start to shed light on SMs we previously could not study. It is also apparent that the field is now beginning to recognize that, in order to understand the roles of these SMs, we must look for and characterize them in their natural environment, and not necessarily in a laboratory fermentation. The future of this field is very promising. Given the convergence of resources and understanding, we feel that the study of Dothideomycete secondary metabolism is about to come of age.

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