Microscopy reveals disease control through novel effects on fungal development: a case study with an early-generation benzophenone fungicide†

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Abstract: The benzophenones are a new class of agricultural fungicides that demonstrate protectant, curative and eradicative/antisporulant activity against powdery mildews. The chemistry is represented in the marketplace by the fungicide metrafenone, recently introduced by BASF and discussed in the following paper. The benzophenones show no evidence of acting by previously identified biochemical mechanisms, nor do they show cross-resistance with existing fungicides. The value of microscopy in elucidating fungicide mode of action is demonstrated through identification of the effects of an early benzophenone, eBZO, on mildew development. eBZO caused profound alterations in the morphology of powdery mildews of both monocotyledons and dicotyledons, affecting multiple stages of fungal development, including spore germination, appressorial formation, penetration, surface hyphal morphology and sporogenesis. Identification of analogous effects of eBZO on sporulation in the model organism Aspergillus nidulans (Eidam) Winter provides a unique opportunity to elucidate important morphogenetic regulatory sites in the economically important obligate pathogens, the powdery mildews. Benzophenones provide a further example of the benefits of whole-organism testing in the search for novel fungicide modes of action. © 2006 Society of Chemical Industry

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1 INTRODUCTION

Discovery and subsequent development of novel antifungal agents require evaluation of a complex set of criteria, including disease-control efficacy and spectrum, environmental and toxicological impact, cross-resistance with existing products, manufacturing costs, patent coverage and market potential, in addition to identification of the most suitable analogue within a chemical series. An agrochemical, however, can be developed and successfully marketed without a full understanding of its mode of action (MOA). Instances in which the precise MOA of a novel fungicide is known prior to product launch, as with the QoI (e.g. strobilurin) fungicides, are rare. However, once a novel antifungal MOA is identified, it becomes possible to develop and implement more efficient assays for compounds affecting that target, thus aiding in the identification of additional materials that may affect the same target site. Information about the target site may also help guide resistance risk assessments and the development of appropriate antisporulant management strategies.

Identification of an unknown but novel biochemical target site for a new class of fungicides from among the universe of potential targets can be a challenging task. MOA studies are particularly problematic when the fungicides are only active against obligate biotrophs, such as the powdery and downy mildews, which cannot be cultured separately from their host. Gustafson et al.1 made a significant, although ultimately unsuccessful, effort to identify a tractable fungal species to use for comparing the modes of action of two related phenoxyquinolines, one of which (quinoxyfen) showed activity against only powdery mildews. Microscopic studies suggested that quinoxyfen did not prevent disease development by inhibiting growth of the pathogen, but instead acted by preventing an essential developmental/morphological
transition involved in appressorial formation, thereby preventing infection.\(^2\)

Microscopy has been a valuable tool in examining the effects on infection processes of many novel fungicides, including melanin biosynthesis inhibitors such as tricyclazole on rice blast,\(^3\) respiratory (Q,I) inhibitors such as kresoxim-methyl on *Blumeria, Uncinula* and *Venturia*,\(^4\) and various azole inhibitors of sterol biosynthesis.\(^5\)

In this paper we demonstrate an approach to understanding the biological effects of a new powdery mildew-specific fungicial chemistry by microscopically identifying its unique effects on pathogen morphogenesis. The demonstration of analogous morphogenetic effects of benzophenones (BZOs) on a biochemically/genetically tractable model organism points to a potential route to the identification of the benzophenones’ ultimate biochemical target, emphasising the utility of microscopy in MOA investigations.

### 2 MATERIALS AND METHODS

#### 2.1 Chemistry

Initial studies with the early benzophenones\(^6\) reported here were conducted with eBZO, an analogue that showed promising activity against powdery mildews. Subsequent to this research, greenhouse and field testing of additional BZOs resulted in the identification of a superior analogue, recently introduced as metrafenone (3′-bromo-2,3,4,6′-tetramethoxy-2′,6-dimethylbenzophenone, Flexity\(^7\); Fig. 1), for the control of powdery mildews.\(^7\) eBZO and metrafenone have qualitatively comparable biological effects on powdery mildews, although metrafenone demonstrates greater inherent potency as well as other commercially desirable properties.

#### 2.2 Pathogens

Experiments were conducted using winter barley (*Hordeum vulgare* L. cv. Halcyon) and spring wheat (*Triticum aestivum* L. cv. Alexandria) inoculated with barley powdery mildew (*Bgh, Blumeria graminis* Speer f. sp. *hordei* Marchal, isolate 23D5) and wheat powdery mildew (*Bgt, B. graminis* f. sp. *tritici* Marchal, isolate W26) respectively. Additional experiments were conducted on *Pisum sativum* L. (*Argenteum* cv. Alexandria) inoculated with pea powdery mildew (*Erysiphe pisi* DC) and *Vitis vinifera* L. (cv. Ugni Blanc) infected with *Uncinula necator* (Schw) Burr.

For protectant treatments, seedlings were sprayed to run-off using an atomiser containing either 5 mg litre\(^{-1}\) eBZO formulated as an emulsifiable concentrate (EC) or a formulation blank. The treated plants were incubated in a controlled environment chamber (20°C, 70% relative humidity (RH), 400 µmol m\(^{-2}\) s\(^{-1}\) photosynthetic photon fluence rate at 400–700 nm, 12 h photoperiod). Plants were inoculated at 1 day after treatment (DAT) and observed at 1–3 days after inoculation (DAI).

For curative treatments, wheat and barley plants were inoculated as above and then treated with 125 mg litre\(^{-1}\) eBZO (EC) or a blank formulation at either 2 or 5 DAI.

#### 2.3 *Aspergillus nidulans* (*Eidam*) Winter (= *Emericella nidulans* (*Eidam*) Vuill)

*Aspergillus nidulans* (strain A26, carrying the biA mutation) spores were flood inoculated onto maltose/glucose agar plates. Droplets (5 µl) of BZO (1 mg ml\(^{-1}\) in dimethyl sulfoxide) were applied to the agar surface, incubated at 37°C and the results observed at 3 DAI.

#### 2.4 Microscopy

Observations were made at intervals after treatment using either low-temperature scanning electron microscopy (LTSEM) of frozen/hydrated samples\(^9\) or Nomarski differential interference contrast (DIC) microscopy of tissues cleared with ethanol + acetic acid (3 + 1 by volume) followed by lactic acid + glycerol + water (1 + 1 + 1 by volume).\(^8\)

For fluorescence microscopy, fresh tissues were treated with fluorescent brightener (diethanol, gift from Prof. Kurt Mendgen, Konstanz University, Konstanz, DE), DAPI (Sigma, Gillingham, UK), acridine orange (Sigma), Nile red (Molecular Probes, Invitrogen, Paisley, UK) or fluorescein isothiocyanate (Sigma) to stain cell walls, nuclei, RNA, lipids and proteins respectively.\(^10,11\) Samples were then viewed by epifluorescence microscopy using appropriate filters. Autofluorescence of papillae was observed in cleared tissues with UV excitation (365 nm).

For transmission electron microscopy (TEM) of fungal penetration sites, leaf samples were collected at 30 h post-inoculation, fixed with glutaraldehyde and osmium tetroxide and embedded in epoxy resin.\(^12\) Infected leaves were prepared for TEM by glutaraldehyde fixation, osmium tetroxide staining and image analysis.

![Figure 1](image-url) Structures of early benzophenones and metrafenone.
embedding in epoxy resin. Multi-lobed appressoria were targeted by light microscopy prior to ultrathin sectioning and staining.\textsuperscript{13}

3 RESULTS
Early MOA studies with eBZO showed that it was not cross-resistant with sterol demethylase (azoles) or adenosine deaminase (ethirimol) inhibitors. It did not inhibit or uncouple respiration in mouse liver mitochondrial assays and there was no evidence of inhibition of adenosine deaminase activity extracted from \textit{Blumeria} conidia (data not shown). Thus eBZO did not appear to act via common biochemical MOAs known to affect powdery mildews. In order to gain some insight into the biological activity of eBZO, we examined its effects on development and morphology of mildews infecting several major monocotyledonous and dicotyledonous crops.

During normal disease development a mildew spore landing on a susceptible host leaf will germinate, forming a primary germ tube followed by a secondary germ tube. The secondary germ tube will then form a specialised infection structure, the appressorium. From the appressorium an infection peg will develop and attempt to penetrate the host epidermal cell directly through the cuticle and cell wall. Successful infections result in the formation of a haustorium, a specialised structure involved in obtaining nutrition from the host. Subsequently, epicuticular hyphae will develop and spread over the leaf epidermis, producing secondary appressoria and secondary haustoria and eventually mildew sporulation (for a more detailed account see Green \textit{et al.}\textsuperscript{14}).

3.1 Protectant activity
Protectant treatments against Bgh and Bgt showed that eBZO significantly altered the fungal infection process for both pathogens (Fig. 2). It substantially reduced, but did not completely block, conidial germination in both pathogens. In control (formulation blank) treatments, slightly over 80\% of the germinated conidia of both mildews formed primary appressoria with a single apical lobe. In the eBZO treatments, however, both mildews showed a shift from single-lobed to multi-lobed appressoria. At 24 h post-inoculation, protectant treatment with the benzophenone reduced haustorial formation in both pathogens (from 60 and 20\% haustorial formation for Bgh and Bgt in control treatments to 10 and 2\% respectively). This reduction in haustorial formation was associated with increased deposition of papillae by host epidermal cells at the sites of attempted pathogen penetration.

These effects of eBZO were confirmed using LTSEM. At 2 days after treatment with the blank formulation (control), Bgh colonies consisted of germinated conidia with both primary and secondary germ tubes, primary appressoria and numerous epicuticular hyphae and secondary appressoria (Fig. 3). Secondary appressoria are seen as small globular structures arranged singly or in pairs on opposite sides of hyphae (Fig. 3A). At 3 days after treatment with the blank formulation, leaves showed large colonies with a dense surface mycelium and abundant conidial chains of normal appearance arising from swollen basal cells (Fig. 3B). In contrast, germlings arising from conidia that germinated after a protectant eBZO treatment formed normal primary and secondary germ tubes but did not usually develop past the formation of

![Figure 2. Effects of protectant applications of eBZO on barley (BPM) and wheat (WPM) powdery mildews.](image-url)
primary appressoria. Primary appressoria commonly formed multiple (two or three) lobes or occasionally more complex structures (Fig. 3C). Light microscopy showed that penetration hyphae were formed beneath each lobe and penetrated through the cuticle and host cell wall but were then encased by a host papilla and developed no further (data not shown).

TEM analysis of four multi-lobed Bgh appressoria which formed after protectant treatment showed that penetration hyphae had penetrated through the host cuticle and cell wall beneath every appressorial lobe. All the penetrated epidermal cells appeared dead, exhibiting electron-opaque condensed cytoplasm and collapsed cell walls, whereas adjacent uninfected cells had normal ultrastructure. In one case (Fig. 4) the host cell had collapsed completely, so that the upper and lower periclinal cell walls were closely appressed.

Protectant treatments against Bgt generally showed the same features as Bgh, except that a few primary appressoria continued to develop, forming a primary haustorium and surface hyphae. These external hyphae, however, were usually malformed, having the swollen or bifurcated hyphal tips commonly seen following curative eBZO treatments (see Section 3.2). Thus it appears that those infections that escape the protectant effects of the BZOs may still succumb to the curative activity of the compounds.

3.2 Curative activity

A notable feature of eBZO is its relatively wide therapeutic window, which adds significant curative and antisporeulant activity to excellent protectant disease control (data not shown). Curative treatment with EC formulations of eBZO induced a number of

![Figure 3. LTSEM of Bgh development. (A) Mildew development at 2 days after inoculation following a protectant treatment with a formulation blank. (B) Dense surface mycelium and abundant conidial chains of normal appearance arising from basal cells at 3 days after inoculation. (C) Development at 3 days after inoculation following a protectant treatment with eBZO.](image1)

![Figure 4. TEM of Bgh penetration structures derived from multi-lobed appressoria following a protectant treatment with eBZO (A, appressorium; W, host cell wall; H, penetration hypha; P, papilla).](image2)
morphological changes that were identical in Bgt and Bgh. After curative treatments, secondary appressoria became more abundant and more closely spaced than normal. They were also larger and more elongated than normal and frequently had bifurcated tips. Occasionally, multiple bifurcation events produced complex branched structures (Fig. 5). Haustoria were never seen beneath these abnormal secondary appressoria. Rarely, epicuticular hyphae exhibited a localised proliferation of lateral lobes (not shown), but it is unclear whether these structures represent secondary appressoria. In addition to bifurcated appressorial tips, the tips of epicuticular hyphae were also sometimes bifurcated (Fig. 6). DAPI staining revealed that large clusters of fungal nuclei were located close to the tips of epicuticular hyphae, while simultaneous staining with diethanol showed that these clusters of nuclei were not associated with septa (results not shown). Hyphal tips were frequently associated with globules of a material that sometimes appeared to spread outwards over the plant surface as a thin film and is frequently associated with collapse of the subtending hyphae (Fig. 6). The globules lacked a cell wall but stained positively with DAPI, acridine orange, Nile red and fluorescein isothiocyanate, indicating the presence of numerous nuclei, RNA, lipids and proteins respectively\(^{10,11}\) and suggesting that these globules comprised leaked cytoplasmic material. Similar globules of material were sometimes associated with bifurcated appressoria (results not shown).

### 3.3 Effects on other powdery mildews

Treatment of other powdery mildews, including *E. pisi* and *U. necator*, with eBZO resulted in morphological and developmental effects generally similar to those seen following treatment of the cereal mildews. Protectant treatments blocked fungal development after formation of the primary appressoria, and no surface hyphae were observed. Appressorial morphology (size, shape and number of lobes) was more complex and variable in control treatments than that seen in *Blumeria*, so conclusions about eBZO effects on appressorial development were difficult to draw. Curative treatments against *E. pisi* and *U. necator* produced bifurcated hyphal tips, hyphal collapse and release of cytoplasmic globules similar to those seen in Bgh.
3.4 Effects on sporulation
In B. graminis, chains of conidiospores are borne on a swollen basal cell (Fig. 3B). DAPI and diethanol staining of untreated Bgh showed that the conidia were uninucleate and separated by brightly stained, regularly spaced septa. After curative treatments with eBZO, the mildew formed basal cells of normal morphology, but subsequent formation of conidia was disrupted. Instead, the basal cells gave rise to elongating tubes of more or less uniform diameter that were either aseptate or showed sparse, irregular septation (Fig. 7A). DAPI staining of nuclei within the malformed conidial chains showed that the cells often contained several nuclei (data not shown), and where a swollen apical cell was present (resembling a spore), it was always multinucleate.

In contrast to Blumeria, where repeated cell divisions produce chains of conidia borne on a swollen basal cell, E. pisi and U. necator undergo pseudoidial sporulation, in which a single conidium is borne on a much thinner basal cell. In these fungi, treatment with eBZO disrupted sporulation in a qualitatively different manner: curative treatment of infected pea or grape leaves resulted in production of conidiophores of uniform diameter, sometimes dramatically elongated, but without formation of the terminal spore (Figs 7B and 7C).

3.5 Effects on Aspergillus nidulans
Interestingly, despite the relatively narrow disease control spectrum, and in striking contrast to other powdery mildew-specific fungicides such as quinoxyfen, BZOs also produced morphogenetic effects in A. nidulans. While Aspergillus lacks specialised infection structures, and no effects of BZO on its vegetative growth were noted, dramatic effects were seen on asexual sporulation. In Aspergillus, as in Blumeria, a swollen basal or mother cell (vesicle) provides the base upon which conidiation occurs. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia. In contrast to Blumeria, however, Aspergillus conidia are not borne directly on the basal cell. Rather, two cell divisions forming additional cells (metulae and phialides) occur, with the characteristic conidial chains formed as the phialides divide to generate individual conidia.

4 DISCUSSION
Early research into the mode of action of a new chemical series during discovery and development occurs in several phases. As is commonly the case where initial evaluations are based on whole-plant disease control activity, the first studies into the BZO mode of action looked both for activity at known commercial MOAs and for cross-resistance with pathogen strains showing reduced sensitivity to existing fungicides. These experiments did not demonstrate any activity at known fungicide target...
Disease control through novel effects on fungal development

Figure 8. Effects of BZO treatment on sporulation of Aspergillus nidulans. (A) DIC image of A. nidulans showing the vesicle, metulae, phialides and only a few conidia. (B) DAPI staining showing nuclei in same field.

sites or any evidence of cross-resistance. These results were seen as indicating a potentially novel MOA and hence were viewed as positive indicators for subsequent fungicide development. Such studies are commonly undertaken early in the discovery process, since the experiments can be completed quickly and relatively inexpensively and their results are critical for decisions on compound advancement. However, subsequent biochemical or genetic studies to identify the precise molecular site of action of a novel fungicide chemistry are both substantially more time- and resource-intensive as well as more technically complex, especially for obligate biotroph-specific chemistries such as the benzophenones.

A complementary approach that is equally applicable to obligate biotrophs as well as other plant pathogens and which provides direct information on the biological effects (sensu lato) of novel fungicide candidates is that of microscopic observation of compound effects on fungal growth and the infection process. While not providing detailed biochemical or genetic information about a compound’s activity, observational studies can be invaluable for identifying those aspects of a pathogen’s growth, development or pathogenicity that are affected by fungicide treatment. This information can be used to better understand the broad biological activity of the compound and thus potentially help fine-tune application methodology, formulation or other practical aspects of fungicide use. It may also suggest general classes of compound activity (spore germination, vegetative growth, morphogenesis, etc.) that can be subsequently investigated using biochemical or genetic approaches. In some instances the effects observed may even suggest specific processes or pathways for detailed study.

The data presented in this paper show that the early benzophenone analogue eBZO has a range of effects on mildew development. Its morphogenetic effects are unique and thus distinct from those seen following application of other fungicides, including strobilurins, sterol C-14 demethylase inhibitors, quinoxyfen and hydroxypyrimidines, but are qualitatively the same as were seen subsequently with the commercialised benzophenone metrafenone.

The range of effects that microscopy reveals corresponds well with the broad application window (protectant, curative, eradicative and antisporeulant) found with early benzophenone fungicide candidates in disease control testing in the greenhouse. Multiple morphogenetic effects on vegetative growth, infection structure differentiation and asexual sporulation were seen.

Some of the most striking effects of eBZO treatment are the multiple morphogenetic abnormalities observed in both primary and secondary appressoria, which are accompanied by impaired appressorial function, i.e. reduced penetration of host epidermal cells and establishment of intracellular haustoria. Abnormal appressorial structure included atypical swelling with increased numbers of lobes, and apparent loss of cell polarity regulation, which resulted in bifurcated lobes and unusual, multiply branched structures. Similar effects on cell polarity control were seen during vegetative growth of epicuticular hyphae, including anomalous hyphal tip bifurcation and subapical proliferation of lateral lobes. The structural integrity of the apical cell wall also appears to be compromised, as evidenced by the leakage of cytoplasmic droplets from hyphal tips, which is often accompanied by hyphal collapse. Cytokinesis and nuclear positioning in epicuticular hyphae appear to be affected too, with many nuclei concentrated near hyphal tips. Older, subapical regions frequently showed uneven nuclear distribution.

Effects are not restricted to vegetative growth: sporulation is dramatically affected in both the cereal and dicot mildews as well as in A. nidulans, though the aberrant sporulation morphologies differ among species. These differential sporulation morphologies after BZO treatment are perhaps not unexpected, since normal sporulation morphologies also differ among the species.

Sporulation in the euoidial Bgh and Bgt begins with the differentiation of a swollen basal mother cell from the epicuticular hyphae; this process is apparently unaffected by eBZO. Normally, a series of well-coordinated nuclear divisions and septation events follows, adding a new spore to the basal cell and elongating the conidial chain. As the chain
elongates, developing conidia containing a single, well-formed nucleus pinch in at the septum and eventually separate. eBZO substantially disrupts this process: nuclear division continues and the basal cell elongates, but nuclei are distributed unevenly along the tube, and septation occurs irregularly and appears to be incomplete. Instead of the regular divisions seen in control treatments, an unevenly septate tube is formed, with each compartment containing a different number of nuclei. No discrete conidia are produced.

In the pseudomidal mildews (U. nectar and E. pisi), where a single terminal spore is normally borne at the tip of a narrow basal cell, eBZO blocked the differentiation of the terminal spore. In the absence of differentiation of this terminal conidium, the subtending basal cell continued to elongate, resulting in a dramatically elongated structure.

In contrast to the sporulation defects seen in the euoidal and pseudomidal mildews, initial events in asexual sporulation in the non-pathogen A. nidulans appear unaffected by BZO. Formation of conidiophores, vesicles, metulae and phialides all appear normal. However, ultimate conidial production is nonetheless blocked in Aspergillus, as is seen in each of the four plant pathogens examined. Hence, although the sporulation structures and developmental sequences are fundamentally different among the several fungi examined, there appears to be a common effect of BZO across fungal species on formation of uninucleate, correctly differentiated conidiospores.

Control of conidiation in A. nidulans has been studied extensively, in marked contrast to the relative dearth of knowledge about sporulation in the powdery mildews. In Aspergillus, conidiation is controlled by a core regulatory pathway involving interacting fluffy, bristle, abacus and wet genes. In addition, other genes such as apsA and apsB (anucleate primary sterigmata) also modify the morphology of the Aspergillus conidial development. The effects of mutations in the different regulatory genes are well characterised, with fluffy mutants producing a proliferation of aerial hyphae, and bristle mutants leading to extreme elongation of the Aspergillus foot cell without formation of the swollen vesicle and subsequently differentiated sporogenous cells. Abacus mutants exhibit a failure of conidial differentiation from the proximate conidiogenic cells (phialides), producing a chain of incompletely septate cells that resemble the ancient counting instrument. WetA mutants develop a faulty cell wall structure in conidia, resulting in the leakage of cellular (conidial) contents and a wet-appearing phenotype. The apsA and apsB mutants are both defective in nuclear positioning, with inappropriate nuclear distribution in vegetative tissues and failure of the nuclei to move from the vesicle into the metulae (primary sterigmata). As such, while the effects of eBZO on sporulation morphology of the pathogenic fungi are not identical among the four species and do not correspond to any single Aspergillus mutant, many of the effects in individual species have characteristics suggestive of one or another of the Aspergillus sporulation mutants.

Curative treatment of E. pisi and U. nectar with eBZO results in a conidial morphology superficially similar to that of the bristle mutation in Aspergillus, where conidiation is blocked and the subtending stalk elongates dramatically. In eBZO-treated Blumeria, conidiophores somewhat resemble those in the abacus mutation of Aspergillus in that mother cells fail to successfully differentiate individual conidia, producing instead an elongate tube with incomplete septation and uneven nuclear distribution. eBZO treatment also results in leakage of the cellular contents as in the case of the WetA mutation, although this is seen from Blumeria surface hyphae, not from the conidia. Nuclear positioning, the primary defect in apsA/apsB mutants, is defective in both Blumeria conidiophores and vegetative hyphae.

The range of effects seen across the four pathogens and Aspergillus has several interesting ramifications. The first is that the clear BZO effects on Aspergillus sporulation provide a feasible means to dissect and clarify the cognate morphogenetic systems in powdery mildews. Aspergillus can be easily cultured in vitro, is amenable to genetic manipulation and has a large number of well-characterised mutants, particularly regarding regulation of asexual sporulation. These effects on Aspergillus sporulation may enable identification of the BZO target site in the model species. However, a second aspect of the data presented here, critical to using information gleaned from studies with Aspergillus to understand the effects on the pathogens, is that the fungal morphologies resulting from BZO treatment differ between the pathogens and Aspergillus as well as among pathogens. That is, even though, as discussed above, there appear to be common themes in the morphogenetic effects, it appears that the steps in the signal transduction chains downstream from the BZO target site or the morphological effects they regulate may diverge somewhat amongst the several fungal species examined. These interspecies differences in morphogenesis make interpretation of BZO regulation of sporulation in pathogens and model systems both more challenging and more interesting.

The effects of eBZO on appressorial development and conidiation in powdery mildews also potentially provide a new perspective on the activity of the phenoxyquinoline fungicide quinoxyfen on mildew morphogenesis. Quinoxyfen, a fungicide specific for powdery mildew, controls disease primarily by interfering with appressorial differentiation. Scanning electron micrographs (SEMs) of powdery mildews treated with quinoxyfen show that formation of the appressorial hook is blocked following protectant quinoxyfen treatments, thus stopping host infection. Recent studies have identified several signalling pathway protein kinases involved in powdery mildew germination and appressorial hook formation. RT-PCR of mildew germlings treated with quinoxyfen shows altered accumulation of transcripts of several
signal transduction genes. Differential display RT-PCR comparing wild-type and quinoxyfen-resistant mutants of Bgh also identified a transcript of the Ras-type GTP-binding protein. Ras and other GTP-binding proteins have been implicated in a number of important process defects, including germination of A. nidulans spores and hyphal proliferation and conidiation in Colletotrichum.

While both eBZO and quinoxyfen interfere with normal development in Blumeria during early infection, the biological effects of these two chemistries appear quite different. In contrast to the multiple eBZO effects involving many developmental stages of the Blumeria infection process and sporulation, quinoxyfen effects seem largely limited to blocking the hooking of the primary appressoria. While the activities of these two fungicide classes sometimes appear to affect similar processes, the effects generally appear to be in opposite directions. For example, benzophenones stimulate appressorial lobe development and proliferation while quinoxyfen blocks this step. Also, quinoxyfen-resistant mildew strains exhibit defects in conidiophore production and sporulation that are somewhat reminiscent of the sporulation defects seen in wild-type (BZO-sensitive) Blumeria after BZO treatment (Fig. 7). Comparative study of the contrasting activities of these chemistries could be very useful in clarifying pathogen responses and the significance of the multiple signalling pathways in mildew. Although the precise molecular targets for either the benzophenone or the phenoxyquinoline fungicides are currently unknown, it appears likely from the data above and from the literature that a site in a signalling or regulatory pathway is affected in either case. Interestingly, in unrelated work that was published after the research for this paper was completed, a benzophenone isolated from an unknown fungal species and structurally distinct from either eBZO or metrafenone was suggested to modulate Ras protein activity by inhibiting its farnesylation by a recombinant human farnesyl protein transferase. Potential inhibition of Ras protein farnesylation by eBZO has not been examined.

The novel fungicidal activities for the phenoxyquinolines and benzophenones were each discovered in whole-plant disease prevention tests. Such greenhouse assays are notoriously resource-demanding. However, whole-plant disease protection tests do have the frequently overlooked benefit of being able to identify useful fungidal activity at new antifungal target sites, often before the sites themselves are precisely known. They may also identify useful disease control where there are significant taxon-specific differences in target–inhibitor interactions, such as for materials with antifungal activity specific to a limited class of pathogens, as in this case for the powdery mildews.

The economics of the current crop protection marketplace puts stringent constraints on the discovery process, forcing increased research efficiencies and driving research organisations to explore the benefits of cutting-edge ‘omics’ technologies (genomics, proteomics, metabolomics, etc.). Within these constraints, though, the benefits and role of traditional whole-organism testing to identify and validate novel, effective MOAs for disease control should not be ignored. Indeed, pharmaceutical researchers, traditionally in the forefront of utilising advanced technologies for discovering biologically active molecules, appear now to be emphasising use of integrated, whole-organism approaches (‘systems biology’) for discovery purposes. Recently, a pharmaceutical researcher (James Stevens, Lilly Research Laboratories) has noted that ‘identifying mechanisms of drug action … linked to modulation of complex signalling pathway can only be determined from the examination of intact multicellular organisms’ (see http://drugresearcher.com/news/newsng.asp?n=59354-drug-researchers-adopt, accessed 15 April 2005). The challenge, then, for research organisations seeking to implement efficient programmes to discover new agents for crop protection is to capture the inherent benefits of traditional whole-organism greenhouse testing while exploiting the complementary advantages of cutting-edge technologies in order to provide the needed tools for agricultural production.

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NOTE ADDED IN PROOF

Harris, S.D. and Momany, M. (2004. Polarity in filamentous fungi: moving beyond the yeast paradigm. Fung Genet Biol 41, 391–400) review the literature relating to signal transduction pathways controlling morphogenesis in yeast and filamentous fungi. Their discussion of the Ras and Rho (including Cdc42) families of small GTPases, their regulators and downstream interactions (including actin) provides a useful framework within which to consider the potential mechanisms of morphogenetic control exerted by the benzophenones and quinoxyfen.

REFERENCES
