12. Towards a molecular understanding of Mycosphaerella/banana interactions - Balint-Kurti, P.* and A.C.L. Churchill**

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Abstract

A critical understanding of the molecular factors required by *Mycosphaerella* pathogens of banana to parasitize their hosts is needed to devise novel methods for control of these diseases. As a first step towards developing a molecular 'tool-box' to study pathogenicity and virulence factors of *M. fijiensis*, *M. musicola*, and *M. eumusae*, we developed a DNA-mediated transformation system, which confers on transformants stable resistance to the selective agent hygromycin B and constitutive expression of green fluorescent protein. These markers serve as a valuable means for detecting and monitoring transformed isolates *in planta* - on hosts or non-hosts - in the laboratory or greenhouse. We have also examined biological factors that may affect the ability of these fungi to infect their plant hosts. We provide results suggesting the presence of self-inhibitors of germination in vegetative spores, describe an assay used to detect effects of fungal phytotoxins on banana suspension cultures, and characterize fungal resistance to oxidative stress, specifically caused by singlet oxygen. As a result of these studies, we have gained a greater appreciation for the complexities of the interaction between *Mycosphaerella* pathogens and their banana hosts.

1. INTRODUCTION

Mycosphaerella fijiensis, the causal agent of the foliar fungal disease black Sigatoka, is the major worldwide constraint to banana and plantain (*Musa* spp.) production, and results in yield losses estimated at 33-76% [1]. Yellow Sigatoka disease, caused by the closely related species *M. musicola*, has been largely supplanted by black Sigatoka in many banana-producing areas, but remains a significant problem at higher altitudes and cooler temperatures [2]. The imperfect states of *M. fijiensis* and *M. musicola* are of the *Paracercospora* and *Pseudocercospora* types, respectively [3,4], although Stewart et al. [5] have suggested reducing *Paracercospora* to synonymy with *Pseudocercospora*. Recently, a *Musa* pathogen with a *Mycosphaerella* teleomorph and an anamorph of the *Septoria* type was identified [6]. This species, called *M. eumusae*, causes Septoria leaf spot, a disease with symptoms similar to those of black Sigatoka.

Previous studies [e.g. 7-13; reviewed in 1] indicate that the methods of pathogenesis used by *M. fijiensis* and *M. musicola* are broadly similar. The symptoms they cause and the structures of their conidia and conidiophores, as well as molecular techniques, are used to differentiate them [4,5,9,10,14,15]. *In vitro* production of several fungal phytotoxins by *M. fijiensis* and *M. musicola* has been reported [16-20], but the role that such compounds play in the disease is unclear.

Cultural, morphological, and pathogenic characteristics of the *Mycosphaerella* pathogens of banana are summarized in Carlier et al. [1].

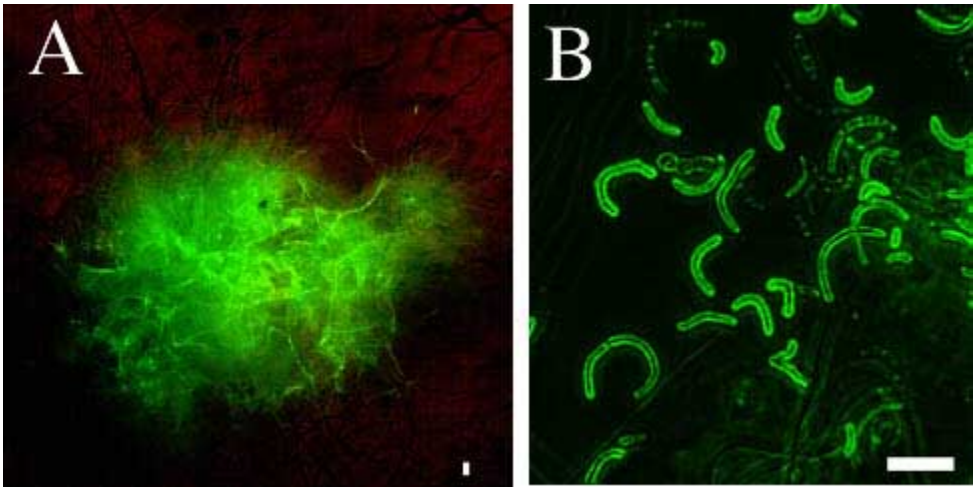
Knowledge of the mechanisms used by *Mycosphaerella* pathogens to parasitize *Musa* remains inadequate. Our primary goal is to understand the mechanisms of pathogenicity and virulence of *Mycosphaerella* pathogens of banana at the molecular level. Towards this end, we have developed a genetic transformation system for these pathogens, and have used transformants expressing green fluorescent protein (GFP) to study and compare growth of the pathogens on a compatible host [21]. We have also investigated other aspects of the biology of these fungi, such as self-inhibition of spore germination, the phytotoxicity of fungal extracts, and fungal resistance to oxidative stress. In this chapter, we document our progress in these efforts and discuss future directions.

2. RESULTS

2.1. Development of a transformation system for *Mycosphaerella* pathogens of banana

Methods were developed to enable the transformation of *M. fijiensis, M. musicola* and *M. eumusae* [21]. Isolates of each species were transformed with a construct carrying a gene encoding green fluorescent protein (GFP) [22] and a gene conferring Hygromycin B resistance. Transformants of each species expressed GFP constitutively (Figure 1) and were used to examine and compare infection processes on the susceptible banana cultivar Grande Nain (Figure 2). All observations were made with a fluorescence microscope using FITC fluorescence (excitation range 455-490 nm; 515 nm long pass barrier/emission filter; dichroic mirror wavelength of 500 nm). For all three species, the process of infection appeared similar, except that symptoms developed faster and were more severe on bananas infected with *M. fijiensis* and *M. eumusae* than with *M. musicola* strains. We hypothesize that this was because incubation conditions for *M. musicola* were not optimal, i.e. incubation temperatures were higher than typically found in natural infections. Leaf penetration was exclusively via stomata (Figure 2A), often with the formation of hyphal swellings (stomatopodia) above the stomata prior to penetration (Figure 2B-E). Hyphae grew between rather than into cells. All three species grew extensively within leaf tissue that eventually turned necrotic, at which point they grew saprophytically on the dead tissue (Figure 2F). Additionally, leaf necrosis and chlorosis was often observed in advance of saprophytic growth of the mycelium on necrotic tissue, suggesting secretion of a phytotoxin (Figure 2G). This work has been reported in greater detail by Balint-Kurti et al. [21].

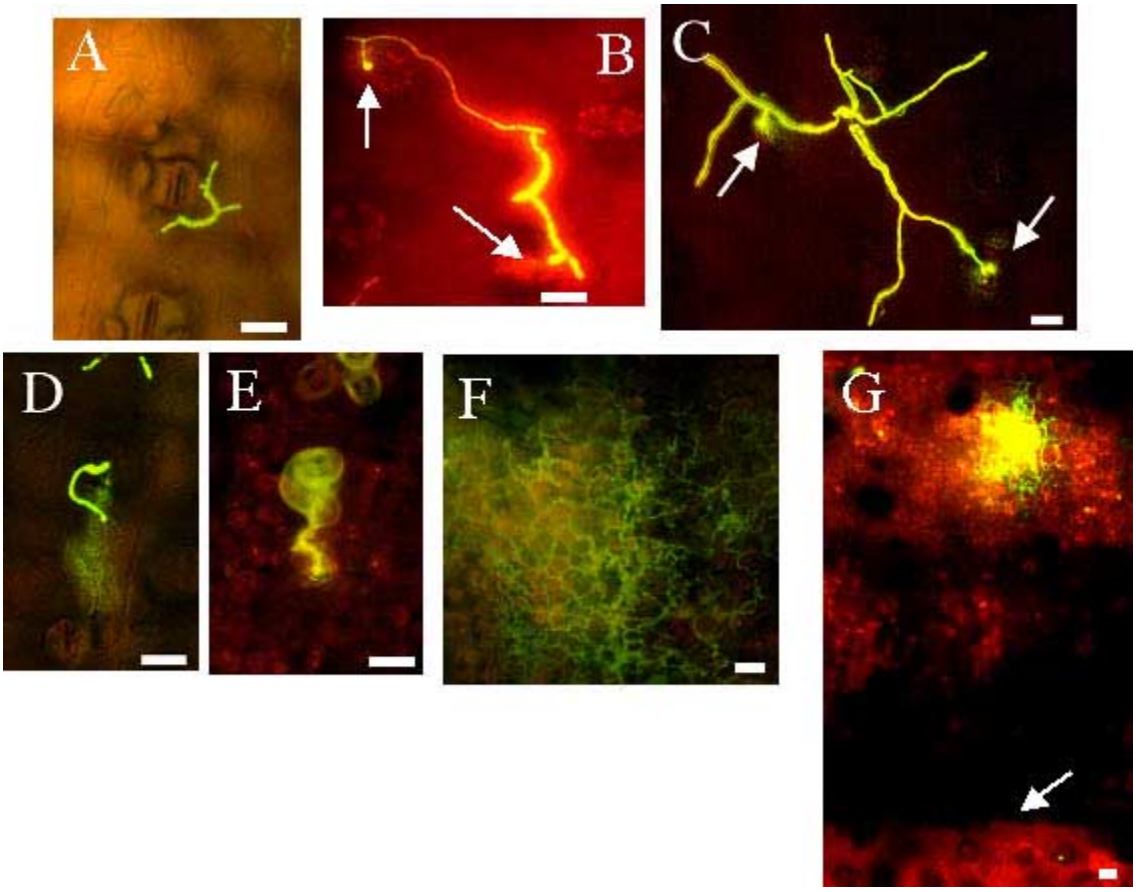
Figure 1 *Mycosphaerella* spp. expressing green fluorescent protein. (A) *M. fijiensis* transformant grown in regeneration agar with hygromycin B selection. (B) Conidia brushed from the mycelium of a *M. eumusae* transformant. The white scale bar in each photograph represents 25 µm. Reprinted with permission from Balint-Kurti, P.J., May, G.D. and Churchill, A.C.L., FEMS Microbiology Letters, 195, 9-15. Development of a transformation system for *Mycosphaerella* pathogens of banana: a tool for the study of host/pathogen interactions. © 2001 Elsevier Science.



2.2. Growth of *M. fijiensis* and *M. eumusae* on non-host species

Many fungi have been reported to form appressoria with equal frequencies on susceptible, resistant, and non-host plant species [23-26]. However, in most instances, further fungal development is arrested prior to, or just after, entrance into the resistant or non-host plant. With the exception of some well-documented examples [27-30], plant non-host resistance remains poorly understood. It is clear from the published literature that several qualitatively different mechanisms are involved in non-host resistance, depending on the specific interaction.

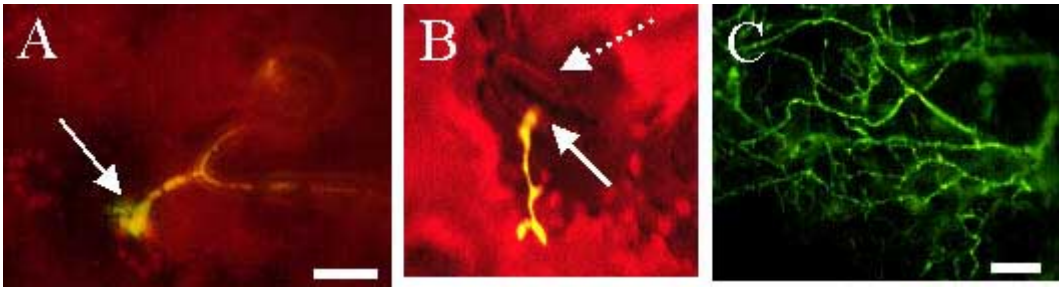
Figure 2 GFP-expressing *Mycosphaerella* spp. observed under FITC fluorescence. (A) & (B) *M. fijiensis* conidia germinating on the underside of a banana leaf; arrows indicate stomatopodia. (C) *M. eumusae* growing on a leaf surface 3 weeks post-infection (PI); arrows indicate stomatopodia. (D) & (E) *M. eumusae* leaf penetration via a stoma 3 weeks PI. Both pictures show the same field. In D, hyphal growth on the leaf surface is in focus and the under surface of the leaf is out of focus; in E the reverse is the case. (F) Extensive growth of *M. fijiensis* within the leaf at 4.5 weeks PI. (G) The border between necrotic and green tissue (indicated by an arrow) on a plant infected with *M.* eumusae, 5 weeks PI. An *M. eumusae* transformant is growing about 250 µm inside the necrotic area. The white scale bar in each photograph represents 25 µm. A, C, D, E, and G reprinted with permission from Balint-Kurti, P.J., May, G.D. and Churchill, A.C.L., FEMS Microbiology Letters, 195, 9-15. Development of a transformation system for *Mycosphaerella* pathogens of banana: a tool for the study of host/pathogen interactions. © 2001 Elsevier Science



To examine growth of *Mycosphaerella* banana pathogens on non-host species, plants of the tomato cultivar Moneymaker and the tobacco cultivar Sampson were inoculated with *M. fijiensis* and *M. eumusae* transformants expressing GFP. Conidia of both species germinated and formed stomatopodia over the stomata of both non-host plants (Figure 3A and 3B). Subsequent stomatal penetration was not observed. However, abundant growth of an *M. eumusae* transformant was observed in a large necrotic spot on an inoculated tobacco leaf approximately 6 weeks post-inoculation (Figure 3C). In the absence of observed stomatal penetration on tobacco, it is likely that this growth represented saprophytic growth on already dead or severely senescent tissue.

The ability of *M. fijiensis* and *M. eumusae* to locate stomata on the very distinct surfaces of banana, tobacco and tomato leaves indicates that these fungi do not rely upon host-specific topographical features of the leaf surface to guide them to the stoma, as is the case in some other plant-pathogen interactions [31,32; reviewed in 33]. The failure of the pathogens to proceed beyond this point on the non-hosts is intriguing. Localized browning of tomato leaf cells directly under stomatopodia (Figure 3A), which resembled a classical hypersensitive defence response, was observed at a few sites. However, on tobacco and in most cases with tomato, no such response was observed.

Figure 3 Growth of *M. eumusae* transformants expressing green fluorescent protein on non-host plants. (A) Formation of a stomatopodium (arrow) 2 weeks post-inoculation over a stoma on the underside of a tomato leaf. (B) Formation of a stomatopodium (solid arrow) 2 weeks post-inoculation over a stoma (dashed arrow) on the underside of a tobacco leaf. (C) Saprophytic growth of a transformant on necrotic tissue of tobacco approximately 6 weeks post-inoculation



In the interactions documented here, it is possible that the pathogen needs a host signal, which is absent in non-host plants, to progress to the next stage of pathogenesis. Determination of the existence and identity of this signal would be of great value for understanding the pathogenicity mechanisms of these and other fungal plant pathogens. Alternatively, if one could demonstrate an active defence response in the non-host species, it might be possible to isolate genes involved in triggering this response from a genetically tractable non-host and use them to confer black Sigatoka resistance in transgenic banana.

2.3. Self-inhibition of spore germination

Spores of many fungi are inhibited from germinating when in a dense suspension or crowded upon a surface. Germination inhibition is readily lifted when the spores are dispersed. This phenomenon, known as self-inhibition of germination, ensures that spores do not germinate in the sorus or under conditions of intense intra-species competition. Germination inhibitors from several plant pathogenic fungi have been identified [34-36]. Typically, these chemicals are small, variably water-soluble, organic compounds found on the spore surface. They are active at nanomolar levels and are often specific to a single species or to a group of closely related species. Genes involved in the production of germination self-inhibitors remain unidentified, and the mode of action of these self-inhibitors is not well understood. Liu and Kolattukudy [37] recently determined that spore germination self-inhibitors of the rice blast fungus *Magnaporthe grisea* inhibit expression of the calmodulin gene, which is believed to be associated with appressorium formation by the fungus.

Initial *in planta* observations using transgenic conidiospores expressing GFP suggested that *M. fijiensis*, *M. eumusae* and *M. musicola* spores germinated well on the leaf when at low concentrations (Figure 2A and B) but that germination frequency was significantly inhibited at high spore concentrations (Figure 4A). *In vitro* spore germination assays displayed a similar phenomenon (Figure 4B and C; Figure 5). At high spore densities germination was inhibited, while at low spore densities this inhibition was lifted. We reasoned that, if a self-inhibitor existed, it might be possible to wash some or all of the self-inhibitor from the spore surface and produce higher frequencies of spore germination at high spore densities. Aqueous spore suspensions were prepared (~10^7 spores/ml) and divided into two aliquots. Half of the spore suspension was plated at serial fivefold dilutions while the other half was washed four times in large volumes of water and then similarly plated. The washed spores of both *M. eumusae* and *M. fijiensis* germinated at a higher frequency than the unwashed spores over a range of dilutions (Figure 5) implying that a self-inhibitor had been removed from the spore surface. It is likely that some self-inhibitor remained on the washed spores, as they still did not germinate well at very high concentrations. These experiments suggest the existence of a self-inhibitor of spore germination in these species.

Figure 4 Spore density affects the ability of conidia of *Mycosphaerella* spp. to germinate. (A) High density of ungerminated *M. musicola* conidia on a banana leaf 2 weeks post-inoculation. (B) *M. fijiensis* spores plated at 6.5 × 10^6 spores/ml remain ungerminated 2 days after plating (spores were diluted just prior to being photographed). (C) Identical

spores to those in (B) were diluted to a 25-fold lower concentration prior to plating and had germinated by two days later

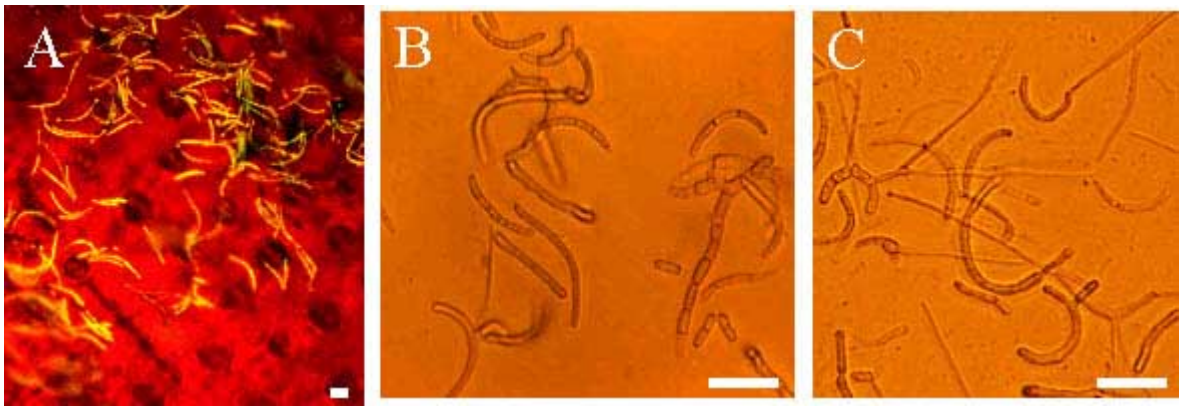
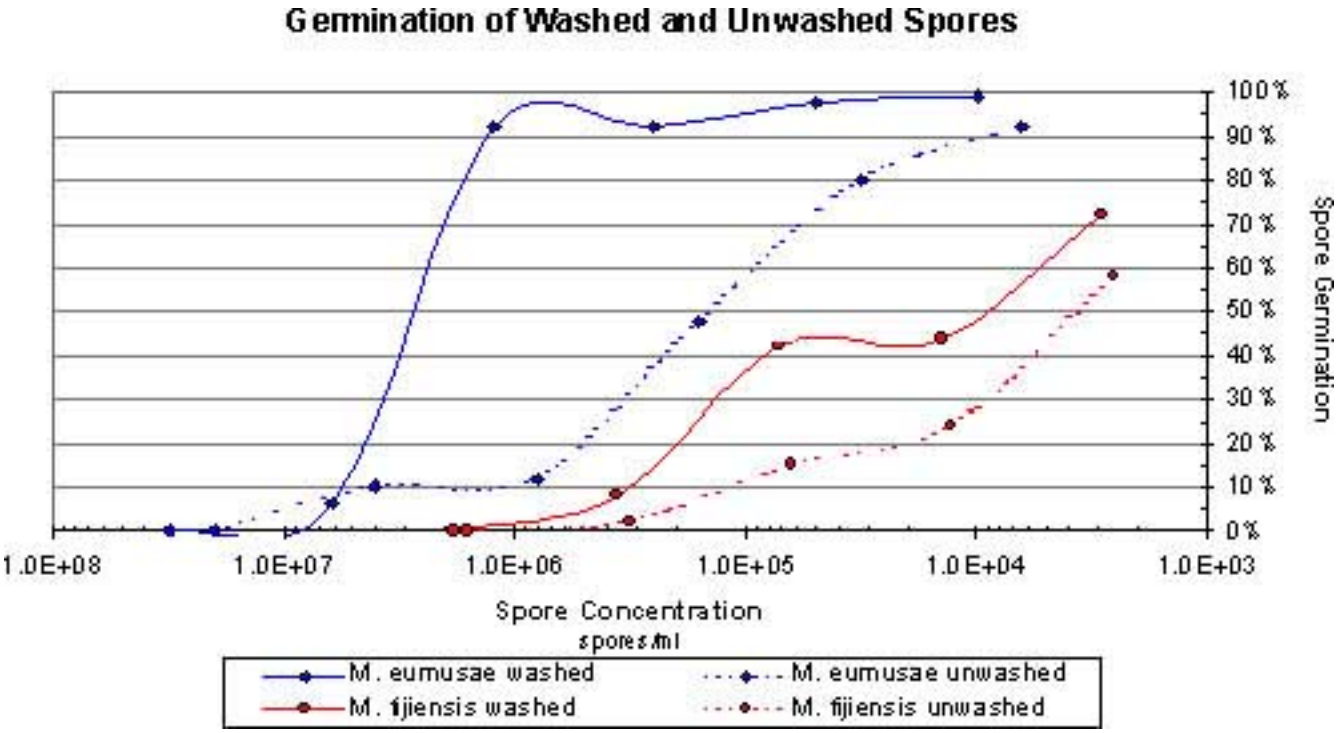


Figure 5 Spores of *M. fijiensis* and *M. eumusae* were plated in water at high densities and at a series of fivefold dilutions. Concurrently, aliquots of untreated spores were washed in water to remove putative germination inhibitors and plated in the same way as the untreated spores. After approximately 24 h, the percentage of germinated spores for each treatment was determined



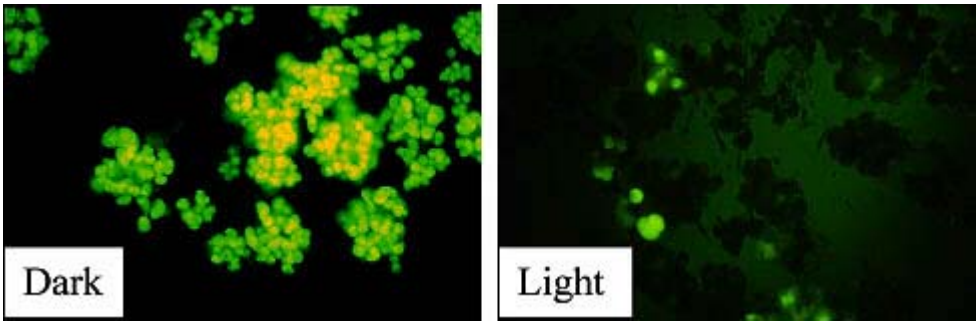
2.4. Light-activated phytotoxins of *Mycosphaerella musicola*

The asexual stages *M. fijiensis* and *M. musicola* are in the closely related *Paracercospora* and *Pseudocercospora* genera, respectively [3-5]. Many fungal plant pathogens in the genus *Cercospora* [38] produce the non-specific toxin cercosporin. When activated by light, cercosporin causes the production of activated oxygen species, particularly the highly toxic singlet oxygen. Although it has been suggested that cercosporin production is limited to fungi within the

Cercospora genus [4], it is possible that related species produce toxins with similar properties. Cercosporin production has not been reported in *M. fijiensis* or *M. musicola* [4; M. Jin, J. Clardy, P. Balint-Kurti, and A.C.L. Churchill, unpublished data].

It has long been observed that the symptoms of black and yellow Sigatoka are ameliorated in plants grown in the shade [1,39-41], suggesting the production of light-activated toxins by these fungi. Indeed, there are several references in the literature suggesting that *M. fijiensis* and *M. musicola* produce toxins that are phytotoxic in *in vitro* assays [16-20] though the exact role such toxins play *in planta* remains unclear. In collaboration with Dr. Jon Clardy's group (Dept. of Chemistry and Chemical Biology, Cornell University), we identified fractions of fungal extracts of *M. musicola* that were phytotoxic and exhibited light activation (Figure 6). Others have also detected light-activated toxins associated with these pathogens (see Chapter 14 by J.-P. Busogoro in this volume). It is only through the use of genetic techniques combined with analytical chemistry that we will definitively determine how any one toxin or family of toxins contributes to the virulence of the *Mycosphaerella* pathogens of banana.

Figure 6 An extract from an *M. musicola* shaking culture was added to banana cell suspensions. After 4 h in the light or dark, the vital dye Fluorescein Diacetate was added. Living cells stained green, whereas dead cells were no longer fluorescent



2.5. Resistance of *Mycosphaerella* pathogens of banana to oxidative stress

Although the *Mycosphaerella* pathogens of banana have not been reported to produce cercosporin [4; M. Jin, J. Clardy, P. Balint-Kurti, and A.C.L. Churchill, unpublished data], there is evidence for the production of light-activated toxins by *M. fijiensis* (see Chapter 14 by J.-P. Busogoro in this volume) and *M. musicola* [42; Section 2.4]. We reasoned that if these fungi have the potential to produce light-activated toxins that could cause oxidative damage to plant cells, then they must also have the ability to resist such toxins. We tested isolates of *M. fijiensis* (seven isolates), *M. musicola* (two isolates), and *M. eumusae* (three isolates) for relative levels of resistance to eight photosensitizer dyes, following the general approaches of Daub [43]. Each dye produces highly toxic, membrane-damaging singlet oxygen upon exposure to light. Not all fungi are resistant to singlet oxygen-generating dyes [43].

Conidia of each fungus were suspended in water at a concentration of approximately 250 spores per microlitre. For each isolate at each concentration of dye, an aliquot of 20 ml (approximately 5000 spores) was transferred to triplicate wells in 12-well microtiter dishes. Each well contained 1 ml Potato Dextrose Agar (PDA) containing one of the following photosensitizer dyes at various concentrations: Toluidine Blue (0,10,50,100 mM), Methylene Blue (0,10,50,100 mM), Erythrosin B (0,1,5,10 mM), Rose Bengal (0,1,5,10 mM), Phloxine B (0,1,5,10,100 mM), Eosin Y (0,1,5,10,100 mM), Cercosporin (0,1,5,10 mM), and Hematoporphyrin (0,1,5,10 mM). Appropriate volumes of organic solvent used for dissolving the dyes were added to control media. Duplicate plates of each treatment were prepared and incubated in the dark overnight. We found it necessary to germinate the spores in the dark after plating, since direct exposure to high light levels at that

time killed the spores. The next day, one set of duplicate plates was incubated at 20ºC under cool white fluorescent light (70 $mE \cdot m^{-2} \cdot sec^{-1}$), and the second set was incubated in the dark at 20ºC. All plates were examined for relative growth at approximately one-week intervals for 3 weeks. Plates were examined under a dissecting microscope, and at each evaluation, a qualitative rating of 0 (no germination), 1 (germinated spores), 2 (good hyphal growth), or 3 (extensive hyphal growth) was given. A rating of '2' or '3' suggested a degree of resistance at a particular concentration, although the growth rate may have been slowed in comparison with that on the same concentration of dye in the dark or on nonamended medium. Spores that germinated within the first week (rating of '1') but failed to grow thereafter were deemed sensitive to the dye at that concentration.

All isolates grew extensively at all concentrations of photosensitizer dyes assayed when incubated continuously in the dark. In the light, variability in relative resistance to the photoactivated dyes was observed between isolates of the same species (Table 1). Interestingly, all isolates were resistant to photoactivated cercosporin at the highest concentration tested (10 mM), which is sufficient to inhibit the growth of several other unrelated fungi [43]. In contrast, relative resistance to the other photosensitizer dyes was variable, depending on the isolate. Most *Mycosphaerella* isolates were as resistant as *Cercospora nicotianae* to the panel of photosensitizer dyes tested, with a few exceptions. Rose Bengal and Eosin Y were generally more toxic to the *Mycosphaerella* banana pathogens than to *C. nicotianae*. Additionally, a few *M. fijiensis* isolates (e.g. Mf 294, Mf 435, Mf 301) were notably more sensitive overall to some of the dyes than other isolates of the same species. Most isolates were quite sensitive to Erythrosin B and Rose Bengal with the exception of Mf 436, Mf 303, and Mm 90, which showed resistance to 1 mM Erythrosin B, and Mm 90, which was resistant to 1 mM Rose Bengal. Although these data are not quantitative, we observed distinct variability among isolates of the same and different species in their responses to the panel of photoactivated dyes. Quantitative analyses, measured by enumerating the number of colonies surviving on each medium in both light and dark, might detect additional differences not shown here.

Oxidative burst, i.e., the release of active oxygen species (AOS), is one of the earliest responses in plant resistance to pathogen attack [44]. Relatively little is known about the range of mechanisms that fungi use to counteract AOS-mediated plant defences. However, it has been well documented that bacterial pathogens of plants and animals can suppress AOS-mediated defences by secretion of AOS-scavenging enzymes such as superoxide dismutase and catalase [45, 46]. There is growing evidence that some phytopathogenic fungi have the ability to actively suppress AOS-mediated plant defences nonenzymatically by quenching AOS via induced synthesis of mannitol [47]. It is likely that phytopathogenic fungi employ multiple mechanisms to defend themselves against AOS-mediated plant defences. Our preliminary data reported here demonstrate that the *Mycosphaerella* pathogens of banana are generally highly resistant to several potent singlet oxygen-generating compounds, but that variability in resistance to such dyes exists. These differences among strains may suggest variability in the response of pathogens to plant oxidative burst. Can overall resistance or sensitivity to potent photosensitising dyes give us clues that might predict the virulence of a particular isolate? Specifically, does enhanced resistance to photoactivated dyes generally confer on the pathogen greater resistance to AOS generated by the host plant? Are pathogens that exhibit reduced resistance to photosensitizer dyes generally less virulent on their plant hosts due to a deficiency in the ability to quench plant-produced AOS? By extension, is there variability between banana cultivars in the oxidative burst generated in response to pathogen attack?

Three distinct genes that confer resistance to cercosporin have been cloned from cercosporin-producing *Cercospora* sp. *CRG1* (Cercosporin Resistance Gene 1) appears to be a membrane protein but has no strong homology to any gene in the databases [48]. Under low stringency hybridization conditions, a *CRG1* homologue was not detected in genomic DNA of *M. fijiensis* [48], or of *M. musicola* and *M. eumusae* (P. Balint-Kurti and A.C.L. Churchill, unpublished data), suggesting that this gene is absent in these fungi. Therefore it is unlikely to play a role in

resistance of the *Mycosphaerella* pathogens of banana to cercosporin or other photosensitizer dyes. *SOR1* (Singlet Oxygen Resistance 1) [49], now known as *PDX1* [50], is a novel gene involved in *de novo* biosynthesis of vitamin B6 (pyridoxine), which confers resistance to cercosporin and other photosensitizer dyes. Unlike *CRG1*, *PDX1* is present in numerous plants, fungi, and bacteria [51]. The *CFP* (Cercosporin Facilitator Protein) gene is related to members of the major facilitator superfamily of integral membrane proteins involved in efflux of and resistance to both bacterial and fungal toxins [52]. Disruption of this gene caused a loss of cercosporin production in light-grown cultures and a 50% reduction in growth of *C. kikuchii* on medium containing exogenously added cercosporin. It is unknown whether homologues of *PDX1* or *CFP* are present in the *Mycosphaerella* banana pathogens. However, the presence of *PDX1* would not be surprising since it is involved in pyridoxine biosynthesis in many organisms. It has been suggested that the only cercosporin-resistant fungi so far identified are those that produce perylenequinone toxins such as cercosporin, phleichrome, altertoxin I, elsinochrome A, and hypocrellin A [51]. Our results might then imply that the *Mycosphaerella* pathogens of banana produce light-activated toxins of this type. However, until such a toxin can be identified, we can only speculate on this topic.

Table 1 Response of *M. fijiensis*, *M. musicola*, and *M. eumusae* to light-activated, singlet oxygen-producing dyes

CIRAD Isolate[a]	Photo-sensitiser[b]							
	TB(100)	MB(100)	EB(10)	RB(10)	PB(100)	EY(100)	C(10)	H(10)
Mf 294	R50[c]	S	S	S	S	R1	R	R5
Mf 301	R	R50	S	S	R5	R10	R	R
Mf 302	R	R	S	S	R10	R10	R	R
Mf 303	R	R	R1	S	R10	R10	R	R
Mf 435	R	R50	S	S	S	R5	R	R5
Mf 436	R	R	R1	S	R10	R10	R	R
Mf 722	R	R	S	S	R10	R10	R	R
Mm 90	R	R	R1	R1	R10	R10	R	R
Mm 318	R	R	S	S	R5	R10	R	R
Me 458	R	R	S	S	R5	R10	R	R
Me 485	R	R	S	S	R10	R10	R	R
Me 548	R	R	S	S	R1	R5	R	R
Cn[d]	R	R	NT	R	NT	R	R	R

[a] Mf = *M. fijiensis*, Mm = *M. musicola*, Me = *M. eumusae*. For each isolate, the same suspension of conidia was used for each dye tested

[b] TB = Toluidine Blue; MB = Methylene Blue; EB = Erythrosin B; RB = Rose Bengal; PB = Phloxine B; EY = Eosin Y; C = Cercosporin; H = Hematoporphyrin; the number in parentheses is the maximum concentration (mM) of dye tested

[c] R = resistant to the highest dye concentration (mM) tested in the light (indicated in parentheses at top of column); R# = resistant to the concentration of dye indicated and all lower concentrations tested in the light; S = sensitive to all concentrations of dye tested in the light.

[d] Cn = *Cercospora nicotianae*, data from [43, 53, 54]; NT = not tested

3. DISCUSSION

The above examples from our own work suggest several areas of research related to pathogenesis or virulence of the *Mycosphaerella* pathogens of banana that would benefit from further analyses. The establishment of a transformation system for these fungi, in which GFP is used as a reporter gene, is an important first step towards developing the tools to dissect the mechanisms of pathogenesis used by these fungi. The next challenge is to identify and characterize specific genes involved in pathogenicity and virulence.

Several genetic approaches have been employed successfully to gain an understanding of pathogenicity and virulence factors in plant pathogenic fungi. Forward genetic approaches include mutant complementation and insertional mutagenesis. In mutant complementation, an untagged mutation is used to isolate a gene by DNA-mediated transformation and restoration of a particular phenotype. A complementation approach generally requires an efficient transformation system for the pathogen of interest. For fungi with low transformation rates, gene isolation by insertional mutagenesis may be possible. In this method, a mutagenic plasmid is introduced randomly into the fungal genome, transformants are screened for altered phenotypes of interest, and genomic DNA flanking the mutagenic plasmid is recovered. Three approaches for insertional mutagenesis have been most successful to date and include Restriction Enzyme-Mediated Integration (REMI) mutagenesis, *Agrobacterium*-mediated integration, and Signature Tagged Mutagenesis (STM). Each of these approaches has been reviewed by Gold et al. [55]. We have taken initial steps in adapting REMI for use in *Mycosphaerella* banana pathogens [21], but further work is required to make this approach useful. A critical component of any insertional mutagenesis approach is the availability of an efficient system for screening large numbers of fungal transformants for alterations in pathogenicity or virulence. This is not an easy task in the *Mycosphaerella*/banana system since symptoms are often not visible for several weeks after inoculation, and there are logistical problems associated with cultivating, maintaining, and inoculating large numbers of relatively large banana plants. The most efficient system will likely be based upon use of an *in vitro* leaf piece assay [56].

Alternatively, reverse genetic approaches could be used, in which specific genes predicted to be involved in pathogenesis are cloned by homology using hybridization with genes from other organisms or PCR using degenerate oligonucleotide primers. Multiple genes involved in critical signalling pathways of phytopathogenic fungi (e.g. MAP kinase, cAMP-dependent protein kinase, and mating pathways) have been cloned in this manner [reviewed in 57]. Other types of pathogenesis genes that have been isolated using this approach are those for cell-wall degrading enzymes [58] and gene families encoding chitin synthases [59]. Alternatively, a protein of interest could be purified and the gene cloned by PCR, based on partial amino acid sequence or antibody screening [60]. A necessary component of a reverse genetics approach is an efficient means for targeted gene disruption by homologous recombination to confirm gene function [reviewed in 61]. Alternatively, homology-dependent post-transcriptional gene silencing techniques could be used. This approach has been of value in many organisms including animals, plants and fungi [62].

Finally, methods that rely on differential gene expression have been of great value in identifying pathogenicity and virulence genes in plant pathogenic fungi. In differential cDNA screening [63], subtractive cDNA hybridization [64], and more recently, suppression subtractive hybridization [65], it is possible to isolate genes that are specifically regulated during developmental changes that occur in the pathogen during infection, as well as at other times in the interaction of the pathogen with its host. Alternatively, differential display of mRNA, a PCR-based technique that generates bands visualized on sequencing gels, has been used to isolate genes that are expressed in phytopathogenic fungi only under specific growth conditions [66].

The *Mycosphaerella*/banana system is not an easy one with which to work, due to the slow growth of both the host and the pathogen and the difficulties associated with performing controlled infections. Black Sigatoka is, however, one of the most important worldwide plant

diseases and this, we believe, makes the study of the interaction at a molecular level imperative. We hope that by pursuing some of the approached outlined here we can make significant progress towards this goal in the next few years.

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