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FEMS Microbiology Letters 195 (2001) 9–15

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Development of a transformation system for *Mycosphaerella* pathogens of banana: a tool for the study of host/pathogen interactions

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Received 24 October 2000; received in revised form 30 November 2000; accepted 1 December 2000

Abstract

A genetic transformation system has been developed for three *Mycosphaerella* pathogens of banana and plantain (*Musa* spp.). *Mycosphaerella fijiensis* and *Mycosphaerella musicola*, the causal agents of black and yellow Sigatoka, respectively, and *Mycosphaerella eumusae*, which causes Septoria leaf spot of banana, were transformed with a construct carrying a synthetic gene encoding green fluorescent protein (GFP). Most single-spored transformants that expressed GFP constitutively were mitotically stable in the absence of selection for hygromycin B resistance. Transformants of all three species were pathogenic on the susceptible banana cultivar Grand Nain, and growth in planta was comparable to wild-type strains. GFP expression by transformants allowed us to observe extensive fungal growth within leaf tissue that eventually turned necrotic, at which point the fungi grew saprophytically on the dead tissue. Leaf chlorosis and necrosis were often observed in advance of saprophytic growth of the mycelium on necrotic tissue, which supports previous reports suggesting secretion of a phytotoxin.

Keywords: Fungal transformation; Plant disease; Green fluorescent protein; *Musa*

1. Introduction

Mycosphaerella fijiensis (anamorph *Paracercospora fijiensis*), the causal agent of the foliar fungal disease black Sigatoka, is the major worldwide constraint to banana and plantain (*Musa* spp.) production. Leaf necrosis caused by black Sigatoka results in yield losses estimated at 33–50% [1,2]. Yellow Sigatoka disease is caused by the closely related species *Mycosphaerella musicola* (anamorph *Pseudocercospora musae*). Although yellow Sigatoka has been largely supplanted by black Sigatoka in many banana-producing areas, it remains a significant problem at higher altitudes and cooler temperatures [3]. Septoria leaf spot of banana, caused by *Mycosphaerella eumusae* (anamorph *Septoria eumusae*) has been only recently described [4]. This pathogen appears to be the predominant agent causing Sigatoka-like leaf spots on banana in southern India, Sri Lanka, western Malaysia, Thailand, and Vietnam.

Previous studies (e.g. [5–11]) indicate that the methods of pathogenesis used by *M. fijiensis* and *M. musicola* are broadly similar. They are differentiated by the symptoms they cause and the structure of their conidia and conidiophores. Molecular techniques have been used to distinguish these species [12–14]. Stewart et al. (1999) [14] recently used rDNA sequence analyses to support reducing *Paracercospora* to synonymy with *Pseudocercospora*. In vitro production of several fungal phytotoxins by *M. fijiensis* and *M. musicola* has been reported [15–18], but the role such compounds play in pathogenesis is unclear.

Knowledge of the mechanisms used by *Mycosphaerella* pathogens to parasitize *Musa* spp. remains inadequate. This is due, in part, to the lack of molecular tools available to characterize pathogenicity and virulence factors of these fungi and an incomplete understanding of specific host/pathogen interactions. In this study, we describe methods for the genetic transformation of three *Mycosphaerella* banana pathogens and characterize the growth of transformants expressing green fluorescent protein throughout multiple stages of plant infection.

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0378-1097/01/$20.00
PII: S0378-1097(00)00537-1

2. Materials and methods

2.1. Sources of fungi, plants, and transformation vector

All *Mycosphaerella* isolates utilized in this study were obtained from CIRAD (Montpelier, France) and are referred to by their CIRAD accession numbers prefaced with an abbreviated species name (e.g. *Mf* 743 is the *M. fijiensis* CIRAD isolate 743). Grand Nain plants, obtained from Rahan Meristem (Rosh Hanikra, Israel), were used as compatible hosts. The plasmid gGFP [19], which was used for transformation of *Mycosphaerella* spp., carries the *sGFP*(*SER65T*) gene [20], which encodes GFP, and the *hph* gene conferring hygromycin B resistance. Both genes are under the control of the *Aspergillus nidulans gpd* promoter.

2.2. Preparation of spheroplasts and transformation

Mycosphaerella isolates were cultured on modified V8 agar [21] at 21°C under continuous fluorescent ('daylight') white light. For initiation of agar plate cultures, an approximately 1 cm^2 piece of 2 weeks old mycelium from a V8 agar plate was mixed with 750 μl water containing the equivalent of ~300 μl sterile glass beads (0.5 mm diameter) and homogenized in a BeadBeater™ (BioSpec Products, Inc., Bartlesville, OK, USA) for 5 s at 5000 rpm. An aliquot was spread over the surface of a V8 agar plate with a glass rod and incubated as above. For initiation of liquid shake cultures for spheroplast isolation, the same maceration procedure was used but the inoculum was transferred to 200 ml of potato dextrose broth (PDB) in a 500 ml baffled flask. After 10 days growth in shaking culture (150 rpm) under continuous fluorescent white light at room temperature, the mycelium was pelleted by centrifugation for 15 min at 5000×*g*, resuspended in ~50 ml of fresh PDB, ground in a Waring blender for 30 s, transferred to 200 ml of fresh PDB, and shaken overnight (18 h) at 180 rpm at room temperature under fluorescent light.

The procedure for spheroplast production was a modified version of a previously published protocol [22]. Cells were harvested by centrifugation (5000×*g*, 10 min) and resuspended in 100 ml osmoticum (0.7 M KCl, 0.2 M $CaCl_2$, pH 6.0) containing 10 mg ml^{-1} Driselase (Inter-Spex Products, Inc., San Mateo, CA, USA) and 5 mg ml^{-1} Cellulase Onuzuka R-10 (Kanemetsui USA, Inc., Los Angeles, CA, USA). The mixture was incubated for 3 h at 30°C on an orbital shaker at 50 rpm, filtered through three layers of cheesecloth and pelleted by centrifugation (4000×*g*) for 10 min at 4°C. The spheroplasts were washed once in 10 ml of osmoticum and twice in 30 ml STC (1.2 M sorbitol, 10 mM Tris–HCl (pH 7.5), 10 mM $CaCl_2$), then pelleted by centrifugation (2000×*g*) for 10 min at 4°C. All procedures were performed under sterile conditions.

The procedure for transformation was a modified version of previously published protocols [22,23]. Spheroplasts (1×10^6) in 100 μl of STC were mixed with 10–40 μg of circular or *Bgl*II-digested gGFP plasmid [19] in 25 μl of sterile distilled water. For REMI mutagenesis (e.g. [22]), 10, 50, or 100 U *Bgl*II restriction enzyme was added to the transformation reaction. The mixture was kept on ice for 5–10 min before the addition of 1 ml PTC (40% PEG (4000 MW), 10 mM Tris (pH 7.5), 10 mM $CaCl_2$). The solutions were gently mixed and incubated for 2–20 min on ice. Samples were plated undiluted or as 1:10 or 1:100 dilutions in STC in 20 ml molten (50°C) regeneration agar [24]. After 18 h of incubation at 21°C in the dark, the plates were overlaid with 10 ml of 1% agar in PDB containing 60 μg ml^{-1} hygromycin B, giving a final hygromycin B concentration of 20 μg ml^{-1}, and incubated in the dark at 21°C. Transformants appeared within 2 weeks and were transferred to V8 agar plates supplemented with 20 μg ml^{-1} hygromycin B. For long-term storage, isolates were maintained as mycelial discs in 10% glycerol at −70°C.

2.3. Mitotic stability tests

Mitotic stability was examined by growth of single spore isolates of hygromycin B-resistant transformants

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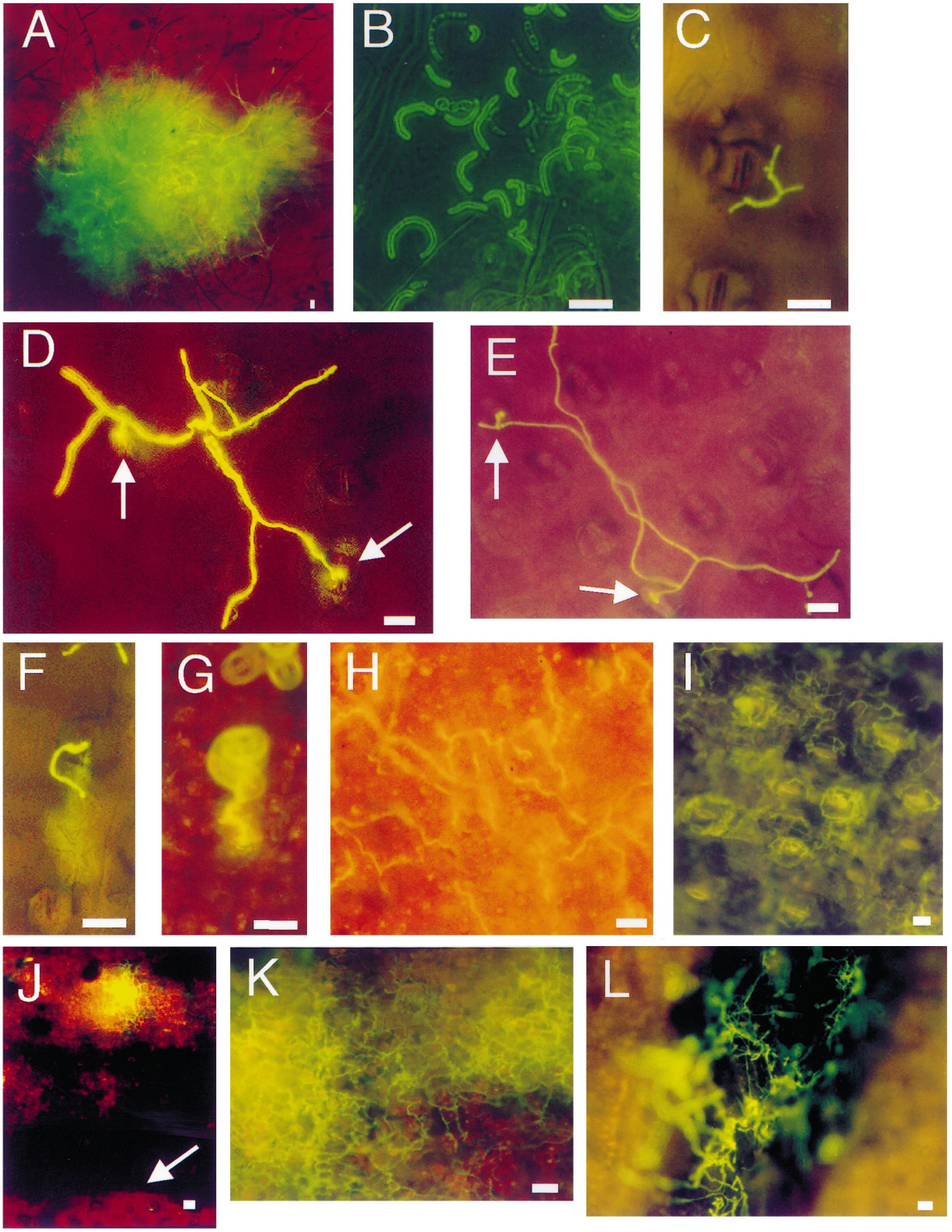
Fig. 1. GFP-expressing *Mycosphaerella* spp. observed under FITC fluorescence. All growth is on or in leaves of the banana cultivar Grand Nain with the exception of A and B, which show transformants from the original selection plates. The white bar in each photograph represents 25 microns. A: *M. fijiensis* transformant 743C10 grown on regeneration agar with hygromycin B selection. B: Conidia brushed from the mycelium of *M. eumusae* transformant 548C1. C: Conidium of *M. fijiensis* transformant 743U7 germinating on the underside of a banana leaf. D: Growth of *M. eumusae* transformant 548C1 on the undersurface of a leaf 3 weeks post-inoculation (PI); arrows indicate stomatopodia. E: Growth of *M. musicola* transformant 318U1 on the undersurface of a leaf 3 weeks PI; arrows indicate stomatopodia. F and G: Leaf penetration via a stomate by *M. eumusae* transformant 548C1 3 weeks PI. Both pictures show the same field in which hyphal growth can be seen both on the undersurface (F) and within leaf tissue (G). In F, the hyphal segment on the leaf surface is in focus, and the fungus within the leaf tissue is out of focus; in G, the reverse is the case. H: Extensive growth of *M. fijiensis* transformant 743U7 within the leaf 4 and 1/2 weeks PI. I: Growth of *M. musicola* transformant 318U1 within the leaf 7 weeks PI. Circular hyphal growth within the substomatal cavity is evident. J: The border (indicated by an arrow) between necrotic and living tissue on a plant infected with *M. eumusae* transformant 548C1 5 weeks PI. Green fluorescent hyphae at the top of the photo indicate the presence of a *M. eumusae* transformant approximately 250 μm inside the necrotic area (i.e. surrounded by necrotic tissue). K: Saprophytic growth of *M. eumusae* transformant 548C1 within a necrotic area 5 weeks PI. L: Growth of *M. fijiensis* transformant 743U7 above a black necrotic area of the leaf. This observation would have been very difficult to make using conventional hyphal staining because of the lack of contrast between the hyphae and necrotic plant tissue.

on a nonselective medium for two sequential 2 week periods, followed by transfer back to the selective medium at the end of each 2 week period. Transformants were cultured for 2 weeks on V8 agar medium lacking hygromycin B. Inoculum was prepared from these plates by maceration of fifteen 5 mm diameter discs of each transformant in a BeadBeater™ as previously described. Aliquots of the 1× concentration of macerated cells, as well as serial dilutions, were spread over the surface of V8 agar medium plates with or without hygromycin B (20 μg ml^{-1}). Plates were incubated in the dark at room temperature for 2 weeks, and the number of colonies growing on each



medium was determined. The lawn of growth arising from the 1× concentration of fungus cultured on nonselective medium for 2 weeks (4 weeks cumulative time) was used as the source of inoculum for the second round of mitotic stability assays.

2.4. Isolation of fungal genomic DNA

Mycosphaerella isolates were grown as shaking cultures in PDB (supplemented with 20 μg ml^{-1} hygromycin B where appropriate) for 2 weeks, with preparation of inoculum and incubation conditions for shaking cultures as described above for spheroplast isolation. The mycelium was collected by centrifugation (5000×*g*, 10 min), frozen, and lyophilized. Tissue (2–3 g) was ground to a fine powder in a mortar and pestle with liquid nitrogen and extracted in 15 ml 2×CTAB Isolation Buffer, as described previously for the extraction of banana DNA [25]. Southern analyses were performed using Nytran Plus membrane following the manufacturer's instructions (Schleicher and Schuell, Keene, NH, USA). Hybridization conditions were as described by Church and Gilbert [26].

2.5. Inoculations of banana plants with Mycosphaerella spp.

After 2 weeks of growth on V8 agar (supplemented with 20 μg ml^{-1} hygromycin B as appropriate) at 21°C under continuous fluorescent white light, conidiospore suspensions were prepared by adding ~5 ml sterile distilled water to each Petri dish culture and brushing the surface with a glass spreader to dislodge spores and hyphal fragments. The suspension was strained through Miracloth (Calbiochem, San Diego, CA, USA) and adjusted to approximately 10^5 conidia ml^{-1}. Because isolate *Mf* 730 does not produce detectable conidiospores in vitro, mycelial fragments were used as inoculum.

The undersides of the two youngest unrolled leaves of each plant (~25 cm tall) were inoculated using a Nalgene hand-held sprayer to apply a uniform covering of fine droplets. The leaves were allowed to dry for 1 h, sprayed a second time, then placed in a growth chamber with misting for 30 min h^{-1} for 3 days. Over the next week, misting was gradually reduced to 10 min h^{-1} and maintained at this level for the remainder of the infection period. This inoculation procedure was based on previous reports [21,27–29].

2.6. Microscopy

Mycosphaerella transformants expressing GFP were observed and photographed using an Olympus IMT-2 inverted microscope with FITC fluorescence and Kodak Ektachrome slide film (400 ASA). Infected leaf tissue was excised from plants and viewed directly mounted in water.

3. Results and discussion

3.1. Development of a transformation system for Mycosphaerella banana pathogens

Five *M. fijiensis* isolates (CIRAD accession numbers 743, 730, 301, 294, 282), one *M. musicola* isolate (318), and one isolate of the newly identified *M. eumusae* species (548) were transformed with the gGFP plasmid [19], which encodes hygromycin B resistance and GFP expression. The efficiency of transformation for all three fungal species was 0.5–4 transformants μg^{-1} gGFP DNA using either circular or linearized plasmids. If the gGFP plasmid was omitted from the transformation, no significant growth of the spheroplasts was observed on hygromycin B-containing plates. Approximately 75% of the hygromycin B-resistant colonies displayed the expected fluorescence phenotype associated with GFP expression, which was constitutive and visible throughout the mycelia and conidia (Fig. 1A and B). The majority of single-spored transformants that expressed GFP constitutively were mitotically stable in the absence of selection for hygromycin B resistance. Transformations utilizing either linearized or circular plasmids yielded transformants that expressed GFP and were used in plant studies (Fig. 1). This is the first report of genetic transformation of *Mycosphaerella* pathogens of banana. The procedure is straightforward and robust in that we isolated stable transformants from all seven of the isolates tested.

3.2. Analyses of transformants

In other fungal transformation systems, restriction enzyme-mediated integration (REMI) (e.g. [22,30]) has been useful in increasing transformation efficiencies 5- to 10-fold and decreasing plasmid copy number. REMI involves the addition of the restriction enzyme used to linearize the plasmid to the transformation reaction. The addition of *Bgl*II enzyme during the transformation of *Mf* 282 and *Mf* 301 spheroplasts with *Bgl*II-digested gGFP did not have a significant effect on transformation efficiency or copy number at any of the enzyme concentrations tested (data not shown). Southern analyses of 39 randomly chosen single-spored transformants (28 REMI transformants and 11 non-REMI transformants) indicated that more than half had single insertions of the gGFP plasmid and that few had more than four insertions (Fig. 2A; data not shown). We conclude that, in our hands, the number of vector insertions in a standard transformation reaction (i.e. without the addition of excess restriction enzyme) is not significantly different than that seen with REMI using *Bgl*II restriction enzyme. Furthermore, the majority of transformants analyzed contained one or two insertions. The fact that most transformants generated, using either REMI or standard transformation methods, carried few

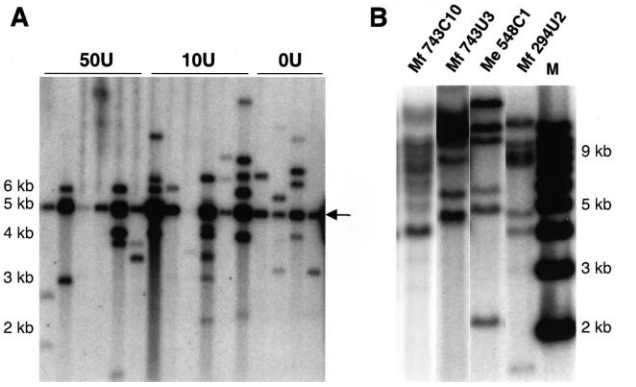


Fig. 2. Southern analyses of GFP-expressing transformants of *Mycosphaerella* spp. A: A Southern blot of *Eco*RI-digested genomic DNA from single-spored *M. fijiensis* transformants of *Mf* 282 isolated after transformation with *Bgl*II-digested gGFP. The entire gGFP plasmid was used as a probe. An arrow indicates a 4.8 kb band common to all transformants. Because there are two *Eco*RI sites in gGFP, each insertion event should result in two novel bands in addition to the 4.8 kb band, in cases where the integration pattern is simple and no rearrangements have occurred. The numbers at the top of the figure indicate the number of units of *Bgl*II enzyme that were included during the REMI mutagenesis transformation reaction. In some lanes, more bands could be discerned after a longer exposure than is shown here. 16 of 39 transformants analyzed are shown. The positions of molecular size markers are indicated on the left side of the figure. B: A Southern blot of *Hin*dIII-digested genomic DNA from *M. fijiensis* (*Mf* 743C10, *Mf* 743U3, *Mf* 294U2) and *M. eumusae* (*Me* 548C1) transformants. The entire gGFP plasmid was used as a probe. Because there is a single *Hin*dIII site in the gGFP plasmid, a single integration of the vector should result in the presence of two bands if a simple integration event with no recombination occurred. The lane marked M shows molecular size standards; the figure is a composite of lanes from a single gel.

insertion events should facilitate insertional mutagenesis screens. With the availability of a robust transformation system, we expect that systematic molecular analyses of the genetics of pathogenesis by *Mycosphaerella* pathogens of banana will be possible. It should be noted that in the plant studies reported here transformants that had the greatest fluorescence usually carried multiple copies of the gGFP plasmid (Fig. 2B).

3.3. Observations of plants infected with transgenic fungi

Leaves of the susceptible banana cultivar Grand Nain inoculated with conidia from GFP-expressing *Mycosphaerella* transformants were observed at 1 week intervals for 8 weeks. Using FITC fluorescence, GFP-expressing transgenic fungi appeared bright green, while background red fluorescence from chlorophyll made it possible to see the plant cells. Untransformed fungi did not exhibit autofluorescence under these conditions. Growth on and under the leaf surface was readily apparent (Fig. 1C–L). Macroscopic symptoms observed on plants infected with transgenic fungi were indistinguishable from the symptoms on plants infected with the original untransformed isolates, suggesting that the transformation process did not markedly affect fungal pathogenicity or virulence.

Small ($\sim$2 mm) necrotic lesions were apparent on the underside of leaves of plants inoculated with *M. fijiensis* or *M. eumusae* 3–4 weeks post-inoculation (PI). After 2 more weeks, the lesions enlarged so that most of the leaf surface was necrotic. *M. eumusae*-infected plants showed slightly (up to 1 week) faster symptom expression than the *M. fijiensis*-infected plants. No symptoms were observed on uninoculated leaves. Symptoms were less severe on *M. musicola*-infected plants in which inoculated leaves showed distinct chlorosis but fewer necrotic lesions. Those that appeared took approximately 2 weeks longer to form than on *M. fijiensis*-infected leaves and did not expand to cover the entire leaf.

In contrast to *M. fijiensis* and *M. eumusae* strains, *M. musicola* displayed low virulence in this study. It is possible that the temperature of the humidity chamber contributed to the low virulence of *M. musicola*. The growth conditions used were those reported to be optimal for *M. fijiensis* infection of banana [21,27–29]. *M. musicola* is generally found in cooler climates than *M. fijiensis* [3].

3.4. Comparison of the three Mycosphaerella species in planta

Expression of GFP in *M. fijiensis* and *M. musicola* allowed more detailed and rapid microscopic analyses of black and yellow Sigatoka disease than was previously possible. In particular, growth under the leaf surface and on necrotic tissue was significantly easier to observe using GFP-expressing transformants. All three species formed stomatopodia (hyphal swellings) above stomata (Fig. 1D and E) 1–2 weeks PI, and leaf penetration through stomata was observed on leaves infected with *M. fijiensis* or *M. eumusae* 2–3 weeks PI (Fig. 1F and G). In most cases, a stomatopodium was apparent above the penetrated stomate, and in no case was cell penetration or formation of haustoria observed (Fig. 1G–I). Markedly less leaf penetration was observed on leaves infected with *M. musicola* transformants, although stomatopodia were formed readily (Fig. 1E). These observations agree with previous findings using conventional hyphal staining techniques [5,8–11] although we observed more growth and branching of hyphae in the mesophyll than was reported previously [5,8,9].

In the later stages of leaf infection with *M. fijiensis* or *M. eumusae*, significantly more growth was apparent within the leaf than on its surface (Fig. 1H, I). Hyphae generally grew parallel to the leaf surface rather than perpendicular to it. No hyphae were observed growing on or just under the uninoculated adaxial leaf surface. *M. musicola* transformants grew largely, though not entirely, on the leaf surface. Circular patterns of hyphal growth, just under stomata in the substomatal cavity, were often observed on leaves infected with all three species (Fig. 1I).

3.5. Observations in support of a diffusible toxin

4–5 weeks PI, necrotic lesions were visible on leaves infected with *M. fijiensis* and *M. eumusae*. Microscopic observations revealed the presence of abundant, saprophytic growth in parts of the necrotic area (Fig. 1K) of infected leaves. Other regions, comprising more than half of the necrotic area, were entirely devoid of hyphae. Furthermore, the limits of the necrotic area were often in advance of the fungal hyphae (Fig. 1J). These observations suggested that *M. fijiensis* and *M. eumusae* were producing diffusible phytotoxins. Phytotoxin production by *M. fijiensis* has been the subject of several reports [15–18,31] in which a number of unique toxins have been identified from in vitro grown cultures. However, the role of such toxins in pathogenesis remains unclear. Our observations are supportive of the hypothesis for a role of phytotoxins in the Sigatoka diseases.

Acknowledgements

We would like to thank X. Mourichon, J. Carlier and colleagues (CIRAD, Montpelier, France) for the provision of fungal strains and helpful advice; G. Nesbit and colleagues (Zeneca Agrochemicals, Berks, UK) and Ronald Romero (CORBANA, San Jose, Costa Rica) for helpful advice; A. Sharon (Tel Aviv University, Tel Aviv, Israel) for providing the gGFP vector; Eli Khayat (Rahan Meristem, Rosh Hanikra, Israel) for provision of Grand Nain plants; T. Ganapathi, N. Higgs, H. McLane, M. McMillan, and S. Mungekar (Boyce Thompson Institute, Ithaca, NY) for assistance with plant care and fungus cultures; and P. Beetham, B. Dugdale, and P. Kipp for helpful discussions. This work was supported in part by the Boyce Thompson Institute.

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