

Introduction

Banana–*Mycosphaerella fijiensis* interactions

P. Lepoivre¹, J. P. Busogoro¹, J. J. Etame¹, A. El Hadrami^{1,2},
J. Carlier², G. Harelimana¹, X. Mourichon², B. Panis³,
A. Stella Riveros⁴, G. Sallé⁵, H. Strosse³ and R. Swennen³

Abstract

Using standard testing procedures, banana genotypes were classified as 1) highly resistant cultivars characterized by an early blockage of leaf infection (incompatible interactions), 2) partially resistant cultivars exhibiting a slow rate of symptom development (compatible reactions) and 3) susceptible cultivars, characterized by rapid development of necrotic lesions (compatible reaction).

Most information on incompatible reactions comes from observations of early necrosis of stomatal guard cells and the deposit of electron-dense compounds around the penetration sites of *M. fijiensis* on the cultivar 'Yangambi km5'. Such rapid death of a few host cells, associated with the blockage of the progression of the infecting agent is usually defined as a hypersensitive reaction. Such a reaction often operates within a gene-for-gene relationship and as a consequence the resulting resistance may be unstable.

As regards compatible interactions, cytological studies showed that *M. fijiensis* behaves first as a biotrophic parasite which colonizes exclusively the intercellular spaces without the formation of haustoria. Two main mechanisms have been investigated to explain the slow development of a lesion in partially resistant genotypes: preformed antifungal compounds and tolerance to putative toxin(s) produced by *M. fijiensis*.

The mechanisms will be presented in relation to their possible use as early screening markers for selecting banana genotypes for durable resistance to *M. fijiensis*.

¹ Faculté Universitaire des Sciences Agronomiques, Gembloux, Belgium

² CIRAD, Montpellier, France

³ Katholieke Universiteit Leuven, Leuven, Belgium

⁴ CATIE, Costa Rica

⁵ Université Pierre et Marie Curie, Paris, France

Resumen - Interacciones banano - *Mycosphaerella fijiensis*

Utilizando los procedimientos de evaluación estándar, los genotipos de banano fueron clasificados en tres categorías: 1) cultivares altamente resistentes caracterizados por un bloqueo temprano de la infección foliar (interacciones incompatibles), 2) cultivares parcialmente resistentes que exhiben una evolución lenta de los síntomas (reacciones compatibles), y 3) cultivares susceptibles, caracterizados por un desarrollo rápido de las lesiones necróticas (reacción compatible).

La mayor parte de la información sobre las reacciones incompatibles proviene de los estudios del cultivar 'Yangambi km5'. Se observaron la necrosis de las células de guarda estomatales y los depósitos de los compuestos con alta densidad de electrones alrededor de los sitios de penetración de *M. fijiensis*. La muerte tan rápida de unas cuantas células hospedantes asociada con el bloqueo de la progresión del agente infectante se define usualmente como una reacción hipersensible. Esta reacción a menudo opera dentro de una relación de gen por gen y podría convertir la resistencia en inestable.

Con las reacciones compatibles, los estudios citológicos revelaron que *M. fijiensis* se comporta primero como un parásito biotrófico que coloniza exclusivamente los espacios intercelulares sin formar los haustorios. Dos mecanismos principales podrían estar involucrados en el desarrollo lento de las lesiones observado en los genotipos resistentes parcialmente: compuestos antifúngicos sintetizados de manera constitutiva o tolerancia a la(s) toxina(s) putativa(s) producidas por *M. fijiensis*.

Estos mecanismos se presentarán en relación con su posible utilidad como marcadores de cribado temprano en la selección de los genotipos de banano con respecto a la resistencia duradera a *M. fijiensis*.

Résumé - Interactions bananier-*Mycosphaerella fijiensis*

En utilisant des procédures de test standard, des génotypes de bananier ont été classés en : 1) cultivars hautement résistants caractérisés par un blocage rapide de l'infection foliaire (interactions incompatibles), 2) cultivars partiellement résistants montrant un développement lent des symptômes (réactions compatibles) et 3) cultivars susceptibles caractérisés par un développement rapide de lésions nécrotiques (réaction compatible).

L'essentiel des informations sur les réactions incompatibles provient d'observations de nécrose précoce des cellules de garde des stomates et du dépôt de composés denses en électrons autour des sites de pénétration de *M. fijiensis* chez le cultivar 'Yangambi km5'. La mort aussi rapide d'un petit nombre de cellules hôtes, associée avec le blocage de la progression de l'agent infectieux, est habituellement définie comme une réaction hypersensible. Une telle réaction se produit souvent dans le cadre d'une relation gène pour gène et, en conséquence, la résistance qui en résulte peut être instable.

Pour ce qui concerne les interactions compatibles, les études cytologiques ont montré que *M. fijiensis* se comporte d'abord comme un parasite biotrophique qui colonise exclusivement les espaces intercellulaires sans formation d'haustoria. Deux mécanismes principaux ont été étudiés pour expliquer le développement lent des lésions chez les génotypes partiellement résistants : des composés antifongiques préformés et la tolérance à une(des) toxine(s) putative(s) produite(s) par *M. fijiensis*.

Les mécanismes sont présentés en relation avec leur utilisation possible comme marqueurs lors de criblage précoce pour sélectionner des génotypes de bananiers possédant une résistance durable à *M. fijiensis*.

Introduction

Black leaf streak disease is the most devastating disease of banana and plantain worldwide. The fungus induces foliar leaf streaks which, in highly susceptible cultivars, leads to the total collapse of the plant.

Just as host plants evolved several defence mechanisms, pathogens have ways to evade or suppress these defence mechanisms. The response of the host and the

pathogen are crucial to the outcome of infection. A knowledge of the interactions is increasingly important for the rational selection of genotypes resistant to plant pathogens. The interactions between banana and *Mycosphaerella fijiensis* remained unknown for a long time.

Although the field performance of an accession is the ultimate reference for evaluating its resistance, the method is not suitable for the study of host pathogen interactions. Nevertheless, the reaction to black leaf streak disease of about 50 *Musa* species belonging to various genetic groups was studied under natural infection conditions (Fouré *et al.*, 1990). The study led to the grouping of the banana genotypes in three categories: 1) highly resistant (HR) cultivars characterized by an early blockage of leaf infection (incompatible interactions); 2) partially resistant (PR) cultivars exhibiting slow rates of symptom development (compatible interactions); and 3) susceptible (S) cultivars characterized by a rapid development of necrotic lesions (compatible interactions). Later, banana-*M. fijiensis*-interactions were studied under controlled conditions of inoculation (Mourichon *et al.*, 1987) which reproduced the behaviour in the field of three reference cultivars: 'Yangambi km5' (AAA; HR), 'Fougamou' (ABB; PR) and 'Grande naine' (AAA; S).

These preliminary results, which were presented at the International workshop held in San José in 1989, were the start of investigations into banana-*M. fijiensis* interactions. They began with the microscopic events that take place in banana tissues and were followed by the analysis of the biochemical processes that culminate in the expression of resistance or susceptibility.

Host-pathogen interactions

Cytological studies of the interactions between *M. fijiensis* and the three reference cultivars 'Yangambi km5', 'Fougamou' and 'Grande naine' revealed that *M. fijiensis* enters banana leaves by the stomata.

In compatible interactions ('Grande naine' and 'Fougamou' inoculated with *M. fijiensis*, strain 049 HND from Honduras), the pathogen colonized exclusively the intercellular spaces between mesophyll cells, without forming haustoria. There was a long period of biotrophy before the observation of the first cytological alterations to the mesophyll cells. Hyphae were observed between living cells ahead of the necrotic zone, a faster growth rate of hyphae being the main difference between susceptible ('Grande naine') and partially resistant ('Fougamou') cultivars (Beveraggi, 1992; Beveraggi *et al.*, 1995).

In contrast, early necrosis of stomata guard cells and appositions around the penetration sites were observed with incompatible interactions ('Yangambi km5' inoculated with *M. fijiensis* strain 049HND) (Beveraggi *et al.*, 1995).

The behaviour of partially and highly resistant genotypes of banana can be linked to major groups of plant-parasite interactions. The rapid death of only a few host cells, associated with the blockage of the progression of the infecting agent in the highly resistant cultivar 'Yangambi km5', is usually defined as an hypersensitive reaction. These often operate within a gene-for-gene relationship giving rise to resistance that is unstable. In comparison, the partial resistance of the reference cultivar 'Fougamou', for example, is usually considered polygenic and durable.

Incompatible interactions: Highly resistant cultivars

The hypersensitive reaction operating within a gene-for-gene relationship is generally explained, either by the presence of a specific avirulence factor (or elicitor) or by the coordinated action of non-specific elicitor(s) and a specific suppressor (de Wit, 1992; Atkinson, 1993). In banana-*M. fijiensis* interactions, there is no experimental evidence of a gene-for-gene relationship because it is difficult to study the genetics of triploid genotypes such as 'Yangambi km5'. But that such a relationship exists is supported by laboratory tests in which isolates of *M. fijiensis* overcame the resistance of 'Yangambi km5' (Fullerton and Olsen, 1995).

Riveros and Lepoivre (1994) did preliminary experiments to identify the elicitors that induce resistance. Intercellular fluids (IF) from leaves of 'Yangambi km5' (incompatible) and 'Grande naine' (compatible) inoculated with 049HND, and crude eliciting fractions (CEF) prepared from germinating spores of virulent and avirulent *M. fijiensis* isolates, elicited necrosis and appositions in banana cultivars (Riveros and Lepoivre, 1994).

Regardless of the eliciting preparation (IFs or CEFs prepared with avirulent or virulent *M. fijiensis* isolates), the reaction was more intense and quicker in 'Yangambi km5' than in the susceptible 'Grande naine'. The behaviour of 'Yangambi km5' cannot be explained by a race-specific eliciting activity in the IFs or the CEFs. However, the eliciting activity present in the IFs of 'Yangambi km5' inoculated with the avirulent strain 049 HND appeared to be higher than that in the compatible relationship between 'Grande naine' and the same isolate. Thus, we speculate that 'Yangambi km5' could have a higher sensitivity to the elicitor(s) but could also have a host-mediated effect on the release, production or stability of specific elicitor(s) produced by the fungal isolates. Such host-mediated effects have been reported in soybean tissues where plant enzymes are responsible for the release of elicitors from hyphal walls of *Phytophthora megasperma* (Boller, 1987).

A wide range of fungal compounds have been implicated as elicitors of HR: polysaccharides (Sharp *et al.*, 1984), glycoproteins (Schaffrath *et al.*, 1995), peptides (de Wit *et al.*, 1985) and hydrolytic enzymes (Boller, 1987). In banana-*M. fijiensis* interactions, polysaccharide compounds may be involved in eliciting activity (Riveros, unpublished data).

Evidence of a hypersensitive-like-reaction to *M. fijiensis* represents a first step towards a better characterization of that reaction. Independently of the mechanisms of resistance, there is the problem of the durability of resistance. Durability is of outmost importance because breakdown of resistance for a staple crop such as plantain would have dramatic effects. Because of the difficulties inherent in improving triploid bananas, breeding for resistance to black leaf streak disease often did not take into account host-pathogen interactions. The existence of *M. fijiensis* isolates able to overcome resistant 'Yangambi km5' in laboratory tests (Fullerton and Olsen, 1995) shows that highly resistant parents should not be used without appropriate management procedures, such as mixtures of cultivars, choice of the "right gene combination" and co-ordinated regional deployment of genes.

Compatible reaction: partial resistant cultivars

With partially resistant cultivars (compatible interactions), cytological studies showed that *M. fijiensis* behaves at first as a biotrophic parasite that colonizes exclusively the intercellular spaces (Beveraggi *et al.* 1995). Two possible mechanisms have been investigated to explain slow lesion development in partially resistant genotypes: preformed antifungal compounds and tolerance to putative toxin(s) produced by *M. fijiensis*.

Constitutively synthesised antifungal compounds

Many antimicrobial compounds produced by plants play an important role in the response to infection by cellular pathogens. Defence compounds may be classified into phytoanticipins, which are constitutive, and phytoalexins, which are synthesised in response to microorganisms. The two groups of secondary metabolites include a wide range of chemical families. However, phytoanticipins are primarily involved in non-host rather than varietal resistance.

For 'Fougamou', histological analysis revealed the presence, in mesophyll layers, of many specialized cells containing vacuoles rich in polyphenol. The contents of the vacuoles were released into the intercellular spaces. The contents had a high affinity for fungal cell walls and their presence around hyphae seemed to be correlated with the slow growth of mycelium in parenchyma tissues (Beveraggi *et al.*, 1992, 1995). Gire (1994) identified soluble phenols in the leaf tissues of several banana cultivars with different levels of partial resistance. He also observed a close correlation between flavane (protoanthocyanidins) content and the level of partial resistance. However, a study conducted on a larger number of genotypes suggested that the role of these constitutive compounds in partial resistance is restricted to a limited number of cultivars (El Hadrami, 1997).

The role of toxins in pathogenesis

Pathogen toxins could constitute an alternative technique for rapidly screening resistant banana genotypes as *in vitro* plant tissues or young plants. The symptoms of black leaf streak disease suggest a possible involvement of phytotoxic compounds. Such compounds were found in culture filtrates of *M. fijiensis* (Molina and Krausz, 1989; Lepoivre and Acuna, 1989; Upadhyay *et al.*, 1990; Strobel *et al.*, 1993). Stierle *et al.* (1991) reported that 2,4,8-tetrahydroxytetralone and juglone were the most abundant and most phytotoxic compounds.

If toxins are involved in the development of black leaf streak disease, it may be possible to use them to identify resistant genotypes. During the previous workshop at San José, the use of *M. fijiensis* toxins for screening had four major limitations: 1) a lack of quantitative and sensitive bioassays to measure the effects of *M. fijiensis* metabolites on banana genotypes; 2) insufficient characterization of the variability in toxin production of *M. fijiensis*; 3) a lack of experimental evidence for the role of the metabolites in the disease; and 4) the assurance that the susceptibility and/or resistance of cultured tissues reflected the reaction of the whole plant.

Bioassay to quantify the effect of *M. fijiensis* metabolites

A set of bioassays was developed to quantify the toxic effects of the metabolites obtained from *M. fijiensis* culture filtrates. The induction of necrosis by a leaf puncture bioassay on detached banana leaves, or the injection of ethyl acetate crude extract (EaCE) into the leaves are easy to perform but neither method is sensitive (injections of 250 ppm EaCE are required for 'Grande naine') or quantitative.

The electrolyte leakage assay represented a quantitative but rather insensitive assessment of the toxicity of *M. fijiensis* metabolites. The test did not distinguish between cultivars. The most sensitive and accurate toxin assay was based on the measurement of chlorophyll fluorescence. The vitality index seemed to be the most sensitive method for early assessment of the effects of EaCE and a specific indicator of photosynthetic activity.

Purification of the EaCE revealed the presence of different fractions with similar properties to the crude extracts. Juglone, a purified metabolite previously shown to be present in extracts of *M. fijiensis* culture filtrates, was identified in the extracts of all the strains analyzed. Injection of juglone into banana leaf tissues gave similar results to EaCE for ranking cultivars (Etame, unpublished data).

Chloroplasts as target site of juglone

The involvement of the photosynthetic apparatus in reaction to EaCE and juglone is in agreement with observation of light-dependent toxicity irrespective of the bioassay. The observation of swelling chloroplasts as the first abnormality observed by electron microscopy of EaCE-treated leaves also fits this pattern.

Busogoro (unpublished data) developed a bioassay using isolated chloroplasts and measuring their capacity to reduce 2,6-dichlorophenolindolphenol (DCPIP) as a marker of the Hill reaction, which expresses electron transport from water to any electron acceptor by intact chloroplasts when exposed to light (Allen and Holmes, 1986).

Juglone inhibited the Hill reaction in suspensions of banana chloroplasts. In addition, 'Fougamou' chloroplasts appeared to be less affected by juglone than 'Grande Naine' chloroplasts. These results suggest that chloroplasts are one of the primary action sites of juglone.

The role of toxins in banana-*M. fijiensis* interactions

The electrolyte leakage assay and chlorophyll fluorescence were used to compare the sensitivity to EaCE of different banana cultivars with their behaviour in the field (highly resistant, sensitive or partially resistant) as scored using the rank of the youngest leaf spotted with necrotic lesions.

These toxin assays confirmed that the incompatible interactions of 'Yangambi km5' were not related to resistance to EaCE. The toxicity of EaCE preparations was independent of the virulence of the strain (unpublished data). Mechanisms of resistance in highly resistant cultivars were definitely not related to the action of these toxic metabolites.

Considering just the susceptible 'Grande naine' and the partially resistant 'Fougamou' cultivars, susceptibility to EaCE was correlated with sensitivity to infection, suggesting that slow lesion development is associated with a lower sensitivity to *M. fijiensis* toxins.

Such quantitative assessment is difficult to interpret because the concentrations of toxin(s) that were used in the bioassay could exceed the *in vivo* concentration and affect the mode of action of EaCE, hence affecting the rating of the cultivars.

The hypothesis that *M. fijiensis* metabolites have a secondary role as determinants of pathogenicity agrees with cytological studies, which showed no evidence of an early effect of toxic compounds in the long period of biotrophy before observing the first cytological alterations in the mesophyll cells.

Selection of banana tissues resistant to juglone

The work was done with 'Three hand planty', a genotype susceptible to black leaf streak disease, and juglone for which an embryo cell suspension was available. Juglone was toxic to embryogenic cell suspensions and somatic embryos of the cultivar. Necrosis of all cell suspensions and somatic embryos was quickly observed at 50 ppm or more of juglone, with the exception of some somatic embryos that continued development after treatment with 50 ppm of juglone.

The plants regenerated from the surviving embryos showed a higher resistance to juglone: 250 ppm was required to induce necrosis in the leaf puncture bioassay with selected plantlets in comparison with 100 ppm for non selected plants. However, the selected plants did not show higher resistance to black leaf streak disease than the mother 'Three hand planty' genotype following inoculation with *M. fijiensis* (El Hadrami, unpublished data).

Daub (1986) advised caution when using metabolites from pathogens to screen tissue cultures for resistance. Nevertheless, fungal toxins have been proposed to screen banana *in vitro* (Strobel *et al.*, 1993). There have been claims that resistant material has been produced by selecting callus of banana that survived increasing concentrations of *M. fijiensis* toxins (Okole and Shulz, 1993). Our results confirm the possibility of selecting banana plants resistant to *M. fijiensis* metabolites but this approach did not result in higher resistance to black leaf streak disease.

References

- Allen J.F. and N.G. Holmes. 1986. Electron transport and Redox Titration. Pp. 103-141 *in* Photosynthesis energy transduction. A practical approach. (M.F. Hipkins and N.R. Baker, eds). IRL PRESS, Washington.
- Atkinson M.M. 1993. Molecular mechanisms of pathogen recognition by plants. *Advances in Plant Pathology* 10:35-59.
- Beveraggi A. 1992. Etude des interactions hôte-parasite chez des bananiers sensibles et résistants inoculés par *Cercospora fijiensis* responsables de la maladie des raies noires. Thèse de 3^{ème} cycle, Université de Montpellier II, USTL.
- Beveraggi A., X. Mourichon and G. Salle. 1995. Etude comparée des premières étapes de l'infection chez les bananiers sensibles et résistants infectés par *Cercospora fijiensis*

- (*Mycosphaerella fijiensis*), agent responsable de la maladie des raies noires. Canadian Journal of Botany 73:1328-1337.
- Boller T. 1987. Hydrolytic enzymes in plant disease resistance. Pp. 385-413 in Plant-Microbe Interactions, vol.2 (Kosuge, T. and E.W. Nester, E. W. eds.). MacMillan, New York.
- Daub M.E. 1986. Tissue culture and the selection of resistance to pathogens. Annual Review of Phytopathology 24:159-186.
- de Wit P.G.J.M. 1992. Molecular characterization of gene-for-gene systems in plant fungus interactions and the application of avirulence genes in control of plant pathogens. Annual Review of Phytopathology 30:391-418.
- de Wit P.G.J.M., E.E. Hofman, G.C.M. Velthuis and J. Kuc. 1985. Isolation and characterization of an elicitor of necrosis isolated from intercellular fluids of compatible interactions of *Cladosporium fulvum* (syn. *Fulvia fulva*) and tomato. Plant Physiology 77:642-647.
- El Hadrami A. 1997. Protoanthocyanidines constitutifs des feuilles de bananiers et résistance partielle vis-à-vis de *Mycosphaerella fijiensis*, l'agent causal de la maladie des raies noires. Thèse de DEA, Faculté universitaire des Sciences Agronomiques de Gembloux.
- Fullerton R.A. and T.L. Olsen. 1995. Pathogenic variability in *Mycosphaerella fijiensis* Morelet cause of black Sigatoka in banana and plantain. New Zealand Journal of Crop and Horticultural Science 23:39-48.
- Fouré E., A. Mouliom Pefoura and X. Mourichon. 1990. Etude de la sensibilité variétale des bananiers et plantains à *Mycosphaerella fijiensis* Morelet au Cameroun. Caractérisation de la résistance au champ de bananiers appartenant à divers groupes génétiques. Fruits 45:339-345.
- Gire A. 1994. Relation entre la résistance partielle du bananier à *Cercospora fijiensis* et une composante cellulaire constitutive de nature polyphénolique. DEA, Université Montpellier II.
- Lepoivre P. and C.P. Acuna. 1989. Production of toxins by *Mycosphaerella fijiensis* var. *difformis* and induction of antimicrobial compounds in banana: their relevance in breeding for resistance to black Sigatoka. Pp. 201-207 in Sigatoka Leaf Spot Diseases of Bananas. Proceedings of an international workshop, San José, Costa Rica, March 28-April 1, 1989 (Fullerton, R.A. and R.H. Stover eds). INIBAP Montpellier, France.
- Molina G., and J.P. Krausz. 1989. A phytotoxic activity in extracts of broth cultures of *Mycosphaerella fijiensis* var. *difformis* and its use to evaluate host resistance to Black Sigatoka. Plant Disease 73:142-144.
- Mourichon X., D. Peter and M.F. Zapater. 1987. Inoculation expérimentale de *M. fijiensis* Morelet sur jeunes plantules de bananier issues de culture *in vitro*. Fruits 42:195-198.
- Okole B.N. and F.A. Shulz. 1993. Selection of banana and plantain (*Musa* spp.) resistant to toxins produced by *Mycosphaerella* species using *in vitro* culture techniques. Pp. 378 in Breeding banana and plantain for resistance to diseases and pests. Proceeding of the International Symposium on Genetic Improvement of Bananas to Diseases and Pests. (Ganry J. ed.). CIRAD-FHLOR, Montpellier, France, 7-9 septembre 1992.
- Riveros A.S. and P. Lepoivre. 1994. Early induction of non specific cultivar glucanase eliciting activity in the apoplastic fluids of banana infected by *Mycosphaerella fijiensis*. Arch. Int. Physiol. Bioch. Biophys. 102(5):9.
- Schaffrath U., H. Schelinpflug and H.J. Reisner. 1995. An elicitor from *Pyricularia oryzae* induces resistance response in rice: isolation, characterization and physiological properties. Physiol. and Mol. Plant Pathol. 46:293-307.
- Sharp J.K., M. McNeil and P. Albersheim. 1984. The primary structures of one elicitor-active and seven elicitor-inactive hexa (β -D-glucopyranosyl)-D-glucitols isolated from mycelial walls of *Phytophthora megasperma* f. sp. *glycinea*. J. Biol. Chem. 259:11321-11336.

- Stierle, A.A., R. Upadhyay, J. Hershenhorn, G.A. Strobel and G. Molina. 1991. The phytotoxins of *Mycosphaerella fijiensis*, the causative agent of black Sigatoka disease of bananas and plantains. *Experientia* 47:853-859.
- Strobel G.A., A.A. Stierle, R. Upadhyay, J. Hershenhorn and G. Molina. 1993. The phytotoxins of *Mycosphaerella fijiensis*, the causative agent of black sigatoka disease, and their potential use in screening for disease resistance. Pp. 93-103 in *Biotechnology applications for banana and plantain improvement*, 27-31 janvier 1992. INIBAP, Montpellier, France.
- Upadhyay, R., G.A. Strobel and S. Coval. 1990. Some toxins of *Mycosphaerella fijiensis*. Pp. 231-236 in *Sigatoka Leaf Spot Diseases of Bananas: Proceedings of an international workshop San José, Costa Rica, March 28-April 1, 1989* (Fullerton, R.A. and R.H. Stover eds). INIBAP Montpellier, France.