Euphytica **96:** 125–128, 1997.
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Use of *Mycosphaerella fijiensis* toxins for the selection of banana cultivars resistant to Black Leaf Streak

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Key words: banana, *Musa* spp., *Mycosphaerella fijiensis*, black leaf streak , resistance, selection, toxin

Summary

The results of our experiments suggest that toxin(s) of *Mycosphaerella fijiensis* would be involved neither in infection initiation, nor in the hypersensitive reaction in highly resistant cultivars but could serve at most as secondary determinant of the pathogenicity, contributing to the lesion expansion in cultivars exhibiting partial resistance to Black Leaf Streak disease. Moreover, the effects of toxin(s) on chlorophyll fluorescence, as well as preliminary electron microscopy observation, suggest that chloroplasts could be a precocious site of action of the toxin(s). Therefore, *in vitro* heterotrophic tissues would not be a suitable target to perform the screening with such toxin(s). The prospects and limitations of *M. fijiensis* toxin(s) for screening banana for resistance to Black Sigatoka are highlighted.

Introduction

Black Leaf Streak or Black Sigatoka caused by *Mycosphaerella fijiensis* Morelet [perfect form of *Paracercospora fijiensis* (Morelet) Deighton] is increasingly becoming the most devastating disease of banana and plantain worldwide. The fungus induces foliar leaf streaks which, in the case of highly susceptible varieties, can lead to the total collapse of the plant.

Chemical control and breeding for disease resistance cultivars are the two dominant strategies to control Black Leaf Streak disease. While banana genotypes resistant to *M. fijiensis* might not be a priority for commercial plantations, it is the most suitable and environment- friendly method in sustainable agriculture.

Creation of genetic variability and a suitable procedure to reliably identify resistant genotypes constitute two prerequisites to disease resistance improvement.

Field evaluation under natural infection conditions was for a long time the only method for selecting banana cultivars with resistance to Black Leaf Streak agent (Fouré, 1982). Among the different parameters used to evaluate the host plant response to Black Sigatoka, the youngest leaf spotted (YLS) with necrotic lesions appears to be highly correlated with disease development and has the advantage of being a simple trait to score. However, a large number of observations is required because of the uneven inoculum pressure and the irregular epidemic development. Therefore, although the field performance of an accession remains the ultimate reference for evaluating host plant resistance, epidemiological characteristics of Black Leaf Streak make field evaluation unsuitable as a rapid and first screening. Artificial inoculation in controlled conditions reproducing the behaviour of the cultivars in the field (Mourichon et al., 1987), represents the first step towards a simplification of selection procedures but remains time- and labour- consuming.

On the other hand, plant tissue culture techniques combined with the use of toxins produced by a pathogen offer a very attractive opportunity to increase the genetic variability and to screen new genetic material among regenerates from explants, cells or protoplast cultures (Daub, 1986). This approach overcomes one of the problems encountered with pathogen selection, as cultured cells can be exposed easily and uniform-

ly to toxins by dispersing the cells in a toxin solution or plating them on toxin-containing media. However, despite the popularity of the use of pathogen toxin(s) as a selection agent for Black Leaf Streak resistance (Molina & Krausz, 1988; Upadhyay et al., 1989), no resistant variety has been derived through such method because this approach encounters two major limitations: (1) the lack of characterised toxins that play a role in the disease and (2) the assurance that the susceptibility and/or resistance of cultured tissues to the toxin(s) reflect those of the whole plant.

The aim of the present paper is to highlight the prospects and limitations of *M. fijiensis* toxin(s) for screening banana for resistance to Black Sigatoka.

Toxins as factors in pathogenesis of M. fijiensis

The sensitivity to Black Sigatoka disease of about fifty musaceous plants belonging to various genetic groups was studied under natural infection conditions by Fouré et al. (1990) and led the grouping of the banana genotypes in three categories: (1) highly resistant cultivars (HR) characterised by an early blockade of leaf infection, (2) partially resistant cultivars (PR) exhibiting a slow evolution of the symptoms, and (3) susceptible cultivars (S), characterised by a rapid development of necrotic lesions. *M. fijiensis*- *Musa* interactions were subsequently studied under controlled conditions of inoculation (Mourichon et al., 1987) which reproduced the behaviour of three reference cultivars in the field: Yangambi Km5 (AAA)(=HR), Fougamou (ABB)(=PR) and Grande Naine (AAA)(=S).

Cytological studies of the interactions between *M. fijiensis* and these three reference cultivars, revealed that *M. fijiensis* behaves as a biotrophic parasite which enters banana leaves through stomata and colonises exclusively the intercellular spaces between mesophyll cells, without forming haustoria (Beveraggi et al., 1995). Grande Naine (susceptible cultivar) or Fougamou (partially resistant cultivar)- *M. fijiensis* (strain 049 HND) interactions show a long period of biotrophy before observing the first cytological alterations of the mesophyll cells (Sallé et al., 1989). On the other hand, early necrosis of the stomatal guard cells and fluorescent appositions observed around the penetration sites of *M. fijiensis* at 7 days after the inoculation are associated with the incompatible interactions in the highly resistant cultivar Yangambi Km5 (Beveraggi et al., 1995). Such rapid death of only a few host cells, which limits the progression of the infecting agent is usually defined as an hypersensitive reaction, which usually operates within a gene-for-gene relationship. Experimental evidences of such plant-fungus interaction is still missing in the case of banana- *M. fijiensis* system. Indeed, characterisation of such relationship for a pathogen-host system requires genetic studies of the host and the pathogen, and a lot of banana genotypes are recalcitrant for such study.

The specific induction of hypersensitive reaction is usually explained either by specific elicitor(s) that mimic the response of the host to the microbial attack, or by non-specific elicitor(s) in concert with specific suppressor(s) (Atkinson, 1993) but toxins are generally not involved in such elicitation.

Correlation between banana genotypes sensitivity to the toxin(s) of M. fijiensis*, and their susceptibility to the disease*

Phytotoxic compounds were found in culture filtrates of *M. fijiensis* (Molina & Krausz, 1988; Upadhyay et al., 1989), extracted with ethylacetate and dissolved in 10% methanol before using them as crude toxic extract (CTE) for bioassay tests (Upadhyay et al., 1989).

The role of toxins in pathogenesis is usually assessed by evaluating the correlation between toxin production and pathogenicity of the organism, and between the sensitivity of different banana genotypes to the toxin(s) and their susceptibility to the disease (Yoder, 1980). In this respect, a set of bioassays were developed to quantify the toxic effects of metabolites obtained from *M. fijiensis* culture filtrates.

Induction of necrosis by a leaf puncture bioassay on detached banana leaves or the injection of CTE into the leaves is easy but neither sensitive nor quantitative. An electrolyte leakage assay was also developed and allowed a more sensitive and precise assessment of the effect of *M. fijiensis* toxin(s). Leaf disks of 0.5 cm in diameter were excised from the first two leaves of banana plantlets cultivated in greenhouse, infiltrated with CTE and incubated for 48 h in a rotary shaker at 25 °C. The incubation solutions were then filtrated (Millipore, 0.22 μm), their volume adjusted to 5 ml with distilled water and their conductivity (C) measured as an evaluation of the electrolyte leakage from CTE-treated leaf disks. Incubation of heat- killed disks (120 °C for 20 min) gave the total conductivity (C_t) and allowed to calculate the % of integrity (I) of the cell permeability [$I = (1 - C/C_t)\ 100$] from which the % of mortality (M_t) was deduced ($M_t = 100 - I$). Lastly, embryogenic cell suspensions of the cultivar Grande Naine as well as mesophyll cell suspensions produced

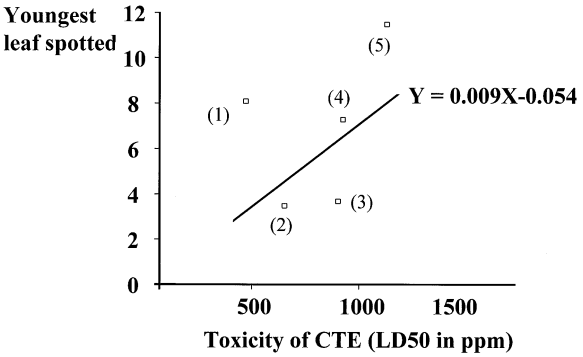

Figure 1. Relationship between sensitivity to CTE (LD 50) (as measured by the electrolyte leakage assay) and the susceptibility to the Black Leaf Streak Disease (as expressed by the youngest leaf spotted with a necrotic center). Cultivars tested : (1) Pisang madu; (2) Pisang glintong ; (3) Grande Naine; (4) Pisang berlin; (5) Fougamou.

Table 1. Vitality index (Rfd at 690 nm) of leaf tissues (cv.Grande Naine) injected with CTE (50 ppm)

Incubation time (min)	Treatment	
	Control (10 % methanol)	CTE (50 ppm)
30	2.12	2.09 (98.4%)[a]
60	2.47	1.44 (58.4%)
180	1.76	0.39 (22.2%)

[a] = Rfd expressed in percent of the control injected with 10 % methanol

by pectinase digestion of laminar tissues of banana leaves (Lepoivre & Acuna, 1989) were also used to quantify the toxicity of CTE as revealed by fluorescein diacetate stain.

The electrolyte leakage assay was used to evaluate the sensitivity to CTE of different cultivars of banana, either highly resistant [Yangambi (AAA), Calcutta 4 (AAA)], sensitive [Grande Naine (AAA), Pisang glintong (AAA)], or exhibiting partial resistance [Fougamou (ABB), Pisang berlin (AA) and Pisang madu (AA)] to Black Leaf Streak. Firstly, the electrolyte leakage assay confirmed that the highly resistance of the cultivars Yangambi and Calcutta 4 was unrelated to the resistance to CTE. Secondly, if we take into consideration susceptible (Grande Naine and Pisang glintong) and partially resistant (Fougamou, Pisang berlin and Pisang madu) cultivars, no significant correlation (r = 0.45) existed between the susceptibility to CTE and the sensitivity to the infection (Figure 1). However, such quantitative assessment is difficult to interpret because the concentrations of toxin(s) that are used in the bioassay could exceed the level required for the disease development, influence both the mode of action of CTE and modify the rating of the cultivars. Resistance to *M. fijiensis* toxin(s) is however certainly not the single mechanism of partial resistance of banana to Black Leaf Streak as Mourichon et al. (unpublished results) observed a high correlation between the level of partial resistance of banana cultivars and their polyphenolic contents. When several mechanisms are involved in the host resistance, a significant correlation could only mean that the mechanism concerned constitutes the limiting factor of the global resistance exhibited by the plant. While a high correlation can provide a very persuasive line of evidence, the absence of correlation renders inconclusive the role of that factor in the resistance. In the future, as *M. fijiensis* can be subjected to genetic manipulations, genetic analysis of different isolates which differ by the production of toxin(s) could be also used as complementary experimental approach to assess the importance of such toxin(s) in disease development.

Mechanisms of action of the toxin(s)

Beside the role played by toxin(s) in symptom development, the second limitation encountered in the use of toxin(s) as *in vitro* selecting agent is the mode of action of the toxin(s) which can differ on cultured tissues and on the whole plants.

The induction kinetic of the chlorophyll fluorescence, in particular the variable fluorescence expressed as Rfd-value [Rfd =fd/fs, the ratio of the fluorescence decrease (fd) to the steady state fluorescence (fs)] is a rapid indicator for the vitality of the leaf and plant (Lichtenthaler & Rinderle, 1988) and provides a non-destructive and specific method to document stress effects on the photosynthetic apparatus. This approch has successfully been applied in recent years in the forest decline research (Rock et al., 1986). In this respect, injection of CTE (50 ppm) into the leaves of Grande Naine (or Yangambi) led to a rapid decrease in Rfd values at 690 nm (10% of the control values after 3 h incubation) (Table 1). Moreover, preliminary observations with electron microscopy showed that the first abnormality observed in CTE-treated leaves was the swelling of the chloroplasts after 1 h of incubation.

These results suggest that the chloroplasts could be a precocious site of action of the CTE. The susceptibility of *in vitro* heterotrophic tissues to these toxins could therefore rest on other mechanisms than those

revealed on chlorophyllic plant tissues. This hypothesis was corroborated by the fact that the sensitivity of the genotype Grande Naine was significantly higher when measured with mesophyll cell suspension (DL50 = 28 ppm) than with embryogenic cell suspensions cultured *in vitro* (DL50 = 539 ppm).

Conclusions

The successful implementation of cell selection by *M. fijiensis* toxins requires an awareness and understanding of the different factors dealing with the role of the toxins in the Black Leaf Streak disease development and with the mechanisms of action of these toxic metabolites.

Our overall results suggest that *M. fijiensis* toxins, even if involved in the resistance of banana to Black Leaf Streak disease, cannot be applied *in vitro* as screening agent on heterotrophic tissues (cell suspension, embryo suspension) but should be used on photosynthetic tissues either *in vitro* or on plantlets after their transfer to the soil.

It is important to realise, contrary to the many claims made over the last few years, that using *M. fijiensis* toxins as screening agent is not an easy way to develop disease-resistant plants. It must be re- emphasised that our lack of knowledge of the basic events that occur in diseased banana and our knowledge of compounds that play a pivotal role in the pathogen virulence or host resistance are undoubtedly the major obstacles in the efficient use of tissue culture technology for crop improvement.

Acknowledgements

This work was supported by the third programme on Life Sciences and Technologies for Developing countries (STD3) of the Commission of the European Communities. The authors wish to thank E. Hardy for her contribution to the experimental work and F. Cote (CIRAD-FLHOR, Montpellier) for providing the embryogenic cell suspension of the cultivar Grande Naine.

References

Atkinson, M.M., 1993. Molecular mechanisms of pathogen recognition by plants. Adv Plant Pathol 10: 35–59.

Beveraggi, A., X. Mourichon & G. Sallé, 1995. Etude comparée des premières étapes de l'infection chez des bananiers sensibles et résistants infectés par *Cercospora fijiensis* (*Mycosphaerella fijiensis*), agent responsable de la maladies des raies noires. Can J Bot 73: 1328–1337.

Daub, M.E., 1986. Tissue culture and the selection of resistance to pathogens. Ann Rev Phytopathol 24: 159–136.

Fouré, E., 1982. Etude de la sensibilité variétale des bananiers et plantains à *M. fijiensis* au Gabon (Part I). Fruits 37: 749–770.

Fouré, E., A. Mouliom Pefoura & 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.

Lepoivre, P & 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. In : Sigatoka Leaf Spot Diseases of Bananas: Proceedings of an international workshop held at San José, Costa Rica, March 28-April 1, 1989 (Fullerton RA, Stover RH, Eds) pp 201–207. Montpellier, France, INIBAP.

Lichtenthaler, H.K. & U. Rinderle, 1988. The role of chlorophyll fluorescence in the detection of stress conditions in plants. CRC Critical Review in Analytical Chemistry 19 (suppl. 1) : S29-S85.

Molina, G. & J.P. Krausz, 1988. 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: 142–143.

Mourichon, X., D. Peter & M.F. Zapater, 1987. Inoculation expérimentale de *M. fijiensis* Morelet sur jeunes plantules de bananier issues de culture *in vitro*. Fruits 42: 195–198.

Rock, B.N., T.Hoshizaki, H.K. Lichtenthaler & G. Schmuck, 1986. Comparison of *in situ* spectral measurements of forest decline symptoms in Vermont (USA) and the Schwarzwald (FRG). In: Proceedings of the International Geoscience and Remote Sensing Symposium (IGARSS), Zürich 1986, ESA Scientific & Technical Publication branch. pp 376, Noordwijk.

Sallé, G., V. Pichard & X. Mourichon, 1989. Cytological study of the interaction between *Mycosphaerella fijiensis* Morelet and three cultivars of *Musa* presenting different levels of resistance. In: Sigatoka Leaf Spot Diseases of Bananas: Proceedings of an international workshop held at San José, Costa Rica, March 28 - April 1, 1989 (Fullerton R.A., Stover R.H., Eds). pp 180–190, Montpellier, France. INIBAP.

Upadhyay, R., G.A. Strobel & S. Coval , 1989. Some toxins of *Mycosphaerella fijiensis*. In : Sigatoka Leaf Spot Diseases of Bananas: Proceedings of an international workshop held at San José, Costa Rica, March 28 - April 1, 1989 (Fullerton R.A., Stover R.H., Eds). pp 231–236 , Montpellier, France. INIBAP.

Yoder, O.C., 1980. Toxins in pathogenesis. Ann Rev Phytopathol 18: 103–129.