European Journal of Plant Pathology **110:** 601–609, 2004.

Toxin profile, fertility and AFLP analysis of *Fusarium verticillioides* from banana fruits

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Key words: AFLP, banana, fumonisins, *Fusarium verticillioides*, *Gibberella fujikuroi*, maize

Abstract

Gibberella fujikuroi is composed of at least nine mating populations (MPs), corresponding to biological species and assigned letters (from A to I). Each MP possesses a specific toxicological profile and a preferential host. Members of *Fusarium verticillioides* and *F. thapsinum*, anamorphs respectively of MPs A (*G. moniliformis*) and F (*G. thapsina*), share identical morphological traits, but they have a different preferential hosts (maize and sorghum, respectively) and toxin profiles, being able the only member of MP A to produce fumonisins and the only member of MP F to produce moniliformin. Isolates from banana fruits were identified morphologically as *F. verticillioides*. The isolates were analyzed for fumonisin and moniliformin production. While none of the isolates produced fumonisin, all the isolates produced moniliformin. The isolates were crossed with tester strains of MPs A and F, showing ability to produce fertile perithecia only when crossed by MP A tester strains isolated from maize. However, the time required for the formation of fertile perithecia and their size differed significantly from the usual fertile crosses of strains belonging to MP A. Pathogenicity tests using such isolates of *F. verticillioides* isolated from banana and a set of *F. verticillioides* isolates isolated from maize were also performed on banana fruits. The data showed that the isolates from banana were more pathogenic. Finally, isolates from banana and maize were compared using AFLP. The results revealed that isolates from banana and maize produced two distinctly different clusters. In conclusion, isolates of *F. verticillioides* from banana showed specific traits (toxin production, *in vitro* fertility, pathogenicity and molecular profiles), that were different to those of the same species from maize. This could reflect important differences in the ecology and natural history of the population from banana and should encourage further investigations into the mechanisms of toxin production and pathogenicity within the same MP.

Introduction

Banana plants (*Musa sapientum*) are attacked by several *Fusarium* species that could be responsible for diseases such as the Fusarium wilt (Panama disease) caused by *F. oxysporum* (Ploetz et al., 1994). Moreover, banana fruits can also be affected by some post-harvest diseases such as 'Fusarium fruit rot' (Hirata et al., 2001) and 'Fusarium crown rot', which is a major cause of ripe fruit losses in the consuming countries (Ploetz et al., 1994). The crown and peduncles of boxed bananas are subject to rot in transit and *F. verticillioides* (syn. *F. moniliforme*) is often present along with other *Fusarium* species (Ploetz et al., 1994). In a survey of the occurrence of *Fusarium* species in banana fruits marketed in Italy (imported from Panama, Ecuador and the Canary Islands, Spain), *F. verticillioides*, together with *F. semitectum*, were identified as the most frequently isolated species (Jimenez et al., 1993). *Fusarium verticillioides* is one of the anamorphs of the *Gibberella fujikuroi* species complex (*Fusarium* sections *Liseola* and *Elegans*), which is composed of at least nine reproductively isolated biological species (mating populations, MPs), assigned letters A to I. However, the definition of species within this group varies. Separate *Gibberella* species names have been assigned to all but one

of these mating populations (Samuels et al., 2001; Zeller et al., 2003a, b) and numerous additional *Fusarium* anamorphs within the *Liseola* and *Elegans* sections have been defined on the basis of morphology and DNA sequence differences (Nirenberg and O'Donnell, 1998; O'Donnell et al., 1998, 2000; Marasas et al., 2001b). This suggests that additional biologically significant entities remain to be identified. These species also produce an array of mycotoxins and secondary metabolites and, in particular, each MP generally possesses a specific toxicological profile and a preferential host (Leslie, 1995). In this respect, *F. verticillioides* is known for being a highly toxigenic species and it is a reported producer of fumonisin B_1 (FB_1), a toxin associated with human oesophageal cancer (Marasas et al., 2001a), FB_2, and moniliformin (MON), a toxin associated with damage to the myocardium (Marasas et al., 1986). However, members of *F. verticillioides* and *F. thapsinum* (originally classified as *F. moniliforme*), which are the anamorphs respectively of MPs A (*G. moniliformis*) and F (*G. thapsina*), share identical morphological traits, but they have a different preferential host (maize and sorghum, respectively) and also a different mycotoxin profile. In this respect, while members of MP A produce FB_1 and FB_2 and low or little amounts of MON, members of MP F are considered not to produce FB_1 or FB_2, but produce MON at high levels (Leslie et al., 1996).

The main objects of this study were (i) to characterize the toxin profile of the isolates of *F. verticillioides* obtained from banana fruits; (ii) to assess their fertility; (iii) to characterize their pathogenicity on the banana fruits; and (iv) to provide a molecular characterization for such isolates using AFLP analysis.

Materials and methods

Isolation and identification

Banana fruits affected by 'Fusarium crown rot' were collected from a market in Bari, Italy. The origin of the fruits was Ecuador, Panama and the Canary Islands, Spain. Tissue fragments were taken from the inside of the fruit (pulp) at the apical end, the centre and base and several were placed aseptically in Petri dishes containing a Fusarium-selective peptone-PCNB medium. The dishes were incubated at 25 °C for 5–7 days under fluorescent lamps for 12 h per day. Colonies were single-spored and transferred to PDA and water agar and carnation leaves for the morphological identification (Nelson et al., 1983). Twenty-eight isolates of *F. verticillioides* were produced (Table 1). Maize isolates were obtained from the Institute of Sciences of Food Production fungal collection (ITEM; http://www.ispa.cnr.it/Collection/) (Table 1). Tester strains of the MPs were obtained by J.F. Leslie, Kansas State University (accession numbers of the ITEM Collection: 3621–3634).

In vitro *toxin production*

Single-conidium isolates of fungal cultures (Table 1) were cultured on 100 g of autoclaved yellow maize kernels that were adjusted to about 45% moisture in 500-ml Erlenmeyer flasks and inoculated with 2 ml of an aqueous suspension containing approximately 10^7 conidia/ml. Cultures were incubated at 25 °C for 4 weeks. The harvested culture material was dried in a forced draft oven at 60 °C for 48 h, finely ground and stored at 4 °C until use. Controls were treated in the same way, except that they were not inoculated.

Toxin analysis

Extraction and analysis of FB_1 and FB_2 were performed by HPTLC (Munkvold et al., 1998). Extraction and analysis of MON were performed by TLC (Bottalico et al., 1982). Representative isolates isolated from banana were also analyzed by HPLC (Moretti et al., 1995) in order to confirm the lack of production of FB_1 and FB_2.

Crosses

Isolates isolated from banana were crossed with tester strains of MPs A to G (Cross A). Three further types of crosses were performed: (i) a set of MP A isolates from maize with MP A tester strains (Cross B), also isolated from maize (Leslie et al., 1992); (ii) MP F tester strains with a set of MP F strains isolated from sorghum (Cross C); (iii) isolates isolated from banana were crossed with each other (Cross D). These crosses were performed in order to obtain and compare data on the size of perithecia and the time of maturation of isolates belonging to MP A and MP F from maize and

Table 1. ITEM[a] Accession number, host and origin of strains

Species and plant host	NO strains investigated	Origin	ITEM accession number
Fusarium verticillioides	6	Ecuador	1142, 1143, 1144, 1145, 1146, 1147
	12	Panama	1113, 1121, 1126, 1129, 1132, 1139, 1140, 1141, 1148, 1149, 1150, 1151
Banana	10	Canary Islands, Spain	1243, 1244, 1245, 1246, 1247, 1248, 1249, 1250, 1251, 1252
Fusarium verticillioides	20	Italy	504, 1495, 1497, 1501, 1502, 1510, 1511, 1512, 1744, 1745, 1746, 1747, 1755, 1757, 1758, 1759, 1773, 1774, 1776, 1777
Maize	24	Slovakia	2617, 2618, 2619, 2622, 2625, 2628, 2629, 2636, 2637, 2638, 2640, 2649, 2650, 3412, 3413, 3415, 3416, 3417, 3418, 3419, 3420, 3421, 3422, 3423
	20	Iowa, USA	2282, 2285, 2288, 2289, 2290, 2390, 2395, 2396, 2397, 3970, 3985, 3993, 3998, 4015, 4029, 4032, 4034, 4037, 4038, 4040
	4	USA	1331, 1332, 3621[b], 3622[b]
	4	Italy (dead larvae in maize field)	2006, 2007, 2009, 2011

[a] Institute Sciences of Food Production fungal collection.
[b] *Gibberella fujikuroi* complex mating population A tester strain (Leslie et al., 1992).

sorghum respectively. These data were compared with those related to the perithecia obtained from positive crosses isolated from maize by MP A tester strains and from fertile crosses within the banana set of isolates. The crosses were performed according to Klittich and Leslie (1988). Plates were observed until the growth of fertile perithecia was evaluated under stereoscope. For each cross, fertility was confirmed by the observation of cirrus on the top of the perithecia. Spores were scored for viability by spreading agar plates with cirrus suspension and assessing germination with a microscope.

Pathogenicity assay

Healthy banana fruits were inoculated with 20 isolates of *F. verticillioides* from banana and 20 from maize. The assay was repeated three times. Healthy fruits were selected, weighed and inoculated with 7-day old cultures of the fungus on PDA at 25 °C. The fruits were surface sterilized with 75% (v/v) ethanol and washed twice with sterile water. Five holes were made on the sterilized skins by toothpicks previously submerged in a fungal spore suspension (10^8 spores/ml) in sterile water containing 0.01% Tween 80. The toothpicks were placed on each of the bruises. Noncolonized toothpicks were used as controls. The inoculated fruits were incubated at 25 °C for 6 days in a moist chamber. The rot diameters were measured after 2, 4 and 6 days. The symptoms of the disease were also estimated gravimetrically. Banana fruits were weighed at the end of each experiment; then rotted tissues (skins and pulps) were removed with a scalpel and the remaining tissues were weighed again.

AFLP analysis

DNA was extracted from fungal cultures (Mulè et al., 2004). For analysis using Fluorescent AFLP (fAFLP), simultaneous restriction–ligation reactions were done according to the manufacturer's instructions (AFLP Microbial Fingerprinting, Applied Biosystems – PE Corporation), in a single tube to prepare DNA template for nonselective amplification. Endonucleases EcoRI and MseI (New England Biolabs, Hitchin, UK) were used to digest approximately 10 ng genomic DNA from each isolate, and restriction fragments were ligated to double-stranded restriction site-specific adaptors. A preselective PCR was carried out in a 20 µl (final volume) mixture and PCR products of each reaction were diluted 20:1 with TE. For the selective PCR, 1.5 µl of the resulting diluted PCR sample was amplified in a 10 µl (final volume) mixture using selective primers. Fluorescent AFLP was done with 6-carboxyfluorescein (FAM; blue) fluorescent dye-labelled *Eco*RI primer (Applied

Biosystems – PE Corporation) with two base selection (*Eco*RI + AC) and unlabelled *MseI* primer with two base selection (*MseI* + CC). PCR reaction was done according to the AFLP microbial fingerprinting protocol by using a model 9700 GeneAmp PCR system. The fAFLP fragments were separated on an ABI Prism 310 automated DNA sequencer according to the manufacturer's instructions with reference to using Performance Optimized Polymer 4 (POP-4) for microsatellite analysis (Applied Biosystems). The products of fAFLP (1 μl) were added to 26 μl of loading dye (a mixture containing 25 μl of deionized formamide and 0.5 μl of GeneScan-500 (ROX) size standard (Applied Biosystems). The sample mixtures were heated to 95 °C for 2 min, cooled on ice and rapidly loaded. Reproducibility tests were based on repeated analysis of identical samples. DNA samples from five isolates were submitted to the AFLP procedure repeated in triplicate, while DNA samples from other samples were analyzed in duplicate. Moreover, three subcultures from each of the five isolates were made and analyzed. For processing data and dendrogram construction, GeneScan collection version 3.1.2 software (PE Applied Biosystems) was used to automatically size and quantify individual fragments by using the internal lane standards. Peak height thresholds were set at 50 and any peak height lower than this value was not included in the analysis. Genotyper software (PE Biosystem) automatically interpreted the GeneScan data after the analysis parameters were set to medium smoothing. The binary matrix was analyzed with NTSYS software using the band-based DICE similarity coefficient and the clustering of fingerprints was performed with the unweighted pair group method by using average linkages (Nei and Li, 1979).

Results

Toxin production

Isolates from banana did not produce detectable amounts of FB_1 and FB_2, while all produced MON at a range between 100 and 1400 μg g^{-1} (Table 2). The isolates from maize produced FB_1 (20–5645 μg g^{-1}) and FB_2 (25–850 μg g^{-1}), but did not produce MON (Table 2).

Crosses

Twenty-four of the 28 isolates from banana which were crossed showed the ability to produce fertile perithecia with MP A tester strains isolated from maize (Leslie et al., 1992), while four of them were unfertile (Table 3). All 28 isolates were unfertile when crossed with tester strains of the other MPs. The results of the three other crosses are summarized in Table 3. The perithecia of Cross A were larger (350–360 (356) μm diameter) than those of the other crosses. In comparison, Cross B produced perithecia with a smaller size

Table 2. Toxin production, mating types and pathogenicity on banana fruits of strains from banana and maize of *Fusarium verticillioides*

Host and no. of strains	MP A[a] strains	FB_1[b] range	FB_2[b] range	MON[b] range	MATA-1/ MATA-2	*Pathogenicity assay**	
						Diameter (cm)	Weight loss (g)
Banana 28	24	0/24	0/24	24/24 100–1400	13/11	1.93	13.13
Maize 72	72	72/72 20–5645	72/72 25–850	0/72	33/39	1.52	5.63

* The diameter of infection value for control was 0.2818 cm; the weight loss for control was 0.3 g. For diameter of infection, Duncan test showed that the values in table were significantly different for $P = 0.01$ and not for $P = 0.001$. For weight loss, Duncan test showed that the values in the table were significantly different for $P = 0.001$.

[a] Number of strains fertile on total number analyzed *in vitro* crosses by tester strains ITEM 3621 and ITEM 3622 of mating population A of *Gibberella fujikuroi* complex (Leslie et al., 1992).

[b] Number of positive strains on total number of strains analyzed; the range is expressed in μg g^{-1} of toxin produced on 4 week old maize kernel cultures.

Table 3. Comparison of perithecia diameter and time of perithecia maturation

Crosses	Diameter range (μm) (mean)*	Maturity[a] (days) (mean)**	Ascospore viability (mean)
Cross A[b]	350–360	47–50	99–100
	(356) **a**	(48) **a**	(99)
Cross B[c]	291–305	15–19	93–100
	(298) **b**	(16) **b**	(98)
Cross C[d]	216–229	15–17	98–100
	(220) **c**	(16) **b**	(99)
Cross D[e]	220–260	18–21	99–100
	(240) **c**	(19) **b**	(99)

*According to Duncan's test values followed by the same letter are not statistically different for $P = 0.01$.
**According to Duncan's test values followed by the same letter are not statistically different for $P = 0.001$.
[a] Mature perithecia were observed when the ascospores started to exude in a cirrhus.
[b] Values from 20 positive *in vitro* crosses between banana strains and tester strains ITEM 3621 and ITEM 3622 of mating population (MP) A of *Gibberella fujikuroi* complex (Leslie et al., 1992).
[c] Values from 20 positive *in vitro* crosses between maize strains and tester strains ITEM 3621 and ITEM 3622 of MP A of *G. fujikuroi* complex.
[d] Values from 20 positive *in vitro* crosses between strains belonging to MP F and MP tester strains ITEM 3631 and ITEM 3632 of mating population F of *G. fujikuroi* complex (Leslie et al., 1992).
[e] Values from 20 positive *in vitro* crosses among fertile banana strains of Cross A.

(291–305 (298) μm), while Cross C produced perithecia of 216–229 (220) μm. The successful crosses between the banana isolates (Cross D), produced perithecia which were smaller (220–260 (240)μm) than those of Cross A. Statistical analysis, performed according to the Duncan's test, showed that the size of perithecia from Cross A was significantly different ($P = 0.01$) to those produced in Crosses B, C and D. The time needed for the perithecia maturation (cirrus extrusion from the top of the perithecia) was also compared. The data showed a significant difference in the time of the perithecia maturation for the set of crosses between banana isolates and tester strains of MP A, when compared to all the others (Table 3). Indeed, this kind of cross produced a mean of 48 days for the maturation of the perithecia, compared with a mean of 16–19 days for all the other kinds of crosses. The viability of ascospores was high, varying from 93% to 100% (Table 3).

Pathogenicity assay

Pathogenicity tests compared the effects of the banana and maize isolates on banana fruits. Typical coloured spots developed on the fruit skins and the fungal mycelia grew over the holes. The data, summarized in Table 2, showed that isolates from banana had a greater ability to cause infection (Figure 1). Both sets of isolates were pathogenic on banana, although with a different degree of severity. The mean rot diameters on banana fruits after 6 days of inoculation were 1.93 cm for banana isolates and 1.52 cm for maize isolates; the fruits used for the control showed a mean rot diameter of 0.2818 cm. Duncan's test showed that these values were significantly different for $P = 0.01$ while they were not for $P = 0.001$. The rotted pulps were also measured after 6 days showing that the mean weight loss was 13.13 g for the banana isolates, 5.63 g for the maize isolates and 0.30 g for the control ($P = 0.001$).

AFLP polymorphism

AFLP bands were scored from amplification with two primer pair combinations. Based on the resulting AFLP polymorphism, at least two subgroups were identified within *F. verticillioides*. These results are supported by a DICE coefficient of similarity that is 51% between the two subgroups (Figure 2).

Discussion

The worldwide distribution of *F. verticillioides* and the wide range of hosts from which it has been isolated supports the existence of variability within

Figure 1. Pathogenicity assay on banana fruits: left, isolates of *Fusarium verticillioides* from maize; centre, isolates of *F. verticillioides* from banana; right, control.

this species (Leslie, 1995). On the other hand, Huss and Leslie (1996) did not detect any variability within *F. verticillioides* by using isozymes, suggesting that this species could be a homogenous biological population characterized by a high incidence of sexual reproduction (Leslie and Klein, 1996). The data provided here showed that members of the *F. verticillioides* population isolated from banana have specific and homogenous biological, toxinogenic and molecular traits compared to the other members of the same species. Hirata et al., (2001) investigated seven isolates of *F. verticillioides* isolated from banana imported from Mexico, in order to elucidate their possible differentiation with strains previously described for this species. Although some morphological features were consistent with Wollenweber's original concept of *F. moniliforme* var *minus* (Wollenweber, 1931), the morphological and phylogenetic analyses suggested that the isolates could be assigned to *F. verticillioides*. On the other hand, isolates of *F. verticillioides* from banana showed different abilities to produce toxins (Jimenéz et al., 1997). In both these reports, fertility tests were not performed, although mating has been a very useful tool in defining the biological species in the *G. fujikuroi* species complex (Leslie et al., 1995). Moreover to belong to a specific MP could reflect differences in secondary metabolite production and host preference, and in colonization of different ecological niches (Leslie, 1995). Our study provided for the first time a wide set of data on a population of isolates from banana, which had been identified morphologically as *F. verticillioides*. All the isolates produced MON, while none produced either FB_1 and FB_2. Our fertility tests showed that most of the isolates belonged to MP A. Thus, we obtained a set of isolates belonging to MP A which were characterized by a toxin profile that was inconsistent with the MP A: the specific toxin profile included FB_1 and FB_2, but not MON (Leslie et al., 1992; Moretti et al., 1995). Indeed, the toxin profile of the strains from banana was typical of members of MP F (syn. *G. thapsina*)

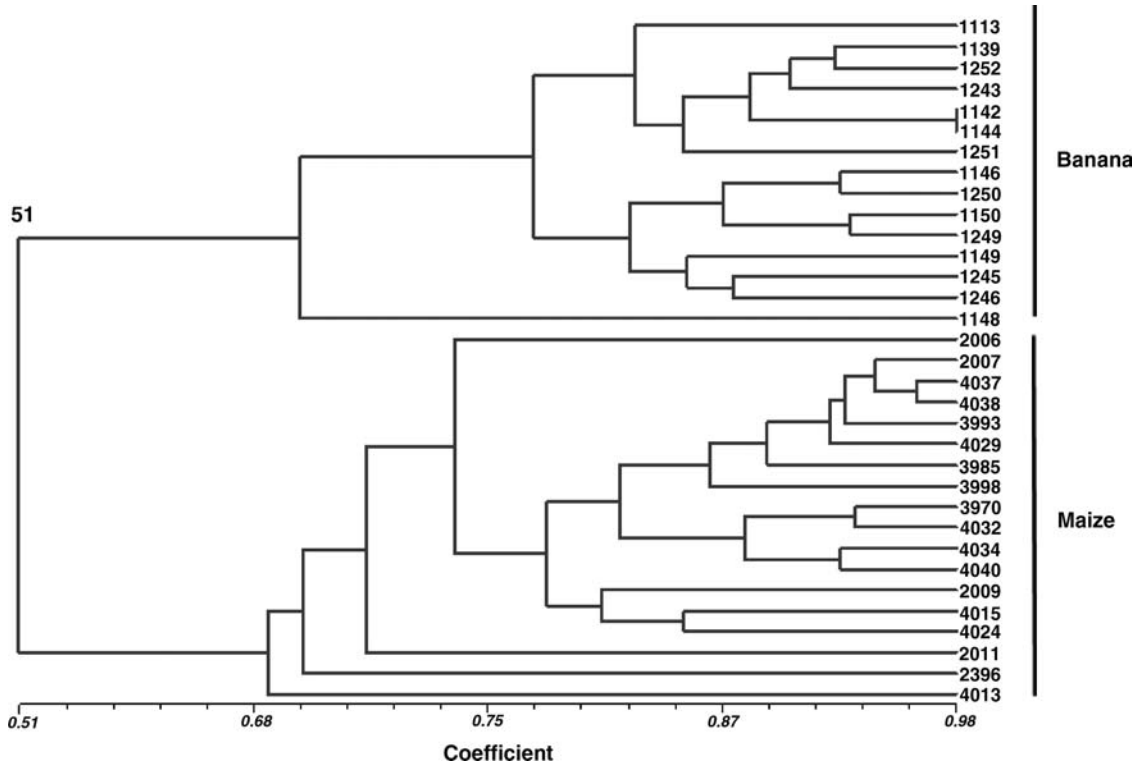

Figure 2. Dendogram generated from UPGMA cluster analysis of 33 *F. verticillioides* strains using DICE similarity coefficient, showing two groups according to the host (banana and maize).

(Leslie et al., 1992; Moretti et al., 1995; Leslie, 1995). This MP has an anamorphic stage, *F. thapsinum*, which until recently was considered as *F. verticillioides* (Klittich et al., 1997). Therefore, the *F. verticillioides* strains from banana showed a toxin profile typical of MP F, although most of them belonged to MP A. However, the size of the perithecia obtained from these crosses was unusually large and, combined with the very long time needed to obtain the maturity of the sexual structures, clearly differentiated such population for the sexual structure formation from other members of the same MP A. According to Leslie et al., (2001), although the concept of populations, as opposed to an individual, is required to delimit and define clearly the extent of variation within species, the interfertility between individuals within a population is not the definitive criterion for subdividing species. In this respect, Desjardins et al., (1997) and Zeller et al., (2003a, b), reported that some isolates from two different MPs, C and D, can interbreed and complete meiosis to produce viable progeny. We suspect that the anomalous traits of the fertile crosses of banana isolates by the MP A testers strains from maize, could reflect genetic differences between such populations and typical members of MP A. Such a hypothesis seems to be confirmed by the different toxin profiles and the pathogenicity assay that showed a different degree of aggressiveness between the two set of isolates on the banana fruits. Further support is provided by the fAFLP analysis that clustered the isolates from maize and from banana in two groups, with a 51% coefficient of similarity. According to Leslie and Marasas (2002), isolates that share ~65% or more of the AFLP bands are in the same species while isolates in different species rarely share more than 40% of the bands. Interestingly, isolates of banana here analyzed are ambiguously located, stimulating several questions on their genetic specificity. Analysis of two partial genomic sequences from the IGS region and the EF-1α carried out on the same set of isolates by Mirete et al., (this issue), revealed the existence of variability between banana and maize isolates and detected a cluster of

isolates from banana which could be considered a distinct population within *F. verticillioides*. From these data and our studies, the populations from banana and maize seem to be distinct, with low if any genetic interchange. Reproductive isolation of the banana population is apparently taking place, since the crosses performed *in vitro* showed some features which are different from those observed in normal crosses within MP A (Cross B). Although the ability of isolates from banana to cross *in vitro* with MP A members from maize could offer a means of moving genes from one of these populations to another by classical genetic methods, the most reliable hypothesis is that *F. verticillioides* from banana has coevolved with its host, undergoing variation in the toxin production ability and virulence with respect to the ancestral progenitor. Moreover, the results of the pathogenic assay indicated that FB_1 and FB_2 could not play a role in the symptom expression on banana fruits, suggesting that the relevance of these toxins in the virulence is probably dependent on the host and the ecological conditions (Leslie et al., 1995).

In conclusion, isolates of MP A from banana showed different traits than members of the same MP from maize. Of particular interest are the data for toxin production. Isolates of MP A rarely produce MON. The toxin profile seems to be that of MP F. The differences in formation of perithecia in the *in vitro* crosses, could mean that genetic differences do exist between the populations from maize and banana. The pathogenicity assay results and the molecular profiles seem to confirm these observations. These could reflect important differences in the ecology and natural history of the two populations from banana and maize and trigger further investigations on the mechanisms of toxin production and pathogenicity within the same MP. Finally, the data obtained by the AFLP analysis could provide useful markers for the development of diagnostic PCR for this species and for its subgroups from maize and banana by sequencing the conserved and polymorphic bands of their different AFLP profiles.

Acknowledgements

This work was supported by an EU project: Early Detection of Toxigenic *Fusarium* Species and Ochratoxigenic Fungi in Plant Products (QLK1-CT-1998-001380); we thank Gaetano Stea and Vincenzo Ricci for their valuable technical assistance.

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