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OCCURRENCE OF *FUSARIUM VERTICILLIOIDES* AND *FUSARIUM MUSAE* ON BANANA FRUITS MARKETED IN HUNGARY

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Fusarium strains were isolated from rotten banana fruit imported into Hungary from some African and some Neotropical countries. The strains were identified using morphological features, 2-benzoxazolinone tolerance, translation elongation factor (EF-1α) sequences and inter simple sequence repeat (ISSR) analysis. All strains from Africa proved to be *F. verticillioides* whereas the strains from the Neotropics are *Fusarium musae*. According to the PCR proof and the fumonisin toxin measurement *F. musae* strains cannot produce any fumonisins (FB_{1-4}).

Keywords: *Fusarium musae,* phylogenetic analysis, maximum likelihood, inter simple sequence repeat (ISSR) analysis, fumonisin

Introduction

Crown rot (crown mold or pedicel rot) is a common post-harvest disease of banana which causes ripe fruit loss in the consuming countries. Banana fruits are often reported to be infected by *Fusarium* species such as "Fusarium fruit rot" [1] and "Fusarium crown rot", however, little attention has so far been paid to the presence of *Fusarium* spp. within and on the fruit. This is an interesting issue not only because of the potential role of such species in banana decay during storage and marketing [2] but also because of the fact that some *Fusarium* species have been found to produce mycotoxins. *F. verticillioides* (Sacc.) Nirenberg is frequently associated with maize worldwide and cause ear and stalk rot and produces fumonisin mycotoxins [3]. Nevertheless, *F. verticillioides* strains were also identified in other host plants including banana [1, 2, 4]. Although

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several *Fusarium* strains recovered from banana were identified as *F. verticillioides* in these studies, a number of differences have been highlighted between some of these strains isolated from banana and typical *F. verticillioides* strains primarily originating from maize. Van Hove et al. [5] reported morphological and biochemical evidence as well as multilocus phylogenetic analyses based on elongation factor (EF-1α), calmodulin, β-tubulin, and the second largest subunit of RNA polymerase II (RPB2) sequences indicating that these isolates represent a new species, *Gibberella musae (F. musae)* within the *Gibberella fujikuroi* species complex [6, 7] related to but distinct from *F. verticillioides.*

The main objectives of our study were (i) to characterize *F. verticillioides*-like isolates obtained from banana fruits marketed in Hungary using morphological, physiological and molecular attributes (ii) to study the mycotoxin-producing potential of the above isolates.

Materials and Methods

Banana fruits affected by "*Fusarium* crown rot" were collected from different markets in Budapest, Hungary. The fruits originated from Ecuador, Ivory Coast, Columbia and Costa Rica (Table I). Tissue fragments were taken from the inside of the fruits (pulp) at the apical end and several were placed aseptically in Petri dishes containing a *Fusarium* selective peptone-PCNB medium [8]. 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 with a piece of sterilized filter paper for the morphological identification. The species determination was made on the basis of the protocols of Leslie et al. [8]. Strains isolated from the same fruits were eliminated from the investigation. Additionally, some strains originated from Agri-Food Toxigenic Fungi Culture Collection, Institute of Sciences of Food Production, Bari, Italy and *F. verticillioides* strains from our collection were included.

2-Bezoxazolinone (BOA) tolerance was determined on PDA medium amended with 1 mg/ml BOA (Sigma-Aldrich, Bornem, Belgium) as described by Glenn et al. [9].

DNA was extracted from subcultures grown from single conidia on potato dextrose agar. Mycelium was scraped off and DNA was isolated using ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). PCR amplification of the *FUM8* gene and the flanking region of the fumonisin gene cluster (ΔFGC) were carried out with primers according to Van Hove et al. [5].

PCR amplification of the EF-1α gene was carried out with primers EF-1H (5'-ATGGGTAAGGAAGACAAGAC-3') and EF-2T (5'-GGAAGTACCAGT-

GATCATGTT-3') based on O'Donnell et al. [10]. PCR conditions were according to Mirete et al. [11].

EF-1α genes were after PCR amplification directly sequenced. Electropherograms were processed and analysed with the Staden Program Package [12]. The resulted DNA sequences and sequences derived from the GenBank (Acc. numbers in Fig. 1) were aligned using PRANK [13] using the PRANKSTER interface. The EF-1α sequence of a *Fusarium andiyazi* strain (Table I) served as outgroup. Maximum likelihood (ML) phylogenetic analyses were carried out with the online version of PHYML 3.0 [14]. The GTR nucleotide substitution model was used with the ML estimation of base frequencies. Six substitution rate categories were set and the gamma distribution parameter and the proportion of invariable sites were estimated. The statistical supports of the branches were tested by ML bootstrap analyses with 1000 replicates. Phylogenetic trees were viewed and edited by the Tree Explorer of the MEGA 5 program [15] and a text editor.

Anchored ISSR primers were designed based on the results of Ren et al. [16] and Mahfooz et al. [17] (Table I). All ISSR PCR amplifications were done in a final volume of 25 µl with the following components: DreamTaq Green PCR Master Mix 2× (Fermentas) 12.5 µl, 30 ng of template DNA, 0.6 µM primer and water. PCR parameters were according to Baysal et al. [18]. Annealing temperatures were fitted to the ISSR primers (Table II). The DNA fragments were separated by electrophoresis in 1.5% agarose gel containing 0.5× TBE and then visualized and photographed under ultraviolet light. ISSR markers were considered as dominant markers and the presence or absence of bands with the same size was scored by eye. Only well-reproducible bands from 150 to 3000 bp were taken into consideration. A dendrogram was constructed using the unweighted pair-group method with arithmetic average (UPGMA) method using TREECON [19] based on the coefficient of Nei and Li [20]. Samples were additionally analysed using the QIAxcel system together with the QIAxcel DNA High Resolution Kit and the OM700 method. The QX Alignment Marker 15 bp/10 kb, the QX DNA Size Marker 250 bp/4 kb was included in the run.

Rice cultures were used for the fumonisin production by *Fusarium* isolates. Long-grain rice (50 g, Uncle Ben's) and HPLC-grade water (50 ml) were added to Erlenmayer flasks (500 ml) and kept room temperature overnight. The excess water was decanted, and the flasks were subsequently autoclaved at 121 °C for 15 min on each of two consecutive days. Conidial suspension (10^4 cells/ml) of *Fusarium* strains were used for inoculation (5 ml per flask). The cultures were shaken once daily for the first 3 days after incubation in order to distribute the inoculum and to prevent the grains from adhering. After two weeks of incubation at 25 °C in the dark, the cultures were frozen immediately after removal

from the incubator and freez-dried, the finely milled samples were stored in a deep freezer at –80 °C until analysis. Extraction of fumonisins, HPLC conditions and ESI-MS analyses were described in detail earlier [21, 22].

Results

A total of 16 strains were isolated from banana fruit during our investigation. According to the BOA-tolerance test (Table I), the analysis of the EF-1α sequences (Table I, Fig. 1) and the ISSR analysis (Fig. 2) the strains could be classified into two species: *F. verticillioides* and *F. musae*. All strains from Africa belong to *F. verticillioides* (FVB 8–13) whereas the strains from the Neotropical countries proved to be *F. musae* (FVB 1–7, 14, 18, 19).

In the PCR test FVB 8–13 shoved positive PCR result for the *FUM8* gene whereas FVB 1–7, 14, 18 and 19 did not. In the case of the PCR amplification of ΔFGC amplification they behaved reversed. During the survey of the fumonisin production of a total of 16 bananas colonizing strains no surprising result was found. All analyzed *F. verticillioides* strains (FVB 8–13) produced high amounts of toxins of the fumonisin B series as expected (Table I) with FB_1 predominating. We could not measure any fumonisins in case of the *F. musae* strains (Table I).

Discussion

F. verticillioides and *F. musae* strains sharing identical EF-1α, β-tubulin, calmodulin and *RPB2* combined sequences have been grouped in nine and six haplotypes by Van Hove et al. [5], respectively. Using only the sequences of the EF-1α, we could not distinguish all these haplotypes, so we could not unequivocally classify our strains to these haplotypes. The sequences of our *F. musae* isolates belong to two types: one of them is the sequence identical through the haplotypes 1–4 and 6 (Type 1). This type includes the isolates FVB 5–7, 14, 18, and 19, ITEM 1113, 1139 and 1142. The other type belongs to the haplotype 5, which includes the isolates FVB 1–4 (Type 2). The ISSR analysis reveals more genetic diversity among *F. musae* strains. However, the four strains of the haplotype 5 produced distinct ISSR patterns based on the UPGMA analysis.

Concerning the *F. verticillioides* isolates, the sequences belong to seven types. First type (T1) includes the haplotypes 1 and 2 and none of the newly sequenced strains belongs here. The second type (T2) includes the haplotypes 3–6 and the EF-1α sequences are identical through these types. The sequence of hap-

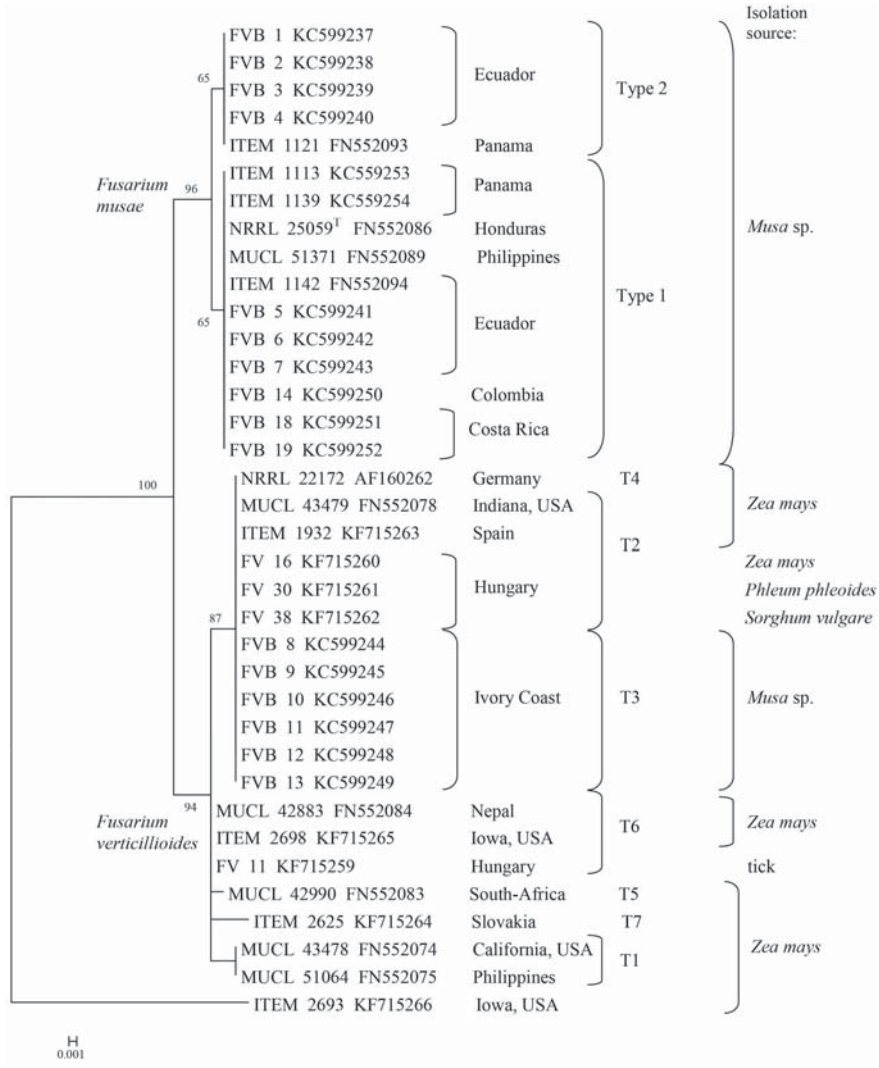

Figure 1. Maximum likelihood (PHYML) tree based on translation elongation factor (EF-1α) sequences of 36 *Fusarium* strains. The EF-1α sequence of *F. andiyazi* ITEM 2693 was used as outgroup. Bootstrap values presented as percentages are on the branches (below 60% are not shown). Bar indicates 0.001 expected change per site per branch. Types 1 and 2 within *F. musae* and T1–7 within *F. verticillioides* indicate individual EF-1α sequences

ITEM: Agri-Food Toxigenic Fungi Culture Collection, Institute of Sciences of Food Production, Bari, Italy; MUCL: Mycothèque de l'Université catholique de Louvain, Université catholique de Louvain, Louvain-la-Neuve, Belgium; NRRL: Northern Regional Research Laboratory, NCAUR, Peoria, Illinois; FV and FVB: our isolates. Accession numbers of the sequences, geographic origins and isolation sources of the strains are also indicated

Table I. *Fusarium* strains used in this study

Strain[a]	Geographic origin	Isolation source	BOA[b]	Acc. nr. EF-1α	*FUM8*[c]	ΔFGC[d]	FB_1; FB_2; FB_3; FB_4[e]
F. musae							
FVB 1	Ecuador	*Musa* sp.	–	KC599237	–	+	nd
FVB 2	Ecuador	*Musa* sp.	–	KC599238	–	+	nd
FVB 3	Ecuador	*Musa* sp.	–	KC599239	–	+	nd
FVB 4	Ecuador	*Musa* sp.	–	KC599240	–	+	nd
FVB 5	Ecuador	*Musa* sp.	–	KC599241	–	+	nd
FVB 6	Ecuador	*Musa* sp.	–	KC599242	–	+	nd
FVB 7	Ecuador	*Musa* sp.	–	KC599243	–	+	nd
FVB 14	Colombia	*Musa* sp.	–	KC599250	–	+	nd
FVB 18	Costa Rica	*Musa* sp.	–	KC599251	–	+	nd
FVB 19	Costa Rica	*Musa* sp.	–	KC599252	–	+	nd
ITEM 1113	Panama	*Musa* sp.	–	KC599253	–	+	nt
ITEM 1121	Panama	*Musa* sp.	– [5]	FN552093	– [5]	+ [5]	nd [5, 23]
ITEM 1139	Panama	*Musa* sp.	–	KC599254	–	+	nt
ITEM 1142	Ecuador	*Musa* sp.	–	FN552094	–	+	nd [5, 11, 23]
NRRL 25059[T]	Honduras	*Musa* sp.	– [5, 9, 24]	FN552086	– [25]	+ [26]	nd [5, 26]
MUCL 51371	Philippines	*Musa* sp.	– [5]	FN552089	– [5]	+ [5]	nd [5]
F. verticillioides							
FVB 8	Ivory Coast	*Musa* sp.	+	KC599244	+	–	9450; 2175; 1460; 458
FVB 9	Ivory Coast	*Musa* sp.	+	KC599245	+	–	5657; 1198; 672; 221
FVB 10	Ivory Coast	*Musa* sp.	+	KC599246	+	–	6137; 1462; 800; 279
FVB 11	Ivory Coast	*Musa* sp.	+	KC599247	+	–	6342; 1712; 1111; 493
FVB 12	Ivory Coast	*Musa* sp.	+	KC599248	+	–	5579; 1254; 620; 212
FVB 13	Ivory Coast	*Musa* sp.	+	KC599249	+	–	5879; 1321; 703; 236

Table I. (cont.)

Strain[a]	Geographic origin	Isolation source	BOA[b]	Acc. nr. EF-1α	*FUM8*[c]	ΔFGC[d]	FB_1; FB_2; FB_3; FB_4[e]
FV 11 (PPI-F111)[m]	Hungary	tick	nt	KF715259	nt	nt	277; 49; 22; 7 [22]
FV 30 (PPI-F130)[m]	Hungary	*Phleum phleoides*	nt	KF715261	nt	nt	197; 20; 30; 2 [22]
FV 38 (PPI-F138)[m]	Hungary	*Sorghum vulgare*	nt	KF715262	nt	nt	2550; 746; 238; 68 [22]
ITEM 1932	Spain	*Zea mays*	nt	KF715263	nt	nt	nt
ITEM 2625	Slovakia	*Zea mays*	nt	KF715264	nt	nt	nt
ITEM 2698	Iowa, USA	*Zea mays*	nt	KF715265	nt	nt	nt
NRRL 22172	Germany	*Zea mays*	nt	AF160262	nt	nt	nt
MUCL 42883	Kathmandu, Nepal	*Zea mays*	+ [5, 9]	FN552084	+ [5, 26]	– [5]	nd; nd; nt; nt [26] trace; nd; nt; nt [28]
MUCL 42990	South-Africa	*Zea mays*	+ [5, 9]	FN552083	+ [5, 26]	– [5]	364; 164; nt; nt [26] 2170; 670; nt; nt [28]
MUCL 43478	California, USA	*Zea mays*	+ [5, 9]	FN552074	+ [5, 27]	– [5]	638; 216; nt; nt [5] 57; 8,1; nt; nt [26] 6160; 1845; nt; nt [28]
MUCL 43479	Indiana, USA	*Zea mays*	+ [5, 9]	FN552078	+ [5, 26]	– [5]	150; 26; nt; nt [5] 673; 212; nt; nt [26] 1045; 240; nt; nt [28] 3622; 986; nt; nt [11]
MUCL 51064	Philippines	*Zea mays*	+ [5]	FN552075	+ [5]	– [5]	137; 15; nt; nt [29]
F. andiyazi							
ITEM 2693	Iowa, USA	*Zea mays*	nt	KF715266	nt	nt	nt

[a] FVB, FV, and PPI-F = our laboratory enumerations; ITEM = Agri-Food Toxigenic Fungi Culture Collection (Institute of Sciences of Food Production, Bari, Italy); NRRL = Northern Regional Research Laboratory, NCAUR, Peoria, Illinois; MUCL = Mycothèque de l'Université catholique de Louvain, Université catholique de Louvain, Louvain-la-Neuve, Belgium.

[b] 2-benzoxazolinone (BOA) tolerance determined by culture on PDA medium amended with 1 mg/ml BOA. +: growth, –: no groth, nt: not tested.

[c] PCR amplification of *FUM8* partial sequence. +: amplification, –: no amplification, nt: not tested.

[d] PCR amplification of the excized Fumonisin Gene Cluster flanking region sequence. +: amplification, –: no amplification, nt: not tested.

[e] Fumonisin B_1, B_2, B_3 and B_4 production in µg/g. nd: not detectable, nt: not tested.

Table II. Inter-simple sequence repeat markers used in this work

Primer code	Primer sequence (5′→3′)*		§	PCR T_A (°C)
AAG1	AAGAAGAAGAAGAAGAAGAAA	$(AAG)_6AAA$	23	53
AG1	AGAGAGAGAGAGAGAGG	$(AG)_8G$	18	46
AG2	AGAGAGAGAGAGAGAH	$(AG)_7AH$	22	46
GCA3	GCAGCAGCAGCAGCAGCAGCY	$(GCA)_5GCY$	5	75

§: Number of scorable bands (ranging from 150 to 2000 bp)
*: H = A + T + C, Y = C + T
T_A: annealing temperature

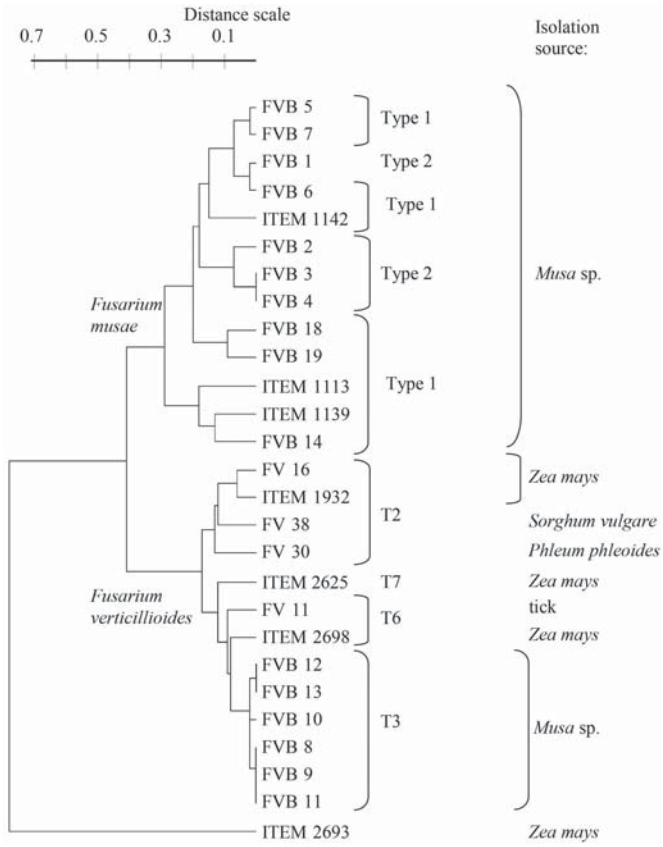

Figure 2. Unweighted pair-group method with arithmetic average (UPGMA) dendrogram showing the clustering pattern of 27 *Fusarium* strains, based on their inter simple sequence repeats (ISSR) patterns determined using four ISSR markers included in Table I. Note the isolation sources indicated. Types 1 and 2 within *F. musae* and T1–7 within *F. verticillioides* indicate individual EF-1α sequences

lotype 9 (NRRL 22172) and the sequences of some of our strains isolated from banana seem also to be identical with those of haplotype 3 based on Figure 1, but the maximum likelihood analysis cannot handle the gaps and do not show the hidden diversity in the phylogenetic tree (Fig. 1). The isolates FVB 8–13 represent a new variation (T3) with one nucleotide less in an intron. On the contrary, the strain NRRL 22172 has a three nucleotide insertion in an intron (T4). The haplotypes 7 and 8 are represented by one and three sequences (T5 and T6), respectively. Lastly, the strain ITEM 2625 has a unique EF-1α sequence with 3 substitutions in different introns (T7).

Regarding the geographic origins final conclusions cannot be drown because of the small number of the isolates. T2 and T6 include more isolates and interestingly they are present worldwide: Asia, North America and Europe. The same is true in the case of the isolation sources. Both T2 and T6 include isolates from different sources: different Poaceae (T2) and corn and tick (T6), respectively.

The UPGMA tree calculated using the ISSR patterns have highlighted the genetic diversity of the investigated strains. The differences are obvious within nearly all strains that had identical EF-1α sequences. The two closely related species *F. verticillioides* and *F. musae* are clearly parted from another but the types shown in the sequence analysis cannot be observed in all cases (Fig. 2). The six *F. verticillioides* strains isolated from bananas imported from Ivory Coast (FVB 8–13) showed much less diversity than the seven *F. musae* strains isolated from bananas from Ecuador (FVB 1–7).

Van Hove et al. [5] detected no fumonisin production of any investigated *F. musae* strains. They justified the lack of fumonisin production of their *F. musae* strains with the excision of the fumonisin gene cluster. They corroborated their hypothesis with PCR amplifications of several *FUM* genes and the ΔFGC. We used *FUM8* and ΔFGC of them, and the PCR amplifications suggested the same.

Acknowledgements

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