Occurrence of Fusaproliferin, Fumonisin B_1, and Beauvericin in Maize from Italy

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The occurrence of fusaproliferin (FP) was investigated in 22 samples of preharvest maize ears *Fusarium* rot, collected in 1994 in Italy. The most frequently recovered *Fusarium* species were *Fusarium moniliforme* and *Fusarium proliferatum*. Chemical analysis by high-perfomance liquid chromatography and gas chromatography/mass spectrometry identified FP (up to 500 mg/kg) in nine samples mostly infected by *F. proliferatum*. FP was often found to be associated with fumonisin B_1 (FB_1) (up to 300 mg/kg) and beauvericin (BEA) (up to 520 mg/kg). The occurrence of FP as a natural maize contaminant and its co-occurrence with FB_1 and BEA is reported here for the first time.

Keywords: *Fusaproliferin; fumonisin B_1; beauvericin; maize; Fusarium; toxins; contamination*

INTRODUCTION

Mycotoxins produced by the *Fusarium* species often occur in agricultural commodities and represent a serious risk to human and animal health (Marasas *et al.*, 1984).

Among agricultural crops, maize is commonly colonized by *Fusarium* species and contaminated by their toxins. In particular, *F. moniliforme* (Sheldon) and *F. proliferatum* (Matsushima) Nirenberg are well known pathogens of maize, causing stalk and ear rot worldwide (Booth, 1971; Leslie *et al.*, 1990), including Italy (Bottalico *et al.*, 1995; Logrieco *et al.*, 1995). Some isolates of these species also can produce potent mycotoxins (Marasas *et al.*, 1984).

Among these, fumonisin B_1 (FB_1; Figure 1A) a toxin produced by both *F. moniliforme* and *F. proliferatum*, is a well-known contaminant of maize (Rheeder *et al.*, 1992; Logrieco *et al.*, 1995); FB_1 was associated with human esophageal cancer in South Africa (Rheeder *et al.*, 1992) and is responsible for several animal diseases, e.g., leukoencephalomalacia in horses (Kellerman *et al.*, 1990) and pulmonary edema in swine (Harrison *et al.*, 1990).

Beauvericin (BEA; Figure 1B), a toxin produced by several *Fusarium* species (Gupta *et al.*, 1991; Logrieco *et al.*, 1995), is a cyclic lactone trimer containing an alternating sequence at three *N*-methyl L-phenylalanyl and three D-α-hydroxyisovaleryl residues. BEA, detected for the first time as a natural contaminant, in a Polish maize (Logrieco *et al.*, 1993) has been found in maize also from Italy (Bottalico *et al.*, 1995; Logrieco *et al.*, 1995), and the USA (Munkvold, personal communication). BEA is highly toxic to insects (Gupta *et al.*, 1991), and to murine and human cells, in which, it induces apoptosis (Ojcius *et al.*, 1991; Macchia *et al.*, 1995, 1996).

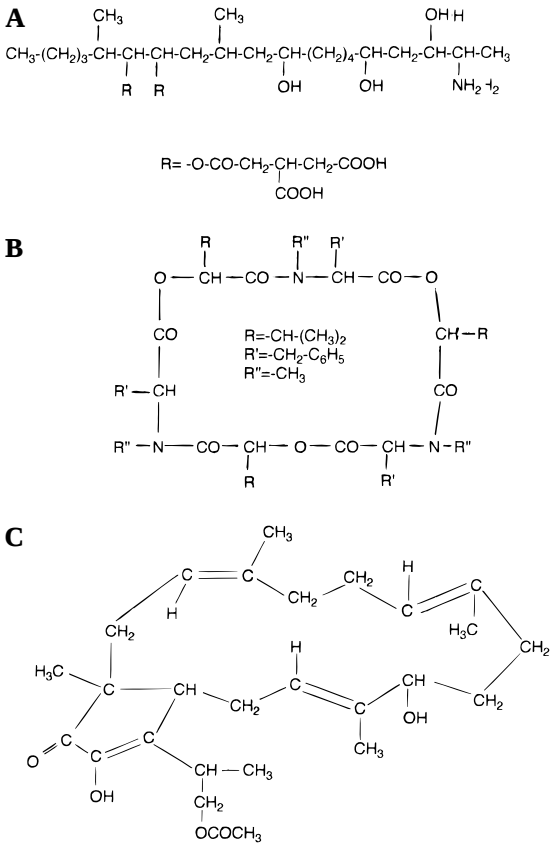

Figure 1. Chemical structures of fusariotoxins: (a) fumonisin B_1; (b) beauvericin; (c) fusaproliferin.

A new bicyclic sesterterpene, fusaproliferin (FP; Figure 1c), was firstly characterized from a toxigenic strain maize culture of *F. proliferatum* (Randazzo *et al.*, 1993; Ritieni *et al.*, 1995). Successively, Moretti *et al.* (1996) found that additional strains of *F. proliferatum* from Italian maize could produce FP *in vitro*, but that

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Table 1. Occurrence of *Fusarium* Species and Associated Mycotoxins Beauvericin, Fusaproliferin, and Fumonisin B_1 in Preharvest Maize Ear Rot in 1994 in Italy

sample	source	*Fusarium* spp.	(%)[a]	BEA[b] (mg/kg[e])	FP[c] (mg/kg[e])	FB_1[d] (mg/kg[f])
17/94	Matera	*F. moniliforme*	30	nd[a]	nd	traces
		F. equiseti	4			
18/94	Matera	*F. moniliforme*	42	nd	nd	traces
		F. proliferatum	3			
19/94	Matera	*F. moniliforme*	65	nd	nd	15
20/94	Matera	*F. moniliforme*	18	nd	25	50
		F. proliferatum	100			
		F. graminearum	7			
21/94	Matera	*F. moniliforme*	18	nd	nd	8
		F. proliferatum	100			
22/94	Matera	*F. moniliforme*	100	nd	nd	175
23/94	Matera	*F. moniliforme*	100	nd	nd	nd
		F. proliferatum	28			
24/94	Matera	*F. moniliforme*	100	nd	nd	10
		F. proliferatum	14			
25/94	Matera	*F. moniliforme*	100	nd	nd	150
26/94	Milano	*F. moniliforme*	66	nd	6	20
		F. proliferatum	100			
		F. equiseti	32			
		F. compactum	3			
27/94	Milano	*F. proliferatum*	78	nd	nd	nd
		F. oxysporum	56			
28/94	Milano	*F. proliferatum*	100	nd	10	50
		F. oxysporum	26			
29/94	Milano	*F. moniliforme*	89	nd	8	traces
		F. proliferatum	40			
30/94	Asti	*F. moniliforme*	23	25	20	33
		F. proliferatum	18			
31/94	Asti	*F. proliferatum*	100	7	35	90
32/94	Asti	*F. moniliforme*	100	nd	nd	185
		F. proliferatum	48			
33/94	Asti	*F. moniliforme*	89	36	20	25
		F. proliferatum	53			
34/94	Asti	*F. moniliforme*	83	520	500	300
		F. proliferatum	100			
35/94	Asti	*F. moniliforme*	13	nd	nd	25
		F. proliferatum	100			
		F. equiseti	7			
36/94	Asti	*F. proliferatum*	100	28	26	100
		F. equiseti	36			
		F. moniliforme	23			
37/94	Asti	*F. moniliforme*	48	traces	nd	60
		F. proliferatum	96			
38/94	Asti	*F. moniliforme*	100	nd	nd	180
		F. proliferatum	36			

[a] Percentages are based on 100 kernels per sample; kernels can be infected by multiples species, and thus the total of isolates for a given sample can be in excess of 100%. [b] Beauvericin. [c] Fusaproliferin. [d] Fumonisin B_1. [e] Data from HPLC analysis. [f] Data from HPTLC analysis. [g] nd, not detected.

30 strains of *F. moniliforme* did not produce this toxin when cultured on maize. Preliminary studies of toxigenicity indicate that FP is toxic to *Artemia salina* L. (Ritieni *et al*., 1995) and mammalian cells (Logrieco *et al*., 1996) and causes teratogenic effects on chicken embryos (Ritieni *et al*., 1997).

Emerging evidence suggests that mycotoxins may have synergistic effects *in vivo* (Javed *et al*., 1993; Bacon *et al*., 1996). Therefore, the detection of all main known toxins occurring in infected maize samples is useful to better evaluate the risk due to human and animal consumption of contaminated maize.

In previous reports, we described the natural co-occurrence of FB_1 and BEA on preharvest maize samples from Italy (Bottalico *et al*., 1995; Logrieco *et al*., 1995). On the other hand, there are not data to support the claim that FP is a natural contaminant. Since the maize samples described here were highly contaminated mostly by *F. moniliforme* and *F. proliferatum*, we were led to investigate on the possible natural occurrence of FP and its co-occurrence with BEA and FB_1, on Italian maize grown in three different areas in Italy.

MATERIALS AND METHODS

Maize Samples. Samples of visibly moldy maize ears were collected at preharvest time from different maize fields in threemaize vocated areas of northern and southern Italy (Table 1), during the 1994 crop season. The samples were transported in sterile plastic bags to the laboratory and stored at 4 °C.

Mycological Analysis. One hundred visibly infected kernels from each of the 22 samples analyzed (20 kernels per ear, from five ears), were placed on Petri plates (five kernels per plate, each kernel broken in two pieces) containing a modified pentachloronitrobenzene medium selective for *Fusarium* (Nelson *et al*., 1983). The plates were incubated in the dark at 25 °C for 1 week. *Fusarium* colonies were transferred to plates of potato dextrose agar and were incubated at 25 °C for 10 days, under fluorescent and black light lamps (2700 lux; 12 h photoperiod). The identification of *Fusarium* species was made according to the taxonomic system of Nelson *et al.* (1983).

Toxin Extraction. For BEA and FP extraction, 100 g of each sample was homogenized in a Waring blender for 5 min with 150 mL of methanol (Baker, Deventer, Holland, 99.5%). Samples were filtered through Whatman no. 4 filter paper and methanol was removed under reduced pressure. This extrac-

tion procedure yielded 1.5 g of raw organic extract that was used to quantify BEA and FP.

For FB_1 extraction, the procedure of Shephard *et al.* (1990) was modified as follows: extracts were prepared by shaking 10 g of each sample in 100 mL of methanol−water (3:1 v/v) for 1 h, and the extracts were then filtered through rapid flow filter paper (Whatman no. 4). A 10-mL aliquot of the extract was applied to a Bond Elut strong anion exchange (SAX) cartridge, and FB_1 was eluted from the column by a solution 99.5−0.5 v/v chloroform (Baker, Deventer, Holland, 99%)−acetic acid (Baker, Deventer, Holland, 100%) (Doko and Visconti, 1994). The dried residue was dissolved in 1 mL of methanol and stored at 4 °C.

Chemical Analysis. Standards of FB_1 and BEA were purchased from Sigma Chemical Co., St. Louis, MO. The FP standard was isolated in our laboratory from maize kernels inoculated by a *F. proliferatum* strain (Ritieni *et al.*, 1995).

Analysis of FB_1 was perfomed by comparing the extracts (spotted from 1 to 20 μL) with pure FB_1 (0.1, 0.5, 1, and 2 μg of standard) by high-performance thin-layer chromatography (HPTLC) on precoated silica gel 60 F_{254} plates (10 × 20 cm, thickness 0.25 mm, E. Merck, Darmstadt) using two different solvent systems: chloroform−methanol−water−acetic acid (55:36:8:1 v/v/v/v) and chloroform−methanol (60:40 v/v). A detection limit of 5 mg/kg was obtained for FB_1 using the extraction procedure described and the HPTLC analysis.

The amount of BEA and FP were determined by high-perfomance liquid chromatography (HPLC). HPLC analyses were carried out with a LDC Constametric 3200 pump equipped with a reverse-phase (RP) C_{18} Accubond 5-μm column (250 × 4.6 mm) (J&W Scientific, Folsom, CA) and a LDC spectrometer 3100 UV detector. FP HPLC analyses were performed with UV detection at 261 nm; acetonitrile−water (65:35 v/v) was used as the eluent at a flow rate of 1 mL/min. BEA HPLC analysis was performed with UV detection at 225 nm using acetonitrile−water (85:15 v/v) as eluent with a flow rate of 1 mL/min. Aliquots of 500 μL of methanolic extracts, corresponding to 500 mg of starting material, were filtered through Acrodisk (0.22 μm) before HPLC injection (20 μL). Toxin quantification was done by comparing the peak areas of the samples with those of the standards.

All analyses are run in triplicated the mean values are reported. Calculated standard deviation was always lower than 5%. From the calibration curve, a detection limit of 20 ng for both BEA and FP was detected, corresponding to 1 mg/kg of dried sample.

Confirmation of the presence of BEA and FP was obtained by spiking the standards with methanolic extracts of samples and analyzing shape and area of HPLC chromatogram peaks. The detection of FP by GC/MS was performed using a Carlo Erba MRC 500 GC coupled with TRIO 2000 MS. The column was a Crossbond 65% phenyl polysiloxane (Restek Corporation, Bellefonte, PA). The injector temperature was 270 °C while the oven was equilibrated at 150 °C rising to 340 °C in 19 min (10 °C/min). The MS detector was set up with a mass range from 50 to 450, a scan time of 0.5 s, and a source temperature of 200 °C; the transfer line was set up at 300 °C. The use of GC/MS can be valuable in lowering detection limits and in obtaining unequivocal identification of metabolites (such as mycotoxins) occurring in low amounts in a complex matrix. This methodology is not directly applicable to FB_1 and BEA, which need a chemical derivatization (FB_1) or direct introduction into the spectrometric source (BEA).

RESULTS

Incidence of *Fusarium* Species. The *Fusarium* species most frequently isolated from the 22 maize samples collected were *F. proliferatum* and *F. moniliforme* (Table 1). Some samples were also infected at a low level by *F. oxysporum* (2 out of 22 samples) and *F. equiseti* (4 out of 22 samples).

Particular attention was paid to the first identification and isolation of *F. moniliforme* and *F. proliferatum*, when grown from the same kernel. For both species,

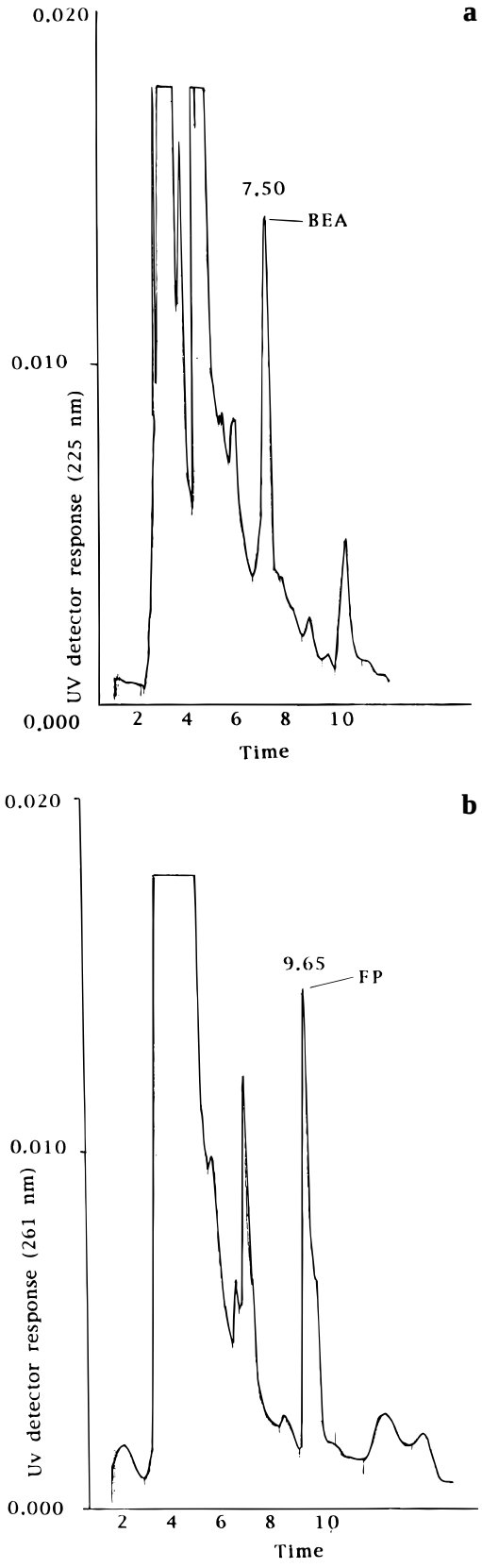

Figure 2. (a) HPLC chromatogram of preharvest sample (34/94) contaminated by beauvericin; (b) HPLC chromatogram of preharvest sample (34/94) contaminated by fusaproliferin.

gross cultural characteristics and pigmentation were highly variable, whereas *F. proliferatum* usually had a more abundant pannose aerial mycelium than did *F. moniliforme*. Definitive identification was based mainly on microscopic observations of micro-conidiogenesis from

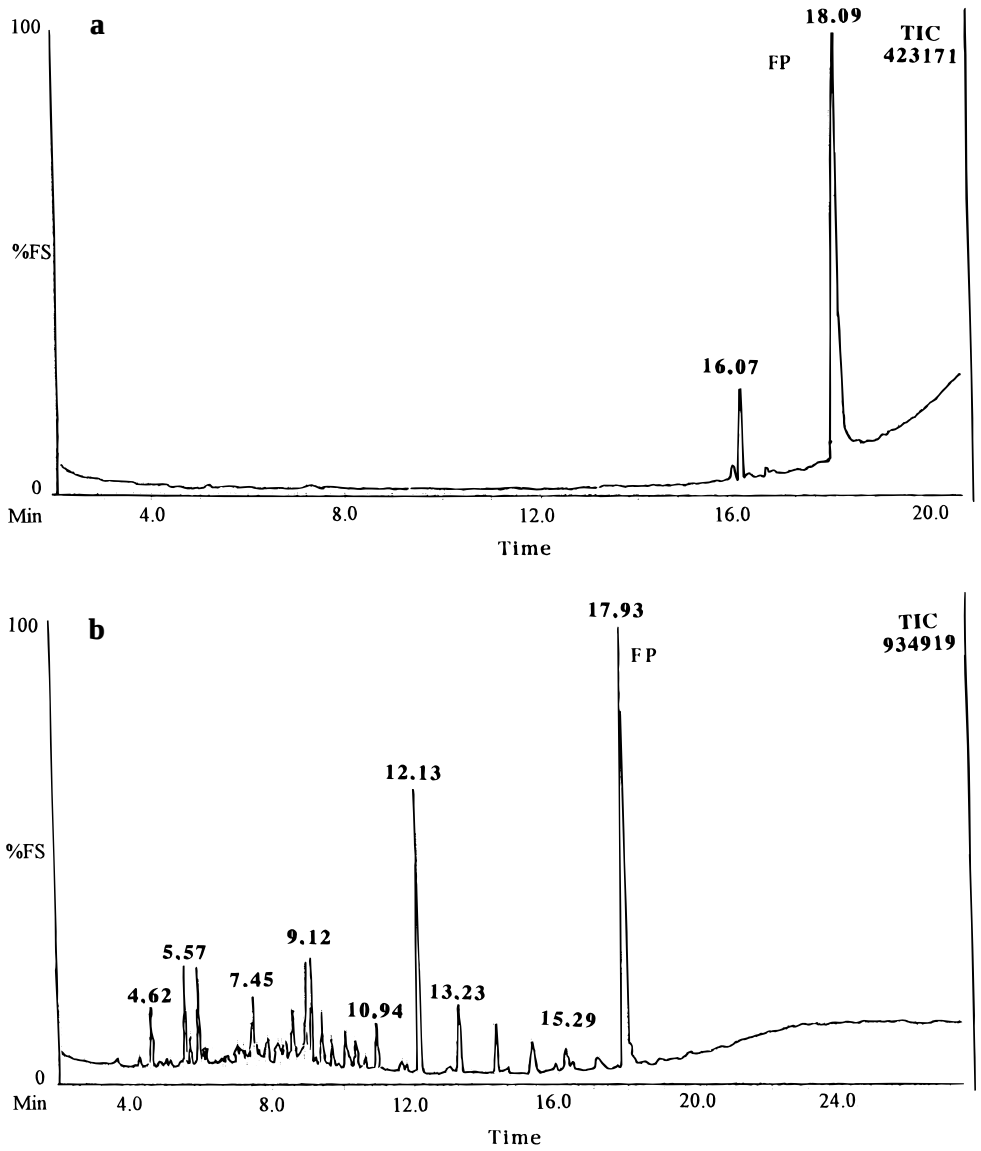

Figure 3. (a) Gas chromatogram of authentic standard fusaproliferin; (b) gas chromatogram of preharvest sample (34/94) contaminated by fusaproliferin.

single-spores cultures. However, regarding *F. moniliforme*, whose isolates can belong to different mating populations (A and F) of *Gibberella fujikuroi* (Sawada) having different profiles of toxic metabolites (Leslie *et al.*, 1992), most of strains from these maize samples were assigned to mating population A (data not shown) by testing them with standard methods (Leslie *et al.*, 1992).

Natural Occurrence of Toxins. Twenty of 22 samples (Table 1) were contaminated with FB_1 (trace to 300 mg/kg), six samples by BEA (trace to 520 mg/kg), and nine samples by FP (6−500 mg/kg). Five samples were contaminated by all three toxins; sample 34/94 was the most highly contaminated (Table 1).

The HPLC experimental conditions used to detect BEA and FP in maize samples gave retention times of 7.50 and 9.65 min, respectively. HPLC analysis gave optimal results on samples containing more than 20 mg/kg each of the two toxins (Figure 2).

Using the HPLC procedure, we are theoretically able to detect 1 mg/kg FP; as our extraction procedure allowed us a recovery of 40% of the toxin occurring in the samples, then the really minimum amount detectable of FP was recalculated as 2.5 mg/kg per sample.

GC/MS analysis was suitable for FP detection, and all natural infected samples were analyzed. Figure 3a shows the gas chromatogram of FP standard with retention time of 18.09 min while Figure 3b reports the gas-chromatogram of sample 34/94 with the main peak of FP at 17.93 min. The mass spectrum of peak at 17.93 min fully agrees with data of Ritieni *et al.* (1995). FP identified by HPLC analysis (Table 1) was confirmed by GC/MS (data not shown).

DISCUSSION

We previously found high levels of FB_1 contamination in Italian preharvest maize from the 1992 and 1993 crop seasons (Bottalico *et al.*, 1995; Logrieco *et al.*, 1995). The data reported here confirm that maize affected by *Fusarium* ear rot in Italy has a high risk of FB_1 contamination.

As already mentioned, FB_1 is a well-known toxic metabolite to animals (Kellerman *et al.*, 1990; Harrison *et al.*, 1990; Rheeder *et al.*, 1992; Gelderblom *et al.*, 1988). Moreover, recently, FB_1 was shown to cause apoptosis in monkey kidney cells (Wang *et al.*, 1996). The maximum level of FB_1 tolerated in cereal foodstuffs

has not been internationally defined; however, in Switzerland, a level of 1 mg/kg has been proposed as the tolerance level for commercialization (FAO, 1995). FB_1 occurred in 14/20 positive samples in our study at levels of at least 20 mg/kg. To the best of our knowledge, this report is the first of the natural occurrence of FP in maize and of the co-occurrence of the above three toxins on the same maize samples.

FP presumably is a common contaminant of maize infected by strains from *Fusarium* section *Liseola*. Previously, all 71 tested strains of *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas and 22/23 strains of *F. proliferatum*, mostly isolated from maize, were able to produce *in vitro* FP up to 1500 mg/kg of dried ground substrate (Logrieco *et al*., 1996; Moretti *et al.,* 1996).

FP, FB_1, and BEA co-occurred in five samples from one location in which *F. proliferatum* was recovered. The level of all three toxins was highest in sample 34/94 (Table 1), which was infested with *F. moniliforme* and *F. proliferatum*. These high levels of natural contamination might reflect the ability of these particular strains to produce the toxins or may indicate that the fungi had colonized more of the kernels and thus had a larger biomass available to produce the toxins. Variation in FB_1, BEA, and FP production has been reported for field strains of *F. proliferatum* (Leslie *et al.*, 1992; Moretti *et al*., 1996).

The present data confirm previous reports (Bottalico *et al*., 1995; Logrieco *et al*., 1995) of the importance of *F. proliferatum* and *F. moniliforme* in ear rot of maize in Italy. The frequent occurrence of *F. proliferatum* is particularly important, since this fungus is often misidentified as *F. moniliforme*. Consequently, *F. proliferatum* significance as both a pathogen and a contaminant of maize has probably been underestimated. Certainly in Italy, *F. proliferatum* is one of the most common species on preharvest maize (Bottalico *et al*., 1995; Logrieco *et al*., 1995).

Strains of *F. proliferatum* are most frequently isolated from samples collected in northern Italy (Asti and Milano; Table 1). In this area, the weather conditions are wetter and cooler than in the southern Italy at harvest time, and *F. proliferatum* appears to adapt to these conditions more readily than *F. moniliforme*.

In conclusion, this investigation confirmed that FB_1 is the main toxin occurring in Italian maize. Nevertheless, since several *F. proliferatum* strains could produce FB_1, BEA, and FP (Logrieco *et al*., 1995; Moretti *et al*., 1996), and since these toxins occurred at significant level in several maize samples from one of the most Italian vocated area to maize cultivation, we consider it of a great concern, the contamination of *F. proliferatum* on maize used for human and animal consumption.

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