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Fusarium species and their mycotoxins in infected corn in Italy

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Abstract

Surveys of corn (infected plants and commercial kernels) for *Fusarium* species and their mycotoxins were carried out on samples collected all over Italy and from some European and mediterranean countries.

Investigations on samples of corn stalk and ear rot standing in the field, mainly collected in southern Italy, proved to be contaminated with zearalenone (ZON), zearalenols (ZOL), and deoxynivalenol (DON). The *Fusarium* species most frequently isolated, and their recorded toxigenic capability (in parentheses), were: *F. moniliforme*; *F. culmorum* (ZON, ZOL, DON, 3AcDON); *F. equiseti* (ZON, ZOL); and *F. proliferatum* (MF). Along with these species, *F. graminearum* group 2 (ZON, DON and/or 3AcDON or 15AcDON); *F. chlamydosporum*; *F. acuminatum* (type-A trichothecene derivatives); and *F. semitectum* were often found to be associated. *F. heterosporum* (ZON, ZOL); *F. solani*; *F. crookwellense* (ZON, ZOL, FUS, NIV); *F. oxysporum* (MF); *F. avenaceum* (MF); *F. sporotrichioides* (T-2 toxin and derivatives); and *F. poae* (DAS, MAS) were occasionally isolated.

Introduction

Several *Fusarium* species are important pathogens of cereals and corn, causing severe crop yield reduction. In addition, some isolates are able to produce mycotoxins which are responsible for some diseases of farm animals and man [22, 25, 42, 44].

The formation and accumulation of the *Fusarium*-mycotoxins can occur both in infected plants standing in the field or in stored products colonized by the toxigenic isolates [4, 24, 28].

According to several investigations, corn turned out to be the dietary staple in which *Fusarium*-mycotoxins are most likely to be encountered, and mycotoxicoses due to the ingestion of contaminated corn are very common [34, 42, 44].

The *Fusarium*-mycotoxins most frequently encountered in corn and often implicated in the natural cases of mycotoxcoses include: zearalenone and zearalenols, some trichothecene derivatives, and moniliformin [42, 44].

Zearalenone (ZON), an anabolic and uterotrophic compound eliciting estrogenic activity, is frequently associated with hyperestrogenism and infertility in swine, poultry and cattle [23]. The same estrogenic activity is shown by zearalenols (ZOL) (α- and β-zearalenol diastereoisomers, often occurring in mixture), which are formed by the reduction of the zearalenone. It has been demonstrated that α-ZOL is about three to four times

more estrogenic than ZON, whereas the activity of β-ZOL is the same or slightly less than that of ZON [14].

Trichothecenes are a group of toxic metabolites responsible for several mycotoxicoses in farm animals and is also implicated in some diseases of man, such as Alimentary Toxic Aleukia (ATA) in the USSR, and Akakabi disease in Japan [33]. Of several trichothecene derivatives produced by *Fusarium* species, only few have been encountered as natural contaminants of plant products. They include: T-2 toxin (T2),HT-2 toxin, neosolaniol (NS), T-2 triol, T-2 tetraol, deoxynivalenol or vomitoxin (DON), nivalenol (NIV), diacetoxyscirpenol (DAS), fusarenone (FUS), 3-acetyldeoxynivalenol (3-AcDON), and 15-acetyldeoxynivalenol (15-AcDON) [4, 8, 24, 28]. The toxicological characteristics of these trichothecenes include skin inflammation (dermatitis and oral lesions), digestive disorders (vomiting, diarrhea, feed refusal), hemorrhagic syndrome, destruction of bone marrow (leucopenia), and nerve disorders [33].

Moniliformin (MF), a mycotoxin with marked toxic effects on animals and plants, was first isolated from *Fusarium* cultures and then found in toxic concentrations in a sample of *Fusarium* -mouldy corn from the Transkeian district of South Africa characterized by a very high rate of human esophageal cancer (EC). Recently, the same toxin was detected together with fusarin-C, another *Fusarium*-mycotoxin, in corn screenings associated with a field outbreak of equine leukoencephalomacia (LEM). However, it appears unlikely that either toxin is responsible for EC, or LEM or hepatocarcinogenicity elicited by *F. moniliforme* cultures [13, 27, 32].

There seem to be some geographical differences in the natural distribution of the *Fusarium* species, as well as of their corresponding mycotoxins, which are influenced primarily by environmental conditions, crop production and storage methods. To this regard, DON is found worldwide in corn infected by *F. graminearum* DON-chemotype often together with its monoacetates 3-AcDON or 15-AcDON. The occurrence of NIV alone or associated with FUS are reported with increasing frequency, in relation to the availability of improved analytical methods [16, 31, 36].

In Italy, the occurrence of toxigenic *Fusarium* species and their mycotoxins in corn has been monitored extensively especially in some irrigated areas of the southern regions, where epidemics of ear and stalk rot are frequent on corn grown for grain but also on fodder corn [6, 8].

Methodology

Isolation and identification of Fusarium *species.* Infected corn kernels or tissue fragments were plated out on *Fusarium*-selective peptone-PCNB medium [6, 19]. *Fusarium* colonies were then transferred on potato-saccharose-agar (PSA) plates, and incubated for seven days at about 24 °C under fluorescent lamps for 12 hours per day. Single-spore cultures were subsequently obtained on PSA, and were finally identified in accordance with the nomenclature of Nelson *et al.* [26]. In addition to PSA medium, observations were also made using water-agar supplemented with sterile soil, or with sterile carnation leaf, or with KCL (2%); the latter medium was used especially for the identification of the isolates of *Liseola* section [19, 26].

Toxin production. The isolates were grown on 200 g of corn kernels brought overnight to about 45% moisture in 500-ml Erlenmeyr flasks, and then autoclaved for 20 min at 120 °C. The substrate was inoculated with pieces of PSA single-spore cultures, and maintained at 27 °C for 4 weeks. Then, the cultures were dried at 60 °C and finely ground [5].

Toxin analysis. A multimycotoxin method, previously described for ZON and trichothecenes [4], was used also for ZOL with a few adaptations [8]. Samples (50 g) of dried corn (kernels or vegetative parts) or dried *Fusarium* cultures (20 g) were extracted with methanol-aqueous NaCl, defatted with hexane, and partitioned with dichloromethane. After the evaporation of the solvent, the residue was brought up to 2 ml with

methanol-water (40:60), passed through a Sep-Pak C-18 cartridge (Water Associates, Inc., Milford, Mass.), and eluted with a new portion (2 ml) of the methanol-water mixture representing the first pure fraction (fraction A). Further elution with methanol (2 × 2 ml) yielded a second pure fraction (fraction B). The two fractions were separately evaporated to near dryness and reconstituted with methanol (0.5 ml). Fraction A was examined for NIV, FUS, DON, 3AcDON, and 15AcDON; and fraction B was examined for DAS, T2, ZON, and ZOL (alpha and beta). Analyses of ZON and trichothecenes were performed by TLC and GLC [4]. Trichothecenes eluted in fraction A, as well as ZOL, were confirmed and quantitated by HPLC [8, 35]. The separation of 3-AcDON and 15AcDON was only possible by TLC or capillary GLC, and not by HPLC. Due to the low recovery of the extraction procedure for polar trichothecenes, particularly NIV [36], the method of Lauren and Greenhalgh [16] was used for NIV (and FUS) in few cases. Analysis of MF was carried out in accordance with the method previously employed [9].

Surveys and results

Occurrence of Fusarium *species*

In several surveys carried out in southern Italy, twelve different *Fusarium* species were isolated from the infected lower internodes of corn plants affected by stalk rot at harvest time. From the same infected stalk fragment very often more than one species was isolated. The species most frequently isolated were, in order of incidence; *F. moniliforme* Sheldon, *F. culmorum* (W.G. Smith) Saccardo, *F. equiseti* (Corda) Saccardo, and *F. proliferatum* (Matsushima) Nirenberg. Along with these species, always found together, *F. graminearum* Schwabe Group 2, *F. chlamydosporum* Wollenweber & Reinking, *F. acuminatum* Ellis & Everhart, and *F. semitectum* Berkeley & Ravenel were often found to be associated. Occasionally also *F. oxysporum* Schlechtendahl emend. Snyder & Hansen, *F. sporotrichioides* Sherbakoff, *F. poae* (Peck) Wollenweber, and *F. solani* (Martius) Saccardo were isolated [12, 17, 18].

The presence of *Fusarium* species in infected ears (peduncles, cobs, and kernels) was almost the same as that found in the stalks with a higher incidence of *F. moniliforme*, *F. equiseti* and *F. proliferatum* early in the season, and with an increasing appearance of *F. graminearum* Group 2 and *F. heterosporum* Nees late in the season [17, 18].

The distribution of *Fusarium* species encountered in commercial domestic corn feed, was almost the same of that found associated with corn ear rot in the field. A relatively higher incidence of *F. graminearum* was found in imported corn.

Surveys carried out on several corn seed lots showed the predominant occurrence of seedborne infections of *F. moniliforme* and, to a lesser extent, of *F. proliferatum*. A positive correlation was found between the incidence of seedborne *F. moniliforme* and the length of the hybrid's vegetative cycle, the late maturing hybrids being the most affected.

The morphological and toxicological characteristics of the *Fusarium* isolated from corn are reported below. The different species are grouped in the following sections according to Nelson *et al.* [26].

Liseola *section.* The *Fusarium* isolates recognized in this section were found to belong mainly to *F. moniliforme* and less frequently to *F. proliferatum*. *F. moniliforme* was isolated more often from corn stalks, ears, and kernels, while *F. proliferatum* was mainly present in soil collected from corn fields. The complete absence of *F. subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas, was unexpected.

F. subglutinans, which differs from both the above mentioned species, since the microconidial chains are absent and microconidia are produced only in false heads, seems to be absent in corn in southern Italy. However, the strictly taxonomic similarities among the species of this section could lead to a misidentification. Confirmations

and further investigations appear to be necessary to increase the knowledge regarding their distribution and epidemiological profile.

Isolates of *F. proliferatum*, but not those of *F. moniliforme*, were able to synthesize MF and this ability appeared to be confined to some isolates colonizing soil and kernels [19].

Eighteen isolates of *F. moniliforme* showing different undersurface coloration on PSA, varying from colorless to dark purple, were assayed for the production of red pigments on autoclaved corn kernels. The analyses, carried out in accordance with the method reported by Steyn *et al.* [30], showed that two isolates produced five pigments, i.e., 8-*O*-methylfursarubin (MF), 8-*O*-methylsolaniol (MS), 8-*O*-methylbostrycoidin (MB), 3-8-*O*-*O*-dimethylfusarubin (DMF), and 8-*O*-methyljavanicin (MJ). Of the other sixteen: one isolate produced four pigments, i.e., MF, MS, MB, and DMF; ten isolates produced only MB, whereas five isolates produced no pigments. When assayed for antibacterial activity, all pigments were bactericidal only to Gram-positive species. The resistance of the Gram-negative species might be in relation to a specific lower permeability of the cell wall [40].

Discolor *section*. The isolates of *Fusarium* from corn stalk and ear rot recorded in this section belonged mainly to *F. culmorum* and, to a lesser extent, to *F. graminearum*; whereas only a few representatives belonged to *F. heterosporum* and *F. crookwellense* Burgess, Nelson & Toussoun. All the isolates of *F. graminearum* were identified as belonging to Group 2 [12].

F. culmorum was isolated more frequently early in the season and with a higher incidence from infected stalks than from ear rot; *F. graminearum* and *F. heterosporum*, on the contrary, appeared late in the season, especially on the ears, and were favoured by a more prolonged corn season, particularly on late maturing hybrids.

All the isolates of *F. culmorum*, collected all over Italy by several surveys of corn and cereals, produced ZON, ZOL (α and β isomers), and the trichothecenes DON and 3-AcDON, but not NIV. On the other hand, all the *F. graminearum* obtained from similar surveys produced ZON but not ZOL, and showed a different ability in the synthesis of trichothecenes. In particular, the isolates from corn belonged to the DON-chemotype (DON and/or 3AcDON or 15AcDON produces); the isolates from barley proved to be of the NIV-chemotype (NIV and FUS producers); while the isolates from wheat and weed cockspur (*Panicum crusgalli* L.) appeared to belong to both the DON and NIV chemotypes [8, 17]. The natural occurrence of NIV-chemotype isolates of *F. graminearum*, alone or together with DON-chemotypes, was also reported in Japan [31].

The ability to produce trichothecenes by the representatives of this section is exhibited also by the recently constituted *F. crookwellense* species [10, 15]. This species, already isolated from a variety of substrates in several countries [10, 26], was recently recorded for the first time also in barley and corn stalk residues in Italy [1]. Of this species, eight isolates from corn, wheat, and other plant products collected in various countries were able to produce considerable concentrations of ZON, FUS, and NIV, together with low amounts of ZOL (α and β isomers) [7]. In relation to trichothecene production, *F. crookwellense* appears to be similar to the NIV-chemotype isolates of *F. graminearum*. These findings suggest that *F. crookwellense* beside *F. graminearum* carry the major responsibility for the worldwide NIV and FUS cereal contamination.

Isolates of *F. heterosporum* from infected corn ears were able to produce high concentrations of ZON and ZOL (α and β diastereomeric mixture) and exhibited strong toxicity on brine shrimps. This activity, not related to the presence of ZON and ZOL nor to any reported trichothecene derivatives, suggests the occurrence of an undescribed toxic metabolite.

Gibbosum *section*. The *Fusarium* species belonging to this section, very often recorded on corn in Italy, was *F. equiseti*. This species was almost always isolated from infected plants during the corn season, both from stalk and ear rot. Several isolates of *F. equiseti* were found to be toxigenic and capable of producing ZON, ZOL (α-ZOL by

some isolates, or α and β diastereomeric mixture by other isolates), but not trichothecene derivatives [8, 17].

Another representative of this section sometimes isolated from corn stalk and kernels as well as from the soil of corn field, was *F. acuminatum*. All the tested isolates of *F. acuminatum* were highly toxic to brine shrimps and produced a new trichothecene named acuminatin (3α-4β-dihydroxy-8α,15-diacetoxy-12,13-epoxytrichothec-9-ene). Besides acuminatin, 8-acetoxyneosolaniol (the major metabolite), neosolaniol, 4,8-diacetoxy T-2 tetraol (NT-1) and three T-2 tetraol monoacetates were also identified. The trichothecene production pattern of these isolates, mainly related to 8-acetoxyneosolaniol, was different from that reported in literature for other isolates of the same species, mainly producing T-2 toxin and its derivatives [39].

Sporotrichiella *section*. The representatives of this section, occasionally found associated to corn stalk and ear rots and to soil taken from corn field, were identified as belonging to *F. sporotrichioides*, *F. poae*, and *F. chlamydosporum*.

All the assayed isolates of *F. sporotrichioides* were found to produce T2 and its derivatives and proved to be highly toxic to brine shrimps. These isolates were able to produce more than one trichothecene derivative, and a toxigenic study on a strain isolated from wheat ear rot in Poland led to the identification of eleven compounds related to T2 in a rice culture [21, 38].

None of the assayed *F. chlamydosporum* isolates produced trichothecene derivatives nor were they toxic to brine shrimps [21]. Nevertheless, observations are in progress on the production of moniliformin by some isolates as reported by Rabie *et al.* [27].

Some isolates of *F. poae* were toxic to brine shrimps and found to produce DAS. Besides DAS, monoacetoxyscirpenol (15-acetoxyscirpenol) (MAS), not before recorded for this fungus, was also identified [11, 21].

According to the Snyder & Hansen *Fusarium* nomenclature [29], many representatives of the three above-mentioned species are grouped under the single name of *F. tricinctum* (Corda) Snyder & Hansen, together with representatives of *F. tricinctum* (Corda) Saccardo. It is well-known that the concurrent use of both these *F. tricinctum* sense, has led to taxonomic confusion, as well as to improper chemotaxonomic assessment. By adopting the nomenclature of Nelson *et al.* [26], which reconsiders the Wollenweber and Reinking taxonomy based on several distinct species for the *Sporotrichiella* section [41], none of the *Fusarium* isolated from corn in Italy was identified as *F. tricinctum* (Corda) Saccardo. On the other hand, toxigenic investigations carried out on nine isolates of *F. tricinctum* (Corda) Saccardo originating from Poland showed that none was able to produce trichothecene derivatives, and the toxicity exhibited by some isolate on brine shrimps seemed to be related to undescribed metabolites [21].

Elegans *section*. The representatives of this section, occasionally isolated mainly from infected corn stalks, were recognized as belonging to the only species *F. oxysporum*. All isolated assayed for toxigenic ability were found to produce high concentrations of MF [17].

Roseum *section*. The few isolates recorded in this section, mainly isolated from corn ear rot, belonged to *F. avenaceum*. All isolates assayed for toxigenic ability were found to produce high concentrations of MF [9, 17].

Arthrosporiella section. The representatives of this section, occasionally isolated from corn stalk and ear rot, belonged to *F. semitectum*. Toxigenic investigations on this isolates showed their inability to produce ZON, ZOL, nor any trichothecene derivatives or MF [17].

Martiella *section*. The few representatives of this section, mainly isolated from corn field soil or from the lower stalk internodes, were recognized as belonging to the only species of *F. solani*. None of these isolates produced ZON, ZOL, nor any trichothecene derivatives or MF, and they were not toxic to brine shrimps [17]. These results

confirmed the reported negligible toxigenic importance of *F. solani* [22].

Occurrence of *Fusarium*-mycotoxins in corn

Several investigations were carried out on the occurrence of *Fusarium*-mycotoxins in samples of freshly harvested corn and commercial feed corn collected all over Italy, also in comparison with samples from central European and mediterranean countries [2, 3, 4, 8, 20, 37].

Observations on *Fusarium* corn stalk rot and associated mycotoxins were essentially made in southern Italy (Basilicata), where the production of corn has increased in recent years [8, 17]. The principal analytical and mycological results are summarized in Table 1. ZON (up to 7,433 ng/g), ZOL (up to 86 ng/g of diastereomeric mixture) and DON (up to 668 ng/g) were found in all infected corn stalk rot samples. No T2, DAS, NIV, FUS, 3-AcDON or moniliformin were detected. The highest concentrations of toxins were found in infected stalk samples showing pink and reddish pith pigmentation. In these samples *F. culmorum* was more often present than *F. equiseti*, which was isolated more frequently from uncoloured samples. *F. equiseti* proved to be less toxigenic than *F. culmorum* and no trichothecenes were produced [8]. In these surveys, ZOL was recorded for the first time as naturally occurring contaminant in *Fusarium* -infected corn stalk before harvest [8].

Surveys of *Fusarium*-infected corn ears in northern Italy (Lombardy) showed high kernel contamination by DON, and in the Austrian samples also DAS, NIV, FUS, and 3-AcDON were found. Before that time, these trichothecene derivatives had never been recorded in infected plants standing in the field, and the natural occurrence of FUS and 3-AcDON was reported for the first time [3, 4].

The results obtained confirmed the diffusion of several toxigenic *Fusarium* species which were able to produce different mycotoxins. This toxigenic ability seems to be almost specific and useful in the chemotaxonomic characterization of several species.

The high incidence of *Fusarium*-mycotoxins both in infected plants in the field and in commer-

Table 1. Occurrence of mycotoxins and *Fusarium* species in Corn stalk rot in southern Italy.

Corn samples	Toxins ng/g[a]			*Fusarium* species				
	Zearalenone	Zearalenols[b]	Deoxynivalenol	*F. moniliforme*	*F. proliferatum*	*F. culmorum*	*F. equiseti*	Others species
Brown stalk rot								
1	883	86	81	+ + +	+	+ +	+ + +	+[c]
2	1,554	10	71	+ + +	+	+	+ + +	+ +[c,d,e]
3	411	20	18	+ + +	+	+	+ + +	+ +[e,f]
Reddish stalk rot								
1	7,433	51	668	+ +	+	+ + +	+ +	+ +[d,e,g]
2	156	10	244	+ + +	+ + +	+ + +	+	–
3	6,900	7	151	+ + +	–	+ + + +	+ +	+[d]
4	668	25	162	+ +	+	+ + +	+ +	+ +[d,e]
5	3,331	83	115	+	+	+ + + +	–	+ + +[c,d,e]

[a] No 3-acetyldeoxynivalenol, fusarerone, nivalenol, T-2 toxin, diacetoxyscirpenol or moniliformin were detected.

[b] Confirmed by GC/MS (full mass spectra) as a diastereomeric mixture of alpha-zearalenol and beta (epi)-zearalenol.

[c–g] Classified in accordance with the nomenclature of Nelson *et al.*: c = *F. acuminatum*; d = *F. oxysporum*; e = *F. chlamydosporum*; *f* = *F. semitectum*; g = *F. sporotrichioides.*

cial corn kernels processed as feedstuffs might represent a real hazard to livestock.

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