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Research Note

Occurrence of Mycotoxin in Farro Samples from Southern Italy

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ABSTRACT

The occurrence of nine mycotoxins and of contamination by pre- and postharvest fungal pathogens of cereals was investigated in samples of stored *Triticum monococcum* L., *Triticum dicoccon* Schrank (emmer), and *Triticum spelta* L. (spelt). In Italy, all three species are collectively referred to as *farro.* The samples examined were harvested in summer 2000 from eight different sites in southern Italy. Conventional fluorimetric and diode array–based high-performance liquid chromatography (HPLC) analyses and HPLC–mass spectrometry analyses were used to identify fumonisin B_1 in five samples (up to 70.00 μg/kg), ochratoxin A in seven samples (up to 4.07 μg/kg), and beauvericin in three samples (up to 4.44 mg/kg). Enniatin B was detected in one sample (30.00 μg/kg), but no zearalenone or fusaproliferin was found. Deoxynivalenol and aflatoxins were not evaluated. The potentially mycotoxigenic fungal species detected were *Alternaria alternata, Fusarium proliferatum, Fusarium tricinctum, Penicillium verrucosum,* and *Penicillium chrysogenum.* This is the first report of the natural occurrence of mycotoxins in farro samples.

Three different hulled wheat species are presently cultivated in the world: *Triticum monococcum* L. (diploid), *Triticum dicoccon* Schrank (tetraploid), and *Triticum spelta* L. (hexaploid). In Italy, these grains are collectively referred to as *farro.*

T. dicoccon and *T. spelta*, also known as emmer and spelt, respectively, and *T. monococcum* had been widely used until the late Roman Empire *(10, 14)*; after that period, their production steadily declined. Recently, renewed interest in these crop species has been based on their health, nutritional, and functional properties, and several farro-based food products are currently being marketed, most of which are derived from organic agriculture *(1, 3, 8, 9, 11).* Pesticide or chemical field treatments are usually limited in farro cultivation because these crops are considered to be relatively resistant to pests and pathogens *(16).*

Mycotoxins are secondary metabolites produced by several fungal genera. These compounds produce acute, subacute, and chronic toxicity in animals and/or humans and some are carcinogenic, mutagenic, and teratogenic. Preharvest and postharvest fungal pathogens of cereals can produce mycotoxins that can then be found in cereal-derived products *(5).* Recent public concern for food safety has made monitoring of mycotoxins in the food chain a major issue to be addressed. Presence or absence of mycotoxins can be considered a safety marker of cereal production and storage. Most of the few scientific reports regarding farro have been focused on genetic or agronomic aspects. No data exist on the possible occurrence of mycotoxins, and very few researchers have addressed mycological issues involving these crops *(7).*

The aim of our study was to investigate the presence of fungal and mycotoxin contamination in farro samples from Molise and Basilicata, two regions in southern Italy, by using a multimycotoxin approach taking into account mycotoxins produced both by preharvest toxigenic fungi, such as the field pathogens *Fusarium* spp., and by storage fungi, such as *Penicillium* spp. To our knowledge, this is the first study that has been focused on mycotoxin contamination of these crop species.

MATERIALS AND METHODS

Farro samples. Samples of grains harvested in July 2000 were collected 2 months later from storage facilities of family farms in Molise (Campobasso County, samples G, C, GRT, and GR) and 2 to 3 months later from a harvesting center in Basilicata (Potenza County, samples PZ1, PZ2, PZ3, and PZ4). In both regions, farro was stored in sacks or small barrellike containers at room temperature. To obtain representative samples of stored product, samples were taken from 10 sacks or containers per location from the different storage facilities. In each sack or container, three imaginary circles were designated perpendicular to the longitudinal axis: 10 cm from the top, halfway along the axis, and 10 cm from the bottom. Four subsamples of approximately 200 g each were withdrawn from each circle using a probe, one in the center and three equidistant at about 120° from each other and about 5 cm from the imaginary outer perimeters. After the subsamples were withdrawn, they were mixed to form 24-kg samples from each site. Farro varieties or populations were named according to local traditions as Guardaregia, Cercemaggiore, Lucanica, Forenza, and Gildone. Samples features are summarized in Table 1. Each sample (24 kg) was divided into 10 subparts and stored in the dark at 4°C in plastic aseptic bags under vacuum

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TABLE 1. *Designation, description and sites of collection of farro samples*

Sample	Harvest region	Species	Variety of population	Utilization	Storage site
G	Molise	*Triticum dicoccon*	Gildone	Feed	Family farm
C	Molise	*T. dicoccon*	Gildone	Feed	Family farm
GRT	Molise	*T. dicoccon, Triticum monococcum*	Guardiaregia	Feed	Family farm
GR	Molise	*T. dicoccon, T. monococcum*	Guardiaregia	Feed	Family farm
PZ1	Basilicata	*T. dicoccon, Triticum spelta*	Lucanica, Forenza	Food	Harvesting center
PZ2	Basilicata	*T. dicoccon*	Lucanica	Food	Harvesting center
PZ3	Basilicata	*T. dicoccon, T. spelta*	Lucanica, Forenza	Food	Harvesting center
PZ4	Basilicata	*T. dicoccon, T. spelta*	Lucanica, Forenza	Food	Harvesting center

before mycotoxin analyses, which were performed within 10 days. Mycological investigations were performed immediately after withdrawal and formation of samples.

Mycological investigations. Farro grains were examined to assess the presence of internal mold infections and the extent of fungal contamination. One hundred kernels were withdrawn from each sample (10 kernels from each of the 10 subparts of each 24-kg sample) for the isolation and identification of fungi according to Pancaldi et al. *(15)* with slight modifications. Kernels for analysis of *Fusarium* spp. were surface disinfected by dipping for 2 min in hypochloride solution (3% of active chlorine), rinsed three times with sterile distilled water, and dried by blotting with sterile paper tissue. Kernels for analysis of other fungi were not surface disinfected. Twenty replicates (10 for internal molds and 10 for fungal contamination) of five surface-disinfected and five nondisinfected kernels were placed in a 100-mm-diameter petri dish containing potato dextrose agar and 200 mg/liter streptomycin sulfate (PDA-S). Petri dishes were incubated for 5 to 10 days in the dark at 23°C, and fungal colonies in each sample were counted. The most representative colonies were transferred to new PDA-S dishes. Isolated fungi were identified to genus level based on colony color and morphology and conidium morphology according to Booth *(4)* and Barnett and Hunter *(2)*. For identification to species, selected fungal cultures were sent to the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands).

Reagents. All organic solvents were high-performance liquid chromatography (HPLC) grade and were purchased from Merck (Darmstadt, Germany). Water for HPLC analyses was purified in a Milli-Q system (Millipore, Bedford, Mass.). Standards of fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), zearalenone (ZON), ochratoxin A (OTA), enniatins (ENNs; enniatin mixture containing 54% enniatin B_1, 19% enniatin B, 20% enniatin A_1, and 3% enniatin A), and beauvericin (BEA) were purchased from Sigma-Aldrich (St. Louis, Mo.). The reference standard for fusaproliferin (FUSA) was obtained from *Fusarium proliferatum* ITEM 1494 as previously described *(17)*, and its purity was confirmed by HPLC analysis *(18)*.

Validation parameters for extraction and analyses of mycotoxins. The following validation parameters were determined for extraction and analysis of the nine mycotoxins: limit of determination (LOD), limit of quantification (LOQ), linearity range, and recovery. For LOD, LOQ, recovery, and linearity range, 10 samples of farro per mycotoxin were selected on the basis of the absence of one of the mycotoxins. These samples were spiked with different amounts of standard for the absent mycotoxin and then extracted and analyzed. LOD was calculated according to the following formula: $\text{LOD} = x + 3 \times \text{SD}$, where x is the mean value of 10 analyzed samples and SD is the standard deviation.

Farro grains for mycotoxin analysis. For analyses of mycotoxins, one aliquot of farro grains (20 g) was withdrawn from each of the 10 subparts in which farro from each site was subdivided. The 10 aliquots were mixed, and different amounts from the resulting 200 g from each sample were deprived of glumes and used for extractions and analyses of the different mycotoxins.

Extraction and analysis of ENNs, BEA, and FUSA. Ten grams of grain was homogenized in a Ultra-Turrax T 25 BASIC (IKA Labortechnik, Staufen, Germany) for 3 min with 50 ml of pure methanol for HPLC (99.5%). Samples were filtered through Whatman no. 4 filter paper and concentrated under reduced pressure at 40°C (Heidolph Instruments, Schwabach, Germany) to 3 ml. Prepurification was performed on a C_{18} column (Varian, Palo Alto, Calif.) preactivated with 3 ml of methanol, and samples were eluted with 2 ml of the same solvent. The eluate was concentrated to 1 ml and filtered through an Acrodisk filter (0.22 μm), and 20 μl was loaded onto the column for HPLC analysis. Analyses of BEA, ENNs, and FUSA were performed according to the method of Monti et al. *(13)* with minor modifications using a Shiseido Capcell Pak C_{18} (250 by 4.6 mm, 5 μm) column, LC-10AD pumps, and a diode array detector (Shimadzu, Tokyo, Japan). The following conditions were used. The solvent system was CH_3CN-H_2O (65:35, vol/vol) with a constant flow at 1.5 ml/min. The starting solvent ratio was kept constant for 5 min and then linearly modified to 70% CH_3CN over 10 min. Mycotoxin identification was performed by comparing retention times and UV spectra of purified samples to those of pure standards and by coinjecting sample and pure standards. Mycotoxin quantification was carried out by comparing peak areas of samples with the calibration curve of reference standards.

Extraction and analysis of FB_1 and FB_2. Five grams of grain was finely ground and added to 50 ml of a methanol:water solution (75:25, vol/vol). These samples were homogenized in an Ultra-Turrax (13,500 rpm for 3 min at room temperature) and then clarified by centrifugation at 2,000 × *g* for 5 min at 4°C. Thirty milliliters of the supernatant (corresponding to 3 g of sample) was dried at 40°C under reduced pressure in a rotary evaporator (RC 10.10, Jouan S.A., St. Herblain, France), redissolved in 1 ml of pure methanol, and then filtered through an Acrodisk filter (0.22 μm). Twenty microliters of this solution was analyzed by HPLC–mass spectrometry (MS).

A bench-top API 100 (Perkin Elmer Sciex, Shelton, Ontario, Canada) single quadrupole mass spectrometer equipped with an atmospheric pressure ionization source and ionspray interface was used. All quantitative results were achieved in positive ion mode with the orifice voltage set at 30 V. The acquired data were processed using Multiview and MacQuan software (Perkin Elmer Sciex). The resolution was set at 0.5 amu (measured at half height), and the mass calibration and resolution adjustments on

TABLE 2. *Validation parameters of analytical procedures used in this study*

Mycotoxin[a]	Recovery (%)		Limit of quantification	Limit of detection	Concentration range
	Mean	SD			
BEA	94	2.8	20 ng	2 ng	1–100 μg/ml
ENNB	67	4.1	100 ng	5 ng	5–100 μg/ml
$ENNB_1$	63	3.9	100 ng	2 ng	5–100 μg/ml
$ENNA_1$	59	4.6	100 ng	5 ng	5–100 μg/ml
FUSA	71	2.4	10 ng	1 ng	0.5–50 μg/ml
ZON	90	1.5	75 pg	7.5 pg	1–50 μg/liter
OTA	70	1.3	1 pg	0.01 pg	0.1–1 μg/liter
FB_1	90	1.2	12 ng	0.1 ng	0.05–1 μg/ml
FB_2	68	1.3	10 ng	0.1 ng	0.01–1 μg/ml

[a] BEA, beauvericin; ENNB, enniatin B; $ENNB_1$, enniatin B_1; $ENNA_1$, enniatin A_1; FUSA, fusaproliferin; ZON, zearalenone; OTA, ochratoxin A; FB_1, fumonisin B_1; FB_2, fumonisin FB_2.

the resolving quadrupole were made in ionspray with 10^{-4} M polypropylene glycol solution. A standard mixture of FB_1 and FB_2 was infused at 10 μl/min to optimize spectrometric performances. Chromatographic runs were performed with an LC-200 (Perkin Elmer Sciex) equipped with a Pecosphere Brownlee C_{18} HPLC column (4.6 by 33 mm, 3 μm) (Perkin Elmer Sciex), using an aqueous solution of 5 mM ammonium acetate acidified with 1% formic acid containing 80% methanol as the mobile phase at a constant flow of 0.8 ml/min. FB_1 and FB_2 were quantified at m/z 722 and 706, respectively.

Extraction and analysis of ZON. Twenty grams of grain was homogenized in a Waring blender at high speed for 2 min with 50 ml of acetonitrile:water (90:10, vol/vol) and 2 g of NaCl. This suspension was filtered through paper, and 10 ml was diluted with 40 ml of water. Cleanup of this solution was achieved through a preactivated immunoaffinity column (ZearalaTest, Vicam, Watertown, Mass.). ZON was eluted from the column with 1.5 ml of pure methanol and then diluted with 1.5 ml of water for HPLC-MS analyses.

Analyses were performed with an atmospheric pressure ionization source set at 450°C in SIM mode. The ion investigated was 319 ± 1 amu. HPLC conditions were a constant solvent ratio (40:60, vol/vol) of 0.1% trifluoroacetic acid in H_2O and acetonitrile. The loop injector was always filled with 20 μl of solution injected onto Phenomenex Rp_{18} (150 by 4 mm, 5 μm), and flow was constant at 0.8 ml/min.

Extraction and analysis of OTA. The method by Solfrizzo et al. *(20)* was used for OTA extraction with slight modifications. Ten grams of each sample was homogenized in a Waring blender at high speed for 2 min in the presence of 200 ml of 1% sodium bicarbonate. The suspension was then centrifuged at 3,500 rpm for 20 min. After filtration of 40 ml through paper, 20 ml was diluted with 20 ml of 0.06 M phosphate buffer solution at pH 7.4. This 40-ml solution was prepurified through preactivated immunoaffinity column Ochraprep (Rhone Diagnostics Technologies, Glasgow, UK). After washing the column with 20 ml of water, OTA was eluted with 1.5 ml of methanol:acetic acid (98:2, vol/vol) and 1.5 ml of water. HPLC conditions were a constant solvent ratio with water plus 1% acetic acid and acetonitrile plus 1% acetic acid (40:60, vol/vol). The loop injector was always filled with 20 μl of sample injected onto Phenomenex Rp_{18} (250 by 4 mm, 5 μm), and the chromatographic run was carried out at a constant flow of 1.0 ml/min. The fluorescence detector RF-10Axl was equipped with a 150-W xenon lamp (excitation, 333 nm; emission, 460 nm). Retention time for OTA was approximately 11 min. For quantitation, peak areas were measured and compared with those of appropriate standard solutions in the range of 2 ng to 0.02 pg.

RESULTS AND DISCUSSION

The objective of this work was to obtain information about fungal and mycotoxin contamination in stored *T. monococcum, T. dicoccon,* and *T. spelta,* collectively known as farro. The mycotoxins FUSA, ENNs, BEA, FB_1, FB_2, ZON, and OTA were selected because they are produced by *Fusarium* spp. and *Penicillium* spp., which are typical field and storage pathogens, respectively.

The four different extraction and analysis methods used in this study were optimized on the basis of protocols routinely used for mycotoxins under investigation and yielded adequate results (Table 2). All analytical performances were comparable to those already reported for the same mycotoxins from other matrices *(6, 12, 13, 19).* The analytical results were considered satisfactory in terms of reproducibility and sensitivity, most likely because farro is similar to the matrices, such as wheat and maize, for which the analytical procedures were originally developed.

Mycological analyses of the samples collected from eight different locations allowed the identification of contaminating fungi at the genus level and determination of the inherent degree of contamination of grains by potentially mycotoxigenic fungal genera (Fig. 1). A large percentage of the farro was contaminated by both field pathogens (*Fusarium* and *Alternaria*) and postharvest fungi (*Alternaria, Aspergillus,* and *Penicillium*). In most of the samples, fungal species detected were *Epicoccum nigrum* Link, *Alternaria alternata* (Fries:Fries) von Keissler, *Aspergillus niger* van Tieghem, *Aspergillus tamari* Kita, *Fusarium proliferatum* (Matsushima) Nirenberg, *Fusarium tricinctum* (Corda) Sacc, *Penicillium verrucosum* Dierckx, *Penicillium polonicum* K. M. Zaleskì, and *Penicillium chrysogenum* Tom (data not shown).

Quantification of *Fusarium* mycotoxins FUSA, ENNs, ZON, FBs, and BEA and the *Penicillium* mycotoxin OTA, which are known to be produced by some of the fungal species listed above, are reported in Table 3 for the various farro samples. These toxins can be considered reliable

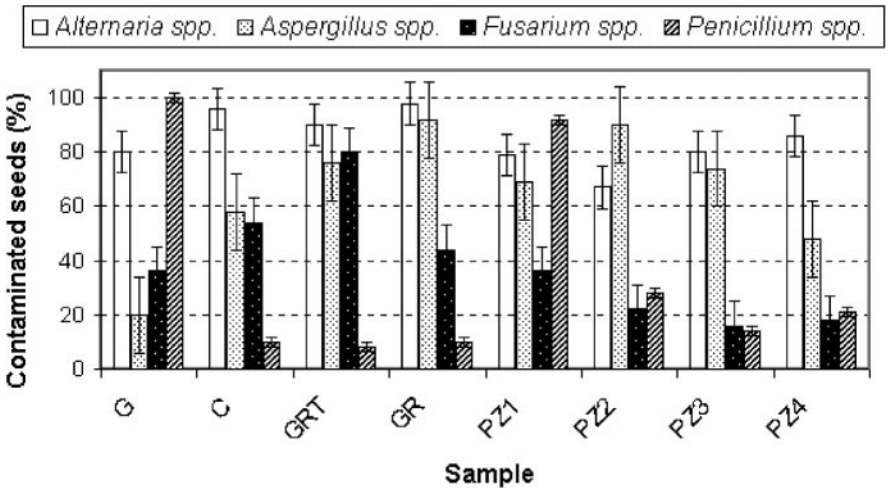

FIGURE 1. *Percentage of farro grains from different samples contaminated with mycotoxigenic fungal genera. Bars represent standard deviation (*n *= 10).*

markers of field contamination of farro plants caused by pathogenic and toxigenic *Fusarium* spp. and of storage contamination by *Penicillium* spp. (and, generally speaking, of other potentially mycotoxigenic postharvest fungal genera). Mycotoxin analyses, therefore, make it possible to identify the step, field, and/or storage area where contamination has occurred, representing possible critical points in a cereal production line. In the farro examined in this study, FB_1 was detected in five samples (up to 70.00 μg/kg). In the same samples, FB_2 also was detected, but concentrations were always below the LOQ. OTA was detected in seven samples (up to 4.07 μg/kg), BEA was detected in three samples (up to 4.44 mg/kg). ENNB (30.00 μg/kg) was detected in only one sample, whereas $ENNB_1$ and $ENNA_1$ were detected in two and one sample, respectively, although at concentrations below the LOQ. No ZON or FUSA were found in any of the samples evaluated. Some of the fungal species identified in the farro samples could be responsible for the mycotoxin contamination detected: *F. proliferatum* for FB_1 and BEA, *F. tricinctum* for ENNB, and *P. verrucosum* and *P. chrysogenum* for OTA. We did not detect other fungal species associated with the other mycotoxins in this study, possibly because of loss of viability of propagules of such fungi or competition by other fungal species during storage. Contamination with different mycotoxins appears to be independent of the species composition of farro samples (see also Table 1). In particular, FB_1 and OTA were detected at comparable concentrations in five and seven of the eight samples, respectively, whereas relatively high concentrations of BEA were detected in three samples, regardless of the *Triticum* spp. An apparent exception is ENNB, which was detected in only one sample consisting of *T. dicoccon.* However, no phytopathological assessment was carried out, particularly during cultivation of the farro plants. Therefore, no conclusions can be drawn on the susceptibility of the different species of farro to pre- or postharvest fungal attacks by *Fusarium* and *Penicillium* spp. and to inherent mycotoxin contamination. Nevertheless, data in Table 3 and Figure 1 suggest that both field and storage contamination occur in the farro production line. The detection of significant concentrations of toxins produced by *Fusarium* spp. suggests that, as for wheat and maize, an important critical point of farro production could be cultivation in the field, although further postharvest development of mycotoxigenic *Fusarium* spp. and production of respective toxins cannot be ruled out. On the family farms and in the harvesting center from which samples were collected, grains were stored in sacks or small containers at room temperature. The presence of widespread contamination with *Aspergillus* (Fig. 1), a fungal genus that includes mycotoxigenic species, suggests that storage conditions of farro grains should be improved.

Possible contamination with deoxynivalenol, which is produced by *Fusarium* spp. (in particular *Fusarium gra-*

TABLE 3. *Occurrence of mycotoxins in farro samples*[a]

Sample	FUSA	BEA	ENNB	$ENNB_1$	$ENNA_1$	FB_1	FB_2	ZON	OTA
G	ND[b]	ND	NQ[c]	ND	ND	70.00	NQ	ND	0.12
C	ND	4,440	30.00	NQ	NQ	20.05	NQ	ND	4.07
GRT	ND	ND	ND	ND	ND	ND	ND	ND	0.52
GR	ND	ND	ND	ND	ND	20.52	NQ	ND	0.06
PZ1	ND	1,800	NQ	NQ	ND	30.12	NQ	ND	0.08
PZ2	ND	1,010	ND	ND	ND	40.21	NQ	ND	NQ
PZ3	ND	ND	ND	ND	ND	ND	ND	ND	0.21
PZ4	ND	ND	ND	ND	ND	ND	ND	ND	0.10

[a] Values are reported as microgram per kilogram. For abbreviations of mycotoxins, see Table 2, footnote *a.*
[b] ND, not detectable.
[c] NQ, not quantifiable.

minearum and *Fusarium culmorum*), was not evaluated in this study. Although these species were not detected in our samples, the presence of conspicuous contamination with *Fusarium* spp. indicates that future assessments of farro samples should take into account this mycotoxin, frequently detected in high concentrations in cereal grains *(5).* The experimental approach used in our study allowed us to identify both cultivation of farro plants and storage of grains as possible critical points for mycotoxin contamination. Although the three grain species collectively referred to as farro are considered to be relatively resistant to pathogen attack *(16),* our results indicate that phytopathological surveillance and proper agronomical practices should be implemented during farro production.

This is the first report on occurrence of *Fusarium* and *Penicillium* mycotoxins in farro. Interventions for prevention of mycotoxin contamination of farro should be implemented in the field and definitely during storage. Farro is perceived by consumers as a natural crop associated with low-ecological-impact agronomic practices and little or no use of chemicals. This perception can be compromised by poor field and storage practices that may allow unwanted mycotoxins to contaminate farro-based feed or food.

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