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Monoculture Maize (*Zea mays* L.) Cropped Under Conventional Tillage, No-tillage and N Fertilization: (II) Fumonisin Incidence on Kernels

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Abstract

Planting maize under no-tillage is an increasing farming practice for sustainable agriculture and sound environmental management. Although several studies on yield of no-till maize have been done, there is few information about the effect of tillage on fumonisin contamination. The present study was done to determine the effect of no-tillage and conventional tillage with two rates of nitrogen on fumonisin content in kernels of continuous maize. Average grain contamination with fumonisins B_1 and B_2 over the years 2004-06 was not significantly different, with mean values of 1682, 1984 and 2504 μg kg^{-1}, respectively. Fumonisin B_1 was the most abundant toxin found in the samples. No-tillage significantly affected the incidence of fumonisins during the first year of the trial, in which fumonisin content was significantly higher with no-till (2008 μg kg^{-1}) compared with conventional tillage (1355 μg kg^{-1}). However, no-tillage did not significantly affect the incidence of fumonisins in the second and third years of the study. Fumonisin content at the rate of 300 kg N ha^{-1} was not statistically different compared to that obtained without N fertilization. The interaction between the soil management system and the rate of applied nitrogen was only evident in the second year. Our results indicate that fumonisin contamination was affected by no-tillage only in the first year. Nitrogen fertilization had no significant effect on fumonisin content in any year. The weather conditions during susceptible stages of maize development have probably overridden the effect of nitrogen fertilization.

Key-words: fumonisins, no-tillage, conventional tillage, N fertilization, *Zea mays* L.

1. Introduction

Conventional tillage practices dominate in maize production, but no-till cropping practices are now recommended for sustainable agriculture. No-till has advantages in terms of soil protection, conservation of water and reduction of energy consumption (Phillips and Phillips, 1984; Sprague and Triplett, 1986). No-till can significantly alter the relative frequency of the pathogens present, as determined for some soilborne pathogens (Bockus and Shroyer, 1998). There are scanty data on the effect of no-till on mycotoxin contamination of maize grain (Munkvold, 2003a), and few of them concern fumonisins (Ramirez et al., 1997; Marocco et al., 2008).

Fusarium ear rot is one of the most common diseases of maize. *Fusarium verticillioides* (Sacc.) Nirenberg is the dominant *Fusarium* species, accounting for up to 60% of the *Fusarium* in freshly-harvested maize cropped under conventional tillage conditions in Italy (Bottalico, 1998; Pietri et al., 2004). *Fusarium verticillioides* causes ear rot disease that typically occurs on random kernels, groups of kernels or insect-damaged kernels. Incidence of kernel infection can be 50-100% with the majority of kernels showing no damage (Munkvold et al., 1997a). When ear rot occurs, kernels can be visible mouldy, darkened, chalky, blistered, cracked, or streaked with white (Miller, 1994). In severe infections, white or pinkish mycelia can progress throughout the entire ear, colonis-

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ing husks and gluing them to the surface of the ear.

There are many infection pathways for *F. verticillioides*, including root, leaf, and stalk infection; systemic infection seems also possible but its contribution to mycotoxin contamination in kernels is not clear (Munkvold et al., 1997b). The most important pathways for kernel infection are European corn borer (*Ostrinia nubilalis* Hb.) damage (Blandino et al., 2008) and silk infection by airborne or rain-splashed microconidia and macroconidia (Miller, 1994; Munkvold and Desjardins, 1997). Furthermore, larvae of the European corn borer have been shown to act as vectors of *F. verticillioides*, transmitting the fungus from maize leaves to kernels (Sobek and Munkvold, 1999). Other insects may act as vectors (Farrar and Davis, 1991). In recent field tests, both visible ear rot symptoms and symptomless kernel infection decreased in maize genetically engineered for resistance to the European corn borer (Munkvold et al., 1997b).

The weather conditions that favour Fusarium ear rot are not well understood. Some studies have associated severe ear rot with dry weather during June and July, followed by wet weather during silking and later in the growing season (Schaafsma et al., 1993; Munkvold and Desjardins, 1997; Vigier et al., 1997). Silks are most susceptible to infection during the first week of silking (Schaafsma et al., 1993) and dry silks favours infection (Reid et al., 1999).

Fusarium verticillioides produces primarily fumonisin toxins. These are a group of at least 15 compounds, the most prevalent being fumonisin B_1. Fumonisin B_1 is implicated in the increased incidence of equine leukoencephalomalacia, porcine pulmonary edema, and is hepatotoxic and carcinogenic to rat (Marasas et al., 2001). Epidemiological investigations have indicated a possible association of *F. verticillioides* and its metabolic products with human oesophageal cancer. Based on existing health hazard data, the EU has issued a regulation containing maximum fumonisin levels, considered adequate to protect human and animal health. The maximum levels for total fumonisins (FB_1 + FB_2) in raw maize is 4000 μg kg^{-1}, and lower levels are fixed for maize-derived foods for direct human consumption (European Commission, 2007). Recommendations have also been published at the European level regarding fumonisin (FB_1 + FB_2) content in animal feeds (European Commission, 2006).

Fumonisins can be produced by almost all strains of *F. verticillioides*. Analyses of shelled kernels have found higher fumonisin levels in visibly mouldy maize than in good-quality maize (Shephard et al., 1996). In one study (Desjardins et al., 1998), the distribution of fumonisin B_1 in symptomatic and symptomless kernels from 116 individual ears of maize was determined. Over a wide range of fumonisin levels, 82-100% of the fumonisin in each ear was localised in symptomatic kernels. However, other studies reported that symptomless kernels were often associated with high fumonisin levels (Bush et al., 2004).

Fusarium verticillioides survive in maize residue, which is probably the most important source of inoculum for kernel infection (Munkvold, 2003b). Macroconidia and microconidia from residue are splashed or carried by wind to above-ground infection sites on the plants. It is not certain whether root infection is caused by conidia or by mycelial growth from infected residue. Tillage has not been shown to decrease Fusarium ear rot intensity, possibly because these fungi can survive for 21 months or more in infected residue (Cotten and Munkvold, 1998).

Because fumonisin contamination in maize depends on dry weather during grain fill (Munkvold and Desjardins, 1997), irrigation to avoid water stress during critical periods for the plant may decrease the risk of fumonisin contamination. The effect of N-fertilization on fumonisin incidence is still controversial and needs to be investigated (Blandino et al., 2008; Marocco et al., 2008).

In this study, our objectives were (i) to determine the incidence of soil tillage and nitrogen fertilization on fumonisins content in kernel of continuous maize; (ii) to investigate the consistency of the result obtained from a previous work (Marocco et al., 2008) carried out in a different site of the same important area for maize cropping.

2. Materials and methods

An experimental trial was carried out on irrigated maize over three years (2004-2006) at Terranova dei Passerini (LO, Po Valley, Northern

Italy). The soil was a *fine-loamy, mixed, mesic Ultic Haplustalf*, under processing tomato the year previous to this study.

The experimental design was a split-plot with four replicates, that was kept unchanged for the three years of the trial. Each sub-plot was 4.5 m wide and 12 m long. The main factor was the soil management system (conventional tillage vs. no-tillage, hereafter indicated as CT and NT respectively), with the rate of applied nitrogen (0, 250 and 300 kg N ha^{-1} $year^{-1}$) as the secondary factor (see the previous Note I: Tabaglio and Gavazzi, 2009). For this study of fumonisins only rates of 0 and 300 kg N ha^{-1} $year^{-1}$ were chosen, identified as N_0 and N_2 respectively. The CT plots were ploughed to a depth of 30 cm, and the NT plots were direct-planted through the chopped residues of the previous crop. The NT plots were treated with Glyphosate before maize planting. For all years, the hybrid PR34N43, FAO 500 (Pioneer Hi-Bred Italia) was sown, 70 cm apart, on April 24th, April 7th and April 14th for 2004, 2005 and 2006, respectively. The grain was harvested on September 21st, September 26th and September 29th for 2004, 2005 and 2006, respectively. The ears were hand-harvested from 2 rows and a sub-sample of 20 ears was taken. The kernels were shelled, dried at 105 °C and grinded with a mill to pass a 2.0 mm sieve.

For fumonisin determination, the flour sample was reduced to approximately 0.6 kg, ground with a laboratory mill to pass through a 1.0 mm sieve and carefully mixed before sub-sampling to ensure homogeneity. A sub-sample of 100 g was drawn from each sample for mycotoxin analysis.

Fumonisin B_1 (FB_1) and B_2 (FB_2) content was analysed according to the method proposed by Visconti et al. (2001). Fumonisins were extracted from 10 g of sample in a plastic centrifuge bottle with 50 mL of acetonitrile: methanol:water (25:25:50 v/v/v). After extraction for 45 minutes using a rotary-shaking stirrer and centrifugation at 4500 *g* for 6 min, the supernatant was poured in a flask; another 50 mL of the same solution was added to the residue in the centrifuge bottle, performing a second extraction for 30 min. The combined extracts were filtered through a folded filter-paper. An aliquot of 2 mL was diluted with 20 mL of 0.1 M phosphate buffered saline (PBS, pH = 7.4) and purified through an immunoaffinity column (R-Biopharm Rhône LTD, Glasgow, Scotland, UK); after washing the column with PBS (2 mL), fumonisins were slowly eluted (0.5 mL min^{-1}) with methanol (6 mL) into a graduated glass vial; subsequently, the eluate was concentrated to 2 mL under a gentle stream of nitrogen. Analysis was carried out using a LC-MS/MS system, consisting of a LC 1.4 Surveyor pump (Thermo-Fisher Scientific, San Jose, CA, USA), a PAL 1.3.1 sampling system (CTC Analitycs AG, Zwingen, Switzerland) and a Quantum Discovery Max triple-quadrupole mass spectrometer; the system was controlled by an Excalibur 1.4 software (Thermo-Fisher). After dilution of the extract (0.1 mL brought to 1 mL) with acetonitrile:water (30:70 v/v, acidified with 0.4% acetic acid), fumonisins were separated on a Betasil RP-18 column (5 µm particle size, 150x2.1 mm, Thermo-Fisher) with a mobile-phase gradient acetonitrile-water (both acidified with 0.4% acetic acid) from 25:75 to 55:45 in 9 min, then isocratic for 3 min; the flow rate was 0.2 mL min^{-1}. The ionisation was carried out with an ESI interface (Thermo-Fisher) in positive mode as follows: spray capillary voltage 4.0 kV, sheath and auxiliary gas 35 and 14 psi respectively, temperature of the heated capillary 270 °C. The mass spectrometer analysis was operated in selected reaction monitoring (SRM). For fragmentation of $[M+H]^+$ ions (722 *m/z* for FB_1, 706 *m/z* for FB_2), the argon collision pressure was set to 1.5 mTorr and the collision energy to 36 V. The selected fragment ions were: 704.2, 352.2 and 334.3 *m/z* for FB_1, 688.3, 336.1 and 318.2 *m/z* for FB_2. Quantitative determination was performed using a LC-Quan 2.0 software. Fumonisin standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). FB_1 and FB_2 (1 mg) were separately dissolved in 10 mL acetonitrile:water (1:1 v/v). These solutions were diluted to obtain HPLC calibrant solutions in acetonitrile:water (30:70 v/v, acidified with 0.4% acetic acid) at individual concentrations of FB_1 and FB_2 between 2.5 and 50 µg L^{-1}. The recovery values were estimated by spiking a blank sample with a measured volume of fumonisin standards, to obtain a contamination level of 1000 µg kg^{-1}. Average recovery values were 95.5 ± 1.9% for FB_1 and 93.6 ± 2.1% for FB_2. Results of analyses were not corrected for recovery. The limits of detection (LOD, signal-to-

noise ratio 3:1) and quantification (LOQ, signal-to-noise ratio 10:1) were 10 and 30 μg kg^{-1}.

Analysis of variance was performed for statistical analysis of all data (MSTAT-C Software); the LSDs were calculated for $P < 0.05$ level.

3. Results and discussion

In the three years of the study air temperatures during the months of June, July, and August were suitable for *F. verticillioides* growth, with monthly minimums and maximums of about 16 °C and 31 °C, respectively (Tab. 1). Available evidence indicates that the disease is negatively correlated with rainfall in June (Munkvold, 2003b). Rainfall during June averaged below the climatological data in the 3 years of the study. Total rainfall in August and September was much lower in 2004 compared with that in 2005 and 2006 (Tab. 1). In 2004, the August-September rainfall was 70% below the long-term average (1987-2006). In contrast, 2005 and 2006 had rainfall of 32% and 46% above normal, respectively. The data indicate that conditions at the experimental site were favourable for the fumonisin production in August and September two years out of three. Several lines of evidence indicate that Fusarium ear rot is positively correlated with rainfall during the period from August to October (Munkvold, 2003b).

The average contamination of grain by fumonisins B_1 and B_2 over the three years was not significantly different, with a mean value of 2057 μg kg^{-1} (Tab. 2). Fumonisin B_1 (FB_1) was the most abundant toxin found in our samples. FB_2 concentration was lower compared with FB_1: the ratio of FB_2 to FB_1 ranged from 0.28 to 0.46 on overall average. The levels of contamination found in the three years examined were about 60% lower compared with those reported through monitoring maize crops in Northern Italy over the period 2004 to 2007 (Battilani et al., 2008). According to EU legislation, these levels are below those of concern in raw maize for human and animal consumption (European Commission, 2006).

No-tillage significantly affected the incidence of fumonisin infection in 2004, but not in 2005 and 2006 (Tab. 2). In 2004, fumonisin content was higher under no-till (2008 μg kg^{-1}), compared with conventional tillage (1355 μg kg^{-1}). This result is probably due to the difficult growth conditions of the plants associated with the change in soil management. This is confirmed by the fact that the production of grain under NT was lower than for CT (see Note I: Tabaglio and Gavazzi, 2009). More information on inoculum amount both on soil surface and on the ear at flowering-ripening stages could help to explain the data obtained.

Table 1. Rainfall and temperatures recorded.

Rainfall (mm)

Month	YEAR			Climatological data (1987-2006)
	2004	2005	2006	
June	10.2	32.0	39.8	47.3
July	64.0	49.2	59.8	45.3
August	5.8	122.6	94.6	59.1
September (up to harvest)	40.2	78.0	127.2	93.1
Total	120.2	281.6	321.4	244.8
June-August	80.0	203.8	194.2	151.7

Monthly mean temperatures (°C)

Month	YEAR								
	2004			2005			2006		
	minimum	mean	maximum	minimum	mean	maximum	minimum	mean	maximum
June	16.0	21.3	29.2	17.2	22.7	28.2	16.0	22.1	27.9
July	17.5	24.2	30.1	17.6	23.3	29.7	19.4	25.0	31.1
August	17.3	23.6	30.5	15.6	20.7	27.3	15.7	20.7	26.7
September (up to harvest)	13.1	19.2	26.4	14.5	18.9	24.8	14.4	20.2	26.6

Table 2. Fumonisin content in maize grains (μg kg^{-1}).

Source	2004			2005			2006		
	FB_1	FB_2	Total fumonisins	FB_1	FB_2	Total fumonisins	FB_1	FB_2	Total fumonisins
Tillage system	0.01	n.s.	0.01	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NT	1361 b	647	2008 b	1514	406	1920	1338	592	1930
CT	948 a	407	1355 a	1574	474	2048	2170	908	3079
N fertilizing	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N_0	1170	460	1630	1992	658	2649	1735	751	2487
N_2	1140	593	1733	1096	223	1319	1773	749	2522
Interaction	n.s.	n.s.	n.s.	0.05	n.s.	0.05	n.s.	n.s.	n.s.
NT-N_0	1029	385	1414	1471	368	1839	1074	523	1596
NT-N_2	1694	909	2603	1557	445	2002	1602	662	2264
CT-N_0	1311	536	1847	2513	947	3460	2397	980	3377
CT-N_2	586	278	863	635	0	635	1944	836	2781
Overall average	1155	527	1682	1544	440	1984	1754	750	2504

NT, no-till; CT, conventional tillage; N_0, 0 kg N ha^{-1}; N_2, 300 kg N ha^{-1}; n.s., not significant at 0.05 level; 0.01 and 0.05, significant at 0.01 and 0.05 levels, respectively; within columns, means followed by different letters are significantly different at 0.05 level, according to LSD test.

During the subsequent two years of no-tillage, the levels of fumonisins were not significantly affected by tillage. The lack of any significant effect agrees with previous reports (Skoglund and Brown, 1988; Flett and Wehner, 1991; Flett et al., 1998). It is also well known that *Fusarium* spp. are able to overwinter in host residues (Shephard et al., 1996) and significant relationships between surface stubble and Fusarium ear rot incidence have also been reported (Byrnes and Carroll, 1986). Reduced tillage results in higher surface residues than conventional tillage. Large amounts of crop residue on the soil surface may particularly favour pathogens, which survive in the infected residue. However, some pathogens are partially or completely controlled by reduced tillage. The increased moisture available to a crop under reduced tillage tends to inhibit disease development (Bailey and Duczek, 1996). Survival of *F. verticillioides* was, in some reports, lower in maize stubble on the soil surface than at a depth of 30 cm, whereas in other studies, *F. verticillioides* was recovered in equal quantities from buried and surface maize residues (Bockus and Shroyer, 1998; Cotten and Munkvold, 1998). Surface residues are exposed to a wider range of temperature and moisture extremes than the buried residues. In our trial maize residues were chopped immediately after harvesting for both systems of soil management and left on the soil until ploughing at the end of winter or in early spring in CT. The chopping of crop residues likely favoured the reduction of inoculum, as suggested by Chervet et al. (2005), increasing the rate of residue decomposition. It therefore appears that surface stubble infected by *Fusarium* would not lead to an increase in inoculum. However, the data from wheat demonstrated that previous crop residues on soil surface increase significantly *F. graminearum* inoculum and deoxynivalenol (DON) contamination (Munkvold, 2003a).

Nitrogen fertilization had no significant effect on fumonisin content over all years (Tab. 2). This result contrasts with that obtained in our previous experiment (Marocco et al., 2008), in which N-fertilization significantly increased fumonisin content in two years out of three. Probably, these findings are due to the higher soil fertility present in the previous trial. In such condition, the control plants without N-fertilization experienced a lower fertility-related stress than the control plants in this trial. The effects of N fertilization on fumonisin contamination reported in the literature are controversial (Ramirez et al., 1997; Munkvold, 2003a; Blandino et al., 2008; Marocco et al., 2008; Blandino et al., 2009). Thus, Ramirez et al. (1997) found no significant difference in any area with respect to the treatment applied, while Blandino et al. (2008) reported that fumonisin increases both with high N rates and with N deficiencies. This may result from a suitably wet microclimate and mechanically weaker, more susceptible leaf tissues. In our study, the weather conditions during susceptible stages of maize

development may have overridden the effect of nitrogen fertilization.

The interaction between soil management system and rate of applied nitrogen was statistically significant in 2005 (Tab. 2). In NT, N-fertilizing with 300 kg ha^{-1} determined an increase in total fumonisins of 9%, while in CT it determined a reduction of 82%. It is probable that 300 kg N ha^{-1} are too much for NT, which is more efficient in its use of N compared with CT. The total fumonisin content in grain from unfertilized (N_0) plots was always lower in NT compared with CT, and in the final year it was almost halved (1596 vs. 3377 μg kg^{-1}). In contrast, when the plants which received 300 kg N ha^{-1} are considered, in the first two years of the trial total fumonisins in the NT plots were approximately three times higher than those found under CT. In any case, these differences are statistically significant only for 2005.

4. Conclusions

Our results indicate that fumonisin contamination was affected by no-tillage only in the first year. A high rate of nitrogen fertilizer did not influence the incidence of fumonisin contamination. The weather conditions during susceptible stages of maize development have probably overridden the effect of nitrogen fertilization. During the three years fumonisin content never reached the high levels that were frequently found in several areas of the Po Valley (Battilani et al., 2008). The highest level found here was 3460 μg kg^{-1} of total fumonisins.

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