Interaction of *Fusarium graminearum* and *F. moniliforme* in Maize Ears: Disease Progress, Fungal Biomass, and Mycotoxin Accumulation

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ABSTRACT

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To investigate the interaction between two major ear-rotting pathogens, maize ears were inoculated with either *Fusarium graminearum*, *F. moniliforme*, or an equal mixture of the two. Silk and kernel tissues were periodically harvested throughout the growing season so that a time course of the experimental variables (disease severity, ergosterol content, fungal DNA content, and mycotoxin concentration) could be recorded. Over the 3 years tested (1992 to 1994), the highest levels of disease and ergosterol were found in the *F. graminearum* treatment, followed by the mixture treatment (*F. graminearum* plus *F. moniliforme*) and, finally, the *F. moniliforme* treatment.

Kernel ergosterol content and disease rating were correlated for both pathogens, but the highest correlation coefficients were obtained in the *F. graminearum* treatment. The DNA analysis revealed that, in the mixed inoculum, *F. moniliforme* had a greater growth rate than did *F. graminearum*. In 1994, appreciable *F. moniliforme* from natural inoculum was found in the *F. graminearum* treatment. Fumonisin B₁ levels did not differ between the *F. moniliforme* treatment and the mixed inoculum treatment. The effect of temperature on the growth rate of the two species explained some of the field results, with temperatures in the silks being more favorable to *F. moniliforme*. Data on the growth rate on silks obtained by the incorporation of radiolabeled precursor to ergosterol demonstrated that *F. graminearum* was able to grow well at 26 to 28°C, whereas *F. moniliforme* grew well over a broader range, including at higher temperatures.

The major ear rot diseases of maize (corn, *Zea mays* L.) in Canada are caused by *Fusarium* spp. Three species of *Fusarium* are predominantly responsible for the disease: *F. graminearum* Schwabe (teleomorph = *Gibberella zeae* (Schwein.) Petch), *F. moniliforme* J. Sheldon (= *F. verticillioides* (Saccardo) Nirenberg, teleomorph = *G. fujikuroi* (Sawada) Ito in Ito & K. Kimura), and *F. subglutinans* (Wollenweb. & Reinking) P.E. Nelson, T.A. Toussoun, & Marasas (teleomorph = *G. subglutinans* (E. Edwards) P.E. Nelson, T.A. Toussoun, & Marasas) (59). All three species can produce mycotoxins in the grain. The toxins of most concern are those produced by *F. graminearum* (deoxynivalenol [DON] and zearalenone) and *F. moniliforme* (fumonisin B₁ [FB₁]). The trichothecene toxin DON causes feed refusal and decreased weight gain in swine (43). This toxin is also an immunosuppressant and thus predisposes animals to other diseases and masks underlying toxicosis (42). Zearalenone causes reproductive problems including reduced litter size, swine estrogenic syndrome, and male infertility (43). FB₁ causes equine leukoencephalomalacia (20), porcine pulmonary edema (14), and liver cancer in rats (10). Fumonisin has also been associated with human esophageal cancer (19).

F. graminearum (causal agent of Gibberella ear rot) produces a pink- to reddish-colored mold on kernels that usually spreads from the tip of the ear downward or outward from an insect wound. *F. moniliforme* (causal agent of Fusarium ear rot) produces a whitish-colored mold growth that tends to be scattered on the ear. Reports indicate that not all *F. moniliforme*-infected kernels are symptomatic (2,21). It has been suggested that this fungal species can be systemic in the maize plant and ubiquitous in nature (28,34).

The entry of *Fusarium* spp. into maize ears can occur through wounds caused by insects or birds (1,56) or by the growth of mycelium down silks to the kernels and cob (rachis) from spores germinating on the silks (17,22,54). *F. graminearum* requires succulent silk tissue, less than 8 to 10 days old, for infection (8,46,55,58). In contrast, colonization of silks by *F. moniliforme* occurs more frequently after the onset of silk senescence (15,16).

The best way of controlling *Fusarium* spp. ear rots is the development of host-plant resistance. Confounding the attempt to breed resistance into maize for both fungal species is the lack of knowledge on how *F. graminearum* and *F. moniliforme* interact in maize ears. The approach favored by some breeders, using a "cocktail" of *Fusarium* spp. to select maize genotypes with resistance to multiple species, is questionable because of possible interspecific competition and varying environmental conditions, especially temperature. Although there is overlap between the temperature ranges for growth of *F. moniliforme* and *F. graminearum*, the diseases of maize caused by these two species are favored by different conditions. *F. graminearum* requires periods of warm temperatures with persistent wetness during July and August (silking and early kernel development) (23,28,54,57). Thus, Gibberella ear rot is associated with growing regions in Illinois, New York, Michigan, and Ontario (28). *F. moniliforme* occurs during higher temperatures and drier years (40,51,59) and is associated with warm, dry conditions of the subtropics and dryland maize production. In Ontario, studies of *Fusarium* spp. on maize show increased recovery of *F. moniliforme* during warmer years (temperatures >25°C) (29,30,59).

Competition between fungi that occupy nominally the same niche has two often-intertwined features: competitive exploitation and interference competition (60). Competitive exploitation refers to the ability of fungi to consume nutrients before other species that might be present, whereas interference competition involves a chemical or behavioral trait that limits the access of the

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competing fungus to resources. For plant-pathogenic species, a third variable, aggressiveness, is important. One component of competitive exploitation is response to temperature. There are data on the temperature response of some strains of *F. moniliforme* and *F. graminearum* (6,26,27,63). However, these data are of limited value in predicting the outcomes under field conditions because they were completed on artificial media under laboratory conditions and strains vary from different climates.

F. moniliforme has been reported to suppress the growth of other maize ear fungi including *F. graminearum* and *Aspergillus flavus* Link:Fr. (17,48,62,65,66). Negative associations between the occurrence of *F. moniliforme* with both *F. graminearum* and *F. subglutinans* have also been reported (25,48).

The objective of this study was to investigate the interaction between *F. graminearum* and *F. moniliforme* in silk channel-inoculated maize ears by monitoring the progress of disease symptoms, ergosterol, mycotoxins (DON and FB₁), and species-specific DNA content. In addition, studies were made of the growth rate on silk tissue harvested from the field of the two species to understand the effect of temperature on the data obtained.

MATERIALS AND METHODS

Inoculation and harvest. From 1992 to 1994, a single *F. graminearum*- and *F. moniliforme*-susceptible maize hybrid (DeKalb DK415) was planted in a 4 × 12 factorial experiment arranged in split-plot design with three blocks at the Central Experimental Farm in Ottawa, Ontario. Four inoculation treatments (*F. graminearum* [G], *F. moniliforme* [M], both species simultaneously [GM], and a sterile water control) were randomized among the main plot units so that each consisted of 12 single-row subplot units consisting of 12 harvest times (2, 4, 6, 8, 10, 12, 14, 18, 22, 35, 49, and 63 days postinoculation). In 1992 and 1993, an additional five subplot harvest dates (1, 3, 5, 20, and 26 days) were used. Each subplot unit consisted of 15 plants in a row (3.8 m long), with 76 cm between rows. The primary ears of the center 10 plants of each row were silk-inoculated.

A macroconidial suspension of *F. graminearum* was prepared as previously described (46,47), using a single isolate, DAOM 180378. In the same manner, a suspension of *F. moniliforme* micro- and macroconidia was prepared from the isolate DAOM 195167. Both isolates were obtained from the Canadian Collection of Fungus Cultures (Agriculture and Agri-Food Canada, Ottawa, Ontario), were previously isolated from naturally infected maize ears, and have a moderately high level of aggressiveness compared with other isolates. Both isolates produced mycotoxins (DON and FB₁) upon inoculation into maize ears. Individual plants were inoculated by injecting 2 ml of a suspension (5×10^5 conidia per ml) into the silk channel of the primary ear approximately 6 days after silk emergence (46,47). For the simultaneous inoculation of a mixture of the two species, equal volumes of the suspensions (5×10^5 conidia per ml) were mixed just prior to inoculation. All inoculations were conducted within 1 to 2 h of each other. After inoculation, humid conditions were maintained by overhead sprinkler irrigation with 2 to 5 mm of water daily in the late afternoon for 4 weeks. Daily minimum and maximum temperatures were monitored by a data logger (model 21x, Campbell Scientific Inc., Logan, UT) located within the plot. The silk channel temperature of 12 plants was monitored by inserting thermocouples into the silk channels. Silk samples were removed each day, until the silk had dried out, to determine percent moisture.

At each harvest date, the 10 inoculated ears were hand-picked and husked (taking care to collect the silk), and the severity of ear rot symptoms was evaluated using a seven-class rating scale, in which 1 = no infection, 2 = 1 to 3%, 3 = 4 to 10%, 4 = 11 to 25%, 5 = 26 to 50%, 6 = 51 to 75%, and 7 = >75% of the kernels exhibiting visible symptoms of infection, such as rot and pinkish- or white-colored mycelial growth (47). Harvested ears were bulked

within each row and hand-shelled, and the cobs (rachises) were discarded. A scalpel was used to remove kernels from young ears. Each sample was mixed thoroughly to obtain a random distribution of the kernels, and a 350-g sample was freeze-dried for 5 days in a Pennwalt Stokes freeze-drier (Vacuum Equipment, Philadelphia), after which a 50-g subsample was ground to a fine powder in a Retsch Ultra Centrifugal mill (type ZM1; Brinkman Instruments, Inc., Rexdale, Ontario) with a 0.75-mm wire mesh. Subsamples were then taken for fungal biomass determination by ergosterol measurement and species identification using molecular analyses of DNA and mycotoxin (DON and FB₁) analysis.

Ergosterol analysis. Ergosterol, a sterol found exclusively in fungal membranes (36), was used to measure fungal biomass (31, 50). Ergosterol was extracted as per Young (64) with slight modifications. A 50-mg subsample of the ground maize tissue was placed in 17-ml culture tubes along with 2 ml of MeOH and 0.5 ml of 2 M NaOH. The tubes were tightly closed with Teflon-lined caps, placed inside capped 500-ml plastic bottles, and irradiated in a microwave oven (Kenmore model #8841; Sears Canada, Toronto, Ontario) at 50% power (2,450 Mhz, 750 watts maximum output) for 20 s and, after approximately 5 min, for an additional 20 s. After cooling, the tubes were removed from the plastic bottles and the samples were neutralized with 1 M aqueous HCl and treated with 2 ml of MeOH. The samples were partitioned with 3 × 4 ml of pentane, and the extracted top pentane layers were combined in 8-ml glass vials and evaporated in a sand bath at 50 to 60°C. The extracts were then redissolved in pentane and passed through a 13 CR PTFE membrane syringe filter, 0.45-μm pore size (Acrodisc; Gelman Scientific, Ann Arbor, MI), into 2.0-ml glass vials and then evaporated to dryness.

Quantification of ergosterol was made using high-performance liquid chromatography (HPLC). MeCN/MeOH/H₂O (80:10:6) was pumped through a 250 × 4.6-mm Keystone Prism RP column (5 μm, 100A pore size; Keystone Scientific, Bellefonte, PA) at a flow rate of 2.0 ml/min. The sample extract was dissolved in a known amount (50 to 1,000 μl, depending upon expected concentration of ergosterol) of ethyl acetate/pentane (1:1), and 25 μl was injected into the column.

A Varian variable wavelength detector (#2050; Varian, San Fernando, CA) was set at 282 nm for peak detection. Ergosterol retention time was used to identify peaks. Retention time and the detector's response (area counts per nanogram of ergosterol) were determined daily with an external standard. Confirmation of ergosterol in the sample was obtained using gas chromatography-mass spectroscopy (GC-MS) and coinjection.

Molecular identification of fungal species and quantification of fungal biomass. Quantification of the fungal biomass for each species was done in two steps. First, a slot blot DNA hybridization assay was used to measure the total fungal biomass for the *Fusarium* spp. and other Hyphomycetes spp. present in the maize samples. Second, a polymerase chain reaction (PCR)-based assay was used to determine the ratio of the fungal species present in each sample. It has been shown that the relative ratio, not the yield, of a mixture of DNA templates is preserved with amplification (12,33,37,39). Fungal biomass for each species in a sample was calculated as follows: total fungal biomass (pg of DNA per mg of tissue) × fraction of the species DNA in the PCR-amplified fungal fragment.

Total DNA was extracted from duplicate subsamples (50 mg each) as described by Möeller et al. (32). Fungal biomass quantification was conducted by slot blot DNA hybridization with the probe FG5B1L, which detects all *Fusarium* spp. and a number of other Hyphomycetes spp. (T. Ouellet, unpublished data). The use of the clone FG5B1L, a nuclear ribosomal DNA fragment present in multicopy in the fungal genome, as a probe increased by several-fold the sensitivity of detection of this hybridization assay (13). Prehybridization was overnight at 65°C in 5 × 0.015 M sodium citrate (SSC, 1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), pH 7.0; 50 mM NaPO₄; 0.1% sodium dodecyl sulfate (SDS); 5 × Denhardt solution; and 250 μg of denatured herring sperm DNA per ml.

The hybridization, also overnight at 65°C, was done in 1× SSC, 20 mM NaPO₄, 0.1% SDS, 1× Denhardt solution, 5% dextran sulfate, 100 µg of denatured herring sperm DNA per ml, and the digoxigenin-labeled probe. After hybridization, the membranes were washed as suggested by the manufacturer (Biotrans; ICN Biomedicals, Inc., Costa Mesa, CA), using 0.1× SSC and 0.1% SDS at 65°C for the final wash. The autoradiograms were scanned and analyzed using the Gel Print 2000i digital camera system and software (BioPhotonics Corporation, Dexter, MI). For quantification, the amount of fungal DNA in each maize sample was calculated by comparison with known fungal DNA controls present on the membranes.

The proportion of each *Fusarium* spp. present in the maize samples was determined by PCR assay using the same DNA extracts as above. A fragment of the nuclear large rDNA gene from fungi was specifically amplified using the primers LROR (45) and LR6 (5'CGCCAGTTCTGCTTACC3'), which are located in conserved areas of the gene (45, public sequence databases). Amplified fragments were then digested with the restriction enzyme *Ava*I to generate species-specific DNA patterns. The enzyme used generated distinct banding patterns for the *Fusarium* spp. used in this study (data not shown). Banding patterns from infected samples were compared with those from pure fungal cultures for identification. When more than one fungal species was detected, the band intensities for the different species were measured by scanning and analysis of ethidium bromide-stained agarose gels with the Gel Print 2000i digital camera system and software.

Mycotoxin analysis. *DON*. A competitive direct enzyme-linked immunosorbent assay (CD-ELISA) determination of DON was conducted according to Sinha and Savard (52). Briefly, samples (1 g)

were placed with 5 ml of methanol/water (1:9) in test tubes, mixed end-over-end for 1 h, and then centrifuged for 5 min at 1,100 × g. The resulting supernatant was then passed through a 0.22-µm filter. For the CD-ELISA, coating of plates with 100 µl of the antibody suspension (100 µg/ml) per well was followed by drying overnight at 35°C and blocking of unbound sites with ovalbumin. The sample extract (50 µl) was mixed with 50 µl of DON conjugated to horseradish peroxidase (HRP; Sigma Chemical Co., St. Louis) solution (2 µg/ml), and this mixture was immediately added to each well. The amount of DON-HRP bound to the antibodies was determined by adding to each well 300 µl of the substrate *o*-phenylenediamine (0.025 M citric acid buffer containing 0.1% H₂O₂, pH 5.0, at a concentration of 0.4 mg/ml), measuring the absorbance at 490 nm after 30 min, and comparing the results with the inhibition produced by known DON concentrations as in Sinha and Savard (52).

FB₁. The FB₁ ELISA (M. E. Savard and R. C. Sinha, *personal communication*) was performed as the DON ELISA described above, with minor modifications. Extraction of the maize was performed with five volumes of 1:1 acetonitrile/water. This solution was diluted to 15% acetonitrile before being added to the ELISA wells. Plates were layered with 3 µg of antibody solution per ml at 125 µl/well. The concentration of HRP conjugated to FB₁ used was 0.125 µg/ml, and that of the *o*-phenylenediamine (OPD) used as a color indicator of HRP concentration was 0.3 mg/ml.

Antibody suspension (125 µl) was dispensed into each well of a microtiter plate, and the plate was dried overnight at 50°C. The next morning, ovalbumin (300 µl, 1%) was added to each well to block unbound sites. Sample extract (50 µl) was mixed with an equal volume of FB₁-HRP, and the mixture was immediately added to each well. The free FB₁ present in the samples inhibited the binding of FB₁-HRP to the antibodies. The amount of FB₁-HRP bound to the antibodies was determined by adding an OPD solution and recording the absorbance of the resulting solution at 490 nm, after stopping the reaction at 30 min with 5 N HCl (50 µl per well). A mixture of equal volumes of FB₁ solutions of known concentration and FB₁-HRP was included in each assay to provide a standard curve correlating percent inhibition of FB₁-HRP binding by FB₁ to FB₁ concentration in solution (52). All data points represent the average of two measurements.

In vivo growth rate measurements. The method described by Newell and Fallon (38) was used to measure fungal growth rates. In 1993, samples of maize silks were removed from 10 *F. gram-*

TABLE 1. Analysis of variance for disease severity after inoculation of the silk channel with *Fusarium graminearum*, *F. moniliforme*, or a mixture of the two species in 1992, 1993, and 1994

Source	1992		1993		1994	
	df ^a	MS ^a	df	MS	df	MS
Block	2	0.09	2	0.56	2	0.59
Species	2	15.7** ^z	2	10.7**	2	5.70**
Error a	4	0.04	4	0.48	4	0.22
Harvest time	16	8.73**	16	4.37**	11	5.14**
Species × time	32	0.98**	32	0.73**	22	0.47**
Error b	96	0.17	96	0.13	66	0.06

df = Degrees of freedom, and MS = mean square error.

** = Significant at the 0.01 level of probability.

Fig. 1. Disease progress curve for disease severity ratings of one maize hybrid silk channel inoculated with *Fusarium graminearum* (G), *F. moniliforme* (M), or an equal mixture of the two (GM). Ratings are based on a scale of 1 to 7, in which 1 = no infection and 7 = >75% of the kernels visually moldy. Bar indicates the standard error of the difference: 0.34 in 1992, 0.29 in 1993, and 0.20 in 1994.

inearum- and *F. moniliforme*-inoculated plants 2 weeks postinoculation and placed into sterile plastic bags. These were immediately taken to the laboratory and approximately 1-g (wet weight) amounts of the fungal-silk material were placed in plastic scintillation vials containing sterile water. A total of 10 mmol of Na acetate per liter (1-14C, 110 mCurie/mmol, ICN Biomedicals, Inc.) was added to each of 10 vials; five of these had formaldehyde added to a final concentration of 2%. Sets of 10 were incubated for 1 h at 24, 26, 28, and 30°C. After 1 h, material from the vials was filtered on 0.45-µm Millipore filters, rinsed with two portions of 10 ml of water and 5 ml of methanol, and transferred to a reflux flask, after which 15 ml of methanol was added. Ergosterol was extracted by refluxing this solution, followed by saponification in methanol/KOH and ethanol, partitioning into pentane, drying down, and redissolving into methanol. Ergosterol concentrations were determined by HPLC with a UV detector at 282 nm after Miller et al. (31). Ergosterol (Sigma Chemical Co.) was recrystallized and was >99% pure as determined by GC-MS. Standards (2.2 µg injected) produced an average daily deviation of ±1.5%. As the ergosterol peak left the detector, it was collected in a scintillation vial and the radioactivity determined. Values were calculated as picometers of acetate incorporated per milligrams of fungal biomass per hour, subtracting the values for the killed cultures. Relative geometric standard deviations were 11% for the killed controls and 12% for the viable cultures. Fungal biomass was converted from ergosterol using the conversion factor of 200 (11).

Statistical analysis. For all dependent variables, residual error terms were generated and tested for normal distribution using the

Kolmogorov D statistic, and error mean squares were tested for homogeneity (SAS Institute, Cary, NC). Any data sets that violated these assumptions were log-transformed. Univariate analysis of variance (ANOVA) for a split-plot design was conducted to assess the significance of block, species, harvest time, and interaction effects on mean disease ratings, ergosterol concentrations, and DNA (mixture treatment only) and mycotoxin content using SAS (version 5, SAS Institute). Mean *Fusarium* spp. DNA recovery of the single inoculation treatments (G and M) were derived from one block and separated using either Tukey's or Kolmogorov-Smirnov tests in SYSTAT (SPSS Inc., Chicago, IL). Pearson's correlation coefficients were generated to determine the relationship between symptoms and ergosterol concentration and the relationship between silk channel temperature and air temperature using SYSTAT (SPSS Inc.), and the *t* test function of this program was used to compare the in vivo growth means. Area under the disease progress curve values (AUDPC) were calculated for each species within each block for disease severity, and ANOVA (and assumptions) was used as above to test the significance of block and species effects for AUDPC values (5). Means were then separated in SAS (SAS Institute) using a Fisher's least significant difference test.

RESULTS

Disease severity and ergosterol concentrations. Disease severity ratings were significantly ($P < 0.01$) affected by species treatment, harvest time, and species-harvest time interaction for all 3 years (Table 1). The G treatment always exhibited the greater

TABLE 2. Analysis of variance for ergosterol concentration (µg/g) in two tissues after inoculation of the silk channel with *Fusarium graminearum*, *F. moniliforme*, or a mixture of the two species in 1992, 1993, and 1994

Source	Silk ergosterol						Kernel ergosterol					
	1992		1993		1994		1992		1993		1994	
	df ^a	MS ^a	df	MS	df	MS	df	MS	df	MS	df	MS
Block	2	8,920.0	2	0.11	2	326.3	2	153.9	2	367.7	2	6.56
Species	2	292,913.7**z	2	51.5*	2	8,091.3**	2	96.2	2	1,633.8	2	278.7**
Error a	4	31,705.6	4	3.26	4	282.4	4	110.6	4	436.7	4	12.4
Harvest time	8	116,654.6**	6	36.6**	6	5,210.6**	10	290.0*	16	1,270.9**	11	291.3**
Species × time	15	47,948.8**	11	12.0**	12	2,600.2**	18	125.9*	32	530.2**	22	103.2**
Error b	34	1,659.7	34	4.09	36	375.1	42	36.6	96	259.1	66	7.4

df = Degrees of freedom, and MS = mean square error.

*z = Significant at the 0.05 level of probability, and ** = significant at the 0.01 level of probability.

Fig. 2. Disease progress curve for ergosterol of one maize hybrid silk channel inoculated with *Fusarium graminearum* (G), *F. moniliforme* (M), or an equal mixture of the two (GM). Bar indicates the standard error of the difference: 33.26 (1992 silk), 4.94 (1992 kernel), 1.65 (1993 silk), 13.14 (1993 kernel), 15.81 (1994 silk), and 2.22 (1994 kernel).

disease severity rating, followed by the GM treatment (Fig. 1). The M treatment usually exhibited the lowest disease severity ratings throughout the experiment. On average, the greatest disease severity was in 1992. Sterile water control treatments had no symptoms, thus indicating little background infection.

For 3 years, species, harvest time, and the species-harvest time interaction were significant for ergosterol levels in both kernel and silk tissues (except for nonsignificant species effects for kernel ergosterol in 1992 and 1993) (Table 2). The trend seen with the disease severity data (highest levels in G, followed by GM and then M) was also seen in the ergosterol data (Fig. 2). Although the trend remained the same over the years, the absolute levels of ergosterol recovered changed, as did the levels in the two different tissues. In 1992 and 1994, the highest amounts of ergosterol were

recovered from the silk tissue, whereas, in 1993, the highest amounts were found in the kernels (Fig. 2). Overall, kernels inoculated with *F. moniliforme* contained significantly less ergosterol compared with *F. graminearum*. Kernel ergosterol was positively correlated to disease ratings (Table 3) for all years, except for M in 1992. Minimal quantities of ergosterol were found in a few samples of the sterile water control, confirming little to no background infection.

Species effects on AUDPC values were significant in all years for disease severity (Table 4). In all years, the highest AUDPC values were found in the G treatment. For only 1 year, disease severity in 1994, was there a significant difference between AUDPC values for all three treatments.

DNA. DNA analysis allowed us to identify and quantify fungal species that were present in kernels at the time of sampling. The total DNA from fungal species of the *Fusarium* or closely related

TABLE 3. Pearson's correlation between disease severity and ergosterol levels in kernels of maize after *Fusarium* spp. inoculation

Year	Treatment ^y	<i>r</i>	<i>n</i>
1992	G	0.897** ^z	12
	M	0.388	13
	GM	0.737**	14
1993	G	0.647**	21
	M	0.552*	20
	GM	0.532*	21
1994	G	0.834**	15
	M	0.567*	13
	GM	0.841**	13

G = *F. graminearum*, M = *F. moniliforme*, and GM = *F. graminearum* plus *F. moniliforme*.

*^z = Significant at the 0.05 level of probability, and ** = significant at the 0.01 level of probability.

TABLE 4. Mean area under the disease progress curve values (% days) for disease severity for a maize hybrids silk channel inoculated with *Fusarium graminearum*, *F. moniliforme*, or a mix of the two species in 1992, 1993, and 1994

Treatment ^y	Disease severity ^z		
	1992	1993	1994
G	255.7 a	206.1 a	179.4 a
M	114.7 b	123.4 b	102.2 c
GM	157.1 b	118.8 b	143.5 b

Inoculation treatments: G = *F. graminearum*, M = *F. moniliforme*, and GM = *F. graminearum* plus *F. moniliforme*.

Means followed by the same letter within a column for a given year and variable are not significantly different at the 0.05 probability level based on Fisher's (protected) least significant difference test.

Fig. 3. *Fusarium graminearum* and *F. moniliforme* DNA recovered from the kernels of maize ears inoculated with *F. graminearum* (G), *F. moniliforme* (M), or an equal mixture of the two (GM) for 2 years, 1993 (top) and 1994 (bottom). Points in the G and M treatments represent one block only. The split-plot analysis of variance was then used to compare recovery of the two species from one treatment (GM), and a significant difference between species was found each year. The standard error of the difference for GM was 33.56 in 1993 and 74.49 in 1994.

genera was detected and identified as originating either from *F. graminearum*, *F. moniliforme*, or other species (Fig. 3). Fungal species not closely related to the *Fusarium* genus were not detected by the DNA assay, but were detected by the ergosterol assay.

DNA data is only presented for 1993 and 1994, because the 1992 sample DNA had degraded by the time of analysis. In 1993, the high amount of fungal biomass detected close to maturity in kernels of the G treatment by ergosterol and DNA analyses was due not only to the *F. graminearum* inoculum but also to the presence of *F. moniliforme* that had become established on the plants. In contrast, the M treatment contained no detectable *F. graminearum* DNA by the end of the experiment (Table 5). In 1994, levels of *F. graminearum* DNA were relatively low, even in the G treatment. By 63 days postinoculation, significantly more *F. moniliforme*

than *F. graminearum* DNA was recovered from the G treatment and, in 1993, no significant differences could be found between the two species, even though only *F. graminearum* was inoculated for this treatment (Table 5). The split-plot ANOVA revealed that, within the GM treatment, significantly more *F. moniliforme* DNA than *F. graminearum* DNA was recovered in both years. In 1994, the amount of *F. moniliforme* DNA at day 35 had a high variance and is not significantly different from the mean at day 49 (Fig. 3). Other fungal species were also detected in both years; however, their biomass varied within and between fungal treatments. In water-inoculated controls (data not shown), the fungal DNA detected was mainly from species other than *F. graminearum* and *F. moniliforme* (in 1994, a variable and small amount of *F. moniliforme* DNA was found).

Fig. 4. Concentrations of the mycotoxins deoxynivalenol (DON) and fumonisin B₁ (FB₁) in the silks of maize ears inoculated with *Fusarium graminearum* (G), *F. moniliforme* (M), or an equal mixture of the two (GM). Bar indicates the standard error of the difference: 6.79 for DON 1993, 0.00 for FB₁ 1993, 1.12 for DON 1994, and 1.29 for FB₁ 1994.

Fig. 5. Concentrations of the mycotoxins deoxynivalenol (DON) and fumonisin B₁ (FB₁) in the kernels of maize ears inoculated with *Fusarium graminearum* (G), *F. moniliforme* (M), or an equal mixture of the two (GM). Bar indicates the standard error of the difference: 73.82 for DON 1992, 10.19 for FB₁ 1992, 57.31 for DON 1993, 7.69 for FB₁ 1993, 4.33 for DON 1994, and 10.48 for FB₁ 1994.

Mycotoxins. With the exception of silk in 1992 and some later silk dates in which insufficient tissue was available, mycotoxin analysis was completed for all years in kernels and silks. Silk mycotoxin levels were not high (<5 parts per million [ppm]), except for DON in the *F. graminearum*-inoculated ears in 1993 (Fig. 4). Figure 4 demonstrates that, in both years as time progressed, DON and FB₁ levels remained relatively stable. As with DON, silk FB₁ levels in the M treatment were higher in 1993 than in 1994. Thus, the trend of higher levels seen in the silk ergosterol in 1994 versus 1993 was not reflected in the toxin concentrations. Partial data from the DNA analysis of silk in 1994 (data not shown) indicated that most of the fungal biomass in the late sampling dates in the G treatment was not *F. graminearum*, but rather *F. moniliforme* and other fungal species. Therefore, a relationship between DON levels in this year and treatment and ergosterol levels was not expected. With simultaneous inoculation (GM), DON levels were lower than levels found in the G treatment and appeared to decrease with time, agreeing with the DNA results. FB₁ levels in GM remained low throughout the time course and, in some cases, were significantly lower than FB₁ levels in the M treatment (Fig. 4).

TABLE 5. Amount of *Fusarium* spp. DNA recovered 63 days postinoculation from the single species treatments^y

Species	Treatment ^z	1993	1994
<i>F. graminearum</i>	G	2,512.5 a	112.5 b
<i>F. graminearum</i>	M	0 c	0 c
<i>F. moniliforme</i>	G	1,237.5 a	446.3 a
<i>F. moniliforme</i>	M	625.0 b	89.0 b

DNA recovered (pg/mg) for each year. Means followed by the same letter are not significantly different ($P > 0.05$) in a Kolmogorov-Smirnov (1993) or Tukey's (1994) test.

Inoculation treatments: G = *F. graminearum* and M = *F. moniliforme*.

In the kernels, very high concentrations of DON were found in the 1992 and 1993 G treatments (Fig. 5) and both of these years had high disease severity. DNA data in 1994 showed more *F. moniliforme* than *F. graminearum* in the G treatment; therefore, the lower DON levels were expected. When both species were inoculated simultaneously, DON levels were less than 20 ppm in 1992 and almost zero in 1993 and 1994. FB₁ levels generally remained the same between the *F. moniliforme* alone and the mixture treatment.

In vivo growth rate. Data on the growth rates of the strains of *F. graminearum* and *F. moniliforme* on silk tissue collected in the field in this study are shown in Table 6. Growth differences between the two species were observed at the various tested temperatures. Although there was no significant difference in growth rate at 28°C, *F. moniliforme* grew significantly faster than *F. graminearum* at the remaining temperatures (Table 6). This trend seemed to be stronger at the extremes of the temperature regime, with growth rates of *F. moniliforme* roughly doubling those of *F. graminearum* at the lowest (24°C) and highest (30°C) temperatures.

TABLE 6. In vivo growth of two species of *Fusarium* cultured under four temperature regimes

Temperature	Mean fungal growth rate ^y	
	<i>F. moniliforme</i>	<i>F. graminearum</i>
24°C	10.6	4.5***
26°C	15.0	19.3*
28°C	20.0	20.5
30°C	22.6	11.6**

Mean growth rate (pm of acetate/mg of fungal biomass/h) for *F. moniliforme* versus *F. graminearum* is compared at each temperature.

* = Significant at the 0.05 level of probability, and ** = significant at the 0.01 level of probability as determined by a *t* test.

Fig. 6. The maximum, mean, and minimum temperature (°C) of the silk channel (top) and of the air (bottom) as measured by a thermocouple, from 1992 to 1994.

Environmental conditions. The temperature of the silk channel was similar across the 3 years (Fig. 6). However, the maximum, mean, and minimum temperatures were generally warmer in 1993 than in 1992 and 1994 for the first 8 days postinoculation. The mean air temperature measured in the plots (Fig. 6) was highly correlated (Pearson's $r > 0.96$, $P < 0.0005$) to the mean silk channel temperature during the 3 years, and it is assumed that air temperature is the largest determinant of ear temperature. Like silk channel temperature, air temperature was generally the warmest in 1993 for the earlier days postinoculation. The mean air temperature in 1993 decreased more rapidly than in other years and, after day 35, was always lower than the corresponding days in 1994 (Fig. 6).

The highest amount of moisture in the silk was recorded in 1992, followed by 1993 and then 1994 (Fig. 7). In 1993, the silk tissue had completely senesced by 8 days postinoculation. Kernel tissue contained the most moisture in 1992, followed by 1994 and then 1993 (Fig. 7).

DISCUSSION

Fungal biomass, as ergosterol, for all treatments remained in the silk tissue in 1992 and 1994, whereas, in 1993, the ergosterol analysis revealed that the fungi quickly invaded the kernels. In this year, silk tissue had almost completely senesced by the eighth day after inoculation. The G treatment consistently rated the highest on the disease severity scale, because it consistently produced the most fungal biomass. However, the ergosterol method identifies total fungal biomass (that is, it is not species specific). Although biomass may be the best predictor of disease ratings, the DNA analysis demonstrated that this biomass was not always produced by the expected *Fusarium* sp. In the GM treatment in both 1993 and 1994, *F. moniliforme* was the predominant species. The main question posed by these results is how does *F. moniliforme* have an advantage over *F. graminearum* and, as a corollary, why in 1994 was there relatively more *F. moniliforme* recovered from the G treatment?

In Ontario and elsewhere, epidemiological and field studies report an association between drier and warmer growing conditions with *F. moniliforme* and wetter conditions with intermediate temperatures with *F. graminearum* (29,30,54,59). This observation is supported by the current studies. The in vivo growth rate data show clearly that temperature conditions strongly affect the infection. In the 2 years when *F. moniliforme* out-competed *F. graminearum* when inoculated as a mixture, the likely major factor is the impact of daytime temperatures. This impact is complex in that, if the conditions are marginal for *F. graminearum* to grow and infect the

plant, *F. moniliforme* colonization after inoculation may occur because of its ability to grow over a wider range of conditions.

It has been suggested that *F. graminearum* predisposes the ear to *F. moniliforme* infection (30). This may address the question of where the *F. moniliforme* infection originally came from in the G treatment in 1994. It is thought that *F. moniliforme* is systemic in maize plants (2,9,34) and ubiquitous in nature (28,35). High rates of *F. moniliforme* recovery from *F. graminearum*-inoculated maize have also been observed in other experiments (49; A. W. Schaafsma, unpublished data). If *F. moniliforme* is ubiquitous, it is not obviously pathogenic every year (2,21,24).

If *F. graminearum* predisposes the plant to *F. moniliforme*, then there is likely an environmental effect, because *F. moniliforme* was isolated from G treatment plots at a greater level in 1994 than in 1993. Our results and previously completed laboratory studies revealed that *F. moniliforme* has an enhanced ability not only to grow over a broader range of temperatures and water activities, but also to germinate. Conditions at the environmental periphery (the maximum and minimum values at which the fungi can still respond) may present *F. moniliforme* with an opportunity to out-compete *F. graminearum*. For example, the minimum water activity (a_w) and temperature needed by *F. moniliforme* for growth is generally reported to be 0.86 to 0.90 a_w and 4°C, respectively (26,63), whereas the same conditions reported for *F. graminearum* are 0.935 a_w and 10°C (6). This trend is even more evident in the minima needed for spore germination. *F. graminearum* is reported to need a minimum a_w of 0.94 to 0.95 at 25°C to germinate (53), whereas, at approximately the same temperature, the ascospores of *F. moniliforme* are reported to be able to germinate down to 0.88 a_w (27).

Growing conditions in 1994 could have theoretically favored *F. moniliforme*. In 1994, the minimum and mean silk channel temperature was lower than in 1993, especially in the first days after inoculation (days 1 to 6). The mean temperatures during this period (1993 versus 1994) ranged from 20 to 25°C versus 15 to 20°C, and the minimum temperatures ranged from 15 to 20°C versus 7 to 15°C, respectively (Fig. 6). Also, the silk tissue was drier in 1994 than in 1993 during this period (Fig. 7). The temperature and water conditions in 1994 may, therefore, have been too low for *F. graminearum* to germinate and grow (in both the G and GM treatments) to a critical level, which may have left the ear open for *F. moniliforme* colonization.

From the results presented here, it can be stated that *F. moniliforme* has at least one competitive advantage over *F. graminearum*: a broader response to temperature that confers direct and indirect benefits. What remains unclear is whether this is the only factor involved or whether there are others. It was thought that the dif-

Fig. 7. The percent moisture content of silk and kernel tissue from 1992 to 1994.

ferent mycotoxins produced by the two species may be a competitive factor (chemical antagonism), because it has been suggested that the production of secondary fungal metabolites are ecologically significant and confer increased fitness to the producing organism (3,4,41). Changes in mycotoxin levels in response to fungal interactions have been shown with aflatoxin in *A. flavus* (18) and with zearalenone and T-2 in *Fusarium* spp. (7,44).

If *F. moniliforme* uses FB₁ as a competitive advantage over *F. graminearum*, then one would expect higher concentrations of this toxin in the GM and G (1994) treatments than in the M treatment. This was not observed in either the silk or kernel tissue. Also, no negative correlation was found between FB₁ concentration and *F. graminearum* DNA levels. As opposed to the other measured variables (disease severity, ergosterol, and DNA), interaction between the two fungal species did not seem to directly influence the mycotoxin production or accumulation. Therefore, the data does not support the hypothesis that *F. moniliforme* gains a competitive advantage over *F. graminearum* via FB₁. Both species produce various toxins, the bioactivity of which has not been thoroughly investigated; therefore, the idea of chemical antagonism between *F. moniliforme* and *F. graminearum* cannot be dismissed.

The results of this study suggest that, for the purpose of breeding maize for resistance to *F. graminearum* and *F. moniliforme*, a "cocktail" mixture of the two species would not be appropriate. The breeder may not know for which species he or she was selecting unless further analyses were conducted, special attention was paid to the environmental conditions, or both. Improved selection can be made by inoculating each species individually, thus avoiding the complicated competitive interactions.

CONCLUSIONS

The experimental data demonstrated that *F. moniliforme* can out-compete *F. graminearum* when the temperature conditions are not favorable for the latter species. This implies that the outcome of the fungal competition will change as growing conditions change from season to season (i.e., the results demonstrated here may not be consistent between years and locations). However, there is evidence suggesting that *F. moniliforme* is a highly competitive colonizer of maize that can successfully compete against fungi in many genera (48,61,66). *F. moniliforme* may, therefore, possess a stable competitive advantage over *F. graminearum* as well as other fungal species.

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