Comparison of Techniques for Inoculating Maize Silk, Kernel, and Cob Tissues with *Fusarium graminearum*

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ABSTRACT

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Six inoculation techniques differing in the method of application of a conidial suspension and in the part of the ear inoculated were evaluated for their effectiveness in assessing maize (*Zea mays*) resistance to ear rot caused by *Fusarium graminearum.* Silk channel injection and ear-tip flooding inoculation techniques were carried out 7 days after silk emergence. The other four techniques (wound-spray, kernel-stab, pipe cleaner, and cob-tip) were carried out 15 days after silk emergence. A 7-class rating scale was used to assess disease severity at harvest. Significant differences ($P < 0.05$) in incidence and severity of ear rot symptoms were detected among the inbred lines and inoculation techniques. There were significant inbred × inoculation technique interactions, but inoculation techniques intended to measure the same resistance mechanism ranked inbred lines similarly in three of the four environments. All inoculation techniques except the ear-tip flooding technique identified CO325 as the most resistant inbred. Among the techniques used, the silk channel and the kernel-stab techniques appeared to be the most effective in measuring silk and kernel resistance, respectively.

Ear rot caused by *Fusarium graminearum* Schwabe (sexual state: *Gibberella zeae* (Schwein.) Petch) is a destructive disease of maize (*Zea mays* L.) in many parts of the world, including maize-growing regions of eastern Canada (11,15). Disease symptoms usually start at the tip of the ear, but occasionally they originate at the butt of the ear (2). Ear rot can also be associated with damage caused by birds or insects such as corn borers. The fungus causes a pronounced reddish discoloration of the rotted grain and husk tissues and produces a pinkish white mold on the surface of colonized grain. The disease has economic implications, in that infection may lead to contamination of grain with mycotoxins, including deoxynivalenol (4). These mycotoxins affect the performance of different species of livestock, with swine being the most susceptible.

Development of resistant maize hybrids could help control this disease. Because of the sporadic nature of epidemics, selection of resistant genotypes requires artificial inoculation. Numerous methods have been used for artificially inducing epiphytotics of maize ear rots (5,12,15). Ullstrup (15) studied two inoculation techniques: spraying silks with a macroconidial suspension 1 to 2 weeks after silking, and inserting a toothpick colonized with mycelium into the silk channel of the ear approximately 1 week before full silk emergence. Both methods established infection at levels that allowed differentiation between genotypes. Sutton and Baliko (12) used the toothpick method, spraying of silks, and a silk-channel injection method. They reported that silk-channel injection was ineffective in differentiating between resistant and susceptible genotypes. However, Reid et al. (5) reported that injection of a conidial suspension into the silk channel gave consistent results and allowed for differentiation between resistant and susceptible genotypes.

In the present study, six inoculation techniques, differing in the method of applying a macroconidial suspension and in the part of the ear inoculated, were evaluated with respect to their effectiveness in assessing genotypic differences in resistance to infection via the silk and to spread of infection on developing kernels of maize.

MATERIALS AND METHODS

Field trials. Experiments were grown in two locations, Macdonald Campus (Ste-Anne-de-Bellevue, Quebec) and Central Experimental Farm (Ottawa, Ontario), in 1992 and 1993. The experimental design was a split-plot design with inbred lines as main-plot factors and inoculation methods as subplot factors. Treatment combinations were replicated four times in both years. Each main plot consisted of seven rows. Rows were 2.4 m long at Ste-Anne-de-Bellevue and 3.8 m long at Ottawa. The experiments were seeded on 12 May 1992 and 1993 at Ste-Anne-de-Bellevue, and on 17 May 1992 and 20 May 1993 at Ottawa. After emergence, plants were thinned to 12 plants per row.

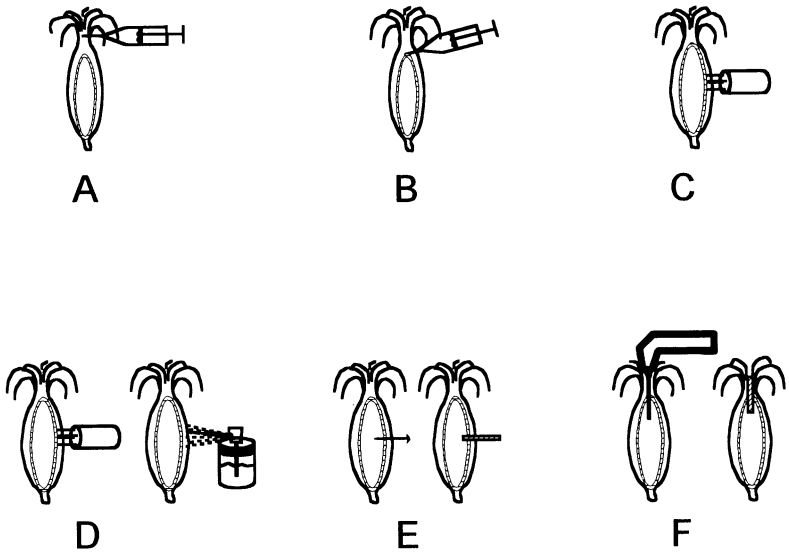

Fig. 1. Diagram of six inoculation techniques evaluated: **(A)** silk-channel injection, **(B)** ear-tip flooding, **(C)** kernel-stab inoculation, **(D)** wound and spray inoculation, **(E)** pipe cleaner inoculation, and **(F)** cob-tip inoculation.

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Maize genotypes. Six inbred lines (A641, CO266, CO265, CO272, CO325, and F2) were evaluated. These lines were chosen to represent a range of levels of resistance to *F. graminearum* based on results reported by Reid et al. (7).

Inoculum preparation. *F. graminearum* DAOM194276, a highly aggressive isolate originally obtained from infected maize in Ottawa and registered in the National Agriculture and Agri-Food Canada Culture Collection, was maintained on Synthetic Nutrient Agar (SNA), a modified form of Bilay's medium (13). A macroconidial suspension was prepared as described by Reid et al. (8).

Ear inoculation techniques. Primary ears of the 10 middle plants of each row were inoculated with a 5×10^5 conidia per ml suspension using the following techniques: (i) Silk channel injection: 2 ml of inoculum was injected into the silk channel using a hypodermic needle (Fig. 1A); (ii) Ear-tip inoculation: 2 ml of inoculum was dispensed with a hypodermic needle to flood the kernels at the tip of the ear (Fig. 1B); (iii) Kernel-stab inoculation: a probe consisting of four nails (1.5 cm) fixed to a cylindrical wooden handle was used to inoculate the ear. The nails were dipped into inoculum and then stabbed through the husks to wound only three to four kernels in the middle of the ear (Fig. 1C); (iv) Wound and spray inoculation: a probe rinsed in sterile water was used to wound the kernels as described above, then approximately 2 ml of spore suspension was sprayed on the wounded area with an atomizer (Fig. 1D); (v) Pipe cleaner inoculation: a 2-cm tunnel was made directly through the kernels and cob in the midear area with a nail. A 2-cm piece of pipe cleaner saturated with spore suspension was then inserted into the tunnel (Fig. 1E); and (vi) Cob-tip inoculation: husks at the apex of the ear were opened to expose the tip of the cob. A tunnel about 1 cm deep was made into the cob tip using a battery-operated drill. A 1-cm piece of pipe cleaner saturated with spore suspension was then inserted into this apical tunnel. The apical husks were then clasped, and a rubber band was used to hold the husks in position (Fig. 1F).

Inoculation methods 1 and 2 were carried out 6 to 7 days after silk emergence (between 29 July and 10 August). The other four inoculation techniques (3 to 6) were carried out 15 days after silk emergence (between 7 and 17 August).

The trials were harvested in mid-October. Disease severity was assessed by rating the percentage of rotted area using a 7-class rating scale where 1 = no symptoms, 2 = 1 to 3%, 3 = 4 to 10%, 4 = 11 to 25%, 5 = 26 to 50%, 6 = 51 to 75%, and 7 = 76 to 100% of the infected ear (1). Disease incidence was calculated as a percentage of inoculated ears that had ratings of 2 or above.

Data analysis. Analysis of variance was performed using SAS PROC GLM (SAS/STAT User's Guide, Version 6, 1994) on subplot means for disease severity values and disease incidence after verifying assumptions for normality of data and homogeneity of variances. The effects of the inbred lines, inoculation techniques, and interactions were evaluated, and mean separation was tested by Duncan's multiple range test (10). Spearman rank correlation coefficients were calculated using

Table 1. Mean squares for disease severity and disease incidence in experiments conducted at Macdonald Campus (Ste-Anne-de-Bellevue) and Central Experimental Farm (Ottawa) in 1992 and 1993

		1992		1993	
Source of variation	df	Severity	Incidence	Severity	Incidence
Ste-Anne-de-Bellevue					
Block	3	0.12	281.07	0.48	159.56
Inbred	5	25.77**[z]	10,288.16**	14.48**	258.50*
Block × inbred	15	1.18**	472.32	0.37	99.15
Inoculation (I)	5	17.13**	4,146.68**	9.38**	179.31
Inbred × I	25	0.93**	591.23**	3.00**	97.16
Error	90	0.45	274.58	0.52	99.86
Ottawa					
Block	3	0.39	595.51	0.57	218.32
Inbred	5	26.14**	7,880.17**	14.81*	513.61*
Block × inbred	15	1.15**	408.95	0.73	118.77
Inoculation (I)	5	20.18**	7,053.16**	16.10**	367.15**
Inbred × I	25	1.85**	1,228.57**	2.39**	216.67*
Error	90	0.47	325.31	0.41	101.55

[z] *,** = Significant at 0.05 and 0.01 probability level, respectively.

Table 2. Mean disease incidence of six inbred lines of maize inoculated with *Fusarium graminearum* using six inoculation techniques at Macdonald Campus (Ste-Anne-de-Bellevue) and Central Experimental Farm (Ottawa) in 1992

Inbred	Silk-channel	Ear-tip flooding	Wound-spray	Kernel-stab	Pipe cleaner	Cob-tip
Ste-Anne-de-Bellevue						
A641	72.5ab[z]	57.5b	72.5b	85.0ab	82.5ab	85.0a
CO265	80.0ab	45.0bc	76.9ab	87.2ab	85.0ab	77.5a
CO266	93.7a	86.1a	100.0a	100.0a	100.0a	100.0a
CO272	58.3bc	42.5bcd	57.5b	60.8c	72.2b	27.5b
CO325	31.9c	15.0d	68.9b	75.0abc	65.8b	26.9b
F2	58.3bc	20.6cd	32.2c	49.0c	77.3b	23.4b
Ottawa						
A641	89.7a	62.3ab	78.9ab	85.0a	100.0a	75.0a
CO265	97.2a	57.5b	35.0c	70.0a	100.0a	81.9a
CO266	97.2a	94.7a	95.0a	97.2a	97.2a	97.2a
CO272	35.3a	35.3bc	25.0c	95.0a	97.5a	30.0b
CO325	35.0b	9.2c	56.9bc	75.0a	97.5a	25.0b
F2	84.4a	35.0bc	57.5bc	81.7a	95.0a	83.9a

[z] Means within column and site followed by same letter are not significantly different at the 0.05 probability level, according to Duncan's multiple range test.

Table 3. Mean disease incidence of six inbred lines of maize inoculated with *Fusarium graminearum* using six inoculation techniques at Macdonald Campus (Ste-Anne-de-Bellevue) and Central Experimental Farm (Ottawa) in 1993

Inbred	Silk-channel	Ear-tip flooding	Wound-spray	Kernel-stab	Pipe cleaner	Cob-tip
Ste-Anne-de-Bellevue						
A641	95.0a[z]	100.0a	100.0a	100.0a	100.0a	100.0a
CO265	100.0a	80.6a	100.0a	100.0a	100.0a	94.4a
CO266	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a
CO272	96.9a	100.0a	91.7a	100.0a	100.0a	100.0a
CO325	83.3a	81.1a	93.7a	100.0a	100.0a	87.3b
F2	92.3a	100.0a	90.0a	100.0a	100.0a	92.7ab
Ottawa						
A641	97.5a	96.9a	100.0a	100.0a	100.0a	100.0a
CO265	97.5a	100.0a	90.0ab	100.0a	100.0a	86.7a
CO266	95.8a	97.5a	100.0a	100.0a	100.0a	100.0a
CO272	86.7a	84.4b	95.0a	97.2a	100.0a	97.5a
CO325	65.0b	92.5ab	85.0ab	97.5a	97.5a	85.0a
F2	97.5a	95.0ab	68.9b	90.0a	100.0a	97.5a

[z] Means within columns and sites followed by the same letter are not significantly different at the 0.05 probability level, according to Duncan's multiple range test.

SAS PROC CORR among inoculation techniques intended to measure the same resistance mechanisms.

RESULTS AND DISCUSSION

Data were analyzed separately for each location and year because of significant location by year interactions for disease incidence and disease severity.

There were significant differences ($P < 0.05$) in disease incidence among inbreds and among inoculation methods in all four environments, and highly significant ($P < 0.01$) interactions of inbreds with inoculation methods in three of the four environments (Table 1). In 1992, disease incidence ranged from 9 to 100% (Table 2). In 1993, disease incidence was higher in both environments, with incidence values ranging from 65 to 100% (Table 3). Inbred CO266 was consistently among those with the highest disease incidence in all four environments. Inbreds CO272 and CO325 exhibited low disease incidence in 1992 at Ste-Anne-de-Bellevue and Ottawa. These lines may have mechanisms that inhibit the initial onset of infection.

Variation in disease severity (Table 1) was highly significant ($P < 0.01$) for inbreds, inoculation methods, and interactions between inbreds and inoculation methods, in all four environments. Symptoms were least severe on inbred CO325, while CO266 and CO265 were among the most severely infected lines over the four environments (Tables 4 and 5).

In both locations, disease severity was higher in 1993 than in 1992. This was probably due to differences in weather conditions between the 2 years. Rainfall was above normal in both years. July and August were wetter and cooler in 1992 than in 1993. Precipitation in September and October was higher in 1993 than in 1992. September and October air temperatures were similar in the four environments, except that it was relatively warm in Ottawa in September 1992. The high disease incidence and disease severity in 1993 relative to 1992 may be explained by warm moist conditions during and shortly after the inoculation period. Tuite et al. (14) reported that mean temperatures of 21°C during August are very conducive to outbreaks of ear rot. The high rainfall during September and October 1993 may also have favored disease development. Koehler (3) postulated that high moisture in these months may facilitate infection by increasing inoculum production and by washing spores between the husks and ears when the ear tip is exposed. He also suggested that high moisture during October slows the natural drying process, making ears more prone to colonization for longer periods.

Table 6 shows rank correlation coefficients among four inoculation techniques that we expected would measure the same resistance mechanisms. For disease severity, significant correlations were obtained between the kernel-stab and wound-spray inoculation techniques and between the kernel-stab and pipe cleaner inoculation techniques in three of the four environments.

Among these inoculation techniques, the wound-spray and the kernel-stab consistently identified CO325 and F2 as the most resistant inbreds. However, the wound-spray technique did not consistently identify inbreds that were intermediate or susceptible in their reaction.

With silk-channel inoculation, inbreds differed in resistance in all four environments. Inbred CO272 showed high resis-

Table 4. Mean disease severity (and rankings) of six inbred lines of maize inoculated with *Fusarium graminearum* using six inoculation techniques at Macdonald Campus (Ste-Anne-de-Bellevue) and Central Experimental Farm (Ottawa) in 1992

Inbred	Silk-channel	Ear-tip flooding	Wound-spray	Kernel-stab	Pipe cleaner	Cob-tip
Ste-Anne-de-Bellevue						
A641	3.7ab[z] (3)	2.9bc (4)	4.5ab (4)	4.2ab (3)	5.9ab (3)	3.8ab (5)
CO265	4.8a (6)	3.3b (5)	5.0a (5)	5.7a (6)	6.6a (4)	3.4b (4)
CO266	4.7a (5)	4.5a (6)	5.5a (6)	5.2a (5)	5.9ab (3)	4.6a (6)
CO272	4.3ab (4)	2.5bcd (3)	3.8bc (3)	5.0a (4)	5.0b (2)	2.4c (3)
CO325	2.3c (1)	1.6d (1)	3.0cd (2)	3.3bc (2)	3.6c (1)	1.8c (1)
F2	3.2bc (2)	2.1cd (2)	2.1d (1)	2.8c (1)	3.6c (1)	2.3c (2)
Ottawa						
A641	3.9bc (3)	2.9bc (4)	3.7b (5)	3.9b (3)	5.8a (3)	3.5bc (3)
CO265	5.0ab (5)	3.5b (5)	3.0a (4)	4.6a (4)	6.0a (5)	4.0b (5)
CO266	6.0a (6)	4.8a (6)	6.1a (6)	6.0a (6)	6.7a (6)	5.2a (6)
CO272	2.9cd (2)	2.8cd (3)	2.3b (1)	5.9a (5)	5.9a (4)	2.5cd (2)
CO325	2.7d (1)	1.6d (1)	2.7b (2)	3.1b (1)	3.8b (1)	2.0d (1)
F2	4.9b (4)	2.4cd (2)	2.8b (3)	3.2b (2)	4.3b (2)	3.4cb (4)

[z] Values are mean severity based on a 1 to 7 scale (1 = no symptoms, 7 = 76 to 100% of each ear infected). Means within columns and sites followed by the same letter are not significantly different at the 0.05 probability level, according to Duncan's multiple range test.

Table 5. Mean disease severity (and rankings) of six inbred lines of maize inoculated with *Fusarium graminearum* using six inoculation techniques at Macdonald Campus (Ste-Anne-de-Bellevue) and Central Experimental Farm (Ottawa) in 1993

Inbred	Silk-channel	Ear-tip flooding	Wound-spray	Kernel-stab	Pipe cleaner	Cob-tip
Ste-Anne-de-Bellevue						
A641	4.1ab[z] (2)	4.3b (2)	4.6b (3)	4.8b (2)	5.8a (4)	3.4b (2)
CO265	4.4ab (3)	4.3b (2)	5.4ab (5)	5.5ab (4)	5.7a (3)	3.7b (4)
CO266	4.5ab (4)	4.2b (1)	5.8a (6)	5.9ab (5)	6.4a (5)	5.9a (5)
CO272	4.8b (5)	6.0a (5)	5.2ab (4)	6.5a (6)	6.5a (6)	3.8b (3)
CO325	3.4b (1)	4.6ab (3)	3.4c (2)	3.6c (1)	4.3b (2)	2.4c (1)
F2	5.3a (6)	5.9a (4)	2.1d (1)	3.6c (1)	3.8b (1)	2.4c (1)
Ottawa						
A641	4.7ab (4)	4.5a (1)	4.8a (3)	5.3a (3)	6.2b (6)	3.5b (5)
CO265	4.2abc (3)	4.6a (2)	4.9a (4)	5.6a (4)	5.7cd (4)	2.0cd (2)
CO266	5.2a (6)	5.5a (5)	4.9a (5)	5.7a (5)	6.0a (5)	4.8a (6)
CO272	3.3c (2)	4.5a (1)	5.1a (6)	5.8a (6)	5.5c (3)	2.6c (4)
CO325	2.8c (1)	4.7a (3)	2.6b (2)	3.4b (1)	3.6b (1)	1.8d (1)
F2	5.3a (5)	5.0a (4)	2.3b (1)	3.6b (2)	4.0cd (2)	2.5cd (3)

[z] Values are mean severity based on a 1 to 7 scale (1 = no symptoms, 7 = 76 to 100% of each ear infected). Means within columns and sites followed by the same letter are not significantly different at the 0.05 probability level, according to Duncan's multiple range test.

Table 6. Rank correlations of disease severity at Ste-Anne-de-Bellevue and Ottawa in 1992 and 1993

	Year	Location	Wound-spray	Kernel-stab	Pipe cleaner
Kernel-stab	1992	Ste-Anne-de-Bellevue	0.89*[z]		
		Ottawa	0.37		
	1993	Ste-Anne-de-Bellevue	0.83*		
		Ottawa	1.00**		
Pipe cleaner	1992	Ste-Anne-de-Bellevue	0.89*	0.87*	
		Ottawa	0.54	0.94**	
	1993	Ste-Anne-de-Bellevue	0.71	0.94**	
		Ottawa	0.43	0.43	
Cob-tip	1992	Ste-Anne-de-Bellevue			0.83*
		Ottawa			0.83*
	1993	Ste-Anne-de-Bellevue			0.89*
		Ottawa			0.77

[z] *,** Correlation coefficients significant at 0.05 and 0.01 probability levels, respectively.

tance at Ottawa but was susceptible at Ste-Anne-de-Bellevue in both years (Tables 4 and 5). Although the silk-channel technique was not consistent in ranking inbreds that were susceptible or intermediate in their reaction, it did identify inbreds CO325 and CO272 as the most resistant.

The ear-tip and wound-spray inoculation techniques were not consistent in how they ranked the inbreds in the four environments. These techniques do not seem to be effective in identifying differences in resistance to ear rot.

The kernel-stab and pipe cleaner inoculation techniques produced relatively severe disease in all four environments (Table 4 and 5). These inoculation techniques produced highly localized infections, probably because inoculum was deposited at a single point. The size of lesions should depend only on kernel resistance and not on passive movement of inoculum as in the ear-tip inoculation method. The high level of ear rots obtained with these two inoculation techniques may be due to the circumvention of a normal physical barrier that serves to reduce the amount of inoculum reaching the kernels. Both techniques were able to identify one inbred (CO325) possessing a high level of kernel resistance. The pipe cleaner technique, however, incited very severe infection in most of the inbreds in all four environments. Better separation of resistant and susceptible inbreds was obtained with the kernel-stab technique, and this method may be the most efficient for identifying true differences in kernel resistance to ear rot.

The kernel-stab technique requires very small amounts of inoculum, is easy to implement, causes minimal damage to kernels, and mimics damage caused by insects or birds. The relative inefficiency of the pipe cleaner technique may have been due to comparatively larger amounts of inoculum and to severe damage caused by piercing the ear. The latter may have resulted in more intense wound-healing reactions, leading to other possible adverse effects that may have affected disease development.

The cob-tip technique was used to evaluate resistance to infection in the cob tissue. It resulted in very low disease severity in both years. It failed to clearly differentiate among inbreds, even in 1993 when the weather conditions were very favorable for infection.

Reid et al. (5,8) reported that the inbred CO272 inoculated through the silk channel had a high level of silk resistance. In the present study, CO272 appeared resistant at Ottawa but susceptible at Ste-Anne-de-Bellevue. This is consistent with the observation of Reid et al. (6) that resistance in silk tissue can be environmentally sensitive. Although CO272 was susceptible at Ste-Anne-de-Bellevue, ears inoculated with the silk-channel injection technique had less disease than ears inoculated by other methods (Tables 2 and 3). Resistance in the silk may have slowed infection enough to limit the development of symptoms. The results of this study support previous reports that CO272 resists infection via the silk. However, this inbred does not appear to have any resistance mechanism to slow the spread of the fungus from kernel to kernel.

Inbred CO325 exhibited a high level of resistance when inoculated by silk-channel injection in all four environments (Tables 2 and 3). Reid et al. (9) also reported that CO325 was more resistant than other inbreds when inoculated in the silk channel. This inbred also had the least disease from wound-spray, kernel-stab, and pipe cleaner inoculation (Tables 4 and 5). The resistance mechanism(s) of CO325 may be located in the kernel and/or cob tissue (7), and perhaps in the silk tissue. An inbred with both silk and kernel resistance would be very useful in maize breeding.

In conclusion, genetic differences for resistance to infection caused by *F. graminearum* in ears of maize were observed among maize inbreds. All inoculation techniques except the ear-tip flooding method identified CO325 as the most resistant inbred. This study also supports the results of previous studies that CO272 possesses silk resistance, and that this resistance seems to be environmentally sensitive. In this study, silk-channel inoculation was effective in measuring silk resistance, while the kernel-stab technique clearly and consistently differentiated among inbreds possessing kernel resistance. To reduce infection via the silk and silk-channel and/or to keep disease from spreading on developing kernels, breeders may need to incorporate both silk and kernel resistance mechanisms into maize hybrids.

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