A Corn Seedling Assay for Resistance to *Fusarium moniliforme*

CHARLES W. BACON, DOROTHY M. HINTON, and MICHAEL D. RICHARDSON, Toxicology and Mycotoxins Research Unit, USDA-ARS, Russell Research Center, P.O. Box 5677, Athens, GA 30613

ABSTRACT

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Fusarium moniliforme, mating population A, causes various disease symptoms and produces several mycotoxins on corn. This fungus is one of the causal agents of seedling blight of corn. A test was designed to determine the pathogenicity of *F. moniliforme* isolates and to test for resistance in corn against this pathogen. The test is conducted conveniently under laboratory conditions. After a 21-day period, either a fungal isolate or a corn cultivar can be tested for pathogenicity or seedling resistance, respectively. The results, obtained from a limited number of corn cultivars, established that there is resistance to the fungus at the seedling stage. Inbreds were more resistant than parental types used in the initial crosses. The data did not suggest a tendency towards physiological specialization within a selected group of isolates from mating population A.

Additional keywords: corn disease, *Gibberella fujikuroi,* mycotoxin, seedborne, toxic corn, *Zea mays*

Seedling blight of corn (*Zea mays* L.) is associated with the systemic infection of kernels and plants with *Fusarium moniliforme* J. Sheld. (*Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura) (6,27,28) and the presence of this fungus in plant residues in the soil (20). The fungus is found on corn throughout the world and is also associated with ear, root, and stalk rots (12). The distribution appears to be host species specific, as it is *F. moniliforme* of mating population A that is primarily found on corn (14).

Establishing *F. moniliforme* as a cause of seedling blight of corn was controversial (12). However, early reports (28,29) that this fungus caused seedling blight of corn have been substantiated by several recent studies (7,24,26,27). This controversy existed because of the complexity of the relationship, including differential reactions of corn cultivars (8,9), variation among *F. moniliforme* isolates (10,13), and environmental influences on infection and disease reactions (21), none of which were considered during the early studies (5,13,28,29). Establishment of a distinct mating group or race pathogenic on corn is very recent (4,14), although the very broad question of physiological specialization within this population, i.e., host specific races, has not yet been attempted.

In addition to plant diseases, *F. moniliforme* has been shown to produce several mycotoxins (16). Corn infected with this fungus has been correlated with human esophageal cancer (23,33), a pulmonary edema syndrome in swine (2), cancer-promoting activity in rats (15), and a variety of other animal toxicities (1,16,31). Thus, the need to control the fungus is important, not only because of the large economic losses caused by stand reductions and poor kernel quality, but also because of the potential threat to human and animal health.

Recent research is directed toward developing corn cultivars resistant to diseases caused by *Fusarium* species (4,8,9, 11,18,25,32). In reference to *F. moniliforme* mating population A, very little progress has been made that reflects the still conflicting information on physiological specialization, seedling resistance, infection, and other aspects of this association. For example, the fungus causes several corn diseases, but it is not known if all isolates can cause one or all of these diseases. Progress can be made toward developing resistance to certain phases of *F. moniliforme* infection of corn only when there are consistent tests to measure corn resistance and fungal variation. We report here a seedling test which incorporates several isolates of *F. moniliforme* that serve the purposes of either screening for seedling resistance in corn cultivars or testing pathogenicity of *F. moniliforme* isolates. Information obtained with this test may allow for possible analysis of physiological specialization within the species.

MATERIALS AND METHODS

Fungus and plant materials. Of the 16 *F. moniliforme* isolates used in this research (Table 1), 15 were obtained from field or sweet corn. One isolate, ATCC 14164, originated from Bakanae-diseased rice (16) and was included because it served as a convenient marker for infection, as all corn seedlings infected with it developed the Bakanae disease. Five isolates (Table 2) served as the basis for this test and also served as tests for variation in aggressiveness among isolates on five corn cultivars. All fungi isolated by the authors were obtained from surface sterilized corn kernels and therefore represented systemic infection of kernels.

The fungi were maintained on silica gel (22) and stored at 5 C. The identity of several isolates was confirmed by P. Nelson, Pennsylvania State University, University Park. Inoculum was prepared for each isolate by incubating this fungus on potato-dextrose agar (PDA) under a 12 hr light/dark cycle at approximately 25–27 C for 15–21 days. Inocula consisted of both conidia and mycelium obtained by flooding a petri plate with 10 ml of sterile distilled water. The conidial concentrations averaged 10^6–10^9 conidia per milliliter for most isolates, but this concentration is not critical (especially since mycelia also contributed to infection); and lower concentrations, e.g., 10^3, gave identical results.

The six cultivars of corn used in this study consisted of inbred and hybrid lines of field and sweet corn. The commercial cultivars included Truckers Favorite, a field corn, and Florida Staysweet, a sweet corn. The inbred and hybrid lines of field corn included the genetic cultivars PR and K61, and K61 × PR and PR × K61, respectively.

Corn sterilization. To completely remove both surface and internally borne fungi from all kernels, the procedure of Daniels (3) was modified. Kernels were placed in sterile plastic cups, covered, and agitated for 3 min in distilled water. The water was removed, and 100% commercial bleach (5.25% sodium hypochlorite) was added to cover all kernels. The kernels were then agitated in bleach for 10 min on a reciprocal shaker. The bleach was removed, and kernels were rinsed twice in sterile water. Kernels then were covered with sterile distilled water and allowed to imbibe for 4 hr at 25 C. They were rinsed twice more in sterile distilled water and covered with sterile distilled water. The plastic cup containing the kernels was placed in a 60 C water bath for 5 min for the cultivars with large kernels, i.e., field corn, and 3 min for the cultivars with small kernels, i.e., sweet corn. After the heat treatment, the water was removed and the kernels were immediately transferred to a covered petri dish, where they were covered with

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sterile distilled water at room temperature.

Several commercial corn cultivars used in this test were treated with a commercially applied fungicide and/or insecticide. These substances were removed prior to the sterilization procedure indicated above by washing kernels in tap water on a rotary shaker for 5 min, then shaking the kernels in 70% ethyl alcohol for 3 min, followed by five rinses in tap water and a 3-min wash in bleach. These kernels were then washed in five changes of distilled water and taken through the procedure above, beginning with agitating the kernel in bleach for 10 min. The removal of pesticides caused an average of 30% reduction in germination. The sterilization procedure for pesticide-treated seeds of field and sweet corn cultivars with small kernels always resulted in a larger reduction in germination than the other cultivars. All kernels treated with pesticide were allowed to sit one additional day in covered petri dishes at 25–27 C before inoculation.

Seedling test. The method used to determine cultivar resistance and pathogenicity of each isolate was a modification of that of Molot and Simone (19). All inoculations and plantings were performed under aseptic conditions. Sterilized kernels (100–150) of each cultivar were placed in a 100 × 15 mm petri dish and inoculated with 5 ml of each fungal inoculum. Inoculated kernels were incubated in the dark for 2 days at 25 C and then for 2 days (no longer than 4 total) at 2–6 C. There was approximately 70–80% germination of all cultivars. Early coleorhizal protrusion was the germination criterion for most cultivars, although for some slow-germinating cultivars, enlargement of the embryo axis was the criterion.

Ten to 15 germinated kernels were carefully placed on a bed of autoclaved perlite in either a 10-cm low-style clay pot or a 32-cell plastic planting flat (each cell 10.2 × 10.2 × 10.2 cm) and covered with 2.5–3 cm of additional autoclaved perlite. The pot and planting flat were placed in a water tray. Pots were then watered lightly with sterile distilled water from above, and subsequent waterings were made from below by adding nonsterile distilled water to a height of 2.5 cm in the water tray. Additional water was added when the tray was dry. Pots were placed in a plant growth room under 16 hr of light (an average of 256 $\mu E \cdot m^{-2} \cdot s^{-1}$, LI-6250, Li-Cor, Inc., Lincoln, NE) at 29–32 C and 8 hr darkness at 21–26 C. Percent emergence was recorded after 21–25 days; only vigorous and typical seedlings were counted (Fig. 1). Each cultivar and inbred line was evaluated for growth after separate inoculations with at least five of the fungal isolates.

Experimental design in the growth room was a split plot with three to four replicates. A replicate consisted of a pot of 10–15 germlings. Cultivars were main plots and fungal isolates were subplots. All experiments were repeated at least twice. Results from all experiments were combined, and the effects of a fungus on all corn cultivars were analyzed by ANOVA (Duncan's multiple range test after analysis). The effects of *F. moniliforme* on emergence of a cultivar were expressed as percent emergence of sterilized but uninoculated corn kernels and analyzed by an unpaired nonparametric test (Mann-Whitney two sample test). All statistical analyses were conducted with an INSTAT software program (GraphPad Intuitive Software, Inc., San Diego, CA).

RESULTS

Seed treatments. Seedling blight was obtained under the controlled environmental conditions of the growth room. Results of the seed treatments used to achieve fungus-free kernels showed that neither percent emergence nor seedling vigor was affected when compared to nontreated kernels (Table 2). Further, fungi did not grow out of treated kernels, even after an extended period on PDA, indicating that the heat treatment used during the sterilization procedure produced fungus-free seed and that the resulting seedling blight was caused by inoculation. Surface sterilized leaf tissue cultured from inoculated corn seedlings yielded *F. moniliforme* when plated out on PDA, indicating that they were systemically infected. Identities of the *F. moniliforme* isolates removed from infected plants cannot be identified with certainty as the ones initially placed there, although one isolate, ATCC 14164, was an ideal marker because it caused the Bakanae disease in corn. ATCC 14164 was recovered from surface sterilized tissue of plants inoculated with this isolate. This isolate was further characterized by its lack of abundant aerial mycelium, wet appearance on PDA, and striking scarlet-purple color on reverse.

Examination of germlings and seedlings indicated that death occurred either during preemergence or a day or two following emergence. Postemergence death usually occurred within 2 wk after emergence and was characterized as either stunted or faciated growth, or a yellowing and browning of leaves (Fig. 1). The disease progressed to a wilting and liquefaction of young stems and leaves, resulting in seedling collapse and finally disintegration. The only exception to these disease symptoms was that pro-

Table 1. Original host, geographical origin, and source of isolates used to inoculate corn cultivars during the seedling test

Isolate	Host	Geographical origin	Source
RRC PAT	Sweet corn	Italy	1[z]
RRC 38	Sweet corn	Italy	1
RRC 374	Field corn	Georgia	1
RRC 388	Field corn	Georgia	1
RRC 408	Field corn	Illinois	1
RRC 415	Field corn	Mississippi	1
RRC 417	Sweet corn	Mississippi	1
RRC 419	Sweet corn	Georgia	1
A149	Field corn	California	2
A498	Field corn	Kansas	2
MRC 826	Field corn	South Africa	3
MRC 1069	Field corn	South Africa	3
ATCC 14164	Rice	Japan	4
F-84	Field corn	Canada	5
37-38	Field corn	California	6
SRRC 1085	Field corn	Louisiana	7

[z] 1 = C. W. Bacon; 2 = J. F. Leslie, Kansas State University, Manhattan; 3 = W. F. O. Marasas, Medical Research Council, Tygerberg, South Africa; 4 = American Type Culture Collection; 5 = J. M. Farber, Health & Welfare Canada, Ottawa; 6 = L. Bjeldanes, University of California, Berkeley; and 7 = M. A. Klich, USDA/ARS, New Orleans, LA.

Fig. 1. Corn seedlings after a typical bioassay. Seedling on right is control, infected seedling on left is an example of a few which emerged but eventually died.

duced by the rice isolate, ATCC 14164, in which infected seedlings were three to four times normal length (Bakanae disease) and died after lodging. There was no correlation between the ability of an *F. moniliforme* isolate to consistently cause seedling blight at the postemerged or preemerged growth stage of any corn cultivar used.

Variation in aggressiveness. The initial series of experiments was designed to evaluate the effectiveness of the procedure in distinguishing the severity of disease reactions produced by different isolates of *F. moniliforme* with seedling emergence of corn cultivars, inbreds, and hybrids. Results showed that there were differences among and within hybrids, inbreds, and cultivars for resistances to seedling blight by *F. moniliforme* isolates (Table 2). The percent emergence of the inbred PR was significantly reduced ($P < 0.005$) by all isolates of *F. moniliforme*. High resistance to seedling blight was shown by the hybrids K61 × PR and PR × K61.

Considerable variation in percent emergence was observed among the five isolates used to test virulence (Table 2). None of the five fungi significantly reduced percent emergence of all six corn types. All fungi produced an 11–77% reduction in emergence after inoculation of all corn cultivars with the exception of the hybrid PR × K61, which was resistant to all five isolates of *F. moniliforme*. One inbred parent used in the crosses of these hybrids, K61, showed moderate susceptibility to all fungi, although more than that of either of the two hybrid crosses. The other inbred, PR, was highly susceptible to *F. moniliforme*. The two field corn hybrids were the most vigorous of the six cultivars, as evidenced by the high percent emergence observed in uninoculated seed (controls). In addition to isolate MRC 826, which caused a significant decrease ($P < 0.005$) in emergence of the hybrid K61 × PR (Table 2), other isolates of *F. moniliforme* also decreased the emergence of hybrids (Table 3). Three isolates, RRC 374, RRC 388, and A149, caused a decrease in the percent emergence of both hybrids (Table 3). Isolate RRC 388 produced a significant decrease in emergence of only one hybrid, K61 × PR. Isolate RRC 374 is distinguished from the other isolates in that it not only caused the greatest percent emergence decrease in the hybrids, but also caused similar decreases in the emergence of all corn cultivars tested (*data not shown*). There were no significant isolate × cultivar effects, suggesting that no physiological specialization occurred within the isolates and cultivars tested (Table 4).

DISCUSSION

Since only germinated seeds were used in this test, kernel rot and seedling vigor were not measured. Results obtained from this seedling test measured the potential of an *F. moniliforme* isolate to cause the seedling disease and of a corn cultivar to resist it. Utilization of several isolates was designed to test for variation in seedling disease expression within each corn genotype by the fungus. The experimental procedures, including storage of the fungus on silica gel, and uniform plant growth room conditions to minimize environmental influences, preclude the association of experimental manipulation with variable disease reactions. This seedling test can be used to screen large numbers of cultivars and provide some differentiation in disease expression within a 3-wk period. In all cases, since fungus-free kernels were used, data reflect only the fungus inoculated, thus distinguishing this test from earlier ones (5,8,13,27,30). Djakamihadja et al (5) reported on a procedure which was designed to evaluate corn cultivars; however, they used one isolate of *F. moniliforme* and did not use surface nor internal kernel disinfectant procedures to control or remove natural kernel-borne fungi. Such omissions are entirely unsuitable as part of a protocol designed to measure the pathogenicity of specific *F. moniliforme* isolates, since most corn kernels may be 90% infected with this fungus (3,6,12,21,24,27,29). Further, since this test (5) used only one isolate, no real information on the variation among isolates could be obtained.

We have no information as to whether there are correlations of seedling infection with other aspects of this disease complex, i.e., ear and stalk rot. However, such correlations have been suggested for resistance between corn ear and stalk rot induced by other *Fusarium* spp., e.g., *F. graminearum* and *F. culmorum* (18).

Since germinating kernels were subjected to a cold treatment, this test could be interpreted as predisposing plants to *F. moniliforme* infection. However, seed quality testing of corn routinely employs a cold test period (17), and preliminary studies conducted without the cold treatment produced broad variations in seedling responses within a cultivar to an isolate, indicating that cold treatment was a requirement for consistency in this seedling infection test.

Corn seedling blight produced by the procedure used in this paper was identical to the descriptions published by Leonian (13) and Voorhees (30), although they inoculated wounds made at the stem bases of emerged seedlings. In

Table 3. Average percentage of plants of two cultivars that died within 21 days or failed to emerge after inoculation with one of 12 different isolates of *Fusarium moniliforme*

	Dead (%)	
Isolate	K61 × PR	PR × K61
RRC 374	90 a[z]	80 a
RRC 388	20 b	50 b
RRC 408	0	0
RRC 417	10 b	10 c
RRC 419	10 b	10 c
A149	50 c	70 c
A498	20 b	20 c
MRC 1069	20 b	30 c
F-84	0	0
37-38	0	10 c
RRC 1085	0	0

[z] Average percentage of plants that died within 21 days or failed to emerge; each is an average of two experiments with two replications (15 seedlings per replication). Within columns (cultivars), means followed by a common letter do not differ significantly ($P < 0.005$). Uninoculated controls averaged 0–5% death during this experiment.

Table 2. Average percent emergence[x] of corn cultivars inoculated with isolates of *Fusarium moniliforme*

	Emergence (%)					
Isolate	Trucker's Favorite	K61	PR	Florida Staysweet	K61 × PR	PR × K61
RRC PAT	70	56**	22**	33	94	96
RRC 38	52**[y]	61*	21**	18*	89*	96
MRC 826	66*	76	19**	17*	88**	93
RRC 415	70	74*	28**	30	94	91
ATCC 14164	59**	87	35**	47	94	98
None (control)[z]	78(85)	90(92)	81(89)	53(60)	99(98)	100(100)

[x] With the exception of cultivar PR × K61, which is replicated twice, each figure is the average of three experiments, and each treatment was replicated at least three times per experiment.
[y] Asterisks denote significant differences within a column from the corresponding uninoculated controls of a cultivar according to the Mann-Whitney's *t* test (*, $P < 0.05$; **, $P < 0.005$).
[z] Numbers in parentheses indicate the percent emergence of seed not heated and not subjected to the cold treatment.

Table 4. Combined analysis of variance *F* tests[z] of differential cultivar experiments

		Seedling symptoms
Source of variation	df	Emergence
Experiment (E)	1	NS
Reps/E	4	...
Isolate (I)	16	**
E × I	16	NS
Cultivar (C)	5	**
E × C	5	NS
I × C	80	NS
E × I × C	80	NS

[z] ** = Significant at $P < 0.01$; NS = not significant.

our study, the entry of the fungus into corn seedlings was probably through the rupture in the pericarp produced by emerging roots during germination. However, this study is distinguished from others (8,13,30) in that it used heat-treated seed (3) to preclude effects from seedborne *F. moniliforme*. Although reports exist on the effects of temperature on expression of seedling disease (12, 13,28), the test as proposed in this paper utilized conditions ideal for cultivation of corn under light room conditions, which may not extrapolate to field conditions.

The results of this study suggest that isolates of *F. moniliforme* from corn consist of a series of aggressive biotypes; therefore, the effect of each on a cultivar varied. The differential aggressiveness of *F. moniliforme* isolates to several corn cultivars demonstrated considerable variability in pathogenicity of the fungus to corn seedlings, which if subjected to additional cultivars and isolates, might indicate physiological specialization or races. With the exception of one, all isolates were from corn and represent only one biological species, *G. fujikuroi* mating population A (10). Only one isolate had the potential to significantly affect the emergence of all corn seedlings used in this study. This suggests that only a few isolates are aggressive. Nevertheless, the lack of an apparent isolate × cultivar interaction indicates that the isolates differ only in aggressiveness and are independent of a corn cultivar, suggesting that physiological specialization is probably not a feature of *F. moniliforme*. Variability of reactions of the seedling disease within a cultivar was observed, and we considered that it may be due primarily to genetic diversity (heterogosity) within an inbred cultivar, although this may not apply to hybrids.

The apparent resistance of certain cultivars to fungal isolates might make it difficult to establish seedling disease ratings within cultivars. Resistance and susceptibility may not be qualitatively expressed in the seedling aspect of the disease complex. However, studies with a large number of cultivars and isolates may define specific corn cultivar–fungal isolate interactions to indicate quantitative differences that can be separated.

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