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Abstract

Species of *Aspergillus* in section Nigri are commonly associated with maize kernels and some strains in this group have the capacity for producing fumonisin mycotoxins, but there is little information about the damage they cause to maize ears, fumonisin (FB) contamination of grain, or their effects on maize seed germination and seedling health. We compared fumonisin-producing and non-producing strains of *A. niger, A. welwitschiae, A. phoenicis, A. tubingensis*, and *A. carbonarius* from the U.S. and Italy in laboratory and field studies to assess their ability to cause maize ear rot, to contribute to FB contamination, and to affect seed germination and seedling growth. In laboratory experiments, some strains of each *Aspergillus* species reduced germination or seedling growth, but there was high variability among strains within species. There were no consistent differences between fumonisin-producing and non-producing strains. In field studies in Iowa and Illinois, strains were variable in their ability to cause ear rot symptoms, but this was independent of the ability of the *Aspergillus* strains to produce fumonisins. FB contamination of grain was not consistently increased by inoculation with *Aspergillus* strains compared to the control, and was much higher in *F. verticillioides*-inoculated treatments than in *Aspergillus*-inoculated treatments. The ratio of FB_2 to FB_1 was altered by inoculation by some *Aspergillus* strains, indicating that FB_2 production by *Aspergillus* strains was occurring in the field. These results demonstrate the pathogenic capabilities of strains of *Aspergillus* in section Nigri but suggest that their effects on maize ears and seedlings are not

related to their ability to produce fumonisins, and that fumonisin contamination of grain by *Aspergillus* is not a major issue in comparison to that caused by *Fusarium* spp.

Introduction

Several species of *Aspergillus* are common among seedborne fungi in maize. The genus *Aspergillus* contains over 200 species, including those in section Nigri, comprising the species which produce black conidia (15). Black *Aspergillus* species are primarily associated with diseases in maize, peanut, grape, and onion (14). Many species of *Aspergillus*, including *Aspergillus* section Nigri, cause ear rot on maize and can also cause damage in storage if grain moisture content is sufficiently high (8). In the United States, they are not typically a major concern as seedling pathogens, although seedborne infection of up to 62% has been reported on maize, and occasional serious outbreaks of *Aspergillus* ear rot have occurred (10). A survey of untreated commercial seed lots in the United States found an overall contamination of 2.8% (16).

It was recently discovered that some *Aspergillus* species contain a cluster of genes similar to those controlling fumonisin production in *Fusarium*. However, *Aspergillus* species do not possess orthologues to all genes in the *Fusarium* fumonisin cluster; in particular, the lack of an orthologue to the *Fusarium fum2* gene results in a lack of fumonisin B_1 production (22). Instead, the primary fumonisin produced in *Aspergillus* is fumonisin B_2 (19), biosynthesis of which requires the *fum8* gene, found in some strains of *Aspergillus*. In-vitro production of fumonisin B_2 in *Aspergillus niger* was first confirmed in 2007 (6). Unlike *Fusarium verticillioides*, which produces fumonisin B_1, fumonisin B_2, and fumonisin B_3, the *A. niger* strains were initially only observed to produce fumonisin B_2 (6). Some strains of Aspergillus isolated from raisins were later reported to produce fumonisin B_1 and fumonisin B_3 as well (23), though this finding has

been disputed (11, 12, 24). The fumonisin B_2 produced by *A. niger* was compared to that produced by *Fusarium* spp., and no differences could be detected in the chemical structures, even though the genes for the synthases differ somewhat (9). Two additional fumonisins are also produced by *A. niger*: fumonisin B_4, which is also present in small amounts in *Fusarium* species (13), and fumonisin B_6, which has the same elemental composition as fumonisin B_1, but differs in structure (9).

It is unclear how fumonisin production benefits fumonisin-producing fungi. Preventing fumonisin B_2 production by disruption of the *fum8* gene in *A. niger* does not affect vegetative growth, sensitivity to temperature, or sensitivity to UV light, suggesting it does not play a role in normal growth of the fungus (19).

In *Fusarium*, the role of fumonisins in pathogenicity is controversial. Fumonisins are not necessary for pathogenicity, since it has been shown that non-producing strains can infect maize (4). However, in a study by Desjardins et al. (5), nearly all highly virulent strains also produced high levels of fumonisin B_1, while many less virulent strains did not. Additionally, disease symptoms and stunting can be induced in maize seedlings by fumonisins alone, though the seedlings eventually outgrew the effects at the lower concentrations tested (2). Maize seedlings have some ability to detoxify low levels of fumonisin, but higher levels have detrimental effects, possibly by inducing premature senescence through their interference with sphingolipid metabolism (2).

It is possible that fumonisins contribute to virulence or pathogenicity in *Aspergillus* species, but this has not yet been experimentally tested. This study is intended to begin an examination of this question by comparing the virulence of a range of fumonisin-producing and non-producing strains of *Aspergillus* section Nigri.

We designed our study to compare a wide selection of strains from *Aspergillus* section Nigri, including both fumonisin-producing and non-producing strains. Warm germination and cold tests were used to evaluate seed germination under ideal and cold stress conditions, and rolled paper towel assays were used to measure effects of inoculation on seedling growth. Field studies were conducted in Iowa and Illinois to assess ear rot development and the effects of inoculation on fumonisin contamination of grain.

Materials and Methods

Germination and seedling disease assays

Three types of assays were conducted to assess the effects of *Aspergillus* strains on maize seeds and seedlings: a warm germination assay, a cold germination assay (AOSA Rules), and a rolled-towel assay (Ellis et al.,). Twenty-six strains of black *Aspergillus* were obtained from maize kernel samples from Iowa, Idaho, Illinois, and Italy, and were selected to provide a broad representation of black *Aspergillus* species occurring on maize, including strains with and without fumonisin B_2 production, as determined in a previous study (21) (Tables 1-3). The strains had initially been placed in *Aspergillus* section *Nigri* based on morphological characteristics, and were further identified to species based on β-tubulin and calmodulin gene sequences (21).

Each *Aspergillus* strain was grown on potato dextrose agar under fluorescent light at 20-25°C for 7 days. The spores were rinsed off the plates, gently dislodging them from the colonies, and suspended in sterile distilled water. The spore concentrations of the suspensions were determined using a hemaocytometer, and they were diluted to a concentration of 10^6 spores/ml. For all spore suspensions, Tween 80 was added as a surfactant at a rate of 0.125 ml to 0.5 ml per liter of water. In the second run of the laboratory experiments, one strain (ITEM 15167) produced insufficient spores to reach the necessary volume of 10^6 spores/ml, so 5 x 10^5 was used instead.

Two maize hybrids were used in the two repetitions of the warm germination, cold, and rolled paper towel assays. Hybrid A was an experimental hybrid obtained from Syngenta, and hybrid B was Syngenta hybrid 85v88-300GT (Syngenta Seeds, Northfield, MN). The maize kernels were first surface sterilized by submerging for 5 minutes in 0.6% sodium hypochlorite,

followed by 3 minutes in 75% ethanol, and finally 2 minutes in sterile water. The kernels were then added to flasks containing the spore suspensions (or the control of Tween water), and placed on a shaker for 12 hours at 130 rpm for the first run, and 80rpm for the second, both at room temperature (20-25°C). For the first run of the warm germination and cold tests, 820 seeds were inoculated for each treatment, divided between two 250ml flasks with 100ml of suspension in each. For the second run, 850 seeds were placed in a single 500ml flask, with 300ml suspension. After the 12 hours, the suspension was drained off, and the kernels spread on paper towels to dry in a biosafety hood.

After the kernels had dried, they were submitted to the Iowa State University Seed Testing Laboratory for a standard warm germination test and a cold test (1). In the warm germination test, the seeds were grown on moist blotter paper at 25°C, and evaluated after one week for the percentage germination. The cold test approximates stresses of early spring planting by covering seeds with a layer of moist sand-soil mixture, chilling for one week at 10°C, and then warming up to 25°C for an additional week before evaluation of the percentage germination. For both assays, four replicates of 100 seeds each were used for each combination of *Aspergillus* strain and hybrid, including noninoculated control treatments.

Maize seeds of the same two hybrids were used for the rolled paper towel assay. Each replicate consisted of 15 seeds, placed two thirds of the way up on two layers of damp paper towel, and covered with a third towel after inoculation. There were 3 replicates for each treatment. In the first run (hybrid A) the U. S. strains were run as one experiment, and the Italian strains were run as a second, due to logistical considerations. In the second run (hybrid B), all strains were run in the same experiment. To inoculate, 0.1mL of spore suspension (or the control of Tween water) was pipetted over the top of each seed. They were covered with a third damp

towel, loosely rolled vertically, and placed in individual unsealed plastic bags. These bags were stood upright in 5-gallon buckets and covered by placing a clear plastic bag over the top of each bucket to prevent contamination or drying. After 7 days of growth in ambient conditions (florescent lighting, 20-25°C), the seedlings were evaluated for growth.

In the warm germination test, seedlings are scored as normal, abnormal, or dead, and the official germination percentage is equal to the percentage of normal seedlings. In the cold test, germination is evaluated on the basis of percentage emergence. In the rolled paper towel assay, the length of the longest root and shoot was measured on each seedling, at the point of the furthest distance from the seed. The roots and shoots were separated and weighed immediately after unwrapping each replicate, to avoid drying. The seed itself was not weighed, so non-germinated seeds were recorded as 0 for both weights. For all measurements, the values for the fifteen seedlings on each towel were averaged and analyzed as a single observation.

All data were analyzed using ANOVA (SAS version 6.1). Fisher's protected least significant difference (LSD) was used to compare treatments, with the level of significance $P < 0.05$. Data for some strains were missing in each experiment due to inadequate spore production.

Field Experiments

Experiments were conducted in Story Co., Iowa, USA, in 2012 and 2013, and in Peoria Co., Illinois, USA, in 2012. Experimental design was similar in both locations, but specific inoculation methods and fungal strains differed slightly.

Iowa field experiments were conducted in a single location in both years with a single maize hybrid. Trials were planted in mid-April 2012 and mid-June 2013 in a reduced-tillage field where maize was the previous crop. Standard commercial fungicide/insecticide seed treatments were used, but no other fungicides or insecticides were applied. Weed control was accomplished

by two applications of glyphosate during the early growth stages of the crop. Field plots were single rows spaced 75 cm apart by 5.2 m in length, with approximately 35 seeds per row. At silk emergence, five ears per row were arbitrarily selected and tagged for inoculation. Two types of inoculation were carried out, as described by Reid et al. (1996): silk inoculation and wound inoculation. For the silk inoculation, 2 ml of a spore suspension was injected by hypodermic needle into the silk channel of the selected ears, 7 to 10 days after silk emergence, without wounding the kernels or cob. For the wound inoculation, ears were wounded by pressing a pin-bar against the side of the ear, approximately 21 days after silk emergence, followed immediately by injection through the husk of 2 ml of a spore suspension into the center of the wounded area. The two inoculation types were applied in separate, adjacent experiments in both years. Within each inoculation type experiment, there were seven treatments, arranged in a randomized complete block design. Treatments consisted of inoculation with one of five strains of black *Aspergillus*, inoculation with a strain of *Fusarium verticillioides*, or a mock-inoculation with sterile distilled water with Tween. *Aspergillus* strains used in the studies were ITEM 15309, 15335, 15337, 15353, and 15375 (Table 1); the *F. verticillioides* strain was ITEM 3927, a fumonisin-producing strain from Iowa maize. Following inoculation the ears were left uncovered; after the plants matured and dried in the field to approximately 20% kernel moisture content, ears were collected, brought to the laboratory and scored for ear rot severity using a 1-7 scale (Reid et al., 1996), in which 1 = no symptoms, 2 = up to 3% ear rot severity, 3 = 4 to 10%, 4 = 11 to 25%, 5 = 26 to 50%, 6 = 51 to 75%, and 7 = 76 to 100%. After scoring, ears were dried in a forced air grain dryer at 38°C until grain moisture was <13%. Ears were then hand-shelled and the kernels were ground using a Romer mill (Model 2A, Romer Labs, Washington, MO, USA).

The Illinois field experiment was conducted in a single location in Peoria Co., IA, in 2012 with a single maize inbred line, B73. Trials were planted on 16 May 2012 in plots where wheat was the previous crop. Neither commercial fungicide/insecticide seed treatments, nor other fungicides or insecticides were used. Weed control was accomplished by a single spot application of 2,4-D / dicamba during the early growth stage of the crop and cultivation. Field plots were composed of rows spaced 100 cm apart by 20 m in length, with approximately 120 seeds per row. At silk emergence, 30 ears per row were arbitrarily selected and tagged for inoculation. Two types of inoculation were carried out: silk inoculation, as described by Reid et al. (1996) and wound inoculation, as described by Dowell et al. (2002). For the wound inoculation, ears were wounded making a 4 cm incision into the side of the ear, approximately 21 days after silk emergence, followed immediately by insertion through the husk of an 8 cm sterile pipe cleaner, saturated in a spore suspension, into the wounded area. The two inoculation types were applied in separate, adjacent experiments in both years. Within each inoculation type experiment, there were six treatments. Treatments consisted of inoculation with one of five strains of black *Aspergillus*, or a mock-inoculation with sterile distilled water. *Aspergillus* strains used in the studies were ITEM 15309, ITEM 15337, ITEM 15353, and ITEM 15375 (Table 1), and ITEM 15333, a fumonisin-producing strain of *A. niger* from Iowa maize. Following inoculation, methods were followed as described for the Iowa experiments.

Grain from the Iowa and Illinois field experiments was analyzed for fumonisins B_1, B_2, and B_3 by LC-MS/MS at the NCAUR laboratory in Peoria, IL. All grain from each field plot was ground, and 10-g subsamples were extracted with 50 mL of acetonitrile / water (1:1, vol / vol). Maize solvent slurries were allowed to steep for 2 h, with gentle shaking, and then extracts were filtered through a Whatman 2V filter. The analytical method utilized a LC-MS/MS instrument

consisting of a ThermoFisher (Sunnyvale, CA, USA) UltiMate 3000 ultra-high performance liquid chromatography (UPLC) system and an AB-SCIEX (Framingham, MA, USA) QTRAP 3200 mass spectrometer. The mass spectrometer was operated in positive mode utilizing an electrospray ionization (ESI) interface. Injections of 10 µL of analyte were eluted from a Phenomenex (Torrance, CA, U.S.A.) Kinetex XB-C18 2.1 x 50 mm column with a 600 µL/min gradient flow of water / methanol (MeOH). Approximately 10% of the column flow was directed to the ESI interface of the mass spectrometer. UPLC solvents were acidified with 0.3% acetic acid. The gradient program consisted of the following steps: 0 to 1 min, 40% MeOH; 1 to 11 min, 40 to 95% MeOH; 11 to 13 min, 95% MeOH; 13 to 14 min, 95 to 40% MeOH; and 14 to 15 min, 40% MeOH. ESI-MS/MS detection of fumonisins was accomplished by monitoring characteristic fragment ions (FB_1: m/z 352, 528 and FB_2, FB_3: m/z 336, 512) from the m/z 722 (FB_1) and 706 (FB_2, FB_3) $[M+H]^+$ ions of the fumonisins in multiple reaction monitoring mode. Quantitation of fumonisins was done on the basis of the integrated intensity of the m/z 352 and 336 fragments compared with a calibration curve generated from fumonisin standard solutions. FB_2 and FB_3 were distinguished by chromatographic retention time.

Data were analyzed using ANOVA (SAS ver. 9.4 or SAS Enterprise ver. 7.1, PROC GLM). Fisher's protected least significant difference (LSD) was used to compare treatments, with the level of significance $P < 0.05$. Prior to data analysis, ear rot severity scores were transformed to percentages by converting the 1-7 scores to the mid-point of the percentage range of each score. The mean percentage of symptomatic kernels was calculated for the five ears from each plot, and those mean values were subjected to ANOVA. Data were analyzed separately for silk-channel and wound inoculations, because these inoculation treatments were done in separate experiments. Grain from the four replicate plots receiving the silk-channel inoculation in the

Illinois field experiment were combined prior to fumonisin analysis; therefore there are no statistical replications for this treatment and these data were omitted from the ANOVA.

Results

Germination and seedling disease assays

Aspergillus strains varied substantially in their effects on warm germination and cold test results for both hybrids. Several strains differed significantly from the non-inoculated control in each test. Some strains ranked more consistently near the high or low end in all four tests, while the ranking of other strains varied widely among experiments.

For hybrid A, the warm germination percentage varied from approximately 75% to nearly 100%, with the control near the high end of the range at 96.5% (Fig 1A). Twelve of the 24 strains reduced warm germination significantly compared to the control. Among the 13 fumonisin-producing strains, seven reduced warm germination significantly. In the cold test, the differences were even more pronounced, with cold test germination ranging from less than 40% to over 80% (Fig 1B). In this case, the control was near the middle of the range at 62.5%, with some strains of *Aspergillus* resulting in significantly higher cold test germination than the control. Eleven of the 26 strains significantly reduced cold test germination, 11 strains did not differ from the control, and four strains had cold test germination values significantly higher than the control. Among the 14 fumonisin-producing strains, seven reduced cold test germination, five did not differ from the control, and two were significantly higher than the control.

In hybrid B, in the warm germination test results were much lower overall, but still showed a wide variation among treatments, from a minimum of approximately 45%, to the control at 71.0% (Fig. 2A). Nineteen of the 25 strains significantly reduced warm germination compared to the control. Eleven of the 13 fumonisin-producing strains significantly warm reduced germination compared to the control. Hybrid B performed even worse than hybrid A in the cold test, with no treatment resulting in cold test germination greater than 60%, and some

near 40% (Fig. 2B). The control was at the lower end of the range at 49.8%. Among the 25 strains, only two strains (both fumonisin-producing) had cold test germination values significantly lower than the control, and four strains had results significantly higher. Among the 13 fumonisin-producing strains, two reduced cold test germination, 10 did not differ from the control, and one was significantly higher than the control.

For both maize hybrids, there were no significant differences in overall means for warm germination or cold test results between the fumonisin-producing *Aspergillus* strains and the non-producing strains (hybrid A: $P = 0.3893$ and $P = 0.8225$; hybrid B: $P = 0.1723$ and $P = 0.2058$). There were significant differences among species for effects on warm germination for hybrid A, and for cold germination for hybrid B. However, the only consistent pattern was significantly lower mean germination following inoculation with *A. niger* strains compared to *A. welwitschiae* and *A. phoenicis*.

In the rolled-towel assays with hybrid A, there were significant differences among the U.S. strains for effects on root weight, shoot weight, and shoot length, but not for root length (Table 1). Three of the 10 strains reduced root weight, including two of the six fumonisin-producing strains. Eight strains reduced shoot weight, including five fumonisin-producing strains. Nine of the strains reduced shoot length, including five fumonisin-producing strains. In the assay with the Italian strains, there were significant differences among the strains in all four variables for maize hybrid A (Table 2). In root weight, all 11 of the strains differed significantly from the control, with one of the six fumonisin-producing strains increasing the root weight, and all other strains decreasing it. Ten strains reduced shoot weight, including five fumonisin-producing strains. Four strains increased root length, including two fumonisin-producing strains. Ten strains reduced shoot length, including five fumonisin-producing strains. For hybrid B, there

also were significant effects on all four variables (Table 3). Twenty-four of the 25 strains reduced root weight, including all 13 fumonisin-producing strains; 22 strains reduced shoot weight, including 12 fumonisin-producing strains; 13 strains reduced root length, including eight fumonisin-producing strains, and 21 strains reduced shoot length, including 11 fumonisin-producing strains.

Differences in overall means of the various measures of seedling growth between fumonisin-producing and non-producing strains were not consistent. For hybrid A inoculated with U.S. strains, there was no detectible difference between fumonisin-producing and non-producing strains with regard to seedling root weight, shoot weight, root length, or shoot length (P values from 0.7303 to 0.8359). However, among the Italian strains, inoculation with fumonisin-producing strains resulted in significantly higher seedling shoot weight and length compared to the non-producing strains ($P < 0.05$), with no difference in root weight or length ($P = 0.3665$ and $P = 0.6318$, respectively). With hybrid B, seedlings inoculated with fumonisin-producing strains resulted in seedlings with decreased root and shoot weight compared to the strains without fumonisin production ($P = 0.0313$ and $P = 0.0363$, respectively). The root and shoot lengths were not significantly different ($P = 0.1565$ and $P = 0.1498$, respectively).

In the rolled paper towel assays, effects on seedling variables did not differ consistently among *Aspergillus* species. For hybrid A inoculated with the U. S. strains of *Aspergillus*, inoculation with *A. phoenicis* resulted in significantly lower root weight, shoot weight, and shoot length relative to *A. welwitschiae*, and root weight relative to *A. niger*. Among the Italian strains, there were no significant differences among species. On hybrid B, inoculation with *A. niger* reduced root weight relative to *A. welwitschiae*, and reduced shoot weight and length relative to

both *A. tubingensis* and *A. welwitschiae*. Inoculation with *A. phoenicis* reduced shoot weight relative to *A. tubingensis* as well.

Field experiments

Ear rot symptoms and signs typical of *Aspergillus* section Nigri occurred in inoculated treatments in all three experiments. In the Iowa experiments, ear rot symptoms were more severe in 2012 than in 2013. Ear rot severity was higher in the experiments with wound inoculations than in those with silk-channel inoculations. *Aspergillus* strains caused ear rot symptoms with severity similar to that caused by the *F. verticillioides* strain. In 2012, ear rot severity for all five *Aspergillus* strains was not significantly different from the *F. verticillioides* strain for both inoculation methods (Fig. 5a); in 2013, severity was significantly greater for strain ITEM 15253 (*A. niger*) than for *F. verticillioides* for the silk-channel inoculation, whereas the other strains were not different from *F. verticillioides* (Fig. 5b). Ear rot severity in inoculated treatments was not always significantly different from the mock-inoculated control. In 2012, the *F. verticillioides* treatment did not differ from the control for either inoculation method, but three and two *Aspergillus* strains had greater severity than the control for the silk-channel inoculation and the wound inoculation, respectively (Fig. 5a). In 2013, the *F. verticillioides* treatment differed from the control only for the wound inoculation, whereas one and three *Aspergillus* strains differed from the control in the silk-channel inoculation and the wound inoculation, respectively. The fumonisin non-producing strain, ITEM 15375, differed from the control only in the 2013 wound inoculation treatment (Fig. 5b); this strain did not differ significantly from the other *Aspergillus* strains in 2012, and in 2013 it differed only from strain ITEM 15353 in the silk-channel treatment.

Fumonisin concentrations in grain were highest in the *F. verticillioides* treatments in 2012, but also were very high in the mock-inoculated, wounded control treatment, which was not significantly different from the *F. verticillioides* treatment (Table 4). In the silk-channel inoculation, concentrations of fumonisin B_2 and total fumonisins did not differ between the control and the *Aspergillus*-inoculated treatments. The ratio of fumonisin B_2 to fumonisin B_1 (a measure of fumonisin production by *Aspergillus* spp.) was significantly higher for *Aspergillus* strains 15335 and 15337 compared to the other treatments. In the wound inoculation, *Aspergillus*-inoculated treatments were significantly lower in fumonisin B_2 and total fumonisins than the *F. verticillioides* treatment and the control. The FB_2/FB_1 ratio was significantly higher for *Aspergillus* strains 15337 and 15353 compared to the control, the *F. verticillioides* treatment, and *Aspergillus* strain 15375 (a non-fumonisin producing strain) (Table 4). Fumonisins were lower in 2013 than in 2012, and no treatments were different from the control in the silk-channel inoculations. In the 2013 wound inoculations, only the *F. verticillioides* treatment differed from the control in fumonisin B_2 and total fumonisins. The FB_2/FB_1 ratio was significantly higher for all four fumonisin-producing *Aspergillus* strains compared to the control, the *F. verticillioides* treatment, and the fumonisin non-producing *Aspergillus* strain 15375 (Table 4).

In the Illinois experiment, the silk-channel inoculation resulted in more severe ear rot symptoms compared to the pipe-cleaner inoculation method. In the silk-channel inoculation treatment, four of the five *Aspergillus* strains showing significantly higher severity than the control. In the wound inoculation treatment, ear rot severity was lower than in the silk-channel inoculation, and only ITEM 15337 (*A. niger*) was significantly different from the control. The fumonisin non-producing strain, ITEM 15375, differed from the control in the silk-channel

inoculation treatment only, and was not significantly different from the other *Aspergillus* strains in either treatment (Fig. 6).

In contrast to the Iowa results, fumonisin concentrations in grain were highest in the silk-channel inoculation treatments, including the mock-inoculated control (Table 4). Statistical comparisons among strains were not possible in the silk-channel inoculation treatments. In the wound inoculations, there were some significant differences among strains in fumonisin concentrations or FB_2/FB_1 ratio, but none differed significantly from the control (Table 4).

Discussion

The results of this study indicate that fungal strains in *Aspergillus* section Nigri can be pathogenic to maize ears, germinating seeds and seedlings. Although storage molds in general are associated with reduced emergence (18) and seedling disease (20), there are no previously published studies reporting the pathogenicity of *Aspergillus* section Nigri on maize seedlings. Windham and Williams (25) reported on ear rot development following the inoculation of maize ears with *A. niger*, but they did not report effects on germination or seedling disease.

There was a wide range in both the warm and cold germination values among the *Aspergillus* strains compared. Results were variable between the warm germination and cold tests, and between the two hybrids, suggesting that the pathogenicity of these *Aspergillus* strains depends strongly on experimental conditions and host genotype. Hybrid B performed poorly, even in the absence of inoculation, suggesting poor quality seed. Results confirm that some *Aspergillus* section Nigri strains can reduce germination and emergence in maize, but other strains have little capacity to do so.

Differences in aggressiveness among *Aspergillus* species in section Nigri were not consistently evident. Among the species compared, *A. niger* inoculation often resulted in reduced

germination, but results varied among experiments. The single strain of *A carbonarius* did not show a high level of pathogenicity, often not differing from the control. However, little can be concluded about this species based on only one strain. In the rolled paper towel assays, nearly all the strains of *Aspergillus* reduced shoot length and weight. Among the species, strains of *A. phoenicis* tended to result in shoot and root lengths and weights lower than the other species, but these differences were not always significant. Root length was less strongly affected than the other variables, and in some cases, root length actually increased with inoculation, even though root weight was reduced. It is possible that growth of lateral roots was reduced by some *Aspergillus* strains, causing more nutrients to be directed to lengthening the radicle and primary roots. If there are significant species differences in aggressiveness, larger number of strains of each species may be required in order to detect this.

There was no clear relationship between fumonisin production and aggressiveness as a seedling pathogen. One of the most aggressive strains overall was ITEM 15114, not a fumonisin-producer, while one of the fumonisin-producing strains, strain 7, produced consistently low levels of symptoms. The lack of association between fumonisin B_2 production and effects on germination for either hybrid indicates that fumonisin production is not necessary for, and probably has little or no role in the aggressiveness of black *Aspergillus* strains as seedborne pathogens. In the rolled-towel assays, there was some evidence for greater aggressiveness of fumonisin-producing strains toward hybrid B, but this was not consistent for hybrid A. For hybrid A, shoot length and weight were reduced to a greater extent by fumonisin non-producing strains from Italy compared to the fumonisin-producing strains. This suggests that the differences observed were due to characteristics of the strains themselves, rather than their ability to produce

fumonisin. It is also possible that hybrid B was more susceptible to fumonisin B_2 than hybrid A, though no effects of fumonisin production were evident for either hybrid in the germination tests.

The fact that inoculation with some strains resulted in higher emergence than the control in both cold tests may be due to competition with other pathogens in the media, as the sand used in the standard cold test is not sterile. It is also possible that other seedborne pathogens that are favored by cool soil conditions played a role. Weakly pathogenic strains of *Aspergillus* may have competed with pathogenic organisms already present in the sand, resulting in a net increase in germination.

Field experiments involved a smaller number of strains than the laboratory experiments, but results regarding pathogenicity, species comparisons, and the role of fumonisins are similar. There was no consistent difference in results between *A. niger* and *A. welwitschiae*, and the fumonisin non-producing strain produced ear rot severity similar to the other strains. Some strains of *Aspergillus* produced ear rot symptoms similar in severity to those caused by *F. verticillioides*, but other strains did not differ from the mock-inoculated control. Ear rot severity was in a similar range as reported by Windham and Williams (2012) for strains of *A. niger,* . In Iowa in 2012, ear rot severity was relatively high in the mock-inoculated control for the wound treatment, and fumonisin concentrations for this treatment were higher than in the *Aspergillus*-inoculated treatments. A similar observation occurred in the Illinois experiment. The wounding procedure (which differed between the two locations) appeared to have promoted infection by other fungi present in the fields, particularly fumonisin-producing *Fusarium* species. Lower fumonisins in the wounded, *Aspergillus*-inoculated treatments may have been the result of competition between the inoculated strains (which produce low amounts of fumonisin B_2) and endemic *Fusarium* strains (which include strains that produce high amounts of fumonisin B_1, B_2,

and other forms). Overall, ear rot severity and fumonisin levels were lower in 2013 than 2012 in Iowa, at least partially because late planting in 2013 resulted in altered ear morphology with more exposed ear tips.

The Illinois results demonstrated very severe ear rot symptoms and high fumonisin levels for the silk-channel inoculation. Environmental conditions obviously differed between the two locations, and the use of an inbred line in Illinois, rather than a hybrid, may have contributed to the relatively higher disease severity compared to the Iowa results. Results for the wound inoculation were similar between the locations in 2012, in spite of the use of different wounding procedures (method of Reid et al., 1996 in Iowa vs. method of Dowell et al., 2002, in Illinois).

Inoculation with *Aspergillus* strains that produce fumonisin B_2 did not result in increases in fumonisin B_2 or total fumonisins in the grain, compared to the mock-inoculated control. However, the elevated FB_2/FB_1 ratio that resulted from these inoculations (Table 4) indicates that these strains were actively producing fumonisin B_2 in the infected kernels. This was not evident by comparing fumonisin B_2 concentrations among the treatments, likely because *Aspergillus* strains produce small amounts of fumonisin B_2 relative to the amounts produced by endemic *Fusarium* spp.

Taken together, the results of this study suggest that fumonisin-producing and non-producing strains of *Aspergillus* spp. have similar levels of aggressiveness as maize pathogens. Pathogenicity tests comparing wild-type *Aspergillus* strains against strains mutated by disruption of the *fum8* gene would provide a more definitive assessment of the role of fumonisin B_2 production in the pathogenicity of fungi in *Aspergillus* section Nigri. Most studies with fumonisin-producing *Fusarium* species have concluded that there is little or no role of fumonisins in disease development (3); however, fumonisins are phytotoxic (2) and some

researchers have concluded that fumonisins play a role in pathogenicity or virulence (7, 17). Fumonisin production by *Aspergillus* spp. does not include fumonisin B_1, and overall levels of production are much lower than in *Fusarium* spp. (21). If fumonisins can influence seedling disease, the effects may not be evident with the low levels of fumonisin B_2 production that occur with *Aspergillus* spp.

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Table 1. Effect of *Aspergillus* strain inoculation on seedling growth in rolled paper towel assay, U. S. strains, hybrid A. Asterisks indicate treatments significantly different from the non-inoculated control ($\alpha = 0.05$).

Strain	Origin	Species	FB_2	Root weight (g)	Shoot weight (g)	Root length (cm)	Shoot length (cm)
Control	NA	NA	NA	1.85	5.24	13.22	9.69
ENDO 3233	Illinois	*A. niger*	+	1.33	3.06*	16.42	5.80*
NRRL 62522	Illinois	*A. phoenicis*	+	1.12*	2.52*	13.33	5.00*
NRRL 62526	Illinois	*A. phoenicis*	+	1.00*	2.28*	12.20	4.44*
ITEM 15304	Iowa	*A. tubingensis*	-	0.87*	1.89*	9.95	3.38*
ITEM 15309	Idaho	*A. welwitschiae*	+	2.05	5.00	14.47	9.26
ITEM 15318	Iowa	*A. tubingensis*	-	1.43	3.93	15.73	6.85*
ITEM 15335	Iowa	*A. welwitschiae*	+	1.36	2.99*	15.91	5.21*
ITEM 15337	Iowa	*A. niger*	+	1.48	2.92*	13.21	5.43*
ITEM 15349	Iowa	*A. tubingensis*	-	1.42	3.56*	15.76	5.87*
ITEM 15375	Iowa	*A. niger*	-	1.72	3.51*	15.47	6.40*

Table 2. Effect of *Aspergillus* strain inoculation on seedling growth in rolled paper towel assay, Italian strains, hybrid A. Asterisks indicate treatments significantly different from the non-inoculated control ($\alpha = 0.05$).

Strain	Origin	Species	FB_2	Root weight (g)	Shoot weight (g)	Root length (cm)	Shoot length (cm)
Control	NA	NA	NA	2.61	6.33	15.88	10.94
ITEM 15065	Marche	*A. carbonarius*	-	1.75*	3.54*	16.84	5.65*
ITEM 15078	Marche	*A. niger*	+	2.15*	3.79*	15.92	6.50*
ITEM 15096	Veneto	*A. niger*	-	1.73*	3.38*	18.05	6.06*
ITEM 15099	Veneto	*A. niger*	+	1.64*	3.60*	17.55	6.12*
ITEM 15114	Veneto	*A. niger*	-	1.64*	2.72*	18.27*	4.83*
ITEM 15129	Veneto	*A. welwitschiae*	-	1.79*	4.25*	19.38*	6.48*
ITEM 15132	Veneto	*A. welwitschiae*	+	1.77*	3.91*	18.74*	6.53*
ITEM 15165	Piemonte	*A. niger*	+	3.24*	6.29	20.28*	10.70
ITEM 15167	Lombardia	*A. niger*	+	1.66*	4.08*	18.24	7.11*
ITEM 15206	E.-Romagna	*A. niger*	+	1.43*	3.28*	15.98	5.21*
ITEM 15225	Molise	*A. niger*	-	2.16*	3.46*	17.87	5.82*

Table 3. Effect of *Aspergillus* strain inoculation on seedling growth in rolled paper towel assay, U. S. and Italian strains, hybrid B. Strain names in italics indicate fumonisin production. Asterisks indicate treatments significantly different from the non-inoculated control ($\alpha = 0.05$).

Strain	Origin	Species	FB_2	Root weight (g)	Shoot weight (g)	Root length (cm)	Shoot length (cm)
Control	NA	NA	NA	4.45	4.89	18.45	8.26
ENDO 3233	Illinois	*B. niger*	+	1.81*	2.10*	12.33*	3.92*
NRRL 62518	Illinois	*A. niger*	+	1.55*	1.98*	12.13*	4.09*
NRRL 62522	Illinois	*B. phoenicis*	+	2.10*	2.48*	15.32	5.00*
NRRL 62526	Illinois	*B. phoenicis*	+	2.04*	2.02*	15.12	4.21*
ITEM 15304	Iowa	*B. tubingensis*	-	2.81*	3.13*	16.29	5.46*
ITEM 15309	Idaho	*B. welwitschiae*	+	3.46*	3.80*	16.43	7.44
ITEM 15318	Iowa	*B. tubingensis*	-	2.78*	3.54*	18.02	6.39*
ITEM 15330	Iowa	*A. tubingensis*	-	2.04*	2.50*	12.54*	4.61*
ITEM 15335	Iowa	*B. welwitschiae*	+	2.35*	2.51*	16.00	4.86*
ITEM 15337	Iowa	*B. niger*	+	1.79*	1.99*	13.88*	4.16*
ITEM 15349	Iowa	*B. tubingensis*	-	2.51*	3.36*	15.61	5.81*
ITEM 15353	Iowa	*A. niger*	+	2.36*	2.22*	14.88*	4.87*
ITEM 15375	Iowa	*B. niger*	-	2.51*	2.27*	14.26*	4.54*
ITEM 15065	Marche	*B. carbonarius*	-	4.06	4.25	18.77	8.04
ITEM 15078	Marche	*B. niger*	+	2.22*	1.69*	14.18*	3.26*
ITEM 15096	Veneto	*B. niger*	-	1.91*	1.82*	13.37*	3.86*
ITEM 15114	Veneto	*B. niger*	-	1.89*	1.78*	13.29*	3.73*
ITEM 15129	Veneto	*B. welwitschiae*	-	1.93*	1.96*	11.11*	3.87*
ITEM 15132	Veneto	*B. welwitschiae*	+	2.13*	2.49*	14.34*	5.04*
ITEM 15165	Piemonte	*B. niger*	+	2.08*	2.41*	13.67*	4.68*
ITEM 15167	Lombardia	*B. niger*	+	2.82*	3.85	18.01	7.30
ITEM 15178	E.-Romagna	*A. niger*	-	2.18*	2.39*	15.58	4.80*
ITEM 15187	E.-Romagna	*A. welwitschiae*	-	3.16*	3.62*	19.34	6.50*
ITEM 15206	E.-Romagna	*A. niger*	+	2.21*	2.32*	14.65*	4.37*
ITEM 15225	Molise	*B. niger*	-	3.53*	3.87	19.26	6.90

Table 4. Fumonisin contamination of grain from field experiments conducted in Iowa and Illinois following inoculation of maize ears with fumonisin-producing or non-producing strains of *Aspergillus*. Fumonisin-producing *F. verticillioides* was included as a positive control in the Iowa experiments. Strain numbers in bold indicate fumonisin B_2 – producing strains.

Location-Year	Inoculation	Strain	Species	FB_2 (µg/g)	Total FB (µg/g)	Ratio (FB_2/ FB_1)
Iowa-2012	Silk-channel	Control	NA[x]	0.13 b	0.52 b	0.61 b
		ITEM 15309	*A. welwitschiae*	0.51 b	3.41 b	0.44 b
		ITEM 15337	*A. niger*	0.87 b	1.24 b	23.84 a
		ITEM 15353	*A. niger*	2.35 b	3.82 b	3.42 b
		ITEM 15335	*A. welwitschiae*	4.06 b	4.99 b	20.16 a
		ITEM 15375	*A. niger*	1.02 b	4.53 b	0.32 b
		ITEM 3927	*F. verticillioides*	20.48 a	76.46 a	0.47 b
	Wound	Control	NA	37.59 a	141.35 a	0.43 b
		ITEM 15309	*A. welwitschiae*	10.89 b	28.57 b	0.90 ab
		ITEM 15337	*A. niger*	9.53 b	18.24 b	1.51 a
		ITEM 15353	*A. niger*	10.18 b	26.28 b	1.90 a
		ITEM 15335	*A. welwitschiae*	8.15 b	21.45 b	0.96 ab
		ITEM 15375	*A. niger*	3.09 b	15.87 b	0.24 b
		ITEM 3927	*F. verticillioides*	42.15 a	159.27 a	0.44 b
Iowa-2013	Silk-channel	Control	NA	1.53 a	3.99 a	0.64 a
		ITEM 15309	*A. welwitschiae*	2.72 a	7.55 a	0.62 a
		ITEM 15337	*A. niger*	3.55 a	8.67 a	0.77 a
		ITEM 15353	*A. niger*	1.40 a	1.43 a	NA
		ITEM 15335	*A. welwitschiae*	1.27 a	1.27 a	NA
		ITEM 15375	*A. niger*	0.28 a	0.70 a	1.30 a
		ITEM 3927	*F. verticillioides*	2.52 a	4.88 a	1.04 a
	Wound	Control	NA	4.43 b	12.25 b	0.65 c
		ITEM 15309	*A. welwitschiae*	8.05 b	15.03 b	8.61 a
		ITEM 15337	*A. niger*	4.42 b	6.67 b	2.55 b
		ITEM 15353	*A. niger*	6.23 b	9.16 b	4.74 b

		ITEM 15335	*A. welwitschiae*	9.18 b	13.57 b	6.81 a
		ITEM 15375	*A. niger*	4.88 b	14.12 b	0.86 c
		ITEM 3927	*F. verticillioides*	15.86 a	36.35 a	0.84 c
Illinois-2012	Silk-channel[z]	Control	NA	56.47	270.24	0.30
		ITEM 15309	*A. welwitschiae*	4.92	6.44	13.90
		ITEM 15337	*A. niger*	41.06	54.29	13.83
		ITEM 15353	*A. niger*	68.07	90.04	12.55
		ITEM 15333	*A. niger*	33.90	50.00	10.6
		ITEM 15375	*A. niger*	ND[y]	ND	NA[x]
	Wound	Control	NA	9.81 ab	47.17 a	0.32 ab
		ITEM 15309	*A. welwitschiae*	5.61 b	17.98 a	0.56 ab
		ITEM 15337	*A. niger*	1.36 b	10.13 a	0.21 b
		ITEM 15353	*A. niger*	8.96 ab	43.55 a	0.32 ab
		ITEM 15333	*A. niger*	17.97 a	91.96 a	0.90 a
		ITEM 15375	*A. niger*	1.47 b	10.79 a	0.21 b

[x] NA = Not Applicable

[y] ND = Not Detected

[z] Grain from all four replicate plots was combined for fumonisin analysis; therefore there are no statistical replications for these treatments

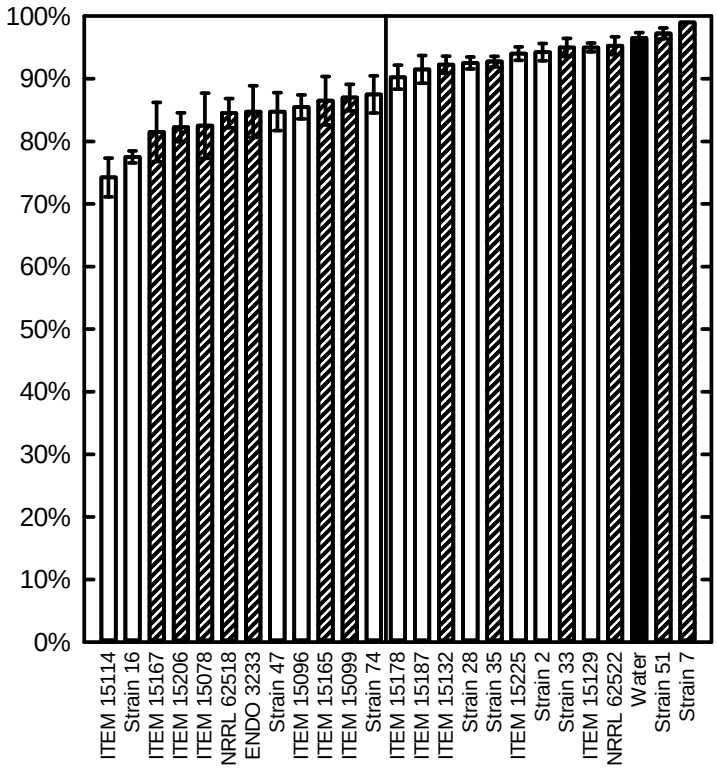

Fig. 1. Germination percentages in warm germination test for maize kernels (hybrid A) inoculated with strains of *Aspergillus* with and without production of fumonisin B_2. Hatch marks indicate strains with fumonisin production. Strains to the right of the vertical line were not significantly different from the water control (LSD = 6.9%, $\alpha = 0.05$). Error bars represent the standard error of the mean.

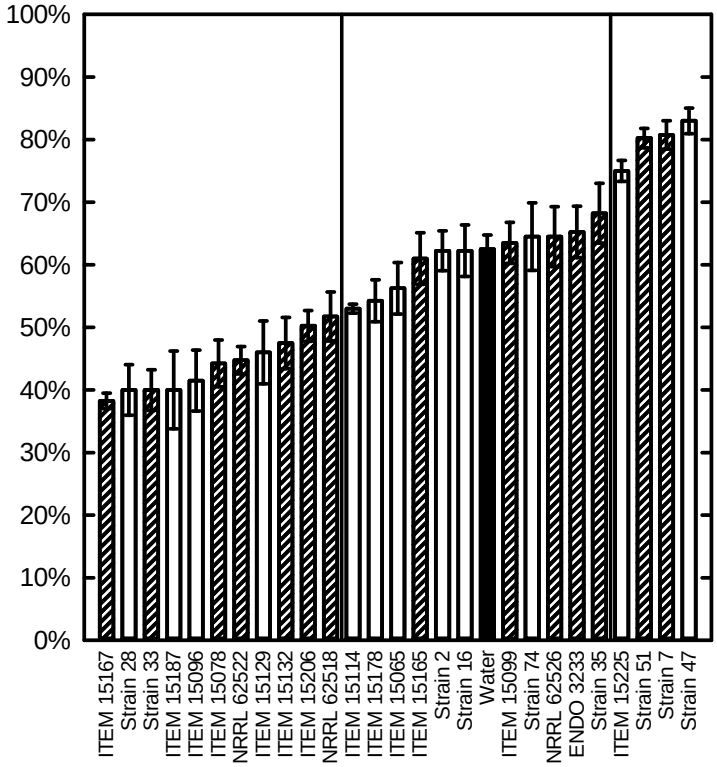

Fig. 2. Germination percentages in cold test for maize kernels (hybrid A) inoculated with strains of *Aspergillus* with and without production of fumonisin B_2. Hatch marks indicate strains with fumonisin production. Strains between the two vertical lines were not significantly different from the water control (LSD = 10.4%, $\alpha = 0.05$). Error bars represent the standard error of the mean.

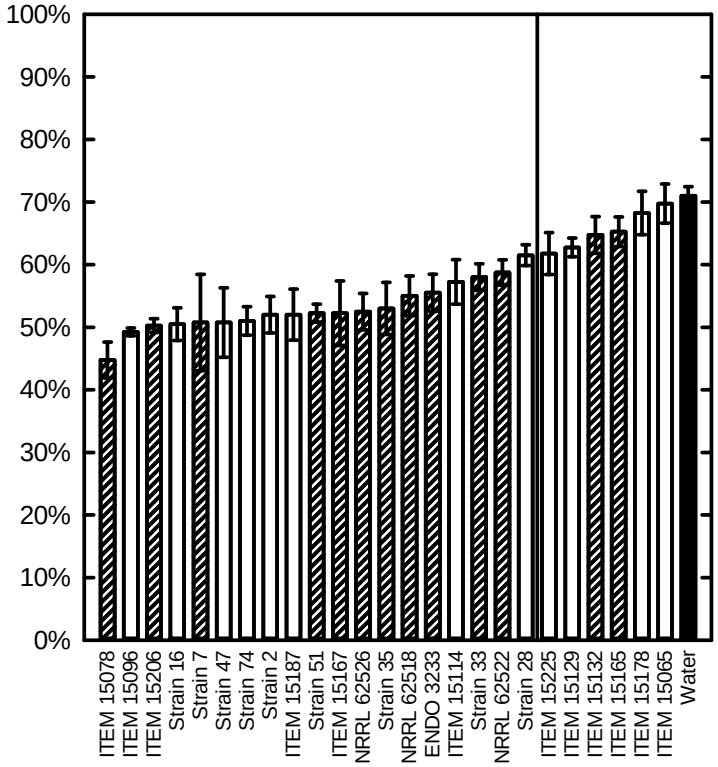

Fig. 3. Germination percentages in warm germination test for maize kernels (hybrid B) inoculated with strains of *Aspergillus* with and without production of fumonisin B_2. Hatch marks indicate strains with fumonisin production. Strains to the right of the vertical line were not significantly different from the water control (LSD = 9.3%, $\alpha = 0.05$). Error bars represent the standard error of the mean.

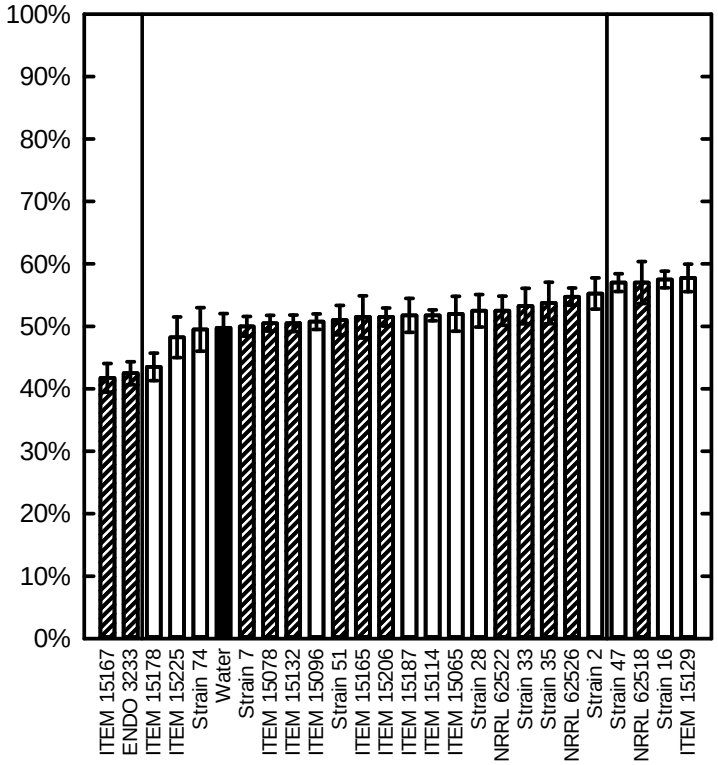

Fig. 4. Germination percentages in cold test for maize kernels (hybrid B) inoculated with strains of *Aspergillus* with and without production of fumonisin B_2. Hatch marks indicate strains with fumonisin production. Strains between the two vertical lines were not significantly different from the water control (LSD = 6.6%, $\alpha = 0.05$). Error bars represent the standard error of the mean.

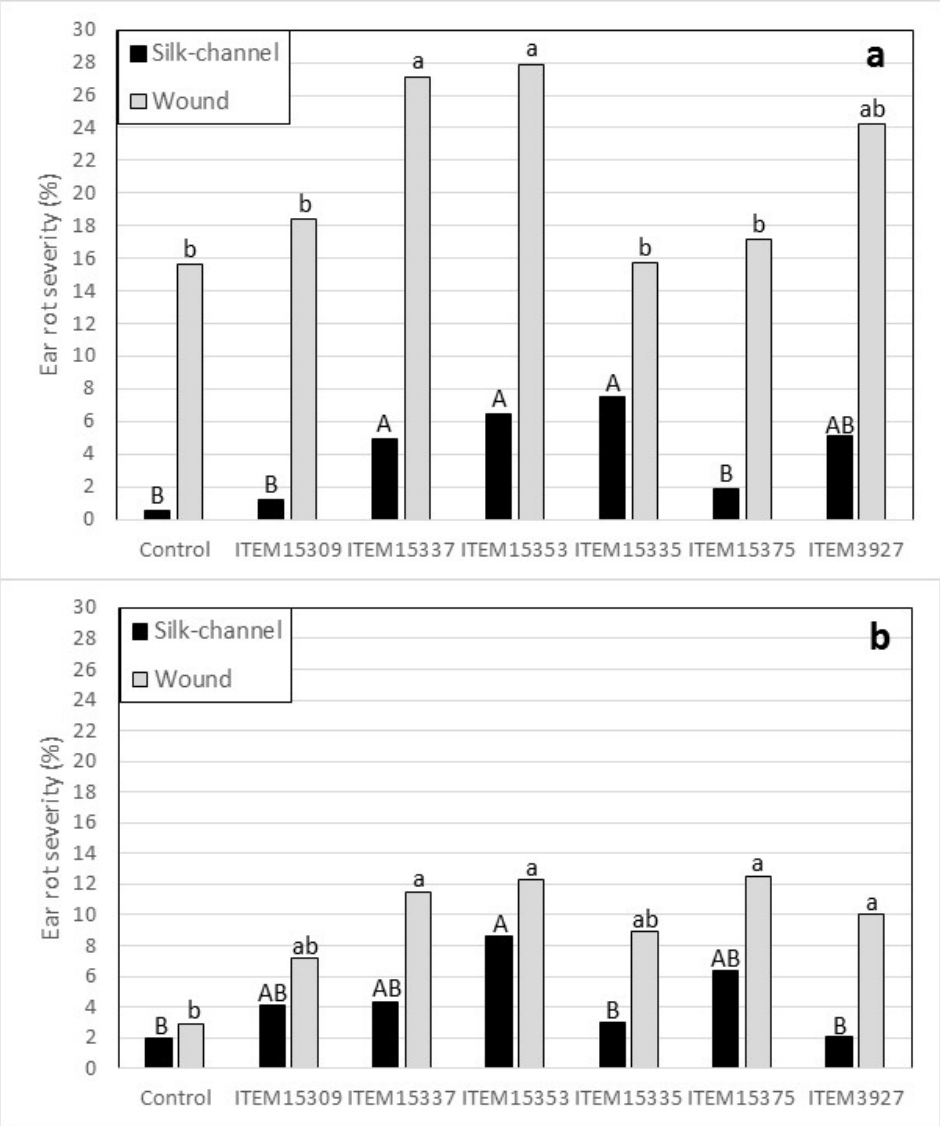

Fig. 5. Ear rot severity for maize ears inoculated with strains of *Aspergillus* section Nigri (ITEM15309 through ITEM15375) or *Fusarium verticillioides* (ITEM3927) in Iowa field experiments in 2012 (**a**) and 2013 (**b**). Inoculation methods (Silk-channel or Wound) were applied in separate experiments. Data are transformed to percentages from original 1 to 7 disease severity scale scores. Capital letters indicate significant differences among strains inoculated by the silk-channel method; lower-case letters indicate significant differences among strains inoculated by the wound method, according to Fisher's protected least significant difference (α = 0.05).

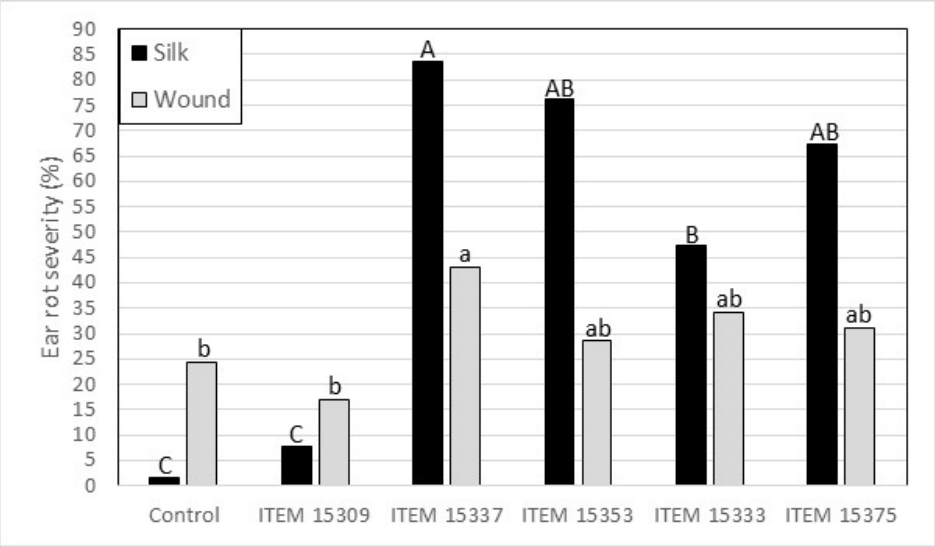

Fig. 6. Ear rot severity for maize ears inoculated with strains of *Aspergillus* section Nigri in an Illinois field experiment in 2012. Inoculation methods (Silk-channel or Wound) were applied in separate experiments. Data are transformed to percentages from original 1 to 7 disease severity scale scores. Capital letters indicate significant differences among strains inoculated by the silk-channel method; lower-case letters indicate significant differences among strains inoculated by the wound method, according to Fisher's protected least significant difference ($\alpha = 0.05$).