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Deanna L. Funnell-Harris

USDA-ARS, Deanna.Funnell-Harris@ars.usda.gov

Jeffrey F. Pedersen

USDA-ARS, jpetersen1@unl.edu

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Inoculation strategies to assess biological interactions between *Fusarium* and *Alternaria* species infecting sorghum

Deanna L. Funnell-Harris and Jeffrey F. Pedersen

Abstract: New cultivars of sorghum are being developed for increased food use, as livestock feed, and for the bioenergy industry by manipulation of secondary metabolic pathways through breeding. Previous work has suggested that such modifications may have an impact on cultivar response to fungal pathogens. We investigated four inoculation strategies that may be used in studies involving pathogens of sorghum. Plants of elite sorghum genotypes 'Wheatland' and RTx430, commonly used in breeding programs, were inoculated with isolates of *Alternaria alternata*, *Fusarium equiseti*, *Fusarium proliferatum*, *Fusarium solani*, *Fusarium thapsinum*, and *Fusarium verticillioides* recovered from field-grown sorghum. Wound inoculation of the peduncles of heads at anthesis demonstrated that isolates of *F. verticillioides* and *F. thapsinum* produced mean lesion lengths that were significantly greater than control inoculations. The length of this assay was decreased by inoculating fungi onto plants of a very early maturing sorghum line (N247). Spraying heads at anthesis with conidial suspensions over three consecutive days in the greenhouse was conducted to attempt to mimic conditions in the field. Results indicated that plants treated with conidia of *F. thapsinum* may develop mature grain with reduced germination. Seed and seedling inoculations produced responses over a relatively short time period. Seed inoculations were conducted in a manner similar to a corn bioassay. Seedling inoculations, conducted in Magenta vessels, allowed for measurement of effects of inoculum on roots, stalks, and leaves. Following inoculation of RTx430 and 'Wheatland' plants grown in Magenta vessels with *F. thapsinum*, nearly all measurements were significantly different from those of the water controls except for percent root infection of RTx430.

Key words: sorghum pathogens, *Alternaria alternata*, *Fusarium equiseti*, *Fusarium solani*, *Fusarium thapsinum*, *Fusarium verticillioides*, *Sorghum bicolor*, inoculation methods.

Résumé : De nouveaux cultivars de sorgho plus productifs sont à l'heure actuelle développés par manipulation des voies métaboliques secondaires durant le processus de sélection. Ces nouveaux cultivars sont destinés à l'alimentation des humains, à celle du bétail ainsi qu'à l'industrie des bioénergies. Des travaux précédents ont suggéré que de telles modifications peuvent influencer la réponse des cultivars quant à leur résistance aux agents pathogènes fongiques. Nous avons étudié quatre stratégies d'inoculation qui peuvent être utilisées dans les études relatives aux agents pathogènes qui s'attaquent au sorgho. Des plants de génotypes élités de sorgho 'Wheatland' et RTx430, communément utilisés dans les programmes de sélection, ont été inoculés avec des isolats d'*Alternaria alternata*, de *Fusarium equiseti*, de *Fusarium proliferatum*, de *Fusarium solani*, de *Fusarium thapsinum* et de *Fusarium verticillioides* obtenus de plants de sorgho cultivés en champs. L'inoculation de blessures causées aux pédoncules des épis arrivés au stade de l'anthèse a démontré que les isolats de *F. verticillioides* et de *F. thapsinum* produisaient des lésions dont les longueurs moyennes étaient passablement plus grandes que celles produites par les inoculations témoins. La durée de ce biotest a été raccourcie en inoculant des plants d'une lignée très hâtive de sorgho (N247) avec des champignons. Afin d'essayer de reproduire les conditions de plein champ, les épis, au stade de l'anthèse, ont été vaporisés, en serre, avec une suspension conidiale, et ce, sur une période de trois jours consécutifs. Les résultats ont montré que les plants traités avec les conidies de *F. thapsinum* peuvent produire des grains matures, mais que leur taux de germination sera réduit.

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D.L. Funnell-Harris,¹ USDA-ARS, Grain, Nebraska, 314 Biochemistry Hall, Lincoln, J.F. Pedersen, USDA-ARS, Grain, Forage and Bioenergy Research, Department of Plant Pathology, University of Nebraska, 314 Biochemistry Hall, Lincoln,

Forage and Bioenergy Research, Department of Plant Pathology, University of Nebraska, 314 Biochemistry Hall, Lincoln, NE 68583-0737, USA. and Bioenergy Research, Department of Agronomy and Horticulture, University of Nebraska, 314 Biochemistry Hall, Lincoln, NE 68583-0737, USA.

¹Corresponding author (e-mail: Deanna.Funnell-Harris@ars.usda.gov).

Les inoculations de graines et de plantules induisent des réponses à brève échéance. Les inoculations de graines furent effectuées selon une méthode semblable à celle utilisée pour les biotests effectués sur le maïs. Les inoculations des plantules, faites dans des boîtes Magenta, ont permis de mesurer les effets de l'inoculum sur les racines, les tiges et les feuilles. À la suite de l'inoculation avec *F. thapsinum* des plants de RTx430 et de 'Wheatland' cultivés dans les boîtes Magenta, presque toutes les mesures effectuées variaient passablement de celles provenant des solutions témoins aqueuses, sauf pour ce qui est du taux d'infection des racines de RTx430.

Mots-clés : agents pathogènes du sorgho, *Alternaria alternata*, *Fusarium equiseti*, *Fusarium solani*, *Fusarium thapsinum*, *Fusarium verticillioides*, *Sorghum bicolor*, méthodes d'inoculation.

Introduction

Sorghum cultivars are being developed for increased use for food, livestock feed, and for the bioenergy industry (Taylor et al. 2006). Characteristics modified include decreased lignin (Funnell and Pedersen 2006) and, in grain, reduced pigments (Pedersen and Toy 2001), increased lysine (Tesso et al. 2006), and decreased amylose ("waxy") starch (Pedersen et al. 2005). Such modifications in the basic structure and biochemistry of the plant may affect interactions with fungi, pathogenic or non-pathogenic (Funnell and Pedersen 2006).

Species of *Fusarium* (*Fusarium verticillioides* (Sacc.) Nirenberg (previously known as *Fusarium moniliforme* Sheldon), *Fusarium proliferatum* (Matsushima) Nirenberg, *Fusarium thapsinum* Klittich, Leslie, Nelson et Marasas sp. nov., *Fusarium solani* (Mart.) Appel & Wollenw. emend. Snyd. & Hans., and *Fusarium equiseti* (Corda) Sacc. sensu Gordon) and members of the *Alternaria alternata* (Fr.:Fr.) Keissl. complex were used in this study. These species are readily isolated from sorghum plants and from sorghum field soil (Leslie et al. 1990, 2005; Funnell and Pedersen 2006). Some of these species, e.g., *F. verticillioides* and *F. thapsinum*, may damage sorghum grain or forage material prior to harvest (Menkir et al. 1996). All of these species can colonize sorghum but not produce obvious symptoms (Funnell and Pedersen 2006). Since all can produce toxins (Leslie et al. 2005), infections by these species pose problems for the yield and quality of the harvested crop and possible problems during storage (Castor and Frederiksen 1980).

Four different bioassays were examined for use to screen sorghum germplasm. One standard sorghum assay involves quantitatively comparing lesions resulting from wound inoculation of peduncles of developing heads (Jardine and Leslie 1992). This assay has been used to characterize pathogenicity or virulence of the *F. moniliforme* complex associated with sorghum (Jardine and Leslie 1992) and to compare the response of sorghum lines to inoculation (Funnell and Pedersen 2006). The second assay was based on a sorghum field assay to assess grain mold responses (Forbes et al. 1989) with modifications to the protocol for inoculum delivery and conditions of infection. The third assay was based on a corn assay (Bacon et al. 1994) in which sorghum grain was incubated with fungal conidia. The last assay was modified from that of sorghum (Leslie et al. 2005), the major modification being that conidia were inoculated onto apparently sterile plants grown in plant growth medium in Magenta vessels.

Materials and methods

Fungal isolates

Fungal isolates were obtained from sorghum or sorghum fields. FRC-M-0900, isolated from a sorghum field soil in Texas, represented *F. solani*. Stalk isolates, FRC-M-1141 from California and FRC-M-3790 from Missouri, represented *F. verticillioides* and *F. thapsinum*, respectively (both formerly known as *F. moniliforme*). These three isolates were obtained from the Fusarium Research Center, Pennsylvania State University, USA. Isolate H02-765S was isolated from sorghum grain grown in Nebraska and represented *F. equiseti*. Two representatives of the *A. alternata* complex, isolates H02-755S and H02-781S-3b, originated from sorghum grain grown in Nebraska. Isolate H02-811L-2, recovered from leaf tissues from sorghum plants grown in Nebraska, represented *F. proliferatum*.

For comparison of plant responses, the *F. thapsinum* isolate had been known from previous work to be highly virulent (Funnell and Pedersen 2006). The *F. solani* isolate served as the weakly virulent isolate since *F. solani* is commonly isolated from sorghum soil and debris (Leslie et al. 1990). Isolates of *F. verticillioides*, *F. equiseti*, *F. proliferatum*, and *A. alternata* were used in some assays to represent different fungal species commonly isolated from sorghum and had differing responses in preliminary assays. *Fusarium* and *Alternaria* spp. isolates were identified by morphological characteristics, and identification was confirmed by sequence analysis of conserved DNA regions (GenBank acc. Nos. EF152426, EF152427, EF152428, EF152430, EF152432, EF152433, EU016680, and EU595566).

Plant materials

Each bioassay was conducted using 'Wheatland' and RTx430, since these elite sorghum genotypes have been commonly used in breeding programs and in other research on sorghum and grasses in general (Rodriguez-Herrera et al. 2006). RTx430 has been shown to be highly susceptible to grain mold diseases (Rodriguez-Herrera et al. 2006) but no published data of response of 'Wheatland' to grain mold disease has been found. Other genotypes used in different bioassays included very early maturing lines N246 and N247, BTx631, which lacks the purple wound response, and Tx2911 and Tx2927, reported to have resistance to grain mold diseases (National Plant Germplasm System, USDA-ARS; <http://www.ars-grin.gov/npgs/>).

Relatively clean, true-breeding seed was generated in the greenhouse by bagging heads at anthesis. No fungal growth or bacterial contamination was detected when seeds were plated onto water agar (0.6 %) or germinated in sterile water. Bioassays were conducted in greenhouses or growth chambers. For bioassays conducted in greenhouses, plants were grown in 25.4 cm diameter pots, 2 plants per pot. Soil mix used was 83% soil (General Excavating, Lincoln, Nebr.), 6% #3 vermiculite, horticultural grade perlite (Therm-O-Rock East Inc., New Eagle, Pa.), 4% peat moss (Conrad Fafard, Inc., Agawam, Mass.), and 3% sand (General Excavating).

Bioassays

Wound-inoculation bioassay

The wound-inoculation assay was essentially as described by Jardine and Leslie (1992). Briefly, broth cultures were prepared by inoculating 50 mL sterile tubes containing 5 mL of full-strength potato dextrose broth (PDB; Becton, Dickinson and Co., Sparks, Md.) and 10 sterile toothpicks with an agar plug from a 1-week-old one-half strength potato dextrose agar (PDA) culture of each fungal isolate, then incubating at 20 °C for 10 days. The day at which plants reached anthesis, defined as approximately half of the plants having panicles with 50% of the anthers exerted, was noted, and two weeks following this date, peduncles were wound inoculated with fungal-infested toothpicks. Toothpicks incubated in sterile broth served as the negative control. Inoculations were randomized so that each plant within a pot was inoculated with different isolates or with a fungal isolate and sterile broth. Eighteen days following inoculation, peduncles were split longitudinally and the total length of the discoloration, which may include regions with discontinuous discoloration, was measured.

Two assays were conducted with RTx430 and 'Wheatland'. Plants were inoculated with *F. solani*, *F. verticillioides*, *F. thapsinum*, *F. equiseti*, and *A. alternata* (H02-781S-3b) or with sterile toothpicks incubated in sterile broth. Levene homogeneity of variance (HoV) tests (SAS 2000) were conducted on data from bioassays with the same plant genotype. Based on results from these analyses, the two bioassays conducted on 'Wheatland' plants were combined and analyzed as randomized complete blocks with individual assays serving as blocks, whereas those from RTx430 plants were analyzed as separate experiments.

Wound inoculations were conducted on very early maturing genotypes N246 and N247 (Pedersen and Toy 1999). Ten plants each of N247 and N246 were inoculated with *F. solani*, *F. thapsinum*, and *A. alternata* or with sterile toothpicks incubated in sterile broth. This assay was conducted twice. HoV tests were conducted on data from bioassays with the same plant genotype. Based on these analyses, the two bioassays conducted on N247 plants were combined and analyzed as randomized complete blocks with individual assays serving as blocks, whereas those from N246 plants were analyzed separately. All analyses of variance were completed using Proc GLM (SAS 2000), and mean lesion lengths and least significant differences (LSDs) among isolates were reported.

Spray inoculation bioassay

The protocol for the spray inoculation bioassay was developed based on a sorghum grain mold field assay (Forbes et al. 1989) with refinements for inoculum delivery and conditions for infection. Two assays were conducted, each with genotypes RTx430 and 'Wheatland'. For the first assay, plants were inoculated with *F. thapsinum*, *F. verticillioides*, *F. solani*, *F. proliferatum*, and *A. alternata* (H02-755S); 'Wheatland' plants were inoculated two weeks prior to plants of RTx430 because of differences in bloom date. Individual plants were sprayed with 10 mL of a fungal conidial suspension (1×10^5 to 1×10^6 conidia mL⁻¹), water, or left untreated. Sprayed plants were immediately covered with plastic bags (Minigrip reclosable bags, 12 in × 12 in, 4 Mil; Minigrip/Zip-pak, Sequin, Tex.) that were closed by folding the open end and stapling near each peduncle. Untreated plants were neither sprayed nor bagged. For the second assay, 'Wheatland' and RTx430 seeds were sown and individual plants were observed until each reached anthesis (>50% of anthers exerted) and inoculated over 3 consecutive days. Treatments included conidial suspensions of *F. equiseti*, *A. alternata* (H02-781S-3b), *F. thapsinum*, or sterile purified water. Inoculations of RTx430 plants were conducted 84 to 104 days after planting (dap), whereas inoculations of 'Wheatland' were conducted 90 to 139 dap. Individual plants were sprayed with a freshly prepared conidial suspension or sterile water then immediately covered with plastic bags. Untreated plants were neither sprayed nor bagged. The treatment was repeated over three consecutive days, and on the fourth day, the bags were removed.

Inoculum was prepared in the following manner. For *Fusarium* isolates, agar blocks from PDA cultures of each isolate were placed in the centre of 60 mm Petri dishes filled with 1.5% agar containing 80 mmol/L KCl. For *A. alternata* isolates, a 2% water agar plate was point-inoculated from a PDA culture followed by placement of -1 cm² filter paper (Whatman #1, Whatman International Ltd., Maidstone, England) over the inoculation site. Fungi were incubated at room temperature or 28 °C for at least 5 days. Conidial suspensions were prepared in sterile filter-purified water and diluted to the concentration 1×10^5 to 1×10^6 conidia mL⁻¹. Sporulation on minimal media was abundant but mycelial growth was reduced; therefore, no filtration step was needed and the conidial suspension could be applied by spraying with 473 mL plastic bottles (National Industries for the Blind, Alexandria, Va.) without clogging the spray apparatus.

Mature grain was harvested and seed threshed and bulked by treatment. The following three measurements were made on each seed sample: (i) mean mass of seed, (ii) percent of seeds appearing damaged, and (iii) percent germination. Mean seed mass was determined by subsampling and obtaining the total mass of 100 seeds (in three replicates). To determine percent of damaged seed, three individuals examined subsamples of 100 seeds (for a total of 300 seeds screened) from each treatment and separated them into the following two classes: (i) healthy appearing or (ii) those that appeared discolored, moldy, small, or deformed. Greenhouse-grown uninfected seed were used for comparison. To determine percent germination, approximately 100 seeds from each treatment were distributed into four Petri dishes between sterile, wet paper towels. Petri dishes containing

seeds were incubated at 25° C, examined daily, and, if dishes appeared dry, sterile purified water was added. Seeds were scored after 72 h as germinated if the radicle and shoot had grown past the length of the seed.

Spray inoculation bioassays were analyzed considering data recorded by each of the three individuals as repeated measurements using Proc GLM. Main effects for treatments were presented where genotype $\times$ treatment effects were not significant. Where genotype $\times$ treatment effects were significant, treatment effects were calculated for each sorghum line used. Correlations among treatments were made using Proc CORR Proc GLM (SAS 2000).

Seed inoculation bioassay

The maize assay of Bacon et al. (1994) served as the basis for the assay in the present work. Approximately 100 surface-sterilized, greenhouse-produced seed of either RTx430 or 'Wheatland' were inoculated with a suspension of conidia of either *F. thapsinum* or *F. solani*, or with sterile purified water. Conidia were produced on KCl agar incubated at room temperature for 7 to 10 days. For the fourth repetition, viable macroconidia of the *F. solani* isolate from an older KCl culture were used. Conidial suspensions were prepared in sterile purified water at a concentration of 1×10^3 conidia mL^{-1} since preliminary assays indicated that this concentration of *F. thapsinum* conidia incubated with grain of RTx430 reduced germination of seed by approximately one-half when compared with seed incubated in sterile purified water. Seeds were incubated for two days in the dark at 25 °C then two days in the dark at 4 °C and the number of germinated seeds per Petri dish were re-recorded. The assay was conducted four times. The data were analyzed using Proc GLM (SAS 2000) as two genotypes $\times$ three treatments design with each experiment considered a replication.

Seedling inoculation bioassay

This assay is a modification of that of Leslie et al. (2005) for sorghum. Seedlings were grown in Magenta vessels on Murashige and Skoog (MS) plant growth medium that was modified to enhance sorghum growth (Medium 11; Elkonin and Pakhomova 2000). Plants were incubated with a fungal isolate and were infected without wounding. Infection of roots was compared with uninoculated controls. The assay was conducted within four to six weeks, depending on plant genotype.

Greenhouse-grown seeds were surface sterilized and transferred aseptically to 0.6% water agar and incubated for 4–7 days in a growth incubator at 25 °C with continuous light. Germinated seeds were transferred to sterile Magenta vessels prepared with 50 mL MS basal medium with Gamborg's vitamins and 87.6 mmol/L sucrose, amended with KH_2PO_4 to increase the concentration to 8.8 mmol/L (Sigma), and solidified with 0.7% phytoblend agar (Caisson Laboratories, Inc., Rexberg, Ind.). Seedlings continued to be incubated at 25 °C with continuous light. Eight to fifteen days following transfer of seedlings to Magenta vessels, plants were inoculated with 0.5 mL of a spore suspension or sterile purified water applied at the base of stalk on the plant growth medium and incubated in a growth chamber at 22 °C, 12 h light.

Fungi used for inoculations were *F. solani*, *F. thapsinum*, and *A. alternata* (H02-755S). Conidia of *Fusarium* spp. were grown on KCl agar while conidia of *A. alternata* were grown on 2% water agar, and suspensions were prepared in sterile purified water at the concentrations indicated in Results.

Sixteen to twenty-one days after inoculation, each plant was removed from the plant growth medium. The mycelial mat was carefully removed so as not to damage the roots. Plants were placed in water-dampened paper towels while the following measurements were made: (i) percent of discolored stalk and leaves; (ii) percent of discolored roots; (iii) fresh mass of stalk and leaves; and (iv) length of discoloration from base of stalk following removal of loose, damaged leaves. To determine the percent of root pieces with fungal growth, approximately 1 cm long non-adjacent root pieces (no root tips) were excised from each plant, surface-sterilized in 1% sodium hypochlorite, and plated onto dichloran (Ultra Scientific, North Kingstown, R.I.) and chloramphenicol (Sigma) medium (DCPA), which was semi-selective for *Fusarium* and *Alternaria* (Funnell and Pedersen 2006). Four to seven days following plating, the percent of root pieces with fungal growth was recorded.

'Wheatland' seeds were plated onto water agar and four days later, seedlings were transferred to Magenta vessels. Thirteen days after plating of seed, randomly chosen plants were inoculated with conidia of *F. thapsinum*, *F. solani*, or with sterile purified water; plants were scored 31 days after plating of seed. Seventeen days after plating of seed, seedlings were inoculated with *A. alternata* conidia or sterile purified water and responses were scored 33 days after seed plating.

Four days following plating of RTx430 seeds, seedlings were transferred to prepared Magenta vessels. Two weeks after seeds were plated, seedlings were inoculated with conidia of *F. thapsinum*, *F. solani*, *A. alternata*, or with water. Plants were scored 31 days after plating of seed. Root pieces were plated onto DCPA.

To examine the response of genotypes bred for grain mold resistance to isolates of *F. thapsinum*, *F. solani*, and *A. alternata*, seedlings of Tx2911 and Tx2927 were inoculated. Seedlings of Tx2911 were transplanted into prepared Magenta vessels seven days after sowing onto water agar, then inoculated after 15 days, and scored 29 days after plating of seed. Seedlings of Tx2927 were transplanted into prepared Magenta vessels six days following plating of seeds onto water agar, inoculated at 14 days, and scored 35 days after plating of seed. Root pieces were plated onto DCPA.

'Wheatland', RTx430, and Tx2911 have the purple wound response, whereas Tx2927 lacks this response (a phenotype called "tan" plant color). To address the possibility that discoloration in roots and other plant parts was due to the accumulation of pigments or other phenolics in plants with the purple phenotype (Huang and Backhouse 2005), another tan genotype, BTx631, was inoculated with conidia from the same three fungi used in the seedling bioassay. BTx631 seedlings were transplanted into prepared Magenta vessels 5 days following plating of seed. Inoculation with conidia of *F. thapsinum* and *F. solani* or with water occurred 13 days following plating of seed, and plants were

scored 32 days after plating of seed. Seedlings were inoculated with conidia of *A. alternata* or with water 20 days following seed plating, and plants were scored 39 days after plating of seed. Root pieces were plated onto DCPA.

Seedling bioassays were analyzed as completely random designs using Proc GLM. Mean percent root discoloration, percent shoot discoloration, shoot mass, lesion lengths, percent infected root pieces, and LSDs among isolates were reported. Correlation coefficients were derived and tested for significance using Proc CORR.

Results

Wound inoculation bioassays

Inoculation of 'Wheatland' and RTx430

HoV tests revealed that the assays with 'Wheatland' were not significantly different ($P = 0.58$); therefore, the results were combined for analysis. Inoculation with *F. verticillioides* resulted in a mean lesion length that was significantly different from that of the sterile broth control and following inoculation with all fungal isolates except *F. thapsinum* (Table 1). Inoculation with *F. thapsinum* yielded a mean lesion length that was significantly greater than that resulting from inoculation with *F. equiseti*, *F. solani*, or the sterile broth control (Table 1).

Mean lesion lengths resulting from inoculation of RTx430 plants were significantly different for treatment in both bioassays ($P < 0.01$). When HoV tests were conducted, the experiments were significantly different ($P < 0.01$); therefore, the bioassays were analyzed separately. In spite of this statistical difference, both assays demonstrated that inoculation of RTx430 plants with either *F. verticillioides* or *F. thapsinum* yielded mean lesion lengths that were significantly different from all other treatments (Table 1).

Inoculation of early maturing lines N246 and N247

These assays were conducted to determine whether very early maturing lines N246 and N247 could shorten the duration of the wound inoculation bioassay and distinguish between highly virulent and weakly virulent fungi. Using the HoV test, it was determined that results from the two assays conducted on N247 plants were not significantly different ($P = 0.16$). Mean lengths of lesions resulting from inoculation of N247 were significantly different ($P < 0.01$; Table 2). Following inoculation with *F. thapsinum*, mean lesion lengths were significantly greater than those resulting from inoculation with *F. solani* and *A. alternata* (Table 2).

The HoV test of bioassays conducted on N246 plants revealed that the results were significantly different ($P < 0.01$). Therefore, the two experiments were analyzed separately. In the first assay, plants inoculated with *F. thapsinum* had a significantly greater mean lesion length compared with other treatments, as expected (Table 2). However, in the second assay, there were no significant differences between treatments (Table 2).

Spray inoculation bioassay

Heads of RTx430 and 'Wheatland' plants were spray-inoculated with conidia of *F. thapsinum*, *F. solani*, *F. verticillioides*, *F. proliferatum*, or *A. alternata* (H02-

Table 1. Mean lesion lengths* resulting from wound inoculation of 'Wheatland' and RTx430 by *Fusarium verticillioides*, *Fusarium thapsinum*, *Fusarium equiseti*, *Fusarium solani*, and *Alternaria alternata*.

Treatment	Mean lesion length (mm)		
	Sorghum genotype 'Wheatland'†	Sorghum genotype RTx430‡	
		Assay 1	Assay 2
<i>F. verticillioides</i>	84.1 a	84.9 a	21.5 b
<i>F. thapsinum</i>	70.0 ab	70.2 b	41.7 a
<i>F. solani</i>	40.3 bc	19.9 c	8.0 c
<i>F. equiseti</i>	24.3 c	13.4 cd	10.3 c
<i>A. alternata</i>	9.3 c	10.5 cd	10.5 c
Sterile broth	8.9 c	2.7 d	8.4 c
LSD(0.05)	33.2	7.8	9.9

*Total length of discoloration, which may include regions with discontinuous discoloration, was measured.

†Combined results of two assays. Means with the same letter are not statistically different.

‡Assays are reported separately because of significant differences in HoV. Means with the same letter are not statistically different within each assay.

Table 2. Mean lesion lengths* resulting from wound inoculation of very early maturing genotypes, N247 and N246, with *Fusarium thapsinum*, *Fusarium solani*, and *Alternaria alternata*.

Treatment	Mean lesion length (mm)		
	Sorghum genotype N247†	Sorghum genotype N246‡	
		Assay 1	Assay 2
<i>F. thapsinum</i>	55.0 a	63.3 a	84.0 a
<i>F. solani</i>	15.7 b	19.7 b	55.1 a
<i>A. alternata</i>	11.0 b	4.9 b	92.8 a
Sterile broth	18.8 b	1.9 b	111.6 a
LSD(0.05)	14.3	21.3	76.3

*Total length of the discoloration, which may include regions with discontinuous discoloration, was measured.

†Combined results of two assays. Means with the same letter are not statistically different.

‡Assays are reported separately because of significant differences in HoV. Means with the same letter are not statistically different within each assay.

755S), then incubated for one day in plastic bags. Both water-treated and untreated controls were also included. There were no significant effects due to treatment for mass per 100 seeds, percent of healthy seed by visual assessment, and percent germination ($P = 0.10$, $P = 0.31$, and $P = 0.44$, respectively). Scorer effects were significant ($P < 0.01$) for visual assessment of seed. There were no significant correlations between the three measurements.

'Wheatland' and RTx430 plants were inoculated by spraying a conidial suspension of either *F. thapsinum*, *F. equiseti*, or *A. alternata* (H02-781S-3b) onto heads as they reached anthesis for three consecutive days. Water-treated and untreated controls were also included. The effects of treatments were significant for percent of healthy appearing seed ($P = 0.02$; Table 3). Untreated seed had the highest mean percent healthy appearing seed, and this percentage was significantly greater than that following treatment with

Table 3. Mean mass, percent healthy seed, and percent germination of mature grain resulting from spray inoculation of heads at anthesis of sorghum genotypes RTx430 and 'Wheatland' with conidia of *Fusarium thapsinum*, *Fusarium solani*, and *Alternaria alternata* as compared with water-sprayed heads or untreated heads.*

Spray treatment	Combined treatments of lines 'Wheatland' and RTx430		Mean mass (g) per 100 mature seeds obtained from fungus-sprayed, water-sprayed, or untreated heads	
	Mean percent healthy seed	Mean percent germination	RTx430	'Wheatland'
None	92.9 a†	75.7 a	3.7 bc	4.3 a
<i>F. equiseti</i>	87.0 ab	61.5 ab	3.6 bc	4.4 a
<i>A. alternata</i>	85.5 abc	70.5 a	3.5 c	4.4 a
<i>F. thapsinum</i>	83.2 bc	55.4 b	3.9 ab	4.1 b
Water	77.3 c	69.7 a	4.0 a	4.3 a
LSD(0.05)	8.7	14.3	0.32	0.14

*Arithmetic means of three measurements for each treatment on each line.

†Comparisons were made on each measurement for each sorghum genotype. Means with the same letter are not statistically different.

F. thapsinum or water (Table 3). There may have been an effect of treatment on percent germination since grain from untreated and water-treated plants germinated at a higher rate than grain from plants treated with *F. thapsinum* ($P = 0.06$; Table 3). Plant genotype $\times$ treatment effects for seed mass were significant ($P = 0.01$), and effects of treatment were significant for both RTx430 and 'Wheatland' ($P < 0.01$ and $P = 0.03$, respectively). Treatment of RTx430 plants with sterile water or *F. thapsinum* conidia yielded seed with significantly higher mean masses (g) per 100 seeds than other treatments, while spraying *F. thapsinum* conidia onto 'Wheatland' plants yielded grain with significantly lower mean masses per 100 seeds than other treatments (Table 3). To confirm that visual inspection could be used as an indication of grain health, correlations were conducted. The percent of healthy appearing seed was positively correlated with percent germination ($R = 0.61$; $P < 0.01$).

Seed inoculation bioassays

Effects of treatments on germination were not significant ($P = 0.06$) following the incubation of seed of sorghum genotypes RTx430 and 'Wheatland' in either sterile water (75.1%), sterile water containing conidia of *F. thapsinum* (71.3%), or *F. solani* (70.5%) (LSD_{0.05} = 5.2). Effects of genotype were significant ($P < 0.01$) with a mean germination of 62.9% for RTx430 seed across all treatments and a mean germination of 81.7% for 'Wheatland' seed across all treatments (LSD_{0.05} = 4.2).

Seedling inoculation bioassays

Inoculation of 'Wheatland' and RTx430

When the genotype 'Wheatland' was inoculated with conidia of *F. thapsinum* or *F. solani*, or with sterile water, the measurements of percent root discoloration and shoot discoloration, shoot mass, and mean lesion length were significantly different among treatments ($P < 0.01$, $P < 0.01$, $P < 0.01$, and $P = 0.02$, respectively) (Table 4). For all measurements, inoculation with *F. thapsinum* conidia resulted in mean measurements that were significantly different from

those obtained from water control plants (Table 4). Following inoculation with *F. solani* conidia, only mean percent root discoloration and shoot mass were significantly different from those found in the water control (Table 4). Seedlings grown in Magenta vessels also were inoculated with *A. alternata* isolate H02-755S, which in preliminary wound inoculation bioassays, resulted in mean lesion lengths similar in length to those of H02-781S-3b (data not shown). Percent root discoloration, shoot mass, and lesion length were significantly different among treatments ($P < 0.01$; $P < 0.01$ and $P = 0.047$, respectively) but not for percent shoot discoloration ($P = 0.054$) (Table 4).

When plants of genotype RTx430 were inoculated with conidia of *F. thapsinum*, *F. solani*, or *A. alternata*, the measurements of mean percent root discoloration, shoot discoloration and infected root pieces, and mass and lesion length were significant among treatments ($P < 0.01$; Table 4). Nearly all measurements resulting from inoculation with conidia from the three fungal species were significantly different from those obtained following application of water to plants. Exceptions were the mean lesion length resulting from inoculation with *F. solani* conidia and the percent infected root pieces following inoculation with conidia of *F. thapsinum* as compared with water controls (Table 4). Additionally, plants treated with *F. solani* conidia had a significantly higher mean mass when compared with that of plants treated with conidia of either *F. thapsinum* or *A. alternata* (Table 4).

Inoculations of genotypes bred for resistance to grain mold pathogens and tan genotypes

When plants of genotype Tx2911 were inoculated with conidia of *F. thapsinum*, *F. solani*, or *A. alternata*, as compared with sterile water controls, measurements of fungal-inoculated plants were significantly different except for percent shoot discoloration following inoculation with *F. solani* conidia (Table 4). The means of percent root discoloration following inoculation with conidia of either *A. alternata* or *F. solani* were significantly higher than the

Table 4. Mean percent root discoloration, percent infected root pieces, shoot mass (g), stalk lesion length (mm), and percent shoot discoloration following inoculation of cultured sorghum seedlings with *Fusarium thapsinum*, *Fusarium solani*, and *Alternaria alternata*.

Genotype	Treatment*	Roots		Shoots		
		Percent discolored	Percent infected	Mass (g)	Lesion length (mm)†	Percent discolored
'Wheatland'	FRC-M-3790	74.4 b ‡	nt§	0.75 b	11.0 a	31.6 a
	FRC-S-0900	92.6 a	nt	0.81 b	5.9 ab	14.5 b
	Water	6.0 c	nt	2.25 a	0.0 b	6.8 b
	LSD(0.05)	18.1	—	0.41	7.0	10.1
'Wheatland'	H02-755S	94.9 a	nt	1.16 b	13.4 a	45.8 a
	Water	8.8 b	nt	2.65 a	0.0 b	14.3 a
	LSD(0.05)	6.8	—	0.571	13.3	32.2
RTx430	FRC-M-3790	46.2 c	9.0 b	0.57 c	19.7 a	65.8 a
	FRC-S-0900	63.8 b	29.1 a	0.77 b	5.3 b	40.4 c
	H02-755S	94.0 a	31.0 a	0.56 c	17.9 a	52.7 b
	Water	2.8 d	0.0 b	1.49 a	0.0 b	21.4 d
	LSD(0.05)	9.3	15.5	0.20	7.0	10.4
Tx2911	FRC-M-3790	58.3 b	62.8 b	0.38 b	44.5 a	51.2 a
	FRC-S-0900	72.9 ab	97.8 a	0.44 b	34.9 a	33.2 bc
	H02-755S	82.3 a	52.9 b	0.38 b	28.8 a	35.8 b
	Water	17.0 c	1.1 c	0.58 a	3.9 b	20.3 c
	LSD(0.05)	20.1	14.3	0.13	16.9	14.7
Tx2927	FRC-M-3790	61.5 b	60.0 ab	0.23 b	74.1 a	91.8 a
	FRC-S-0900	61.5 b	82.3 a	0.28 b	13.2 b	54.4 b
	H02-755S	92.3 a	38.9 bc	0.29 b	25.2 b	64.7 b
	Water	16.8 c	20.8 c	0.61 a	0.0 b	21.3 c
	LSD(0.05)	19.6	37.2	0.10	41.6	13.1
BTx631	FRC-M-3790	17.3 ab	11.8 ab	0.48 b	26.3 a	78.6 a
	FRC-S-0900	33.1 a	27.4 a	0.52 b	3.6 b	49.0 b
	Water	1.0 b	0.0 b	1.86 a	0.0 b	13.0 c
	LSD(0.05)	21.7	21.9	0.31	5.6	15.2
BTx631	H02-755S	69.5 a	49.4 a	0.57 b	7.9 a	59.5 a
	Water	0.0 b	7.4 b	1.94 a	0.0 b	15.3 b
	LSD(0.05)	24.0	40.9	0.575	6.8	29.1

*The approximate number of conidia applied to each plant was as follows: 'Wheatland' (first assay), 2.5×10^5 ; 'Wheatland' (second assay), 3.4×10^4 ; RTx430, 5.0×10^3 ; Tx2911, 1.2×10^4 ; Tx2927, 5.0×10^4 ; BTx631 (first assay), 5.0×10^4 ; and BTx631 (second assay), 6.0×10^3 .

†The length of necrosis or discoloration rising from the base of stalk is defined as the lesion.

‡Comparisons were made on each measurement for each sorghum genotype. Means with the same letter are not statistically different.

§nt, not tested.

same measurement after inoculation with *F. thapsinum* conidia (Table 4).

Plants of genotype Tx2927 were inoculated with conidia from each of the three fungal isolates, and the measurements of percent of root and shoot discoloration and shoot mass ($P < 0.01$) as well as mean lesion length ($P < 0.01$) and percent of infected root pieces ($P = 0.01$) were significantly different among treatments (Table 4). Only plants treated with *F. thapsinum* conidia had stalk lesions that were significantly higher than that of the water control (Table 4). Plants treated with conidia of either of the two

Fusarium spp. had mean percent root infections that were significantly higher than those of the water-treated plants or plants inoculated with *A. alternaria* conidia (Table 4). For means of percent root or shoot discoloration, treatment with conidia of all three fungi yielded results that were significantly higher than those obtained with water alone (Table 4). Application of *A. alternata* conidia resulted in significantly higher mean percent root discoloration than all other treatments, while treatment with *F. solani* conidia resulted in significantly higher mean percent shoot discoloration as compared with all other treatments (Table 4).

Sorghum genotypes 'Wheatland', RTx430, and Tx2911 produce dark pigments in response to pathogens, insects, or injury, whereas Tx2927 has the tan response. To include an additional tan genotype in this analysis, plants of BTx631 were inoculated with conidia of the two *Fusarium* species or with water. Following inoculation with *F. thapsinum* conidia, measurements for shoots (mass, lesion length, and percent shoot discoloration) were significantly higher than those for water, while measurements for roots (percent discoloration and percent infected root pieces) were not (Table 4). Application of *F. solani* conidia to BTx631 plants resulted in significantly higher mean percent shoot discoloration and percent root discoloration, with significantly reduced shoot masses as compared with water-treated plants (Table 4). When plants of genotype BTx631 were inoculated with conidia of *A. alternata*, all measurements were significantly different from those resulting from application of sterile water (Table 4).

In general, when considered across all assays, all measurements were correlated in a biologically relevant manner. For example, percent root discoloration and percent infection of root pieces were directly correlated ($R = 0.23$, $P < 0.01$ when considering only fungal inoculations; $R = 0.44$, $P < 0.01$ when water controls are included).

Discussion

A set of isolates of *Fusarium* and *Alternaria* spp. associated with sorghum were used in four different inoculation strategies. Each of these strategies could be used to assess biological interactions between fungal species and sorghum, or to compare responses of different genotypes of sorghum, under conditions of the particular assay. Table 5 summarizes the four assays, including the length of time to obtain analyzable material, the plant part analyzed, and the extent of analysis required.

The wound inoculation assay of Jardine and Leslie (1992) is valuable for comparison of fungal isolates as well as comparison of sorghum genotypes (Funnell and Pedersen 2006) with regard to fungal colonization of the peduncle of maturing heads following wound inoculation. By using very early maturing lines, such as N247, the duration of this assay could be reduced by nearly eight weeks (Table 5), a strategy that might be used for comparison of colonization by different fungi. An unexpected consequence of this study was the observation that wound inoculation of genotype N246 resulted in mean lesion lengths that were not significantly different by treatment in one assay, while treatment was significant for genotype N247. We used wound inoculation on plants from several genetic backgrounds (Funnell and Pedersen 2006) and only genotype N246 responded in this manner. It has been observed that early physiological maturation in sorghum may be directly associated with senescence (Crasta et al. 1999). In ryegrass, it has been shown that wounding could elicit molecular responses also involved in senescence (Le Deunff et al. 2004). Perhaps, in the present work, wounding of N246 resulted in senescence, even in the broth-only control. The response of the N246 genotype to wound inoculation of peduncles indicates that careful selection of plant genotypes for artificial inoculations is critical before extensive use in studies for biological

characterization of interactions between fungi and plants (Miedaner et al. 2003).

Direct application of fungal conidia to sorghum flowers and developing grain would appear to mimic natural infection (Navi et al. 2005). Sorghum grain mold is likely caused by a species complex that may include *Curvularia lunata*, members of the *F. moniliforme* complex, *Alternaria* spp., *Bipolaris* spp., *Cladosporium* spp., or *Phoma* spp. (Navi et al. 2005). Tarekegn et al. (2006) reported that moist and warm conditions post-flowering, including 3 to 19 days of precipitation, were highly favorable for grain mold infections. Attempts have been made to reproduce conditions that were favorable for grain mold infections in the field, following controlled inoculations with known fungal species, by the use of overhead sprinkler systems and bagging (Bandyopadhyay and Mughogho 1988; Forbes et al. 1989). However, reports of greenhouse spray inoculations, in which conidia had been applied to heads and mature grain assessed, are not available. In the present study, we attempted to provide conditions that would allow for successful infection of flowering heads under greenhouse conditions. When large numbers of conidia were applied to each head at anthesis, over a three-day period, during which time heads remained bagged in plastic, biologically relevant results were achieved. However, it also was observed that plants sprayed with water alone had reduced quality of the appearance of the seed, similar to that of plants sprayed with conidia of *F. thapsinum* (Table 3). In maize, it has been suggested that fungi, particularly *F. moniliforme*, could provide protection to kernels from other invading fungi (Kedera et al. 1994). Previously, it was reported that asymptomatic grain and leaf tissue of field-grown sorghum plants could harbor *Fusarium* and *Alternaria* spp., including pathogenic species (Funnell and Pedersen 2006). Perhaps, during the assay conducted in the present study, conditions provided by bagging heads over three days allowed for infection of the developing grain by pre-existing fungi in water controls. Spraying heads with high concentrations of conidia of weakly virulent species may have somehow prevented these internal infections, perhaps by competition or by induction of defense responses (Little and Magill 2003).

Seed inoculations were conducted to determine whether *F. thapsinum* and *F. solani* isolates could infect and inhibit germination of sorghum grain. In four assays, there were no significant differences due to treatment ($P = 0.06$). However, there appeared to be a trend for lower germination rates in fungal-inoculated grain than water-treated grain. During preliminary assays in which RTx430 seeds were incubated with conidial suspensions of the two fungal species or with water, germinated seeds were transplanted and allowed to grow to maturity. When mature grain collected from each head were plated onto agar medium containing the fungicide pentachloronitrobenzene, there were no significant differences in fungal growth from seeds obtained from plants grown from inoculated grain (data not shown). This suggests that if there was systemic growth in the plant, it did not infect grain or it infected grain at a low level. This is consistent with results from maize, in which field-grown plants emerging from *F. moniliforme*-treated seed did not have significantly higher levels of *F. moniliforme*-infected kernels as compared with water-treated seed (Munkvold et al. 1997).

Table 5. Comparison of four strategies for inoculation of sorghum with *Fusarium* or *Alternaria* spp.

Experimental method	Duration (days)*	Plant part analyzed	Analysis required
Wound inoculation-'Wheatland', RTx430	122-134	Peduncle	Split peduncle, measure lesion length
Wound inoculation-N247	80-91	Peduncle	Split peduncle, measure lesion length
Spray inoculation	135-188	Mature grain	Weigh, examine grain; germination tests
Seed inoculation	4	Germinated seeds	Count germinated seeds
Seedling inoculation	29-35	Roots, shoot	Examine roots and shoots; measure shoot lesion length; plate and score root pieces

*Length of time to obtain analyzable plant material during the assays.

Previous work showed that *Fusarium* spp. isolated from various sources, including grain of sorghum, pearl millet or maize, or sorghum debris, could cause in vitro infection of sorghum seedlings (Gourley et al. 1977; Leslie et al. 2005). In the present study, an assay was developed to infect sorghum seedlings in a highly controlled and rapid manner using seedlings transplanted and grown on MS medium. This medium had been previously modified to enhance growth of sorghum and included a relatively low concentration of sucrose (-90 mmol/L) (Elkonin and Pakhomova 2000; Tomes 1985). All of the sorghum genotypes tested appeared to be susceptible to *F. thapsinum* following application of a large number of spores near the stalk, on the MS medium, as indicated by stalk lesion lengths. In contrast, *F. solani* produced stalk lesions that were not significantly different from the water control on nearly all genotypes except Tx2927 (Table 4). However, at least some genotypes also appeared to be infected by *F. solani* and *A. alternata* isolates that were weakly virulent in other assays. In particular, roots were highly discolored, which could be due to an accumulation of phenolic compounds in response to challenge by these fungi (Huang and Backhouse 2005). Additionally, all of the genotypes tested had roots infected as a result of application of at least one of these fungi (Table 4). *Fusarium solani* is commonly isolated from sorghum field debris (Leslie et al. 1990), whereas sorghum grain and leaf tissue are colonized by *Alternaria* spp. (Funnell and Pedersen 2006). In the present work, whether the high levels of conidia, the sucrose levels in the MS medium, or other conditions in the Magenta vessels resulted in root infections by these fungi is not known. *Fusarium* conidia can be induced to germinate at quite low levels of sucrose (-15 mmol/L; Beyer et al. 2004). On the other hand, Gómez-Ariza et al. (2007) have shown that when 300 mmol/L sucrose was added to liquid MS medium in which rice was being grown, plant defense responses were "primed" prior to infection by pathogens, including *F. verticillioides*. It is not known whether the addition of sucrose to the plant growth medium affected the interaction of fungi with sorghum.

In summary, each of the four bioassays were able to provide information about individual fungal isolates associated with sorghum or plant materials for further study of fungal interactions with sorghum. The use of an early maturing line, such as N247, appeared to considerably shorten the duration of the wound inoculation bioassay (Table 5), which was useful to compare different fungal species associated with sorghum. The spray, seed, and seedling inoculation as-

says may also identify plant materials for investigations of gene expression, enzyme activities, or products of secondary metabolism during sorghum-fungal interactions (Muthukrishnan et al. 2001; Salzman et al. 2005).

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