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Isolation and characterization of the grain mold fungi *Cochliobolus* and *Alternaria* spp. from sorghum using semiselective media and DNA sequence analyses

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ARTICLE

Isolation and characterization of the grain mold fungi *Cochliobolus* and *Alternaria* spp. from sorghum using semiselective media and DNA sequence analyses

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Abstract: Mold diseases, caused by fungal complexes including *Alternaria*, *Cochliobolus*, and *Fusarium* species, limit sorghum grain production. Media were tested by plating *Fusarium thapsinum*, *Alternaria* sp., and *Curvularia lunata*, individually and competitively. Dichloran chloramphenicol rose bengal (DRBC) and modified V8 juice (ModV8) agars, found to be useful, were compared with commonly used agar media, dichloran chloramphenicol peptone (DCPA) and pentachloronitrobenzene (PCNB). Radial growth, starting with mycelia or single-conidia and hyphal tips, demonstrated an effect of media. For isolation of grain fungi, DRBC and ModV8 were similar or superior to DCPA and PCNB. When seedlings were inoculated with conidia of *C. lunata*, *Alternaria* sp., *F. thapsinum*, or mixtures, the percentage of root infection ranged from 28% to 77%. For mixed inoculations, shoot weights, lesion lengths, and percentage of root infections were similar to *F. thapsinum* inoculations; most colonies recovered from roots were *F. thapsinum*. For *Alternaria* grain isolates, 5 morphological types, including *Alternaria alternata*, were distinguished by colony morphologies and conidial dimensions. Sequence analysis using a portion of the endo-polygalacturonase gene was able to further distinguish isolates. *Cochliobolus* isolates were identified morphologically as *C. lunata*, *Curvularia sorghina*, and *Bipolaris sorghicola*. Multiple molecular genotypes were apparent from rRNA internal transcribed spacer region sequences from *Cochliobolus* grain isolates.

Key words: Alternaria, *Cochliobolus*, *Fusarium*, semiselective media, *Sorghum bicolor*.

Résumé : La moisissure produite par des complexes de champignons formés des espèces *Alternaria*, *Cochliobolus* et *Fusarium* limite la production de sorgho. Des milieux de culture ont été étudiés en ensemençant *Fusarium thapsinum*, *Alternaria* sp. et *Curvularia lunata* de manière individuelle et compétitive. La gélose Dichloran Rose-bengale Chloramphénicol (DRBC) et la gélose au jus V8 modifiée (ModV8), qui s'étaient avérées utiles, ont été comparées à d'autres géloses couramment utilisées, la gélose Dichloran Chloramphénicol Peptone (DCPA) et la gélose pentachloronitrobenzène (PCNB). L'examen de la croissance radiale, commençant par les mycéliums ou les conidies uniques et les têtes des hyphes, a démontré que les milieux exerçaient un effet. Les milieux DRBC et ModV8 étaient similaires ou supérieurs au DCPA et au PCNB quant à l'isolement des champignons des céréales. Lorsque les semis étaient inoculés avec les conidies de *C. lunata*, *Alternaria* sp., *F. thapsinum* ou des mélanges de celles-ci, le pourcentage d'infection des racines allait de 28 % à 77 %. La taille des pousses, la longueur des lésions et le pourcentage d'infection obtenus après une inoculation mixte ou une inoculation avec *F. thapsinum* étaient similaires ; la plupart des colonies récupérées des racines appartenaient à *F. thapsinum.* En ce qui concerne les isolats de *Alternaria* présents sur les céréales, cinq types morphologiques dont *Alternaria alternata* se distinguaient par la morphologie des colonies et les dimensions des conidies. L'analyse de séquence d'une portion du gène codant l'endo-polygalacturonase a permis de distinguer davantage les isolats. Les isolats de *Cochliobolus* ont été identifiés morphologiquement comme étant *C. lunata*, *Curvularia sorghina* et *Bipolaris sorghicola*. De multiples génotypes moléculaires étaient mis en évidence par les séquences de la région transcrite de l'espaceur interne de l'ARNr des isolats de *Cochliobolus* provenant des céréales. [Traduit par la Rédaction]

Mots-clés : Alternaria, *Cochliobolus*, *Fusarium*, milieu semi-sélectif, *Sorghum bicolor*.

Introduction

Sorghum has been developed for use in food products and for bioenergy (Carpita and McCann 2008; Sarath et al. 2008; Taylor et al. 2006). The responses of some newly developed lines to fungal pathogens have been unexpected. For example, grain of low-lignin lines was less susceptible to *Fusarium* spp. infecting near-isogenic wild-type grain (Funnell-Harris et al. 2010), and sorghum with reduced production of antimicrobial plant pigments was less susceptible to head smut, caused by *Sporisorium reilianum* (Kühn) Langdon & Fullerton, than near-isogenic pigmented lines (D.L. Funnell-Harris, L.K. Prom, S.E. Sattler, and J.F. Pedersen, unpublished). Thus, further research of fungal pathogens in newly developed sorghum lines is warranted.

A major limitation to sorghum production is grain mold disease (Sharma et al. 2010), caused by a fungal complex (Tarekegn et al. 2006) that includes *Fusarium*, *Alternaria*, and *Cochliobolus* species. Infection can significantly reduce grain yield and quality or contribute to deterioration during storage (Castor and Frederiksen 1982; Stack and Pedersen 2003). Several *Fusarium* species infect sorghum grain (Funnell-Harris et al. 2010; Lincy et al. 2011; Sharma et al. 2011), while relatively few species of *Alternaria* and *Cochliobolus* have been reported (Navi et al. 2005; Tarekegn et al. 2006). Commonly isolated species are *Fusarium thapsinum* Klittich, Leslie,

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Nelson & Marasas 1997 (teleomorph *Gibberella thapsina*); *Alternaria alternata* (Fr.) Keissl. 1912; and *Curvularia lunata* (Wakker) Boedijn 1933 (teleomorph *Cochliobolus lunatus* Nelson & Haasis 1964) (Funnell-Harris and Pedersen 2008; Funnell-Harris et al. 2010; Lincy et al. 2011; Navi et al. 2005; Sharma et al. 2011; Tarekegn et al. 2006).

The major routes of grain infection by these fungi are likely to be via the floret and developing grain (Menkir et al. 1996; Navi et al. 2005). It is also possible that grain can be infected systemically from plants grown from infected grain or infected through roots or crowns, as has been observed in other grains (Al-Sadi and Deadman 2010; Murillo-Williams and Munkvold 2008). Thus, the goal of the present study was to identify media that would allow for isolation of *Alternaria*, *Cochliobolus*, and *Fusarium* spp. from sorghum grain as well as more fragile tissues such as roots.

Previously, peptone-based media containing the fungicide *p*entachloro*n*itro*b*enzene (PCNB) (Nash and Snyder 1962) or the fungicide dichloran with the antibiotic chloramphenicol (*d*ichloran *c*hloramphenicol *p*eptone *a*gar or DCPA) (Andrews and Pitt 1986) were commonly used for screening for *Fusarium* and *Alternaria* spp. in grain (Andrews and Pitt 1986; Rabie et al. 1997). The focus of the current study was (*i*) to establish semiselective media that would allow isolation of *Cochliobolus* as well as *Alternaria* and *Fusarium* spp. from sorghum and (*ii*) to morphologically, using culture media, and molecularly, using sequences from DNA regions, characterize species of *Cochliobolus* and *Alternaria* found in sorghum grain. Besides PCNB and DCPA, 2 other media, *d*ichloran *r*ose *b*engal *c*hloramphenicol (DRBC) agar and a modification of V8 juice agar (ModV8) were compared. Field-grown grains were plated onto the 4 media to determine isolation frequency of *Cochliobolus*, *Alternaria*, and *Fusarium* spp. on each medium. The media DRBC and ModV8 also were tested for recovery of sorghum pathogens *C. lunata*, *Alternaria* sp., and *F. thapsinum* from infected seedling roots. *Cochliobolus* and *Alternaria* isolates from sorghum grain were characterized using colony morphologies on media, conidial dimensions, and by comparing sequences from 3 (*Cochliobolus*) or 4 (*Alternaria*) DNA regions with those in GenBank.

Materials and methods

Maintenance of cultures, production of conidia, and storage of fungi

Medium for maintenance of working stocks of fungi was one-half strength potato dextrose agar (PDA), prepared using potato dextrose broth (Becton, Dickinson and Co. (BD), Sparks, Maryland) and amended with 100 μmol/L ampicillin (Sigma-Aldrich (SA), St. Louis, Missouri). The following techniques have proven to work well in this laboratory for production of conidia for use in assays and for storage of most *Fusarium*, *Alternaria*, and *Cochliobolus* grain sorghum isolates. For production of *Fusarium* conidia, an agar block was aseptically cut from a 5-day-old PDA culture and transferred to 1.5% agar containing 80 mmol/L potassium chloride (KCl), then incubated 5–7 days at room temperature. For production of *Alternaria* conidia, mycelia were transferred from a 5-day-old PDA culture by point inoculation at the center of 2% water agar prepared with purified water (Labconco, Kansas City, Missouri), and sterile 1 cm^2 filter paper (Whatman No. 1; Whatman International, Ltd., Maidstone, Great Britain) was placed over the inoculation site; cultures were incubated at least 2 weeks at room temperature. Production of *Cochliobolus* conidia was conducted by point inoculation onto corn meal agar (CMA) (BD); the colony was allowed to grow at room temperature for 7–10 days, after which sterilized 1 cm^2 filter paper was applied at the growing colony edge. The cultures continued to incubate up to 2 more weeks (Pratt 2006).

For preparations of conidia for use in bioassays, conidia were suspended in sterile purified water. To prepare suspensions of *Fusarium* conidia, a small volume of sterile purified water was applied to the surface of the KCl medium, the spores and mycelium were gently dislodged, and the suspension was retrieved by pipetting. To prepare suspensions of *Alternaria* or *Cochliobolus* conidia for use in bioassays, filter paper was aseptically transferred to microcentrifuge tubes, sterile purified water was added, then the suspension was vortexed briefly to dislodge the conidia.

To prepare conidial suspensions for long-term storage of fungi, isolates of the 3 genera were grown as described above to induce production of conidia. For *Alternaria* and *Curvularia* isolates, the filter paper was aseptically transferred to microcentrifuge tubes with 2 mL of sterile skim milk (BD) and vortexed as above. For *Fusarium* isolates, 1 mL of sterile skim milk was applied to the KCl culture, and a conidial suspension was obtained as above. Conidial suspensions were maintained on ice throughout the process of preparing fungi for long-term storage. For all 3 genera, 300 μL of each conidial suspension was evenly distributed over precooled sterile silica gel (3 mL vol in 15 mL screw cap tubes) (Grace Davison, Columbia, Maryland) (Windels et al. 1988). Another 300 μL of each conidial suspension was added to an equal volume of 80% glycerol in cryovials. Finally, each suspension was aseptically streaked onto PDA slants, using a loop, and allowed to grow 3–5 days at room temperature. The slant cultures and freshly prepared silica gel stocks were stored at 4 °C while the glycerol stocks were stored at −80 °C.

Semiselective media

Semiselective media tested were as follows. DRBC agar medium, a general purpose fungal medium, was prepared with 0.5% (*m*/*v*) peptone, 1.0% (*m*/*v*) glucose, 7 mmol/L potassium phosphate, monobasic, 2 mmol/L magnesium sulfate, heptahydrate, 1.5% (*m*/*v*) agar, with the following added to the warm, sterilized solution, 10 μmol/L dichloran (SA), 25 μmol/L rose bengal (SA), and 300 μmol/L chloramphenicol (SA), each added in solution prepared in either ethanol (dichloran and chloramphenicol) or sterile purified water (rose bengal). A medium made with the nonionic surfactant Tergitol-7 (nonylphenol ethoxylate), previously used in selective medium for *Cochliobolus* spp. (Yang 1973), was prepared by amending Tergitol-7 agar (Fluka BioChemika, Buchs, Switzerland) with 0.011% (*m*/*v*) PCNB; 700 μmol/L streptomycin sulfate (Fisher BioReagents, Fair Lawn, New Jersey, USA) was added to the warm, sterilized medium (designated as Ter). A V8-juice-based agar medium, amended with nalidixic acid and chlortetracycline ($V8^+$), useful for isolating fungi from environmental samples, was prepared as previously described (Wilson 2002). Also prepared was a modification of this medium (ModV8) that had reduced quantities of antimicrobials. It contained 20% (*v*/*v*) V8 juice (Campbell Soup Co., Camden, New Jersey), 30 mmol/L calcium carbonate, 2.0% (*m*/*v*) agar, which is then autoclaved, and to this warm, sterilized solution, 1.0 mmol/L nalidixic acid (SA) and 500 μmol/L chlortetracycline hydrochloride (SA) are added. Nalidixic acid was added in a filter-sterilized (Millex-GV 0.22 μmol/L filter unit, Millipore S.A., Molsheim, France) solution prepared with 100 mmol/L nalidixic acid (SA) and 100 mmol/L sodium hydroxide in sterile purified water. Also utilized in this study were DCPA and PCNB, which have been commonly used for screening for *Fusarium* and *Alternaria* spp. from sorghum (Andrews and Pitt 1986; Nash and Snyder 1962).

Media for morphological characterization of *Alternaria* and *Cochliobolus* spp.

The following media were used for morphological characterization of *Alternaria* isolates (Dugan and Peever 2002; Simmons 2007):V8 juice agar (lacking amendments) (V8), one-half-strength V8 juice agar (also lacking amendments), full-strength PDA (lacking ampicillin), malt extract agar (MEA; prepared from malt extract broth (BD)), dichloran rose bengal yeast extract sucrose agar (DRYES), and potato carrot agar (PCA) (HiMedia Laboratories,

Pvt. Ltd., Mumbai, India). DRYES consisted of 2% yeast extract, 15% sucrose, and 2% agar amended with 10 μmol/L dichloran, 25 μmol/L rose bengal, and 300 μmol/L chloramphenicol. For morphological identification of *Cochliobolus* isolates, V8 and water agar were used.

Selection of *Cochliobolus*, *Alternaria*, and *Fusarium* on different media

Testing of semiselective media for growth of C. lunata, Alternaria *sp., and* F. thapsinum *colonies*

To determine which media among 4 would be potentially useful for isolation of fungi from sorghum tissues, a preliminary screen of different media, for growth and selectivity of representative species from *Cochliobolus*, *Alternaria*, and *Fusarium*, was conducted. The media to be tested were Ter, DRBC, V8$^+$, and ModV8; CMA was also included since the 3 fungi grew well upon this medium. Agar media were prepared in 100 mm Petri dishes. Fungal species commonly isolated from sorghum grain were grown for production of conidia. To represent a common *Fusarium* grain pathogen, isolate M-3790 (Fusarium Research Center, Pennsylvania State University, University Park, Pennsylvania), which was identified morphologically (Klittich et al. 1997) and molecularly (Funnell-Harris and Pedersen 2008) as *F. thapsinum* (teleomorph *G. thapsina*), was used. Throughout the manuscript, this isolate will be known by the more familiar *F. thapsinum*. *Alternaria* sp. grain isolate H02-781S-3b, which was isolated at Lincoln, Nebraska (Funnell-Harris and Pedersen 2008), was used to represent *Alternaria* spp. To represent *Cochliobolus* isolates, LP09-1, which was previously identified morphologically as *C. lunata* (Little and Magill 2003) (teleomorph *Co. lunatus*), was used.

Conidia of each fungus were produced as described above. Conidial suspensions were prepared in sterile purified water, quantified using a haemocytometer, and diluted to 5×10^3 conidia/mL; 150 μL was spread onto each medium. Conidial suspensions of each fungus and a conidial mixture of *C. lunata*, *F. thapsinum*, and *Alternaria* sp. (2:1:1) were prepared. Three plates per treatment were inoculated and Petri dishes were incubated at 25 °C in the dark for 5–6 days. Number of colonies and range of colony diameters were determined for each plate; the mean of each range was calculated.

The experimental design was completely randomized and each treatment combination was replicated 3 times. The experiment was conducted twice. Data were analyzed using the PROC MIXED procedure of SAS/STAT software (SAS 2002–2008). Repetitions of the experiment and replications of treatment combinations within experiment repetitions were considered random effects. Media and inoculum were considered fixed. The model was factorial including all interactions. The KENWARD-ROGER option was specified for estimating degrees of freedom. Least squares means were generated and differences among treatment main-effect least squares means, and among least squares means of treatment combinations were compared using the DIFF option.

Since this was a preliminary screen, the results are summarized here (data not shown). For *C. lunata*, the greatest amount of growth on semiselective media was observed on ModV8 medium. When inoculated with *Alternaria* sp. conidia, the mean colony diameters on DRBC and ModV8 were greater than on other semiselective media. For inoculation with *F. thapsinum* conidia, growth on ModV8, V8$^+$, and DRBC was similar ($P \geq 0.07$).

To determine the ability to distinguish *C. lunata* from the other 2 fungi on the different semiselective media, putative *C. lunata* colonies were transferred from each medium onto half-strength PDA for colony identification. Transferred colonies from all media grew on PDA in the first assay; during the second assay, only 50% of colonies transferred from Ter grew on PDA. *Curvularia lunata* colonies could be readily distinguished on CMA, DRBC, ModV8, or V8$^+$ and less so on Ter. Upon assessing these results, DRBC and ModV8 were chosen for further analyses.

Radial growth bioassays

Radial growth bioassays were performed using the *F. thapsinum*, *Alternaria* sp. and *C. lunata* isolates described above. Two experiments were conducted. Since it previously was demonstrated that mycelium is apparent in mature grain following artificial infection of sorghum heads with *F. thapsinum* (Little et al. 2012), one experiment was designed using mycelial mats as starting inoculum. To account for the possibility of relatively few viable fungal cells remaining in mature grain, a second experiment was designed with single germinated conidia, or hyphal tips from single germinated conidia as starting inoculum.

For the first experiment with mycelial mats as starting inoculum, growth rates were determined on 5 media: half-strength PDA (lacking amendments), DCPA, PCNB, DRBC and ModV8. Mycelium was prepared in the following way. A spore suspension was spread onto half-strength PDA (lacking amendments) then incubated 2–3 days in a moist chamber at 28 °C. At that time a layer of mycelia covered the medium in each plate. A number 2 cork-borer (radius 3 mm) was used to cut agar disks from each inoculum plate. One agar disk was placed on the edge of each medium, prepared in 35 mm Petri dishes. Three plates per treatment were prepared. The plates were incubated in a moist chamber at 28 °C. Measurements of radial distance from the edge of the disk nearest the center of the medium were taken daily for 4 days, using a dissecting microscope.

The experimental design was completely randomized and each treatment combination was replicated 3 times. The experiment was conducted 3 times. Data were analyzed using the PROC MIXED procedure of SAS/STAT software (SAS 2002–2008). Each inoculum was analyzed separately. Repetitions of the experiment were considered random effects. Media were considered fixed effects. Measurements across time on the same plate were considered repeated measures. The KENWARD-ROGER option was specified for estimating degrees of freedom. Slopes were output using the SOLUTION option and the NOINT option was used to force the intercept to zero at the initiation of the experiment. Confidence intervals for the slopes were set at $P = 0.05$ using the CL option. Comparisons among media were made using single degree of freedom contrasts.

Radial growth bioassays also were performed to determine the growth rates of *F. thapsinum*, *Alternaria* sp., *C. lunata* and 2 other *Cochliobolus* isolates (H05-531S-2 and H05-557S-1), from either hyphal tips or germinated single conidia on 3 media: PDA (lacking amendments), ModV8 and DRBC. Conidial suspensions from each fungus were prepared in sterile purified water and spread onto half-strength PDA amended with 200 μmol/L ampicillin (SA) prepared in 100 mm Petri dishes; plates were incubated 16 to 20 h at 25 °C. Agar blocks no larger than 1 mm^3, each containing a germinated conidium or a hyphal tip from a germinated conidium, were aseptically cut from the PDA cultures and placed at the edge of media prepared in 35 mm Petri dishes. Five plates per treatment were prepared and incubated in a moist chamber at 25 °C. Survival on each medium was indicated by the presence of growth, as determined using a dissecting microscope, on each of 5 plates. Measurements of radial distance from the edge of the block closest to the center of the plate were taken daily for 4 days, also using a dissecting microscope.

The experimental design was completely randomized and each treatment combination was replicated 5 times. The experiment was conducted 3 times. Data were analyzed using the PROC MIXED procedure of SAS/STAT software (SAS 2002–2008). Each inoculum was analyzed separately. Repetitions of the experiment were considered random effects. The model used to analyze the spore survival data included media as its single fixed factor. The KENWARD-ROGER option was specified for estimating degrees of

freedom. Least squares means were generated, and the differences among media least squares means were compared using the DIFF option. Media was considered a fixed effect in the model used to analyze radial growth, and measurements across time on the same plate were considered repeated measures. The KENWARD-ROGER option was specified for estimating degrees of freedom. Slopes were output using the SOLUTION option and the NOINT option was used to force the intercept to zero at the initiation of the experiment. Confidence intervals for the slopes were set at $P = 0.05$ using the CL option. Comparisons among media were made using single degree of freedom contrasts.

The data were reanalyzed to compare growth of the 5 fungi on PDA to determine whether isolates grew at similar rates on this medium. The model used for this analysis considered repetitions of the experiment to be random effects. Fungal isolates were considered fixed effects. Measurements across time on the same plate were considered repeated measures. The KENWARD-ROGER option was specified for estimating degrees of freedom. Slopes were output using the SOLUTION option and the NOINT option was used to force the intercept to zero at the initiation of the experiment. Confidence intervals for the slopes were set at $P = 0.05$ using the CL option. Comparisons among fungal inoculums were made using single degree of freedom contrasts.

Isolation of *Cochliobolus*, *Alternaria*, and *Fusarium* spp. from field-grown grain using semiselective media

The media ModV8, DRBC, PCNB, and DCPA were used to screen for *Cochliobolus*, *Alternaria*, and *Fusarium* spp. from field-grown grain. Sorghum was planted at Ithaca and Lincoln, Nebraska, in 2005, and at Corpus Christi, Texas, in 2008. For details of sorghum lines planted see Pedersen and Toy (2001) (Pedersen and Toy 2001). Plots consisted of two 7.6 m rows spaced 0.76 m apart at Ithaca and Lincoln and of two 5.2 m rows spaced 1.0 m apart at Corpus Christi. Plots at Ithaca were irrigated with overhead sprinklers, 5 times through the growing season, 3.8 cm per application. Plots at Lincoln and Corpus Christi were not irrigated. The field experimental design at all environments was a randomized complete block with 4 replications.

At grain maturity, 5 randomly chosen heads were removed from each plot, dried, then the mature seeds were threshed and the chaff was removed. Surface infection of mature grain was not apparent. Therefore, to screen for internal infections, probably due to infections of flowers or developing grain, grain was surface sterilized to remove incidental spores or mycelium. Grain from each field plot was washed for 2 min in 95% ethanol followed by a 10 min wash in 1% sodium hypochlorite with 0.01% Tween 20, rinsed 3 times in sterile purified water then dried in a laminar-flow hood. Five seeds were aseptically applied to each medium. There were 4 replicate plots of each line, and 10 lines, thus, 200 seeds per location, or 600 total seeds were screened per medium.

Individual colonies growing from each seed onto the medium were transferred to PDA. After a preliminary identification to genus, transfers were made to the appropriate media for inducing sporulation, as described above. For preliminary identification, Simmons (1967, 1990, 1999) and Simmons and Roberts (1993) were utilized for *Alternaria* isolates, Barnett and Hunter (1972) and Ellis (1971) were utilized for *Cochliobolus* isolates, and Leslie and Summerell (2006) and Nelson et al. (1983) were utilized for *Fusarium* spp. (Barnett and Hunter 1972; Ellis 1971; Leslie and Summerell 2006; Nelson et al. 1983; Simmons 1967, 1990, 1999; Simmons and Roberts 1993). Colony morphology on PDA and conidiophore structures and conidial types were initially used for identification. Fungi per 100 seeds were enumerated for each medium. Groups analyzed were *Cochliobolus*, *Alternaria*, *Fusarium*, and other fungi ("Other").

The experimental design at each location was a randomized complete block with 4 replications. Data were analyzed using the PROC MIXED procedure of SAS/STAT software (SAS 2002–2008). Each fungal group was analyzed separately. Location and replications within location were considered random effects. The model used to analyze fungal numbers included media as its single fixed factor. The KENWARD-ROGER option was specified for estimating degrees of freedom. Least squares means were generated, and differences among media least squares means were compared using the DIFF option.

Inoculation of seedlings with conidia of *C. lunata*, *Alternaria* sp., *F. thapsinum*, or a mixture of the 3 species

Previous studies had utilized DCPA for root isolations because PCNB medium had been considered too harsh when applied with some plant tissues (Funnell-Harris and Pedersen 2008; D.L. Funnell-Harris and J.F. Pedersen, unpublished). The purpose of the present assay was to test 2 media, DRBC and ModV8, for ability to recover *C. lunata*, *Alternaria* sp., and *F. thapsinum* from infected root tissues. This assay also allowed assessment of competition of the 3 fungi in the root zone of sorghum, under conditions of the assay, by using mixed inoculations.

Seedlings of sorghum 'RTx430' were grown with slight modifications in Magenta boxes with plant growth medium (Funnell-Harris and Pedersen 2008). Briefly, greenhouse-grown seeds were surface sterilized and germinated on 0.6% water agar in a growth chamber for 5–7 days at 25 °C, 16 h light. Germinated seeds were transferred to Magenta boxes prepared with Murashige and Skoog basal growth medium with Gamborg's vitamins (MP Biomedicals, LLC, Solon, Ohio) modified for sorghum plant culture (Funnell-Harris and Pedersen 2008). Seedlings were returned to the growth chamber and grown for 8 more days with the same temperature and light regime. Conidial suspensions of each fungus, *C. lunata*, *Alternaria* sp., and *F. thapsinum*, and of a mixture of *C. lunata*, *Alternaria* sp., and *F. thapsinum* (1:1:1) were prepared in sterile purified water as described above, and 1.25×10^3 conidia were applied at the base of the stalk of each seedling. Control plants were inoculated with sterile purified water. Plants were arranged in a growth chamber in 9 or 11 blocks, each block with 5 treatments. The seedlings were grown at 22 °C, 12 h light, for 15–21 days and then scored. Percent discoloration of roots or shoots, shoot weight, and stalk lesion length were recorded. Percent root infection was determined by plating 1 cm lengths of roots onto 2 media, DRBC and ModV8. After incubation at room temperature, the root pieces were scored 28–42 h after plating, using a dissecting microscope. At 42 h after plating, from infected root pieces resulting from mixed inoculation of the 3 fungal species, colonies were transferred onto half-strength PDA using a dissecting microscope to discern small colonies. The PDA plates were incubated at room temperature, and 5–7 days later the fungal species were identified to determine recovery of fungi from infected roots from each semiselective medium. The entire experiment was conducted 2 times.

The experimental design for each repetition of the experiment was a randomized complete block with 9 replications in 1 repetition, and 11 replications in the other repetition of the experiment. Data were analyzed using the PROC MIXED procedure of SAS/STAT software (SAS 2002–2008). Repetition, replications within repetitions, and position of samples within replications were considered random effects. The model included inoculum as its single fixed factor. The KENWARD-ROGER option was specified for estimating degrees of freedom. Least squares means were generated, and differences among least squares means were compared using the DIFF option. Pearson correlations were generated for all response variables using SAS Proc Corr software (SAS 2002–2008). Chi-square tests for differences in percent root infection attributable to DRBC and ModV8 media were made using SAS Proc Freq software (SAS 2002–2008).

Morphological identification of *Alternaria* and *Cochliobolus* spp.

Identification of *Alternaria* spp. by characteristics when grown on different media was conducted as described by Dugan and Peever (Dugan and Peever 2002; Simmons 2007). The agar media, V8, half-strength V8, full-strength PDA, MEA, DRYES, and PCA, were prepared in 60 mm Petri dishes. Nine sorghum grain isolates (CC06-61S-2, H02-747S-5, H02-755S, H02-781S-3b, M05-1541S-1 (ModV8), M05-1551S-4 (DCPA), M05-1551S-4 (ModV8), M05-1553S-3b (DCPA), M05-1561S-5 (DRBC)), representing differing morphological types when grown on half-strength PDA and water agar, were transferred from half-strength PDA by point inoculation to each medium. Cultures were incubated at 22 °C, 8 h light, 33 cm from fluorescent lamps (daylight conditions). Duplicate DRYES plates were incubated at 25 °C, in darkness. Morphological descriptions of V8, half-strength V8, full-strength PDA, MEA, and DRYES cultures were recorded 4–17 days after inoculation, depending on isolate; light- and dark-grown DRYES cultures were scored the same day for a given isolate. PCA cultures were scored 6–18 days after inoculation. Conidial and conidiophore morphologies, as well as colony morphology, were recorded. Dimensions of 45–71 conidia, taken from 3–5 PCA plates per isolate, were determined, and means and standard deviations of length and width were calculated. For 3 isolates, conidiation on PCA medium was poor, and due to mycelial growth, conidiophore morphology was difficult to distinguish. Therefore, these isolates were also grown on water agar with filter paper, using daylight conditions 18–25 days to determine conidiophore morphology. Using colony, conidia and conidiophore morphologies, and mean conidia dimensions, *Alternaria* isolates were placed into morphologically similar groups.

Morphologically based identification of 4 *Cochliobolus* isolates, representing different morphological types on half-strength PDA and CMA, was conducted in this way. Transfers were made by point inoculation to V8 medium and cultures were grown 5 days, 33 cm under 8 h of fluorescent light at 22 °C. Agar blocks were aseptically cut and transferred to water agar; plates were incubated in the same way 7–11 days before scoring morphologies of conidia and conidiophores and determining conidia dimensions. Species identifications were made in accordance with Sivanesan (1987).

Molecular analysis of *Cochliobolus* and *Alternaria* spp. from sorghum grain

DNA sequences from 3 loci for *Cochliobolus* and 4 loci for *Alternaria* isolates from sorghum grain were analyzed. DNA was extracted, as previously described (Lee and Taylor 1990), from lyophilized mycelia grown in full-strength potato dextrose broth. The internal transcribed spacer (ITS) region was amplified using ITS5 and ITS4 primers and conditions as previously described (O'Donnell et al. 1998; White et al. 1990). A region in the mitochondrial small subunit of the rRNA gene (mt SSU) was amplified using primers NMS1 and NMS2, also as previously described (Li et al. 1994). A portion of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) was amplified using primers *gpd1* and *gpd2* (Berbee et al. 1999). A 50 μL volume reaction was prepared with 1.5 mmol/L $MgCl_2$, 400 μmol/L dNTPs, 20 pmol of each primer, 2.5 units of Amplitaq Gold (Applied Biosystems, Life Technologies Corporation, Carlsbad, California), and 1× buffer. Reactions were incubated at 96 °C for 2 min, then there were 40 cycles of 96 °C for 1 min, 48 °C for 1 min, and 72 °C for 45 s for the first cycle with a 3 s increase per cycle thereafter, followed by a final extension at 72 °C for 7 min. For amplifications of these loci, products of the expected sizes were purified using Amicon Ultra-0.5 Centrifugal Filter Device (Millipore, Billerica, Massachusetts). Occasionally, multiple products resulted; in these cases, bands of the expected size were gel-purified using the E.Z.N.A. Ultra-Sep Gel Extraction kit (Omega bio-tek, Norcross, Georgia).

Amplification of the endo-polygalacturonase gene (endo-PG) from *Alternaria* spp. isolates was conducted using the reaction mixture described above with primers PG2 and PG3 (Peever et al. 2002). The amplification conditions were 5 min of incubation at 95 °C, then 50 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. All products of the appropriate size (Peever et al. 2002) were gel-purified as described above. In most cases, reamplification of PCR products was conducted to obtain adequate product for sequencing, using the conditions just described with 0.5–2.0 μL of the gel-purified eluent and reducing the cycles to 25. Products of reamplification were gel-purified.

Sequencing of purified PCR amplification products was conducted using primers used for amplification. For ITS, *gpd*, and mt SSU, both primers were utilized and sequences from opposite strands were assembled using Sequencher 4.10.1 (Gene Codes Corp., Ann Arbor, Michigan, USA). Assembled sequences were compared with those in the nucleotide collection in GenBank using megablast, and submitted to GenBank (Table 1). In the case of endo-PG, only the 5′ primer (PG3) was used for sequencing. Sequences were trimmed as needed and compared with those in GenBank. In addition to *Cochliobolus* spp. listed in Table 1, sequences of ITS regions from 16 additional *Cochliobolus* isolates also were determined and submitted to GenBank (accession Nos. JX960581–JX960596).

Primers were purchased from Life Technologies (Grand Island, New York) and Bioneer (Alameda, California). Sequencing was conducted at Eton Bioscience, Inc. (San Diego, California) and University of Arkansas for Medical Sciences (Little Rock).

Results

Radial growth of *C. lunata*, *Alternaria* sp., and *F. thapsinum* on semiselective media

Relative growth rates (mm/day) of *C. lunata*, *Alternaria* sp., and *F. thapsinum* from disks of mycelia on the media DRBC and ModV8 were compared with those on PDA, DCPA, and PCNB. There were significant effects of medium on growth of each fungal isolate ($P < 0.01$). For all 3 fungi, growth on ModV8 was faster than on DRBC ($P < 0.01$) (Table 2). Relative growth rates of *C. lunata*, *F. thapsinum*, and *Alternaria* sp., and 2 additional *Cochliobolus* sp. isolates, starting from a single germinated conidium or the hyphal tip of a germinated conidium, on ModV8, DRBC, and PDA, were also compared. There were significant effects of medium on growth of *C. lunata*, and one other *Cochliobolus* isolate (H05-557S-1) ($P < 0.01$). In this assay, when inoculum was relatively small, all *Cochliobolus* isolates and the *Alternaria* sp. isolate grew faster on DRBC than ModV8 (Table 3A). There were no significant effects of medium on survival of single germinated spores or hyphal tips ($P \geq 0.24$) (Table 3B). Percent survival was consistently lower on ModV8, but the differences were not significant in pairwise comparisons ($P \geq 0.27$). Additionally, there were no significant differences in growth rates of the different fungi on PDA medium ($P = 0.61$).

Isolation of *Cochliobolus*, *Alternaria*, and *Fusarium* spp. from field-grown grain using 4 semiselective media

Grain grown at 3 environments were screened on 4 semiselective media (DRBC, ModV8, DCPA, and PCNB). Fungal colonies growing from the grain and onto each medium were collected, and the numbers of *Cochliobolus*, *Alternaria*, and *Fusarium* spp., as well as other fungi, were enumerated. Medium had a significant effect on numbers of colonies collected in each fungal category ($P \leq 0.01$) (Table 4). Selection on DRBC and ModV8 resulted in the greatest number of *Cochliobolus*, *Alternaria*, and *Fusarium* isolates, and both media yielded significantly more *Fusarium* spp. colonies per 100 grains than did PCNB. DRBC and ModV8 did not allow significantly more growth of other fungi than DCPA, another

Table 1. Representative *Alternaria* and *Cochliobolus* isolates from sorghum grain, characterized morphologically and molecularly in this study.

Isolate No.	Medium*	Environment†	GenBank acc. No.‡
Alternaria			
H02-711S-2	DCPA	Lincoln, Nebraska, 2002	JX960578, ITS EF152429, mt SSU JX960574, *gpd*
H02-747S-5	DCPA	Lincoln, Nebraska, 2002	JX960576, ITS EF152432, mt SSU
H02-781S-3b	DCPA	Lincoln, Nebraska, 2002	JX960577, ITS EF152430, NMS JX960575, *gpd*
M05-1541S-1	Mod V8	Ithaca, Nebraska, 2005	JN634835, ITS JN634842, mt SSU JN634821, *gpd*
M05-1551S-4	DCPA	Ithaca, Nebraska, 2005	JN634833, ITS JN634840, mt SSU JN634819, *gpd*
M05-1551S-4	ModV8	Ithaca, Nebraska, 2005	JX960579, ITS JN634843, mt SSU JN634822, *gpd*
M05-1553S-3b	DCPA	Ithaca, Nebraska, 2005	JN634834, ITS JN634841, mt SSU JN634820, *gpd*
M05-1561S-5	DRBC	Ithaca, Nebraska, 2005	JN634836, ITS JN634844, mt SSU JN634823, *gpd*
M05-1557S-4	ModV8	Ithaca, Nebraska, 2005	JN634837, ITS JN634845, mt SSU JN634824, *gpd*
Cochliobolus			
H05-505S-1	DRBC	Lincoln, Nebraska, 2005	JN700926, ITS JN634827, *gpd*
H05-531S-2	DRBC	Lincoln, Nebraska, 2005	JN634839, ITS JN634847, mt SSU JN634826, *gpd*
H05-557S-1	ModV8	Lincoln, Nebraska, 2005	JX960580, ITS JN634848, mt SSU JN634828, *gpd*
LP09-1§			JN634838, ITS JN634846, mt SSU JN634825, *gpd*

*Semiselective media used to isolate *Alternaria* and *Cochliobolus* spp. from sorghum grain were dichloran chloramphenicol peptone agar (DCPA), dichloran rose bengal chloramphenicol agar (DRBC), and modified V8 agar medium containing nalidixic acid and chlortetracycline (ModV8).

†"Environment" is the location and year grain was grown from which fungal isolate came.

‡Accession numbers for sequences from the internal transcribed spacer region from rDNA (ITS), mitochondrial small subunit of the rDNA region (mt SSU), and a portion of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*).

§For details about isolate see Little and Magill (2003).

medium used for isolation of *Fusarium* and *Alternaria* spp. (Table 4).

Inoculation of seedlings with conidia of *C. lunata*, *Alternaria* sp., and *F. thapsinum* and isolation of these fungi from roots

Following inoculation of seedlings with conidia of *C. lunata*, *Alternaria* sp., or *F. thapsinum*, with a mixture of the 3 fungi (1:1:1), or with sterile water, percent fungal recovery from roots were determined by excising root pieces from each plant and plating them onto DRBC and ModV8 agar. Isolation of fungi from roots was highly correlated for the 2 media ($r^2 = 0.84$; $P < 0.01$). Means and standard errors for each measurement are shown in Table 5. Treatment was significant for all measurements ($P < 0.01$). Inoculations of sorghum seedlings with *Alternaria* sp. and *F. thapsinum* conidia significantly increased root and shoot discoloration, shoot lesion length, and percent recovery from roots, as determined on both media, and decreased shoot mass, as compared with the water control ($P < 0.01$), similar to what has been previously observed (Funnell-Harris and Pedersen 2008). When seedlings were inoculated with *C. lunata* conidia, all measurements were significantly different from the water control ($P \leq 0.03$). For lesion length and percent shoot discoloration, measurements were significantly less on plants inoculated with *C. lunata* conidia than on those inoculated with *F. thapsinum* conidia ($P < 0.01$). Mean shoot mass of plants inoculated with *C. lunata* was significantly greater than of plants inoculated with *Alternaria* sp. or *F. thapsinum* conidia ($P \leq 0.02$) (Table 5). Inoculation of seedlings with *C. lunata* conidia resulted in percent root discoloration similar to that following inoculation with *F. thapsinum* ($P = 0.19$) and significantly less than that resulting from inoculation with *Alternaria* sp. conidia ($P < 0.01$). However, mean percent *C. lunata* recovery from roots, as determined on 2 media, was significantly greater than that following inoculation with *F. thapsinum* conidia ($P < 0.01$) and similar to that resulting from inoculation with *Alternaria* ($P \geq 0.10$).

When a mixture of conidia from the 3 fungal species was inoculated onto seedlings, the responses were similar to those obtained when *F. thapsinum* alone was inoculated onto seedlings, especially when considering measurements for mean shoot mass, lesion length, and percent fungal recovery from roots as determined on both media ($P \geq 0.14$) (Table 5). *Fusarium thapsinum* was most frequently recovered (62%) from infected roots resulting from inoculations with conidial mixtures, and a few *C. lunata* colonies (7%) were recovered from DRBC and ModV8 plates while only 1% of the reisolated colonies were *Alternaria* sp. Thirty-one percent of colonies transferred from the semiselective media did not grow. This rate of recovery may have been due to transfer of very small colonies that may have been damaged in the process; transfer from larger single colonies from these media to PDA did not result in loss of viability (see Materials and methods). Chi-square analyses of *F. thapsinum* and *C. lunata* transferred colonies and attempted transfers that did not grow indicated that there were no significant differences between the 2 media ($P \geq 0.32$).

Table 2. Slopes (radial growth (mm)/day) resulting from regression analyses of radial growth bioassays of sorghum grain fungi grown on different media.

Medium*	*Curvularia lunata*	*Alternaria* sp.	*Fusarium thapsinum*
PDA	6.78a±0.40	5.11a±0.36	2.66a±0.39
ModV8	5.02b±0.40	4.03b±0.36	5.04b±0.39
DCPA	4.84b±0.40	3.46c±0.36	5.09b±0.39
DRBC	3.93c±0.40	3.15c±0.36	4.32c±0.39
PCNB	0.64d±0.17	0.60d±0.15	2.20d±0.16

Note: Slopes calculated from estimates for fixed effects, and standard errors, are reported. For a given fungus, slopes with different letters are significantly different at $P \leq 0.05$.

*PDA is half-strength potato–dextrose, commonly used for growing fungi; ModV8 is a modification of a V8-juice-based agar (see Materials and methods); DCPA (dichloran chloramphencial peptone agar) is a semiselective medium for *Fusarium* spp. and *Alternaria* spp. and other dark-spored ascomycetes (Andrews and Pitt 1986); DRBC (dichloran rose bengal chloramphenicol) is another commonly used medium for selection of fungi; PCNB (pentachloronitrobenzene) is semiselective for *Fusarium* spp. (Nash and Snyder 1962).

Morphological and molecular analyses of representative *Alternaria* isolates from sorghum grain

Representative *Alternaria* spp. isolates obtained from sorghum grain were identified morphologically using the system described by Dugan and Peever (Dugan and Peever 2002). Colony, conidia, and conidiophore morphologies were compared by growing each isolate on 6 media. Isolates were compared with descriptions of species complexes commonly found on grain. Three isolates (H02-747S-5, M05-1553S-3b, and M05-1541S-1) had characteristics that placed them in the *Alternaria alternata* species complex, particu-

Table 3. (A) Slopes (radial growth (mm)/day) resulting from regression analyses of radial growth bioassays; and (B) percent survival of sorghum grain fungi grown from germinated single spores or hyphal tips on different media.

Medium*	*Curvularia lunata*	*Cochliobolus* sp. Isolate H05-557S-1	*Cochliobolus* sp. Isolate H05-531S-2	*Alternaria* sp.	*Fusarium thapsinum*
(A) Slopes†					
PDA	7.30a±0.13	6.86a±0.16	5.70a±0.24	5.04a±0.15	4.31a±0.21
DRBC	1.63b±0.31	1.54b±0.36	1.69b±0.58	1.82b±0.36	1.48b±0.50
ModV8	1.16c±0.31	0.98c±0.37	0.51c±0.60	0.62c±0.39	2.41c±0.51
(B) Percent survival					
PDA	91.1±4.21	91.1±4.21	91.5±5.35	91.1±4.21	91.1±4.21
DRBC	90.1±4.21	90.1±4.21	88.2±5.35	90.1±4.21	90.1±4.21
ModV8	81.5±4.37	81.5±4.37	80.0±5.64	81.5±4.37	81.5±4.37

Note: Slopes calculated from estimates for fixed effects, and standard errors, and least squares means of percent survival (growth present on each of 5 plates per isolate in each of 3 assays) and standard errors, are reported.

*PDA is half-strength potato–dextrose, commonly used for growing fungi; ModV8 is a modification of a V8-juice-based agar (see Materials and methods); DRBC (dichloran rose bengal chloramphenicol) is a commonly used medium for selection of fungi.

†For a given fungus, slopes with different letters are significantly different at $P \leq 0.05$. There were no significant differences between mean percent survival on media for any of the fungi.

Table 4. Mean numbers of *Cochliobolus*, *Alternaria*, and *Fusarium* species, and other fungi, isolated from field-grown sorghum grain on 4 semiselective media.

	Mean numbers of colonies per 100 grains			
Medium*	*Cochliobolus* spp.	*Alternaria* spp.	*Fusarium* spp.	Other
DRBC	4.1a±0.8	61.1a±3.1	2.2a±0.5	4.5a±0.8
ModV8	4.4a±0.8	62.2a±3.1	2.3a±0.5	5.0a±0.8
DCPA	3.1ab±0.8	58.9a±3.2	1.0ab±0.5	5.6a±0.8
PCNB	1.1b±0.8	0.1b±3.1	0.0b±0.5	0.0b±0.8

Note: Least square means and standard errors are shown. Within a fungal group, means with different letters are significantly different at $P \leq 0.05$.

*DRBC (dichloran rose bengal chloramphenicol) is a commonly used medium for selection of fungi; ModV8 is a modification of a V8-juice-based agar (see Materials and methods); DCPA (dichloran chloramphenicol peptone agar) is a semiselective medium for *Fusarium* spp. and *Alternaria* spp. and other dark-spored ascomycetes (Andrews and Pitt 1986); PCNB (pentachloronitrobenzene) is semiselective for *Fusarium* spp. (Nash and Snyder 1962).

larly mean conidial dimensions (length: 19.0 ± 4.7 to 23.1 ± 5.3 μm; width: 9.0 ± 2.0 to 10.7 ± 2.0 μm); relatively short, highly branched conidiophores on PCA medium; and pale olive green to tan flocculent growth on DRYES medium (daylight conditions).

The other 6 isolates did not place well in the *A. alternata* species complex nor in 2 other species complexes (*Alternaria infectoria* and *Alternaria tenuissima*) (Dugan and Peever 2002). The 6 isolates could be placed into 4 morphological groups, with 1 to 2 members, as determined by colony morphology on 6 media, conidial dimensions and conidiophores on PCA or water agar. Conidial dimensions ranged from 22.1 to 28.5 μm (length) by 6.4 to 14.0 μm (width). Conidiophores could be relatively short and unbranched or relatively long with multiple branches. Different colony morphologies on DRYES (daylight conditions) had multiple colors (off-white, pale yellow, beige, tan, or brown) and textures (flocculent or powdery). These isolates were designated as *Alternaria* sp.

Amplification, sequencing, and comparisons with sequences in GenBank, using 4 loci, were conducted to determine whether isolates can be further distinguished using molecular tools. Comparisons of ITS sequences from the above listed 9 isolates indicated that all shared high sequence similarity (100%) to *A. alternata* and *Alternaria* sp. Sequence analysis of the mt SSU region from 8 of the 9 isolates had sequences with high similarity (100%) to sequences from 5 species: *A. alternata*, *Alternaria arborescens* E.G. Simmons 1999, *Alternaria destruens* E.G. Simmons 1998, *Alternaria longipes* (Ellis & Everh.) E.W. Mason 1928, and *Alternaria tenuissima* (Kunze) Wiltshire 1933. A portion of the *gpd* gene was amplified and sequenced from 8 of the 9 isolates described above, revealing that this region from 1 isolate (No. H02-711S-2) had high similarity (99%) to sequences from *A. alternata*, *Alternaria dumosa* E.G. Simmons 1999, and *Alternaria limoniasperae* E.G. Simmons 1999, while the sequences from the other 7 isolates had high similarities (100%) to sequences from 3 *Alternaria* species (*A. arborescens*, *A. destruens*, and *A. tenuissima*). Sequences from a portion of endo-PG were obtained from 6 isolates (M05-1561S-5, H02-747S-5, H02-781S-3b, M05-1553S-3b, M05-1541S-1, and M05-1557S-4). Sequences from all six isolates had high similarity (99%) to a sequence from *A. tenuissima* and most from *A. alternata* (all except M05-1541S-1). Additionally, sequences from some isolates had high similarities (99%) to sequences from *Alternaria citriarbusti* E.G. Simmons 1999 (M05-1541S-1), *Alternaria macrospora* (Sacc.) Mussat 1900 (M05-1553S-3b), *Alternaria mali* Roberts 1914 (M05-1553S-3b), and *Alternaria toxicogenica* E.G. Simmons 1999 (M05-1561S-5, H02-747S-5, H02-781S-3b, and M05-1557S-4).

Morphological and molecular analyses of representative *Cochliobolus*-like isolates from sorghum grain

Cochliobolus isolates chosen to represent morphological types obtained from sorghum grain were identified using the system of Sivanesan (1987). *Cochliobolus* includes the 2 anamorph genera, *Curvularia* and *Bipolaris*. Using conidial dimensions and conidiophore structure, isolate LP09-1 was placed in *C. lunata* (as previously described by Little and Magill (2003), while isolate H05-531S-2 had similarities to *Curvularia sorghina* R.G. Shivas & Sivan. 1987. Isolates H05-505S-1 and H05-557S-1 had characteristics consistent with *Bipolaris sorghicola* (Lefebvre & Sherwin) Alcorn 1983.

Analyses of ITS, mt SSU, and *gpd* sequences from the 4 isolates showed that they all had high similarities (99%–100%) to sequences from *Cochliobolus* (*Cochliobolus geniculatus* R.R. Nelson 1964, *Cochliobolus carbonum* R.R. Nelson 1959, *Cochliobolus victoriae* R.R. Nelson 1960, *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur and *Cochliobolus* sp.) and *Bipolaris* (*Bipolaris zeae* Sivan. 1985) species. Additionally, LP09-1 and H05-557S-1 had high sequence similarities to *Curvularia fallax* Boedijn 1933, *Curvularia affinis* Boedijn 1933, *Curvularia brachyspora* Boedijn 1933, and *Curvularia* sp.

ITS sequences from 18 *Cochliobolus* isolates were compared with one another and with sequences in GenBank, and 10 ITS genotypes were distinguished. Two genotypes had high sequence similarity (97% and 100%) to those from *C. lunatus*, 2 genotypes had high sequence similarity (99% and 100%) to those from *Bipolaris* sp.,

Table 5. Means of shoot percent discoloration, mass (g), and lesion length (mm), and root percent discoloration and percent recovery as determined using 2 semiselective media, following inoculation of sterile sorghum seedlings with *C. lunata*, *Alternaria* sp., *F. thapsinum*, a mixture of the 3 fungi, or sterile water.

Measurement	*Curvularia lunata*	*Alternaria* sp.	*Fusarium thapsinum*	Mixture	Water
Shoots					
% Discoloration	48.8c±4.8	54.0c±4.8	81.2a±4.8	69.5b±4.9	39.6d±5.0
Mass (g)	2.58b±0.19	2.01c±0.19	1.13d±0.19	1.50d±0.19	4.25a±0.21
Lesion length (mm)	6.53b±2.83	9.95ab±2.89	12.72a±2.95	12.42a±2.97	1.09c±2.95
Roots					
% Discoloration	59.8bc±3.0	78.0a±3.1	54.2c±3.0	64.0b±3.2	17.9d±3.4
% Recovery-DRBC	72.5a±9.4	76.5a±9.5	37.8b±9.4	27.9b±9.6	1.2c±9.9
% Recovery-ModV8	67.9a±11.6	74.2a±11.7	29.3b±11.6	34.5b±11.7	0.0c±12.0

Note: Conidial suspensions of each fungus, *C. lunata*, *Alternaria* sp., and *F. thapsinum*, and a mixture of 1 *C. lunata*: 1 *Alternaria* sp.: 1 *F. thapsinum* were prepared in sterile purified water, and 1.25×10^3 conidia were applied at the base of the stalk of each seedling. Least square means and standard errors are reported. For each measurement, means with different letters are significantly different at $P \leq 0.05$. DRBC is a commonly used medium for selection of fungi; ModV8 is a modification of a V8-juice-based agar (see Materials and methods).

and 6 genotypes had high sequence similarity (99%–100%) to those from *Cochliobolus* sp.

Discussion

As quoted from Bragulat and associates (Bragulat et al. 1991), "one ideal medium does not exist". In the present study, a systematic approach was undertaken to determine media that would consistently allow for isolation of *Cochliobolus*, *Alternaria*, and *Fusarium* spp. from sorghum tissues. Cultural techniques for enumerating microorganisms allow for organisms of interest to be in-hand for further investigations. A combination of isolation of culturable fungi using semiselective media, as well as culture-independent methods (Chistoserdova 2010), would be ideal for creating a complete picture of the interactions of fungi with different plant tissues.

A combination of nutrient bases to encourage growth of the desired fungi and antimicrobials that would inhibit growth of other fungi were chosen with the goal of increasing efficiency of isolating *Cochliobolus*, *Alternaria*, and *Fusarium* spp. from sorghum grain. This was achieved with DRBC and ModV8 media. DRBC is a general purpose semiselective medium for enumerating fungi. ModV8 is a modification of classic V8 juice agar, used for growing a wide variety of fungi that prefer acidic conditions and for inducing sporulation in many fungi and yeasts. DRBC, as well as DCPA and PCNB, include peptone as a nutritional source, while ModV8 includes vegetable juice for nutrients. To limit growth of unwanted microorganisms, DRBC and DCPA include the fungicide dichloran and the antibiotic chloramphenicol, although the concentrations of both antimicrobials are less in DRBC (Andrews and Pitt 1986; King et al. 1979). In addition, DRBC includes rose bengal, an indicator dye that inhibits the growth of bacteria and the spread of fungi, making it easier to enumerate individual fungal colonies (King et al. 1979; Ottow 1972). It had been suggested that fungal enzymatic release of chloride ions from rose bengal contributed to this inhibition (Hutchison 1990). PCNB, another peptone-based medium, includes the fungicide pentachloronitrobenzene, which is highly selective against many oomycetes and fungi other than *Fusarium* spp. (Nakanishi and Oku 1969). However, in the present study, it was found that mean numbers of *Fusarium* spp. isolated from sorghum grain on DRBC or ModV8 were greater than those isolated on agar medium containing PCNB.

Inoculation of sorghum seedlings with *C. lunata* conidia in a gnotobiotic system demonstrated the response of sorghum seedlings to root infection under these conditions (Table 5). Percent recovery from roots, as determined on DRBC and ModV8 media, was relatively high (73% and 68%, respectively). When a mixture of *C. lunata*, *F. thapsinum*, and *Alternaria* sp. conidia was applied to seedlings, the responses from mixed infection by the 3 fungi was most similar to those resulting from infection by *F. thapsinum* alone (Table 5). This lack of synergism was also observed in roots of wheat or barley infected by a *Fusarium* sp. and a *Cochliobolus* sp. (Hill and Blunt 1994; Scardaci and Webster 1981) and in sorghum grain infected with a mixture of *F. thapsinum* and *C. lunata* (Prom et al. 2003). In the present study, colonies grown from root pieces plated onto semiselective media were transferred to PDA medium for identification; over half of the transfers were *F. thapsinum*. These results suggest that the *F. thapsinum* isolate was competitive against the *C. lunata* and *Alternaria* sp. isolates applied in this system.

Alternaria is one of the most commonly recovered fungal genera from sorghum grain (Funnell-Harris et al. 2010; Funnell and Pedersen 2006; Seitz et al. 1983; Tarekegn et al. 2006). Many small-spored *Alternaria* isolates are grouped together as "*A. alternata*" (Simmons 1999). An attempt was made in the present study to define *Alternaria* isolates from sorghum grain further. Superficially, most isolates appeared to be *A. alternata*, but a morphological screen revealed differences, particularly in configuration (length and branching patterns) of conidiophores formed on water agar. Thus, representatives of these morphological types were analyzed further using the system of Dugan and Peever (2002). By growing isolates on 6–7 different media, it was clear that 3 isolates fit well within the parameters described for the *A. alternata* species complex, while 4 other morphologies were observed; these isolates were designated morphologically as *Alternaria* sp. Analysis using sequence data from ITS, *gpd*, and mt SSU regions neither disputed nor supported these observations. Sequence analyses of these loci provide further evidence for the close relationship between *A. alternata* and *A. tenuissima*, as was observed in previous phylogenetic studies (Hong et al. 2006; Pryor and Bigelow 2003; Pryor and Michailides 2002). In particular, ITS may be problematic as a tool for species identification; because of its repetitive nature, within species as well as within individual variation may be such that difficulties arise in defining a threshold of percent identity that delineates closely related species (Begerow et al. 2010). Therefore, sequence analysis of a portion of the endo-PG gene (Peever et al. 2002) was undertaken for 6 of the isolates; adequate amplification or sequence from 3 other isolates could not be obtained. Taken together, the results allowed a conclusion, supported by morphological and endo-PG analyses, that 2 isolates were *A. alternata* (H02-747S-5 and M05-1553S-3b) and 1 was *A. tenuissima* (M05-1541S-1). Three isolates are likely to be *A. tenuissima* or *A. alternata* (M05-1561S-5, H02-781S-3b, and M05-1541S-1) while other isolates may be outside the *A. alternata*/*A. tenuissima* group, supported by

differences in morphology. Thus, a combination of comparisons of morphological and endo-PG sequences allowed for further characterization of *Alternaria* isolates from sorghum grain. Using the system of Sivanesan (1987), different *Cochliobolus* isolates from sorghum grain were distinguished into morphological species. *Cochliobolus*, which encompasses the sexual genus and 2 asexual forms (*Bipolaris* and *Curvularia*) includes over 90 species (Berbee et al. 1999). Using ITS and *gpd* sequences of *Cochliobolus*, *Curvularia*, and *Bipolaris* spp., as well as species from closely related genera, it had been shown that *Cochliobolus* and its asexual genera grouped in a clade separate from species in closely related genera but the single clade formed 2 groups (Berbee et al. 1999). *Cochliobolus* Group 1 includes *B. sorghicola* as well as other *Cochliobolus* and *Bipolaris* spp. Group 2 includes *C. lunata* and other *Cochliobolus*, *Bipolaris*, and *Curvularia* spp. The other species identified morphologically in this study, *C. sorghina*, had high (99%–100%) sequence homology to *C. carbonum* (*gpd*, ITS), and *C. victoriae* (*gpd*), previously found to be in Group 1 along with *B. sorghicola* (Berbee et al. 1999). In addition to indicating molecular relationships with other species in the *Cochliobolus/Bipolaris/Curvularia* clade, ITS sequences may be useful for describing genotypic diversity, as 10 ITS genotypes were distinguishable among *Cochliobolus* isolates obtained from sorghum grain.

In summary, 2 semiselective media were found to be efficient for isolation of the grain mold fungi, *Cochliobolus*, *Alternaria*, and *Fusarium* spp., from sorghum. Molecular analyses of ITS regions allowed identification of multiple genotypes of *Cochliobolus* isolates from sorghum. The semiselective media and molecular markers are tools to allow for comprehensive, culture-dependent, and culture-independent analyses of the interactions of grain mold fungi with sorghum.

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