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RESEARCH ARTICLE

Response of near-isogenic sorghum lines, differing at the *P* locus for plant colour, to grain mould and head smut fungi

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Alternaria alternata; *Curvularia lunata*; *Fusarium thapsinum*; *Gibberella fujikuroi*; plant pigments; plant secondary metabolites; *Sorghum bicolor* (L.) Moench.

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Abstract

Leaves and stalks of many sorghum genotypes accumulate dark red or purple pigments upon wounding while some plants, called 'tan,' do not. Grains with unpigmented 'white' pericarps grown on tan plants are more desirable for food. The hypothesis tested was that pigments in plants protected grain against the panicle diseases grain mould and head smut. Near-isogenic tan or purple plant colour genotypes with white grain were planted at Lincoln and Ithaca, NE and Corpus Christi, TX. The field grown grain was plated onto semi-selective media to detect the presence of grain colonisation by mould genera *Alternaria*, *Fusarium* and *Curvularia*. More *Fusarium* and *Curvularia* spp. were recovered from grain grown at Corpus Christi than the Nebraska locations; however, there was no indication that the grain from purple plants was more resistant to the three fungal genera. Most fungi were identified morphologically as *Alternaria alternata*. Molecular identification of *Fusarium* species, using translation elongation factor 1-α gene sequences, showed that *Fusarium thapsinum* and *Fusarium proliferatum* infected grain at all three locations. Head smut disease of panicles, caused by the fungus *Sporisorium reilianum*, was assessed at Corpus Christi; surprisingly, purple plants had significantly greater disease incidence than tan plants. We propose that the tan plant colour lines with white grain are promising for development of food-grade sorghums not more susceptible than pigmented lines to grain mould and head smut.

Introduction

Many sorghum genotypes accumulate wound-inducible purple or dark red anthocyanin pigments in response to pathogens, insects or mechanical injury (Dykes *et al*., 2005). If a sorghum genotype lacks this ability, it is described as 'tan.' The pericarps of sorghum grains can also be coloured with anthocyanin pigments; grain with pericarps that lack these pigments are referred to as 'white' (Dykes *et al*., 2005). Grain with white pericarps grown on tan plants are highly desirable for food or animal feed, because it has a neutral flavour, more appealing appearance and higher digestibility, while grain grown on purple or red plants results in products with an off-colour due to leaching of anthocyanin pigments onto the grain (Bach Knudsen *et al*., 1988; Dykes *et al*., 2005; Rooney & Awika, 2005). Because sorghum lacks gluten, flour made from non-pigmented 'food quality' sorghum can be a wheat substitute for individuals who are gluten-intolerant (Rooney & Awika, 2005).

In sorghum and other crops, pigments in grain or vegetative tissues have been implicated in resistance to pathogens (Esele *et al*., 1993; Gandikota *et al*., 2001). In sorghum, two loci, *P* and *Q*, confer plant colour (Smith & Frederiksen, 2000). The *P* allele confers purple plant colour, and *p* confers tan plant colour. The *Q* allele confers a dark blackish-purple trait. The allele *P* is epistatic to *Q*, which suggests that *P* may encode for a

regulatory factor necessary for activation of *Q*. Therefore, control of plant colour in sorghum may be similar to that in maize. In this well-characterised system, *P* acts within a regulatory complex of transcriptional activators that regulate anthocyanin production of red-purple plant pigments in vegetative tissues (Vermerris & Nicholson, 2008). In sorghum, anthocyanin pigments can also be produced in grain but this trait is controlled by two other loci, *R* and *Y* (Smith & Frederiksen, 2000). The genotypes *R_Y_* or *rrY_* have red or yellow pericarps, respectively, while the genotypes *R_yy* or *rryy* have white pericarps (Smith & Frederiksen, 2000).

Panicle diseases of sorghum and other cereals can significantly affect yield, quality and usability of grain as food, feed or fuel (Leslie, 2002; Taylor *et al*., 2006). This study focuses on two major panicle diseases of sorghum: grain mould and head smut. The primary mode of grain mould infection is from airborne spores landing directly onto the flower or developing grain, especially during moist conditions (Navi *et al*., 2005). Numerous species have been reported to be involved in the grain mould disease complex, but commonly detected species include *Fusarium* species, *Alternaria* spp. and *Curvularia lunata* (Wakker) Boedijn (Tarekegn *et al*., 2006). *Fusarium* spp. can be insidious, infecting grain without obvious symptoms (Funnell-Harris *et al*., 2010*a*) but producing mycotoxins during grain development, or after harvest during storage (Sashidhar *et al*., 1992; Leslie *et al*., 2005).

The soil-borne head smut pathogen, *Sporisorium reilianum* (J.G. Kühn) Langdon & Full. (syn. *Sphacelotheca reiliana* (J.G. Kühn) G.P. Clinton 1902), infects seedlings, then grows within the developing plant (Frederiksen & Odvody, 2000). Visible symptoms include stunting, increased tillering and change in plant colour, in addition to colonisation of the reproductive structures, resulting in replacement of grain with a teliospore-filled sorus (Frederiksen & Odvody, 2000). Spores can be prevalent in the soil but abiotic factors, seedling age, and plant host- and non-host resistance factors determine infection and development of head smut (Osorio and Frederiksen, 1998). Because most sorghum germplasm has purple plant colour (Smith & Frederiksen, 2000), no reports of the response of tan plants to *S. reilianum* infection are known.

In this study, the hypothesis that purple pigments in sorghum vegetative tissues protect grain against the panicle diseases grain mould and head smut was tested. Purple (*PPQQ*) and tan (*ppQQ*) near-isogenic lines were planted at three locations, Ithaca, NE (irrigated), Lincoln, NE (dryland) and Corpus Christi, TX (dryland), because precipitation, humidity and irrigation can affect the severity of grain mould (Bandyopadhyay & Mughogho, 1988; Alves dos Reis *et al*., 2010). All the lines had grain with white pericarps, genotype *RRyy*, to eliminate possible pleiotropic effects caused by the *Y* allele (Zanta *et al*., 1994; Pedersen & Toy, 2001). Grains were evaluated for colonisation by the grain mould fungi, *Alternaria*, *Fusarium* and *Curvularia* spp., and plants were scored for incidence of head smut. This study was designed to address whether the tan plant genotype was more susceptible to grain mould and smut fungi than purple plants, using near-isogenic lines that differ only at the *P* locus.

Materials and methods

Field experiments

Experiments were conducted at University of Nebraska Field Laboratories at Lincoln and Ithaca, in eastern Nebraska, during 2004 and 2005, and at Texas A & M AgriLife Research and Extension Center, Corpus Christi, TX, 2006, 2007 and 2008. All plots were planted with grain produced in the greenhouse. In Lincoln and Ithaca, sorghum was planted mid-May to early June and harvested late September through early November. Eastern Nebraska has a humid continental climate with wet springs, warm to hot and humid summers and mild to cold and relatively dry autumns (Table 1). Sorghum was planted in Corpus Christi during early March and harvested from late June to late July. Corpus Christi has a subtropical, semi-arid climate with warm winters, mild springs and hot, humid summers (Table 1).

The 10 sorghum lines used are segregates of S_3 sister lines from the BC_1 generation of the cross (BTx398 *ms3* × BTx630)(*ms3* × BTx630) (Pedersen and Toy, 2001). Among these 10 near-isogenic lines, there were five lines of each plant colour phenotype, purple (*PPQQ*) or tan (*ppQQ*). All 10 lines had white grain (*RRyy*). The purple phenotype lines have University of Nebraska registration numbers N331 through N335 and the tan phenotype lines are numbered N321 through N325 (Pedersen & Toy, 2001). During 2004–2007 growing seasons, S10 generation segregates were planted, except for one tan line (N323) planted in 2007 which was from the S11 generation; all lines planted during 2008 were S11 segregates. Plots consisted of two 7.6 m rows spaced 0.76 m apart at Lincoln and Ithaca and two 5.2 m rows spaced 1.0 m apart at Corpus Christi. The field experimental design at all environments was a randomised complete block with four replications.

During 2004, nitrogen fertiliser was applied at 157 kg ha^{-1} prior to planting on both fields in Nebraska. At the Lincoln location, propachlor [2-chloro-*N*-(1-methylethyl)-*N*-phenylacetamide] and atrazine [6-chloro-*n*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine] were applied at 3.36 and 1.1 kg ha^{-1}, respectively, immediately after planting for weed control.

Table 1 Mean monthly maximum and minimum temperature, relative humidity and total monthly precipitation and irrigation for Lincoln and Ithaca, NE, (2004 and 2005) and Corpus Christi, TX (2006, 2007 and 2008)

Location	Year	Month	Temperature (°C) Maximum	Minimum	Relative humidity (%)	Precipitation (cm)[a]
Lincoln	2004	May	24.7	11.4	68.9	12.0
		June	26.6	14.3	69.5	7.2
		July	28.6	17.2	78.9	13.3
		August	28.3	14.8	70.5	4.5
		September	29.0	13.4	59.8	6.6
		October	19.9	6.1	64.2	1.0
Lincoln	2005	May	24.2	9.5	58.7	8.4
		June	29.9	18.0	66.3	10.9
		July	32.2	19.3	61.8	13.5
		August	29.6	17.3	73.2	4.1
		September	28.9	13.6	61.3	0.4
		October	19.9	5.3	66.9	5.8
Ithaca	2004	May	24.2	11.0	67.1	10.0
		June	25.9	13.6	69.9	6.8
		July	27.9	16.4	80.0	5.9
		August	27.7	13.9	72.8	2.5 + 7.5(I)
		September	28.8	12.5	60.6	8.9
		October	18.9	5.1	64.9	1.8
Ithaca	2005	May	23.7	8.9	59.3	9.0 + 3.8(I)
		June	29.9	17.8	67.2	8.3 + 3.8(I)
		July	32.5	18.4	64.8	10.5 + 11.4(I)
		August	29.4	16.6	76.0	3.0 + 7.6(I)
		September	29.2	12.7	63.3	3.2
		October	19.8	4.9	67.6	4.5
Corpus Christi	2006	March	26.7	16.3	79.8	0.7
		April	30.1	19.8	82.0	0.9
		May	32.3	21.3	80.5	7.1
		June	33.2	23.1	82.8	23.4
		July	32.8	24.1	88.8	14.1
Corpus Christi	2007	March	25.1	16.0	84.7	5.7
		April	25.6	16.3	87.8	3.0
		May	29.8	20.5	87.9	5.3
		June	32.2	23.3	91.0	6.7
		July	32.2	23.6	94.1	37.8
Corpus Christi	2008	March	25.9	12.9	79.8	3.7
		April	28.6	16.9	77.9	3.9
		May	31.3	22.2	86.4	2.4
		June	33.3	23.2	86.8	0.5
		July	32.8	22.7	87.9	23.1

[a] Amounts of irrigation water applied in field at Ithaca, NE, are indicated by number preceding '(I)'. Fields at Lincoln, NE and Corpus Christi, TX were not irrigated.

At Ithaca, atrazine was applied at 2.2 kg ha^{-1} immediately after planting, followed by an application of quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) and atrazine at 0.37 kg ha^{-1} and 1.1 kg ha^{-1}, respectively, approximately 14 days post emergence. At Ithaca, supplemental irrigation (2.5 cm) was applied on August 3, 12 and 19 to avoid water stress (Table 1). During 2005, nitrogen fertiliser application to both Nebraska fields and herbicide applications to the Lincoln field were the same as in 2004. At Ithaca in 2005, atrazine was applied at 2.2 kg ha^{-1} immediately after planting, followed by an application of alachlor [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide] at 4.75 L ha^{-1} and atrazine at 1.1 kg ha^{-1}, approximately 14 days post emergence. Supplemental irrigation (3.8 cm) was applied on 25 May, 28 June, 28 July, 4 August and 11 August (Table 1). At Corpus Christi, TX, for all 3 years, nitrogen, phosphorus and zinc (72:24:0.6) fertiliser was applied at 337 kg ha^{-1} during December prior to the growing season. To control winter weeds, atrazine was

applied in January, at 2.34 L ha^{-1}. Propazine [6-chloro-*N*,*N*′-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine] and Dual® (S-metolachlor) were applied pre-emergence at 2.34 and 0.61 L ha^{-1}, respectively. During late April, pendimethalin (2,6-dinitroaniline) was incorporated two times at 2.34 L ha^{-1} for weed control. There was no noticeable disease, insect or lodging problems noticed during 2004 and 2005 for Ithaca, NE. At Lincoln, there was one plot in 2004 and two plots in 2005 with mild head smut. At Corpus Christi, there was below average rainfall during 2006. During all 3 years, head smut was apparent throughout the plots.

Analysis of grain colonisation by *Fusarium*, *Alternaria* and *Curvularia* spp.

Five randomly chosen heads were harvested from each field plot at physiological maturity, dried at 32°C for 1 week, then the mature grain was threshed and the chaff was removed. Cleaned grain were placed in coin envelopes and labelled with location, year and plot number; plant colour was unknown to laboratory personnel. Grain was stored at 4°C at 25–40% humidity, depending on the season. Screening grain from one field would take 2–6 months. One complete replicate was screened at once, on three media, as described below.

To prepare grain for screening, fifty grains from each plot were placed in a 15-mL polypropylene conical tube then surface sterilised by washing, using continuous gentle rocking, for 2 min in 95% ethanol followed by a 10-min wash in 1% sodium hypochlorite with 0.01% Tween 20 (Sigma Chemical Co., St. Louis, MO, USA), rinsed three times in sterile purified water (Labconco, Kansas City, MO, USA), then dried in a laminar-flow hood. Seeds were aseptically applied to three semi-selective agar media, five seeds per medium. All three media were peptone-based but differed in antibiotics, fungicides or other inhibitory compounds. Pentachloronitrobenzene (PCNB) agar medium (also known as Nash-Snyder) was semi-selective for *Fusarium* spp. and contained streptomycin (Fisher Scientific, Fair Lawn, NJ, USA) and the fungicide PCNB ('Terrachlor'; Uniroyal Co., Middlebury, CT, USA) (Nash and Snyder, 1962). Dichloran chloramphenicol peptone agar (DCPA) medium contained the fungicide dichloran and the antibiotic chloramphenicol and was semi-selective for *Fusarium* spp., and *Alternaria* spp. and other dark-spored ascomycetes (Andrews & Pitt, 1986). Dichloran rose bengal chloramphenicol (DRBC) agar medium, contains dichloran, chloramphenicol, and rose bengal, a stain that also suppresses bacterial growth and slows growth of rapidly-growing fungi (King *et al.*, 1979); DRBC is a general purpose fungal medium. Dichloran, chloramphenicol and rose bengal were purchased from Sigma-Aldrich, St. Louis, MO, USA. A total of 15 grains were screened per plot. Since there were four replicate plots of each line at each location and five lines per plant colour phenotype (purple or tan), 300 grains per location and year were screened, for a total of 1800 grains for each plant colour.

Individual colonies growing from each grain onto the medium were transferred to one-half strength potato dextrose agar (PDA; prepared with potato dextrose broth, Becton, Dickinson and Co., Sparks, MD, USA). Colony morphology on PDA and conidiophore structures and conidial types and morphologies on appropriate media were used to identify *Fusarium*, *Alternaria* and *Curvularia* spp. (Barnett & Hunter, 1972; Nelson *et al.*, 1983; Simmons, 1999; Leslie & Summerell, 2006). To induce sporulation of putative *Fusarium* spp., agar blocks from actively growing PDA cultures were transferred to 1.5% agar medium containing 80 mM potassium chloride. To induce sporulation of putative *Alternaria* or *Curvularia* spp., isolates were transferred by point inoculation to a 2% water agar medium or corn meal agar (Becton, Dickinson and Co.), respectively, followed by aseptic placement of an approximately 1 cm^2 filter paper square (Whatman #1, Whatman International Ltd, Maidstone, England) over the inoculation site. Fungi per 100 seeds were enumerated in each of the following categories: total *Alternaria* spp., *Alternaria alternata*, total *Fusarium* spp., *Fusarium* in the *Gibberella fujikuroi* species complex, *Fusarium* spp. not in the *G. fujikuroi* ('other *Fusarium* spp.'), and *Curvularia* spp. Data from Lincoln and Ithaca, 2005, and Corpus Christi, 2008, were reanalyzed for effects of media on recovery of different fungal genera and reported previously (Funnell-Harris *et al.*, 2013).

Numbers of fungi within a given category, recovered per 100 grains on a given medium were analysed to determine effects of location or plant colour; results obtained from each medium were analysed separately. As stated above, the experimental design for all experiments was a randomised complete block with four replications in each environment (year and location). The data were analysed using the PROC MIXED procedure of SAS/STAT software® (SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc. in the USA and other countries.® indicates USA registration.) (SAS, 2002–2008). Years (two per location), lines (five per plant colour) and replications were considered random variables. Plant colour and location were considered fixed variables. In the MODEL statement, the DDFM = KR option was specified to calculate denominator degrees of freedom using the Kenward-Rogers method because of heterogeneous

Table 2 Mean numbers and standard errors of *Alternaria* spp. colonies per 100 grains, by selection on DCPA or DRBC media, obtained from grain grown on purple or tan sorghum plants at three locations[a]

Fungal group	Medium[b]	Location			Plant colour	
		Ithaca	Lincoln	Corpus Christi	Purple	Tan
		Number of colonies per 100 grains				
Total *Alternaria* spp.	DCPA	51.7 ± 14.3	51.5 ± 14.2	46.2 ± 14.4	50.7 ± 8.7	49.1 ± 8.8
		$P = 0.951$			$P = 0.749$	
	DRBC	53.9 ± 14.9	53.1 ± 14.8	45.9 ± 14.9	51.4 ± 8.8	50.5 ± 8.8
		$P = 0.920$			$P = 0.786$	
A. alternata	DCPA	35.1 ± 13.0	38.9 ± 13.0	34.0 ± 13.1	37.8 ± 7.7	34.2 ± 7.7
		$P = 0.963$			$P = 0.317$	
	DRBC	37.9 ± 13.7	40.7 ± 13.7	33.1 ± 13.8	36.8 ± 8.0	37.6 ± 8.0
		$P = 0.927$			$P = 0.777$	

[a]Least squares means were compared within location or plant colour, for medium selected upon.
[b]Grains were screened for fungal growth by plating onto the media, dichloran chloramphenicol peptone agar (DCPA) and dichloran, rose bengal, chloramphenical (DRBC) agar.

variances and to reduce standard error and F-statistic bias (Kenward & Roger, 1997; Littell *et al.*, 2006). Levene's homogeneity of variance tests were conducted for each class variable, which indicated heterogeneity in covariance structure of some class variables. On the basis of these results, the REPEATED/GROUP option of PROC MIXED (SAS, 2002–2008) was used to specify those structures as appropriate. The numerator degrees of freedom for location was 2 and plant colour was 1; the error degrees of freedom was 2. Numbers per seed of total *Alternaria* spp., *A. alternata*, total *Fusarium* spp., *G. fujikuroi*, other *Fusarium* spp. and total *Curvularia* spp. were dependent variables. In the text and tables, means are expressed as least squares means (LSM) followed by the standard error (SE). To determine the effects of media on recovery of fungi, fungal numbers from DCPA and DRBC media were reanalyzed for each fungal category, as above, except media was included as a fixed effect.

Molecular identification of *Fusarium* spp.

Representative *Fusarium* isolates from grain of each plot were single-spored. A subset of these isolates (52 of 82) was randomly chosen for molecular identifications. For each isolate, DNA was extracted from ground lyophilized mycelium (Lee & Taylor, 1990) and the 5′ region of the translation elongation factor gene (*TEF*) was PCR-amplified using primers EF-1 and EF-2 (Geiser *et al.*, 2004). Amplification products were sequenced and assembled using Sequencher 4.10.1 (Gene Codes Corp., Ann Arbor, MI, USA). Assembled sequences were compared with those in the publically available FUSARIUM-ID database (http://isolate.fusariumdb.org) (Geiser *et al.*, 2004). Sequences were submitted to GenBank, accession numbers JX268961–JX269008 and JX512463–JX512466.

Head smut incidence

Head smut incidence was based on the number of plants within each row with characteristic head smut symptoms [sori, phylloidial miniature leaves with smut gall, excessive tillering, bleached sterile panicle, and/or bleached sterile panicle with smut gall (Frederiksen & Odvody, 2000)] divided by the total number of plants in the row, multiplied by 100. Head smut scores for the two rows within each plot were averaged. Head smut incidence was assessed for the 2007 and 2008 growing seasons at Corpus Christi. Statistical analyses of the data were as described above.

Results

Fungal colonisation of grain

Fungal colonisation of grain collected from three locations over 2 years was determined for each of three semi-selective media. There were no significant differences when analysed by location or colour or for their interactions ($P \geq 0.114$) Similar numbers of fungi were obtained on DCPA and DRBC media. On DCPA, growth per 100 grains ranged from 55.4 (Lincoln) to 64.7 (Corpus Christi) (SE = 14.6) and on DRBC medium, numbers of fungal colonies per 100 grains ranged from 57.7 (Lincoln) to 65.3 (Corpus Christi) (SE = 15.1). Numbers of fungal colonies on PCNB, semi-selective for *Fusarium* spp., ranged from 1.2 (SE = 1.6) (Lincoln) to 4.5 (SE = 1.8) (Corpus Christi). Analyses of different genera colonising grain follow.

Alternaria species

Analyses demonstrated that there were no significant differences in total numbers of *Alternaria* spp. with

Table 3 Mean numbers and standard errors of *Curvularia* spp. colonies per 100 grains, as determined by selection on DCPA or DRBC media, obtained from grain grown on purple or tan sorghum plants at three locations[a]

Medium[b]	Location			Plant colour	
	Ithaca	Lincoln	Corpus Christi	Purple	Tan
	Number of colonies per 100 grains				
DCPA	0.3 ± 0.3	0.3 ± 0.3	3.2 ± 1.1	0.5 ± 0.5	2.0 ± 0.5
	$P = 0.058$			$P = 0.052$	
DRBC	0.3 ± 0.8	1.5 ± 0.8	4.3 ± 0.8	2.9 ± 0.8	1.2 ± 0.5
	$P = 0.010$			$P = 0.063$	

[a]Least squares means were compared within location or plant colour, for medium selected upon.
[b]Grains were screened for fungal growth by plating onto the media, dichloran chloramphenicol peptone agar (DCPA) and dicholoran, rose bengal, chloramphenicol (DRBC) agar.

Table 4 Mean numbers and standard errors of *Fusarium* spp. per 100 grains, by selection on DCPA, DRBC and PCNB media, obtained from grain grown on purple or tan sorghum plants at three locations[a]

Fungal group	Medium[b]	Location			Plant colour	
		Ithaca	Lincoln	Corpus Christi	Purple	Tan
		Number of colonies per 100 grains				
Total *Fusarium* spp.	DCPA	1.3 ± 1.2	1.3 ± 1.0	5.7 ± 1.8	3.4 ± 1.0	2.1 ± 1.0
		$P = 0.204$			$P = 0.272$	
	DRBC	3.7 ± 2.7	1.1 ± 2.6	6.0 ± 2.9	3.9 ± 1.8	3.3 ± 1.7
		$P = 0.721$			$P = 0.237$	
	PCNB	0.3 ± 0.3	0.7 ± 0.4	3.3 ± 0.9	1.8 ± 0.5	1.0 ± 0.5
		$P = 0.015$			$P = 0.253$	
Gibberella fujikuroi[c]	DCPA	0.5 ± 0.5	0.5 ± 0.5	0.8 ± .05	0.7 ± 0.4	0.5 ± 0.4
		$P = 0.933$			$P = 0.768$	
	DRBC	2.2 ± 1.4	0.5 ± 1.4	1.3 ± 1.4	1.8 ± 0.9	0.8 ± 0.9
		$P = 0.721$			$P = 0.237$	
	PCNB	0.3 ± 0.5	0.0 ± 0.5	1.3 ± 0.5	0.3 ± 0.4	0.7 ± 0.4
		$P = 0.249$			$P = 0.544$	
Other *Fusarium* spp.[d]	DCPA	0.8 ± 0.7	0.8 ± 0.7	5.0 ± 1.4	2.8 ± 0.8	1.6 ± 0.8
		$P = 0.112$			$P = 0.270$	
	DRBC	1.6 ± 1.5	0.6 ± 1.4	4.7 ± 1.7	2.1 ± 1.0	2.5 ± 1.0
		$P = 0.379$			$P = 0.677$	
	PCNB	0.0 ± 0.6	0.7 ± 0.6	2.0 ± 0.6	1.5 ± 0.5	0.3 ± 0.4
		$P = 0.109$			$P = 0.071$	

[a]Least squares means were compared within location or plant colour, for the fungal group and medium selected upon.
[b]Grains were screened for fungal growth by plating onto the media, dichloran chloramphenicol peptone agar (DCPA), dicholoran, rose bengal, chloramphenicol (DRBC) agar and pentachloronitrobenzene (PCNB) agar.
[c]Members of the *Gibberella fujikuroi*, including several sorghum pathogens, were analysed as a group.
[d]All other *Fusarium* spp., excluding those in the *G. fujikuroi*, were analysed as a group.

regard to location or plant colour (Table 2). A majority of the fungi collected from grain on DCPA and DRBC media were in the species *A. alternata*, as determined by morphological characteristics. Again, analyses demonstrated that there were no significant differences in numbers of *A. alternata* attributed to location or plant colour (Table 2). Besides *A. alternata*, isolates with morphological characteristics [as determined by colony morphology, conidiophore length and branching pattern, conidial chain length and conidial dimensions (Funnell-Harris *et al.*, 2013)] similar to *Alternaria tenuissima* (Kunze) Wiltshire 1933 also were obtained.

Curvularia *spp.*

Analyses of numbers of *Curvularia* spp. per 100 grains at three locations indicated that *Curvularia* spp. are more prevalent at Corpus Christi, TX, than at either Nebraska location when grains were screened on DCPA and DRBC media (Table 3). Analyses of numbers of *Curvularia* spp. per 100 grains also suggested an effect of plant colour. However, there were more colonies in grain from tan plants when screened on DCPA, in contrast to the results on DRBC medium where there were more colonies in grain from purple plants (Table 3). The data were reanalyzed for each fungal group including media

(DRBC and DCPA) as a fixed effect. In this case, there were significant plant colour × media interactions for numbers of *Curvularia* spp. ($P = 0.006$) but for all other fungal groups in these analyses, there were no such effects ($P \geq 0.268$).

Fusarium *spp.*

Analysis of *Fusarium* spp. per 100 grains showed that total *Fusarium* spp. obtained from grain grown at Corpus Christi, TX, tended to be higher than those from grain grown at the Nebraska locations, and significantly so when grain fungi were screened on PCNB medium (Table 4). Additionally, numbers of *Fusarium* spp. other than those in the *G. fujikuroi* ('Other *Fusarium* spp.') tended to be greater from grain grown at Corpus Christi; when fungi were selected on PCNB, numbers from Corpus Christi were significantly greater than from grain grown at Ithaca, NE. There were no significant differences because of plant colour ($P \geq 0.071$) for total *Fusarium* spp., members of the *G. fujikuroi* or other *Fusarium* spp. not in the *G. fujikuroi*. However, when grown at Lincoln, and selected on DCPA medium, grain from purple plants had significantly more *Fusarium* colonies recovered per 100 grains (2.6) than grain from tan plants (0.0; SE = 1.2; $P = 0.018$). Additionally, when these lines were grown at Corpus Christi and grain was selected on DRBC, there were significantly more *G. fujikuroi* colonies per 100 grains in grain from purple plants (2.6) than from tan plants (0.0; SE = 1.5; $P = 0.048$).The *G. fujikuroi* is a species complex that includes several sorghum pathogens (Leslie *et al.*, 2005).

Representative *Fusarium* isolates from grain were identified based on type species using *TEF* sequences (Table 5). Two members of the *G. fujikuroi* species complex, *Fusarium thapsinum* and *Fusarium proliferatum* (Matsush.) Nirenberg 1976, were detected from grain grown at all three locations. Members of the *F. incarnatum – F. equiseti* species complex (FIESC) (O'Donnell *et al.*, 2009) also were detected at all three locations (Table 5). *Fusarium* genotypes detected only in grain grown at one location were *F. andiyazi* Marasas, Rheeder, Lampr., K.A. Zeller & J.F. Leslie 2001 at Lincoln, and *F. verticillioides* (Sacc.) Nirenberg 1976 and two genotypes in the *F. chlamydosporum* species complex (FCSC) (O'Donnell *et al.*, 2009) at Corpus Christi.

Table 5 *Fusarium* species isolated from sorghum grain grown on plants with either purple or tan plant colour, at Lincoln, NE, Ithaca, NE and Corpus Christi, TX and as determined by comparing sequences from the translation elongation factor (*TEF*) gene with those in the FUSARIUM-ID database

Location Species[a]	Plant colour Purple % Similarity	*n*	Tan % Similarity	*n*
Ithaca, NE				
F. proliferatum	99	1	100	1
F. subglutinans	n/a	0	100	1
F. thapsinum	99	3	99	4
FIESC	100	1	n/a	0
Lincoln, NE				
F. andiyazi	100	1	Not tested	
F. proliferatum	99–100	2		
F. thapsinum	99	3		
FIESC	98	1		
Corpus Christi, TX				
F. proliferatum	99	1	100	1
F. subglutinans	n/a	0	100	1
F. thapsinum	99	7	99	4
F. verticillioides	100	1	n/a	0
Fusarium sp.	97	1	n/a	0
FCSC	100	3	100	3
FIESC	100	5	99–100	6

[a]The 5′ region of *TEF* was amplified and sequenced (Geiser *et al.*, 2004) and compared with sequences of type isolates in the database (http://isolate.fusariumdb.org/index.php). Percent similarities to species greater than 98% are reported. In one case, species similarity was 97%, thus it was reported as '*Fusarium* sp.' FIESC indicates genotypes of the *F. equiseti – F. incarnatum* species complex; four FIESC genotypes were observed in this study. FCSC indicates genotypes of the *F. chlamydosporum* species complex; two FCSC genotypes were observed in this study. n/a, not applicable.

Incidence of head smut

Head smut disease was not observed at Ithaca, NE, during the 2 years of the field study. At Lincoln, NE, head smut was found in 2.5% of the plots during 2004 and 5% of the plots during 2005. However, at Corpus Christi, TX, 100% of the plots exhibited symptoms of head smut during 2007 and 2008, so head smut incidence (percent of heads affected in each row) was recorded. Mean incidence for purple plants (14.8%; SE = 2.5) was significantly greater than mean incidence for tan plants (6.8% SE = 2.1) ($P = 0.023$).

Discussion

Near-isogenic sorghum lines have been developed that segregate for plant colour (purple versus tan) and pericarp colour (red versus white) (Pedersen & Toy, 2001). Analysis of germination, seedling elongation, field emergence, grain yield and test weights, showed that plant colour × pericarp colour interactions were not significant. However, these analyses indicated that grain from purple plants had greater fitness than grain from unpigmented plants, as determined by higher yields, germination under stress conditions and greater seedling

elongation, than tan plants (Pedersen & Toy, 2001), One possible explanation was that the grain, originally grown under field conditions, may have been more resistant to grain mould fungi when it was grown on purple plants than when grown on tan plants (Funnell & Pedersen, 2006). A follow-up study provided evidence that grain from purple plants with either pericarp colour had significantly less *Fusarium* spp. colonisation than grain from tan plants, which indicated that vegetative purple pigments may protect sorghum grain against *Fusarium* spp. (Funnell & Pedersen, 2006). However, this previous study was relatively small in scale. This study sought to test the hypothesis that purple pigments in sorghum vegetative tissues protect grain against the panicle diseases grain mould and head smut. Therefore the presence in grain of three fungal genera involved in grain mould, *Alternaria*, *Fusarium* and *Curvularia* (Tarekegn *et al*., 2006), and symptoms and signs of the head smut fungus, *S. reilianum*, in plants, were screened for.

The results of this study indicated that the purple plant colour did not reduce infection by *Alternaria* spp., including the commonly found species, *A. alternata*. *Alternaria* spp. are associated with 'weathering,' which occurs when maturing grain is exposed to wet weather before harvest, resulting in discolouration (Sauer *et al*., 1978). Although not usable for food-grade sorghum, weathered grain has not been associated with toxicity to livestock.

The results of this study also indicated that purple plant colour did not inhibit fungal colonisation of white grain by *Fusarium* spp., as suggested by a previous study (Funnell & Pedersen, 2006). In this study, only grain with white pericarps was screened to eliminate possible interactions between plant and grain pigmentation (Zanta *et al*., 1994). When fungal colonies grown from grain were selected on three semi-selective agar media (DCPA, DRBC and PCNB), numbers of *Fusarium* spp. in the *G. fujikuroi* species complex, and all other *Fusarium* spp., were statistically the same or less in grain from tan plants when compared with grain from purple plants (Tables 1 and 4). To summarise, the purple plant colour did not result in reduced infection by *Fusarium* spp. for the lines examined.

It is less clear whether or not purple plant colour protects against *Curvularia* spp. Depending on which medium fungal colonies were selected upon, the results indicated that grain from either purple (DCPA) or tan (DRBC) plants had less *Curvularia* isolates. Reanalyzing the data with medium as a fixed effect indicated that for *Curvularia* spp. there were media by plant colour interactions, but not for *Alternaria* and *Fusarium* spp. Differences between DCPA and DRBC include the addition of rose bengal to the latter, a compound that restricts growth of some fungi (King *et al*., 1979), while DCPA has twice the concentration of dichloran and nearly four times the concentration of chloramphenicol as DRBC (Andrews & Pitt, 1986). The more stringent levels of fungicide and antibiotic in DCPA or the addition of another inhibitor in DRBC may impact growth of some genotypes of *Curvularia* spp. or of other fungi potentially competing with *Curvularia* genotypes. In this study, there were more *Curvularia* isolates recovered from grain on DRBC medium (24) than on DCPA medium (14); however the difference was not significant ($P = 0.14$). Additionally, molecular genotyping using the internal transcribed spacer (ITS)-1 region indicated that there were no differences in ITS genotypes obtained on DCPA or DRBC medium ($P = 0.55$) (Funnell-Harris *et al*., 2013). Using semi-selective culture media, as opposed to culture independent methods to analyse microorganisms from environmental samples (Whipps *et al*., 2008), has the advantage of obtaining isolates for further analyses. However, each medium is a new environment for grain-infecting fungi, benefiting some and challenging others (Funnell-Harris *et al*., 2010*b*). Thus, any single medium does not give the complete picture of infection by all fungal genotypes.

A consequence of this study was the opportunity to assess response of these lines to modest disease pressure from *S. reilianum*, the fungus causing head smut. During the early 1960s, the development and deployment of hybrids with race-specific resistance had significantly reduced head smut (Frederiksen & Reyes, 1980). However, less than 10 years later, the existence of at least one resistance-breaking fungal race became evident. The teliospores of *S. reilianum* become incorporated into soil after they are released from the sorus and fall to the ground at the end of the growing season (Frederiksen & Odvody, 2000; Bdliya, 2005). This can result in an intermittent distribution of inoculum through fields (Osorio & Frederiksen, 1998). This factor, the short time period for seedling susceptibility, and a preference for cool dry soils for spore germination, probably contributed to sparse field infections observed at Lincoln, NE, during 2004 and 2005. At Corpus Christi during 2007 and 2008, disease incidence in different plots ranged from 1% to 37%; however all plots during both years had plants exhibiting symptoms. When these near-isogenic lines differing in plant colour were analysed, tan plants had significantly lower disease incidence than purple plants.

This result was surprising and indicated that purple and dark red pigments were not factors in resistance to the pathogen, *S. reilianum*, at least not in these lines under this disease pressure. The parent contributing the tan plant colour trait (*pp*) in the crosses for development of these lines, was reported to have resistance to head smut (Miller, 1986; Pedersen & Toy, 2001). Because most sorghum genotypes have purple or dark red pigmentation (Smith & Frederiksen, 2000), it seems likely that the head

smut pathogen evolved to tolerate anthocyanin pigments in order to infect and spread through the developing plant. In purple plants, it is possible that *P* encodes for a transcription factor that regulates genes for the production of anthocyanin pigments, similar to the *P* gene of maize (Vermerris & Nicholson, 2008). The ability of *pp* (tan) plants to produce pigmented grain (Pedersen & Toy, 2001) suggests that other components in the flavonoid biosynthetic pathway, besides the factor encoded by the *P* gene, are functional. In tan plants, other metabolic pathways may be induced or intermediate metabolites may accumulate during initial stages of *S. reilianum* infection which are not present or are at lower levels in purple pigmented plants.

In plants, phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) catalyze the first committed steps in phenylpropanoid and flavonoid biosynthesis, respectively (Vermerris & Nicholson, 2008). It was previously reported that accumulation of transcripts of the genes for PAL and CHS did not increase in sorghum plants inoculated with *S. reilianum* even though increases in expression were observed following inoculation with a non-pathogenic fungus and a pathogenic oomycete (Cui *et al*., 1996). This suggests that *S. reilianum* can evade these plant defence pathways by not triggering their induction. Results from the current study, and from Cui *et al*. (1996), indicate that, although tan plants lack the ability to synthesise flavonoid pigments within their vegetative tissues, these plants are not without defences against fungal pathogens. Hence, other defences appear to be elevated in the absence of flavonoid pigments. One candidate may be the cyanogenic glucoside, dhurrin, which accumulates to high levels in seedlings (Halkier & Møller, 1989). Following plant cell disruption, the phytoanticipin, hydrogen cyanide, is enzymatically released (Seigler, 1991). However, at the present time there is no evidence that hydrogen cyanide is involved in resistance of sorghum to *S. reilianum*.

The near-isogenic purple and tan lines with white grain used in this study, are potentially valuable to investigate other defences of sorghum besides the well-characterised 3-deoxyanthocyanidin phytoalexins (Lo *et al*., 1999). Alternatively, the tan lines described in this study would be ideal to develop food-grade sorghum hybrids, especially in combination with lines having race-specific resistance to sorghum head smut (Osorio & Frederiksen, 1998).

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