Stalk Rot Fungi Affect Leaf Greenness (SPAD) of Grain Sorghum in a Genotype- and Growth-Stage-Specific Manner

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Abstract

Bandara, Y. M. A. Y., Weerasooriya, D. K., Tesso, T. T., and Little, C. R. 2016. Stalk rot fungi affect leaf greenness (SPAD) of grain sorghum in a genotype- and growth-stage-specific manner. *Plant Dis.* 100:2062-2068.

Stalk rots are among the most prevalent and destructive sorghum diseases worldwide. Although experimental evidence is limited, delayed postflowering senescence due to the staygreen trait is accepted as a physiological means of stalk rot resistance. Staygreen has been shown to be correlated with chlorophyll content (as measured by a soil and plant analytical development [SPAD] meter). Field experiments were conducted to test the effects of *Fusarium* stalk rot and charcoal rot on SPAD readings at three developmental stages, to test whether staygreen genotypes are more resilient to stalk-rot-mediated chlorophyll degradation, and to examine the relationships between SPAD and stalk rot resistance and tolerance when plants were inoculated with causal organisms. Staygreen and nonstaygreen lines (two) and hybrids (two) established in the field were inoculated with *Fusarium thapsinum*, *F. proliferatum*, *F. andiyazi*, and *Macrophomina*

phaseolina at 14 days after flowering. SPAD readings were obtained at soft-dough, hard-dough, and physiological maturity. Most pathogens significantly reduced the SPAD of the genotypes over the mock-inoculated control at three developmental stages. The stalk-rot-resistant and staygreen check line, SC599, showed a remarkable feature of negative senescence from soft dough to physiological maturity under disease pressure. Disease severity was significantly and negatively correlated with SPAD at all developmental stages, revealing the potential impact of the staygreen trait on stalk rot resistance. The difference between control and pathogen-treated total seed weight per panicle (i.e., tolerance) was significantly and positively correlated with the difference between control and pathogen-treated SPAD at physiological maturity, demonstrating the ability of staygreen trait to enhance stalk rot tolerance under disease pressure.

Stalk rots are among the most prevalent and destructive diseases of sorghum (*Sorghum bicolor* L. Moench) worldwide (Tesso et al. 2010). There are two stalk rot diseases of sorghum: *Fusarium* stalk rot, also called “soft rot,” caused by *Fusarium* spp., and charcoal rot, caused by *Macrophomina phaseolina* (Tassi) Goid. (Tarr 1962). Degradation of pith tissue at or near the base of the stalk leads to pith cell death and is the primary symptom of both diseases (Tesso et al. 2012). Infected plants often have damaged vascular and cortical tissues in both the root and stalk systems that reduce nutrient and water absorption and translocation (Hundekar and Anahosur 1994). Under extreme conditions, stalk rot diseases can cause plant lodging and significant yield loss (Tesso et al. 2004). Prolonged postflowering drought stress, heat stress, and unbalanced mineral nutrition aggravate stalk rot diseases (Dodd 1980; Seetharama et al. 1987; Tesso et al. 2004). Jardine and Leslie (1992) have shown that the average yield loss caused by stalk rots in Kansas is about 4% while the loss at specific locations can reach as much as 50%. Stalk-rot-induced lodging of up to 60% is common among high-yielding hybrids in Kansas, and the incidence can be even higher under stressful conditions (Tesso et al. 2004). Tesso et al. (2004) estimated that the annual economic loss associated with this disease in Kansas surpasses \$15 million per year.

Delayed senescence (or “staygreen”) in sorghum is widely accepted as a valuable trait because it improves the ability of a genotype to adapt to postflowering drought stress, particularly when the crop depends on residual soil moisture for grain development and maturity (Rosenow et al. 1977). Greater green-leaf-area duration during grain filling is a result of different combinations of three factors: green leaf

area at flowering, senescence onset time, and rate of senescence (Borrell et al. 2000a; van Oosterom et al. 1996). Furthermore, this trait has a slight, if any, yield penalty when plants are grown in environments where water is not a limiting factor (Borrell et al. 2000b). Xu et al. (2000) have shown that the soil and plant analytical development (SPAD) value (measured by a SPAD meter) is significantly and linearly correlated with total leaf chlorophyll ($R^2 = -0.91$) and with visual staygreen ratings ($R^2 = -0.82$). This instrument determines the relative quantity of chlorophyll present in leaf tissue by measuring red and infrared leaf transmittance (at wavelengths of 650 and 940 nm, respectively) (Yamamoto et al. 2002). Transmittance values are used by the device to derive a relative SPAD meter value that is proportional to chlorophyll content in the sample (Uddling et al. 2007). Therefore, SPAD readings measured from the early reproductive to physiological maturity stages can be used to assess the staygreen capacity of grain sorghum genotypes. Xu et al. (2000) used SPAD values at physiological maturity as the direct indicator of the staygreen trait.

Staygreen has been reported to be associated with reduced lodging (Mughogho and Pande 1984) and lower susceptibility to charcoal rot (Mughogho and Pande 1984; Tenkouano et al. 1993). Moreover, as mentioned above, staygreen in sorghum is known to improve the ability to adapt to postflowering drought stress. By the end of the 21st century, global surface temperatures are predicted to increase by 1.4 to 5.8°C (IPCC 2007). Drought is the most important abiotic stressor that directly affects yield. The increased frequency of prolonged water stress events is both an indicator and a predicted outcome of future climate change (Araus et al. 2002; Meehl et al. 2007). According to Meehl et al. (2007), an increased frequency of drought incidents is an important feature of the forecasted climatic changes along with short episodes of high temperature.

Cool and moist weather conditions at physiological maturity that are preceded by drought and heat during grain formation tend to exacerbate *Fusarium* stalk rot severity while prolonged exposure to drought and high-temperature stress throughout host development increases charcoal rot incidence (Tesso et al. 2012). Under insufficient moisture, sorghum is subject to drought-associated root and stalk rots that could lead to lodging, as well as loss of stover, grain

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This article is contribution number 16-317-J from the Kansas Agricultural Experiment Station, Manhattan.

Accepted for publication 6 June 2016.

<http://dx.doi.org/10.1094/PDIS-02-16-0171-RE>
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quality, and productivity (Borrell et al. 2000b). Lodging poses an additional challenge under mechanized harvesting and contributes to reduced harvestable yields and quality. *M. phaseolina* is considered a high-temperature pathogen, with the severity of the disease increasing as air and soil temperatures rise from 28 to 35°C and when soil moisture becomes limiting (Gray et al. 1991; Smith and Wyllie 1999). Therefore, under predicted future climatic changes, tremendous yield losses due to intensified charcoal and Fusarium stalk rot incidence around the world could be expected in many economically important crops, including sorghum. In that context, staygreen is an increasingly important trait to be pursued and incorporated in future breeding efforts to face these challenges.

Although staygreen is common among certain genotypes of many economically important crop species (Thomas and Smart 1993) that are prone to stalk and root rot diseases, experimental evidence for the degree of association or correlation between staygreen and charcoal rot and Fusarium stalk rot resistance are currently lacking. Further, the effect of stalk rot pathogens on leaf chlorophyll content and the relationship between staygreen and stalk rot tolerance are unknown. Therefore, the objectives of this study were to (i) determine the effects of multiple stalk rot pathogens on SPAD readings at three developmental stages (soft dough, hard dough, and physiological maturity), (ii) test whether staygreen genotypes are more resilient to stalk-rot-mediated chlorophyll degradation, and (iii) investigate the degree of correlation between staygreen and stalk rot resistance and tolerance when plants were inoculated with *Fusarium thapsinum*, *F. andiyazi*, *F. proliferatum*, and *M. phaseolina* by using grain sorghum as a model system.

Materials and Methods

Sorghum genotypes and fungal pathogens. Four sorghum genotypes, including two hybrids and two inbred lines, were used for this study. The two hybrids used were DKS37-07 (Monsanto; staygreen and charcoal rot resistant) and 84G62 (Dupont Pioneer; charcoal rot tolerant). These are commonly grown hybrids in the state of Kansas. SC599R (Fusarium stalk rot and charcoal stalk rot resistant, staygreen) and BTx3042 (Fusarium stalk rot and charcoal rot susceptible, nonstaygreen) were the two breeding lines used. The *M. phaseolina* isolate (r144) utilized in this study was provided by Dr. Gary Odvody, Texas A&M AgriLife Research and Extension, Corpus Christi. The three *Fusarium* spp. used in this study (*F. thapsinum*, *F. proliferatum*, and *F. andiyazi*) were obtained from the culture collection of the Row Crops Pathology Lab at the Department of Plant Pathology, Kansas State University. For information on cultural and molecular identification of the isolates used, please refer to Bandara et al. (2015).

Establishment and maintenance of plants in the field. Two field experiments were conducted during the 2013 and 2014 main seasons at the Kansas State University agronomy research farms near Manhattan, KS (39.22°N, 96.60°W) and Ashland, KS (39.13°N, 96.62°W), respectively (hereafter referred to as the two environments). Field preparation, planting, and crop maintenance were conducted according to standard procedures for sorghum. Seed treated with ethyl mercaptan (Captan; 2 ml kg⁻¹ seed) were planted in rows of 5 m in length by 0.75 m in width. Eighty seeds were planted per plot that resulted in a plant stand of 60 to 65 plants. Seedlings were not thinned. A four-by-five-by-three factorial treatment structure was deployed to investigate SPAD response, where factors one, two, and three consisted of sorghum genotype, inoculation treatment, and developmental stages, respectively, at which SPAD measurements were acquired. For disease severity (lesion length) and yield measurements (total seed weight [TSW] per panicle and 100-seed weight [HSW]), a treatment structure of four-by-five factorial was used, excluding the developmental stage factor (see below). Both experiments were arranged in a split-plot design with randomized complete blocks. Genotypes were assigned to the whole-plot unit, whereas pathogens with control treatments were assigned to the subplot unit. Each experiment was replicated three times (i.e., three blocks). At 4 weeks after planting, three plants in each of the five subplots were tagged using five different-colored tapes (three plants of each color) to demarcate the pathogen or control treatment they would receive at 2 weeks after flowering. There were three subsample plants (observational unit) at the level of each treatment.

Inoculum preparation, artificial inoculation, SPAD, disease severity, and yield measurements. The protocol described by Bandara et al. (2015) was used to prepare *Fusarium* spp. and *M. phaseolina* inocula. Briefly, liquid cultures (potato dextrose broth) were grown at 30°C for 5 days to obtain *Fusarium* spp. conidia and *M. phaseolina* mycelia. Filtrates containing *Fusarium* spp. conidia and *M. phaseolina* hyphal fragments were centrifuged and diluted with sterile 10 mM phosphate-buffered saline (PBS; 2.17 g of NaCl, 8.71 g of Na₂HPO₄·7H₂O, and 0.26 g of KH₂PO₄ dissolved in 1 liter of sterile-distilled H₂O; pH 7.2). All inoculum preparation steps were performed under aseptic conditions. Inoculations were performed at 14 days after anthesis. Plants were inoculated using a 10-ml, 20-gauge, 3.8-cm needle, sterile surgical syringe by injecting 0.1 ml of inoculum with a concentration of 2×10^6 conidia (for the three *Fusarium* spp.) or mycelial fragments (*M. phaseolina*) per milliliter into the basal node of the stalk. PBS was used as the mock-inoculated control treatment.

Chlorophyll content of the flag leaf of each inoculated and control plant was measured using a SPAD meter (Minolta SPAD 502 chlorophyll meter; Spectrum Technologies, Inc.). All SPAD measurements were acquired at the base, center, and the tip of the leaf lamina and averaged. SPAD was measured at the soft dough, hard dough, and physiological maturity stages at 7, 19, and 31 days after inoculation, respectively. Plants were harvested at 42 days after inoculation. The stalks of harvested plants were longitudinally split using a knife and the length (in centimeters) of the pigmented lesion that occurs due to infection of stalk rot pathogens was measured using a tape. Panicles were separated from plants and dried for 10 days at 40°C and threshed to measure TSW per panicle using a weighing scale. HSW was computed by dividing the TSW per panicle by the total number of seed per panicle. An automated seed counter was used to count the seed.

Statistical analysis. SAS software, version 9.2 (SAS Institute 2008) was used for all data analyses. Data were subjected to analysis of variance (ANOVA) using the PROC GLIMMIX procedure. The Kenward-Roger option was used to compute the denominator degrees of freedom in *F* tests. Environment, genotype, inoculation treatment, and developmental stage were considered as fixed factors for SPAD measurements. Environment, genotype, and pathogen treatment were considered as fixed factors for yield (TSW per panicle and HSW) and disease severity (lesion length) traits. Block was regarded as a random factor nested within environment. Because SPAD measurements at the three maturity stages were repeated measures and were correlated with each other, there was a covariance structure to the data at the level of developmental stage. Hence, a time series analysis was conducted by modeling data for the most suitable variance-covariance structure. The data were modeled with first-order antedependence covariance structure. Bayesian information criterion was used as the basis for model selection. In the case of the other response variables (i.e., TSW per panicle, HSW, and lesion length), the model assumptions were tested using Studentized residual plots (for identical and independent distribution of residuals) and Q-Q plots (for normality of residuals). Whenever residuals were not homogeneously distributed, appropriate heterogeneous variance models were fitted to meet the model assumptions. Means separations were carried out using the PROC GLIMMIX procedure of SAS. Main effects of factors were determined with adjustments for multiple comparisons by using the Tukey-Kramer test. Simple effects of factors were determined using the Bonferroni adjustment when higher-order interactions among factors are statistically significant for the trait concerned. Only relevant pairwise comparisons were considered for determining the critical comparison-wise error rate (α_{CER}) and subsequently compared against unadjusted *P* values resulting from Bonferroni correction to test the significance of mean treatment differences. Pearson correlation coefficients and significance levels among measured traits were computed using the PROC CORR procedure of SAS.

Results

ANOVA revealed a statistically nonsignificant four-way interaction effect between environment, genotype, treatment, and developmental stage for SPAD at the 5% significance level (Table 1). The three-way interactions between genotype, pathogen, and developmental

stage and environment, genotype, and developmental stage were significant. Except for the interactions of pathogen with environment and developmental stage, all other two-way interactions were significant. ANOVA conducted for the computed values of mean SPAD difference (SPAD at physiological maturity [SPAD_{pm}] – SPAD at soft dough stage [SPAD_{sd}]) revealed significant genotype main effects (Table 1). The genotype–pathogen interaction was significant for TSW per panicle, whereas significant main effects of all three factors were evident for HSW. The environment–genotype–pathogen interaction was significant for lesion length.

F. andiyazi, *F. proliferatum*, *F. thapsinum*, and *M. phaseolina* significantly reduced the SPAD of 84G62 at hard dough ($P = 0.005$) and physiological maturity ($P < 0.0001$) over the respective controls, while only *F. thapsinum* and *M. phaseolina* significantly reduced the SPAD at soft dough stage ($P = 0.0024$ and $P < 0.0001$, respectively) compared with the control (Table 2). Although no significant SPAD differences among the pathogens were observed for 84G62 at hard dough and physiological maturity, *F. thapsinum* and *M. phaseolina* resulted in significantly lower SPAD readings in comparison with *F. andiyazi* and *F. proliferatum* at the soft dough stage. All four pathogens significantly reduced the SPAD of DKS37-07 at soft dough (*F. andiyazi*, $P = 0.001$; others, $P < 0.0001$), hard dough ($P < 0.0001$), and physiological maturity ($P < 0.0001$) in comparison with their respective controls. At physiological maturity, *M. phaseolina* had a significantly greater SPAD reduction ($P < 0.001$) compared

with *F. andiyazi*, although pathogens were not significantly different from each other at soft and hard dough stages. All four pathogens significantly reduced the SPAD of BTx3042 at hard dough stage (*F. andiyazi*, $P = 0.006$; others, $P < 0.0001$) compared with the control. At soft dough stage, except for *F. andiyazi*, the other three pathogens significantly reduced SPAD compared with the control ($P < 0.0001$). *F. proliferatum* ($P < 0.0006$) and *M. phaseolina* ($P < 0.0001$) significantly reduced SPAD compared with the control at physiological maturity. All four pathogens significantly decreased the SPAD of SC599 at soft dough ($P < 0.0001$), hard dough ($P < 0.0001$), and physiological maturity (*M. phaseolina*; $P = 0.0015$, others; $P < 0.0001$) compared with the respective controls. Across environments and treatments, SC599 showed a positive SPAD difference (SPAD_{pm} > SPAD_{sd}), while 84G62, BTx3042, and DKS37-07 had negative SPAD differences (SPAD_{pm} < SPAD_{sd}) (Fig. 1). The latter three were significantly different from the former ($P = 0.003$, $P = 0.001$, and $P < 0.0001$, respectively).

F. andiyazi, *F. proliferatum*, *F. thapsinum*, and *M. phaseolina* significantly reduced the TSW per panicle of 84G62 (*F. andiyazi*, $P < 0.005$; *F. proliferatum*, $P < 0.001$; others, $P < 0.0001$), DKS37-07 ($P < 0.0001$), SC599 ($P < 0.0001$), and BTx3042 (*M. phaseolina*; $P < 0.0001$, others; $P < 0.001$) in comparison with their respective controls (Table 3). However, pathogens did not significantly differ among themselves for any of the genotypes. *F. andiyazi*, *F. proliferatum*, *F. thapsinum*, and *M. phaseolina* significantly decreased the HSW compared with the control ($P < 0.0001$) (Fig. 1).

All pathogens significantly increased the lesion lengths of all genotypes in both environments in comparison with the control treatment ($P < 0.001$; Table 4). In the 2013 experiment, *F. thapsinum* produced the longest mean lesion length of 11.2 cm in 84G62, which was significantly higher than the stalk lesions caused by the other pathogens in this commonly grown hybrid ($P < 0.005$). In 2014, *F. thapsinum* produced a mean lesion length of 7.5 cm in 84G62. In both years, *M. phaseolina* produced the shortest lesion lengths in this hybrid. *F. thapsinum* produced the longest lesion length in DKS37-07 in both years, while *M. phaseolina* produced the lowest lesion length in both environments and *F. thapsinum* produced the shortest lesion lengths. SC599, in general, showed the shortest lesion length values against tested pathogens in both years. Although all pathogens produced significantly higher lesion lengths compared with the control ($P < 0.001$), the pathogens were not significantly different from each other for the stalk-rot-resistant, staygreen line SC599 (Table 4).

Pearson correlation coefficient analysis revealed significant correlations between SPAD measured at the three developmental stages as well as the mean SPAD across the three developmental stages with TSW per panicle and lesion length in both environments (Table 5). The correlation coefficients (r) between TSW per panicle and SPAD at the soft dough, hard dough, and physiological maturity stages and mean SPAD in 2013 were similar to those in 2014 ($P < 0.0001$). P values for the correlation coefficients between HSW and the above-mentioned SPAD values were much stronger in 2014 ($P < 0.0001$) compared with those in 2013 ($P = 0.013$, $P = 0.018$, $P < 0.0001$, and $P < 0.011$, respectively). Lesion length data in both environments

Table 1. P values of F tests from analysis of variance for soil and plant analytical development (SPAD) meter readings measured at soft dough, hard dough, and physiological maturity stages; and total seed weight (TSW) per panicle, 100-seed weight (HSW), and lesion length (LL) as measured with four sorghum genotypes after inoculation with *Fusarium thapsinum*, *F. andiyazi*, *F. proliferatum*, and *Macrophomina phaseolina* ($\alpha = 0.05$)

Effect	Pr > F ^y				
	SPAD	ΔSPAD ^z	TSW	HSW	LL
Environment (E)	<0.0001	0.1852	<0.0001	0.0002	0.3154
Genotype (G)	0.0001	<0.0001	<0.0001	<0.0001	0.0031
E × G	<0.0001	0.1282	<0.0001	<0.0001	<0.0001
Treatment (T)	<0.0001	0.2430	<0.0001	0.0002	<0.0001
E × T	0.1173	0.0692	0.9362	0.8979	0.6096
G × T	0.0002	0.1006	0.0094	0.7476	<0.0001
E × G × T	0.1348	0.1611	0.0948	0.1236	<0.0001
Stage (S)	0.0002	...	...	...	...
E × S	<0.0001	...	...	...	...
G × S	0.0461	...	...	...	...
E × G × S	0.0002	...	...	...	...
T × S	0.6279	...	...	...	...
E × T × S	0.1517	...	...	...	...
G × T × S	0.0241	...	...	...	...
E × G × T × S	0.0511	...	...	...	...

^y TSW, HSW, and LL were measured at physiological maturity.

^z SPAD meter reading difference between physiological maturity and soft dough stages (SPAD_{pm} – SPAD_{sd}).

Table 2. Mean soil and plant analytical development (SPAD) values (in relative SPAD units) of sorghum hybrids and lines at soft dough (SD), hard dough (HD), and physiological maturity (PM) after inoculation with phosphate buffered saline (mock-inoculated control), *Fusarium andiyazi*, *F. proliferatum*, *F. thapsinum*, and *Macrophomina phaseolina* across the 2013 and 2014 experiments^z

Treatment	Hybrids						Lines					
	84G62			DKS37-07			BTx3042			SC599		
	SD	HD	PM	SD	HD	PM	SD	HD	PM	SD	HD	PM
Control	58.5 a	59.3 a	56.8 a	58.9 a	58.6 a	58.9 a	57.2 a	57.3 a	52.1 a	54.8 a	55.9 a	56.5 a
<i>F. andiyazi</i>	54.2 a	54.6 b	50.1 b	53.2 b	51.9 b	50.7 b	53.4 ab	52.7 b	51.6 a	45.2 b	45.4 c	45.1 c
<i>F. proliferatum</i>	54.1 a	54.2 b	50.5 b	51.5 b	50.4 b	47.3 bc	49.5 bc	46.6 c	46.5 b	47.6 b	49.7 b	47.0 bc
<i>F. thapsinum</i>	53.2 b	53.9 b	51.4 b	51.5 b	50.8 b	48.3 bc	48.6 c	49.1 bc	48.3 ab	46.5 b	48.8 b	46.6 bc
<i>M. phaseolina</i>	51.5 b	52.8 b	48.6 b	50.9 b	50.1 b	45.2 c	47.7 c	47.0 c	45.4 b	47.9 b	49.1 b	48.3 b

^z Standard error for each mean is 1.2. Means within a column followed by the same letter are not significantly different based upon the adjustment for multiple comparisons using Bonferroni correction ($\alpha_{\text{CER}} = 0.005$).

had significant and negative correlations with SPAD at soft dough, hard dough, physiological maturity, and mean SPAD across the three developmental stages (2013, each at $P < 0.0001$; 2014, $P < 0.0001$, 0.0003, 0.0012, and 0.0001, respectively). The difference between TSW per panicle (Δ TSW) after control and pathogen inoculations was significantly and positively correlated to the difference between control and pathogen-treated plant SPAD (Δ SPAD_{pm}) readings at physiological maturity (2013: $r = +0.47$, $P = 0.008$; 2014: $r = +0.35$, $P = 0.014$). Δ SPAD_{pm} explained 22 and 12% of the total observed variation in Δ TSW in 2013 and 2014, respectively (Fig. 2).

Discussion

Stalk rots cause significant global economic loss, and use of resistant cultivars is essential for managing these diseases (Tesso et al.

2012). Despite the absence of reported experimental evidence for the potential correlation between staygreen and stalk rot resistance, staygreen has been accepted as a means of physiological resistance. Most stalk-rot-resistant sorghum genotypes, including ‘SC599’, ‘SC33’, and ‘SC35’, possess the staygreen trait (Bramel-Cox et al. 1988; Woodfin et al. 1988). These reports suggest that indirect selection for staygreen can be used to identify genotypes with superior stalk rot resistance. Visual scoring of the staygreen trait is performed at or soon after physiological grain maturity. However, visual ratings could have some limitations, including disparities in rating scores among observers and individual biases (Rosenow 1994). Xu et al. (2000) showed that staygreen is highly correlated with chlorophyll content (as measured by a SPAD meter). Therefore, SPAD is a reliable and high-throughput method for assessing the staygreen trait. There are five types of staygreen based upon leaf senescence behavior (Thomas and Howarth 2000; Thomas and Smart 1993). The functional staygreen types A and B maintain both leaf greenness and photosynthetic activity much longer, while the nonfunctional staygreen types C, D, and E have persistent leaf greenness without sustaining photosynthetic competence during senescence. Therefore, type A and B sorghum genotypes, compared with other types, are most likely to provide some physiological resistance against stalk rot diseases.

The first objective was to investigate whether stalk rot pathogens affected leaf chlorophyll content. Results revealed the ability of stalk rot pathogens to reduce SPAD in a significant genotype- and developmental stage-specific manner. Currently, although the literature does not describe the potential involvement of stalk rot pathogens in altering sorghum chlorophyll metabolism, Kariola et al. (2005) demonstrated that chlorophyllase 1 (encoded by *AtCLH1*) of *Arabidopsis thaliana* was quickly induced after infection with the necrotrophic fungus *Alternaria brassicicola*, leading to enhanced chlorophyll degradation. It is possible that stalk rot pathogens also decrease chlorophyll biosynthesis while increasing chlorophyll degradation through upregulation of host genes that encode for enzymes such as chlorophyllase.

Our second objective was to test whether staygreen genotypes are more resilient to stalk-rot-mediated chlorophyll degradation. SC599 exhibited a higher SPAD at physiological maturity than at the soft dough stage across two environments and five treatments (Fig. 1). In contrast, the other three genotypes had lower SPAD readings at physiological maturity than at soft dough stage. Green-leaf-area duration during grain filling is a result of different combinations of green leaf area at flowering, senescence onset time, and rate of senescence (Borrell et al. 2000a; van Oosterom et al. 1996). SPAD difference between physiological maturity and soft dough stage (e.g., Δ SPAD) is indicative of the rate of senescence. It is clear that SC599 maintains a greater green-leaf-area duration during grain filling through a negative rate of senescence (i.e., SPAD increases over time from soft dough to physiological maturity), even in the presence of pathogens, whereas the other three genotypes exhibited a positive senescence rate. Consequently, it is apparent that SC599 belongs to

Fig. 1. A, Comparison of Δ SPAD (soil and plant analytical development [SPAD] meter reading difference between physiological maturity [SPAD_{pm}] and soft dough stage [SPAD_{sd}] $\pm$ standard error) of tested sorghum lines (SC599 and BTx3042) and hybrids (84G62 and DKS37-07) across five pathogen treatments and two environments. **B,** Effect of pathogens on 100-seed weight (in grams) across four genotypes and two experiments using Tukey-Kramer's test. Means followed by the same letters are not significantly different based on the adjusted P value for multiple comparisons at $\alpha = 0.05$. CON = mock-inoculated control (phosphate-buffered saline), FA = *Fusarium andiyazi*, FP = *F. proliferatum*, FT = *F. thapsinum*, and MP = *Macrophomina phaseolina*.

Table 3. Mean total seed weight per panicle (in grams) among sorghum genotypes after inoculation with phosphate-buffered saline (control), *Fusarium andiyazi*, *F. proliferatum*, *F. thapsinum*, and *Macrophomina phaseolina* across two environments^a

Treatment	Hybrids		Lines	
	84G62	DKS37-07	BTx3042	SC599
Control	93.6 a	82.1 a	32.9	53.3 a
<i>F. andiyazi</i>	76.8 b	53.9 b	19.5 b	34.6 b
<i>F. proliferatum</i>	69.3 b	53.5 b	18.3 b	27.8 b
<i>F. thapsinum</i>	60.2 b	40.3 b	19.3 b	36.2 b
<i>M. phaseolina</i>	61.7 b	42.4 b	16.8 b	33.0 b

^a Means within a column followed by the same letter are not significantly different based upon the adjustment for multiple comparisons using Bonferroni correction ($\alpha_{CER} = 0.005$). Standard error: ± 4.6 for hybrids and ± 2.8 for lines.

the functional staygreen category and exhibits the characteristics of both A and B staygreen types. Type A staygreen shows delayed senescence compared with wild-type plants yet has the same rate of chlorophyll degradation as in the wild type after senescence begins, whereas type B initiates senescence at the same time but exhibits a much slower decrease of chlorophyll content and photosynthetic activity (Thomas and Howarth 2000; Thomas and Smart 1993). Interestingly, the hybrid DKS37-07, designated as staygreen by the seed company, did not show the same phenomenon. Although the plants that were inoculated with PBS showed constant SPAD through physiological maturity, it exhibited the highest SPAD reduction from soft dough to physiological maturity across pathogen treatments (Fig. 1). This is possibly attributed to the greater chlorophyll catabolism (enhanced chlorophyllase production) and lower anabolism of DKS37-07 under stalk rot pathogen infection at physiological maturity compared with those of SC599 and N remobilization pattern differences among functional and nonfunctional staygreen types.

In functional staygreen genotypes, the C–N transition point is delayed, or the transition occurs on time but subsequent yellowing and N remobilization occur slowly (Thomas and Howarth 2000; Yoo et al. 2007). However, delaying the C–N transition and slowing foliar senescence may compromise yield, particularly in high-N genotypes. Evidence in the literature suggests that the functional staygreen trait can be of limited or even negative value, particularly in oilseed crops such as soybean (Kumudini 2002) and legumes such as cowpea (Ismail et al. 2000). Using five high-yielding sorghum hybrids, Youngquist and Maranville (1992) demonstrated that stem and leaf fractions lost from 45 to 69% of their N content during grain filling. They also found 44 to 57% of plant N in the stem and 43 to 56% in the leaves at anthesis. However, at maturity, 58 to 68% of total plant N was in the grain, while 14 to 17% remained in the stems and 18 to 25% in the leaves. Therefore, with the high demand for N remobilization toward the sorghum panicle, high-yielding hybrids such as DKS37-07 may be unable to maintain higher chlorophyll contents in leaves during later stages of their life cycle, particularly when soil N content is limited. Therefore, DKS37-07 may belong to the nonfunctional staygreen category, representing type C or E. Type C retains chlorophyll almost indefinitely in the senescent leaves, although their photosynthetic capacity typically decreases

during senescence, while type E maintains a much higher chlorophyll level throughout leaf development without increasing photosynthetic competence (Thomas and Howarth 2000; Thomas and Smart 1993).

Significant pathogen–genotype–environment interactions observed for lesion length elucidated the genotype- and environment-dependent aggressiveness of stalk rot pathogens. There were no significant differences among pathogens on SC599 for either year, demonstrating SC599's similar levels of resistance against tested pathogens. Although BTx3042 is known to be highly susceptible to *Fusarium* stalk rot, it showed the shortest lesion length against the *F. thapsinum* isolate used for these inoculations in both environments. *F. thapsinum* is widely recognized as the most aggressive *Fusarium* sp. causing stalk rot (Leslie et al. 2005; Tesso and Ejeta 2011; Tesso et al. 2005, 2010) (Table 4). *Fusarium* stalk rot is usually more severe when drought and heat stresses occur during grain development followed by wet and cool conditions near physiological maturity (Tesso et al. 2012). BTx3042 attained physiological maturity in October under both environments. Although a considerable amount of rain was received during the month of October (2013, 98.3 mm; 2014, 90.2 mm; 30-year average, 65.3 mm), contributing to wet conditions, the monthly mean temperature in October was 23.3°C under both environments (30-year average, 13.5°C). It is possible that much cooler conditions are required by *F. thapsinum* to cause more disease in BTx3042 compared with the other genotypes tested. However, *M. phaseolina* produced the longest lesion length on BTx3042 in both environments. Because *M. phaseolina* is a necrotrophic pathogen and takes advantage of senescing tissues for rapid growth, delayed senescence may provide some protection against the pathogen. Compared with other sorghum genotypes tested in this study, SC599 showed lower lesion lengths against *M. phaseolina* under both environments. As mentioned above, SC599 is capable of maintaining a negative senescence rate from soft dough to physiological maturity. Hence, it can be considered to be a genotype showing strong delayed senescence which, in turn, may contribute to enhanced resistance against *M. phaseolina*.

Compared with the sorghum lines, both hybrids tested in this study showed longer lesion lengths when inoculated with *F. thapsinum* under both environments. This finding supports the idea that high-yielding genotypes are more vulnerable to *Fusarium* stalk rot, which

Table 4. Mean lesion lengths $\pm$ standard error (in centimeters) among sorghum genotypes after inoculation (Inoc) with phosphate-buffered saline (control [Con]), *Fusarium andiyazi* (FA), *F. proliferatum* (FP), *F. thapsinum* (FT), and *Macrophomina phaseolina* (MP) in 2013 and 2014^z

Inoc	Genotype							
	84G62		DKS37-07		BTX3042		SC599	
	2013	2014	2013	2014	2013	2014	2013	2014
Con	0.5 a $\pm$ 0.1	0.5 a $\pm$ 1.0	0.8 a $\pm$ 0.1	0.5 a $\pm$ 1.0	0.5 a $\pm$ 0.1	0.7 a $\pm$ 1.0	0.5 a $\pm$ 0.1	0.6 a $\pm$ 1.0
FA	4.2 bd $\pm$ 1.1	6.3 bc $\pm$ 1.0	8.0 bc $\pm$ 1.1	7.4 b $\pm$ 1.0	6.0 bc $\pm$ 1.1	5.3 b $\pm$ 1.0	3.7 b $\pm$ 1.1	3.0 b $\pm$ 1.0
FP	6.8 d $\pm$ 0.6	9.3 c $\pm$ 1.0	6.5 c $\pm$ 0.6	7.1 b $\pm$ 1.0	7.5 b $\pm$ 0.6	7.1 b $\pm$ 1.0	3.0 b $\pm$ 0.6	4.4 b $\pm$ 1.0
FT	11.2 c $\pm$ 0.7	7.5 bc $\pm$ 1.0	9.7 b $\pm$ 0.7	7.6 b $\pm$ 1.0	3.8 c $\pm$ 0.7	4.0 b $\pm$ 1.0	3.7 b $\pm$ 0.7	5.8 b $\pm$ 1.0
MP	4.0 b $\pm$ 0.4	4.7 b $\pm$ 1.0	5.7 c $\pm$ 0.4	5.8 b $\pm$ 1.0	8.8 b $\pm$ 0.4	7.2 b $\pm$ 1.0	3.8 b $\pm$ 0.4	3.0 b $\pm$ 1.0

^z Means within a column followed by the same letter are not significantly different based upon the adjustment for multiple comparisons using Bonferroni correction ($\alpha_{\text{CER}} = 0.005$).

Table 5. Pearson correlation coefficients and significance levels among yield, severity, and soil and plant analytical development (SPAD) traits for four genotypes after inoculation with *F. thapsinum*, *F. andiyazi*, *F. proliferatum* and *M. phaseolina* in 2013 (top, right, environment 1) and 2014 (bottom, left, environment 2) ($\alpha = 0.05$)^z

	TSW	HSW	LL	SPAD	SD	HD	PM
TSW	1	0.73***	−0.21*	0.59***	0.50***	0.53***	0.62***
SW	0.64***	1	−0.12	0.33*	0.31*	0.31*	0.49***
LL	−0.20*	−0.11	1	−0.58***	−0.49***	−0.57***	−0.54***
SPAD	0.61***	0.64***	−0.49***	1	0.89***	0.96***	0.88***
SD	0.50***	0.57***	−0.48***	0.93***	1	0.82***	0.71***
HD	0.61***	0.63***	−0.45**	0.96***	0.891***	1	0.87***
PM	0.62***	0.61***	−0.41**	0.94***	0.79***	0.87***	1

^z TSW = total seed weight per panicle (in grams); HSW = 100-seed weight (in grams); LL = lesion length (in centimeters); SPAD = mean SPAD across soft dough (SD), hard dough (HD), and physiological maturity (PM); *, **, and *** indicate $P < 0.05$, 0.001, and 0.0001, respectively.

is in agreement with Seetharama et al. (1991). These authors hypothesized that stalk rot is more severe in cultivars that utilize stem carbohydrates for higher yield compared with genotypes that exhibit lower grain yield because they do not mobilize stem carbohydrates. Furthermore, Clark and Miller (1980) showed that stalk sugar content is positively correlated with stalk rot resistance.

The present study provided evidence that the staygreen trait is correlated with decreased disease severity and, thus, increased plant resistance to stalk rot diseases. Because the difference between control and pathogen-treated TSW per panicle (Δ TSW, indicative of host tolerance) was significantly and positively correlated with the difference between control and pathogen-treated SPAD at physiological maturity (Δ SPAD_{pm}), the potential involvement of staygreen in enhancing the plant's tolerance to stalk rot diseases was evident. Although regression analysis did not reveal much stronger R^2 values for models fitted between Δ TSW (the response variable) and Δ SPAD_{pm} (the explanatory variable) under both environments, they were still statistically significant (Fig. 2). Moderate R^2 values were not surprising because only one explanatory variable (Δ SPAD_{pm}) was incorporated into the models.

The results of this study demonstrated the ability of stalk rot pathogens to reduce SPAD in a significant genotype- and developmental stage-specific manner. A commonly used stalk-rot-resistant and staygreen check line, SC599, showed a remarkable feature of negative senescence rate from soft dough to physiological maturity under disease pressure (also under no disease pressure). This phenomenon may be the best manifestation of the staygreen trait that contributes to enhanced stalk rot resistance. Results also indicated that staygreen is strongly and positively correlated with disease resistance and tolerance. Therefore, using grain sorghum as a model plant, we provide evidence of the potential involvement of the staygreen trait in enhancing sorghum stalk rot resistance and tolerance. The staygreen

trait deserves continued consideration in breeding efforts that particularly focus on producing hybrids that yield better under predicted, intensified future drought conditions and associated stalk rot incidence. It is also possible that staygreen is instrumental in reducing yield losses associated with various foliar diseases caused by numerous pathogens, including bacteria, fungi, and viruses, for many economically important crops. Given its various physiological resistance benefits, staygreen should remain a central trait to be pursued in sorghum given the climatic changes, increased disease occurrence, rapid population growth, and threatened food security predicted for our future.

Acknowledgments

We thank the Kansas Grain Sorghum Commission for their financial support of this research.

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Fig. 2. Scatter plots showing the relationship of Δ SPAD_{pm} (in relative soil and plant analytical development [SPAD] units) at physiological maturity (mock-inoculated control – pathogen treatment) with total seed weight per panicle (Δ TSW; mock-inoculated control – pathogen treatment) in the **A**, 2013 and **B**, 2014 experiments.

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