crops

MDPI

Article

# Response of Senegalese Sorghum Seedlings to Pathotype 5 of *Sporisorium reilianum*

**Ezekiel Ahn [1,†], Louis K. Prom [2,†], Coumba Fall [1] and Clint Magill [1,*]**

[1] Department of Plant Pathology & Microbiology, Texas A&M University, College Station, TX 77843, USA; eja85@tamu.edu (E.A.); coumba@tamu.edu (C.F.)
[2] USDA-ARS Southern Plains Agricultural Research Center, College Station, TX 77845, USA; louis.prom@usda.gov
* Correspondence: c-magill@tamu.edu
† These authors contributed equally to this work.

**Abstract:** *Sporisorium reilianum* causes head smut in sorghum. A total of 36 Senegalese sorghum accessions comprised of sorghum lines that have not been explored with response to pathotype 5 of *S. reilianum* were evaluated with 3 different treatments. First, seedling shoots were inoculated while still in soil with teliospores in agar, and then submerged under water at 4 days post inoculation. Signs of infection (noticeable spots) on the first leaf were observed up to 6 days post submergence. Second, seedlings at the same stage were inoculated by placing the teliospore impregnated agar around the stem in pots, moved to a greenhouse and grown to full panicle development stage. Third, seedings were inoculated via syringe inoculation in the greenhouse. Although soil inoculated seedlings grown in the greenhouse did not result in systemic infection as determined by lack of symptoms at panicle exsertion, 88.9% of tested cultivars showed systemic infections when syringe inoculated in the greenhouse. Inoculation of seedlings maintained under water led to broad range of noticeable spots that are assumed to be potential infection sites based on a previous study. In addition, seedling inoculation led to slightly upregulated expression of chitinase and PR10, genes that are associated with defense in aerial parts of plants.

**Keywords:** sorghum; Senegalese accessions; head smut; *Sporisorium reilianum*; seedling inoculation

check for updates

**Citation:** Ahn, E.; Prom, L.K.; Fall, C.; Magill, C. Response of Senegalese Sorghum Seedlings to Pathotype 5 of *Sporisorium reilianum*. *Crops* **2022**, *2*, 142–153. https://doi.org/10.3390/crops2020011

Academic Editors: Marta Nunes Da Silva and Carla Sofia Sancho Dos Santos

Received: 5 April 2022
Accepted: 27 April 2022
Published: 2 May 2022



## 1. Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is considered the fifth most important cereal crop in the world [1]. Sorghum acts as a dietary staple for millions of people living in about 30 countries in the subtropical and semi-arid regions of Africa and Asia [2]. It is a source of food and fodder, mostly in the traditional, smallholder farming sector [2]. Its production worldwide is constrained by several biotic and abiotic stresses [2] Head smut, caused by the soil-borne facultative biotrophic basidiomycete *Sporisorium reilianum* (Kühn) Langdon & Fullerton (syns. *Sphacelotheca reiliana* (Kühn) G.P. Clinton and *Sorosporium reilianum* (Kühn) McAlpine), is a serious global sorghum disease [3]. Although the fungus is a biotroph, the disease is devastating for the plant because it leads to complete harvest loss of each affected plant [4]. Soilborne spores germinate and penetrate the nodal region of the seedling shoot apex [5]. Based on host colonization patterns, the pathogen colonizes the meristematic area shortly after seed germination [5]. After growing within the plant as a dikaryon with opposing mating types, when the host plant enters its flowering stage, smut mycelium grows vigorously to produce teliospores [5] that replace the seed that should be formed. In areas where head smut occurs, planting the most resistant cultivars has been the primary means of control [5]. In addition to previously defined pathotypes P1–P4 of *S. reilianum*, two new pathotypes (P5 and P6) were identified among head smut isolates collected from South Texas in 2011 [3]. *S. reilianum* isolates of the two new pathotypes were syringe inoculated (hypodermic inoculation) separately into seedling plants within

the cultivars included in the Sorghum Association Panel (SAP), and a few cultivars were identified as resistant [6]. Sorghum germplasm from West and Central Africa is cultivated in rainy and high humidity regions and is an important source of resistance genes to fungal diseases [7]. Senegalese sorghum germplasm has an unclear genetic basis of resistance to fungal diseases including anthracnose [8].

In 1992, Craig and Frederiksen described a seedling inoculation method of sorghum; sorghum plants were grown in peat pellets and inoculated by infesting the vermiculite surrounding seedling epicotyls with teliospore cultures. Four days after inoculation, the seedlings were placed in test tubes containing water deep enough to submerge the first leaf [9]. Subsequently, symptoms on the first leaf blade differentiated susceptible and resistant genotypes wherein susceptible genotypes showed brown or dark spots on the first leaf [9] (Figure 1).

**Figure 1.** Seedling inoculation causes noticeable spots on the first leaf of susceptible genotypes. Seedling inoculation followed by submerging first left under water caused dark spots on the first leaves in PI514283.

These authors subsequently identified three different host resistance mechanisms: R1, horizontal, resistance to natural infection but susceptibility to all races following syringe inoculation; R2, vertical, specific resistance to some races of *S. reilianum* and susceptibility to others, with the same response to natural infection as to syringe inoculation; and R3, horizontal resistance to natural infection and syringe inoculation [6,9].

In this study, 36 Senegalese sorghum accessions and two Johnsongrass (*Sorghum halepense* (L.) Pers.) accessions were evaluated for resistance against pathotype 5 of *S. reilianum* based on the inoculation method described by Craig and Frederiksen [9] in the hope that it would provide a rapid test for resistance to pathotype 5. It is hypothesized that the % of sorghum seedlings with susceptible symptoms as well as the timing of confirmation for symptoms reflect variable levels of susceptibility. Comparisons of the results to needle inoculation and soil inoculation were also made. Johnsongrass is a wild relative of sorghum, but unlike sorghum, it is considered one of the most noxious weeds in the world [10]. As Johnsongrass is known to be a reservoir for various sorghum pathogens [11], it was hypothesized that Johnsongrass would show similar spots when treated at seedling

stage. The second inoculation protocol was similar to inoculation in nature where seedling infections rely on interaction with soil borne teliospores. Here, a high concentration of spores was provided to seedlings started in small pots. We tested potential effects on fully grown plants when inoculated seedlings were grown in a greenhouse to the stage of full panicle development. As a control, syringe inoculation was conducted to the same accessions grown in in a greenhouse. Lastly, it was hypothesized that Craig and Frederiksen's inoculation method [9] would induce expression of defense-related genes in aerial parts of sorghum seedlings. Thus, expression of defense-related genes [β-1,3-glucanase, chalcone synthase 8 (CHS8), pathogen induced chitinase, flavonoid-3′-hydroxylase and pathogenesis related protein-10 (PR-10)] were measured with Real-Time qRT-PCR [12] in two accessions at day 1 post inoculation.

## 2. Methods

### *2.1. Sorghum Lines*

Thirty-six accessions of Senegalese sorghum germplasm were obtained from the USDA-ARS, Plant Genetic Resources Conservation Unit, Griffin, Georgia. BTx635 (resistant) and BTx643 (susceptible) were also included to screen against Pathotype 5 of the head smut pathogen. These two accessions are widely used as negative and positive controls for syringe inoculation screening of head smut [3,6], but the accessions were not tested by the seedling inoculation method described by Craig and Frederiksen [9]. Johnsongrass cultivars, SH1136 and SH1152, were included in the screening. These two accessions were grown from fragments of rhizome to the first leaf stage in the greenhouse and brought to a laboratory for inoculation.

### *2.2. Seedling Inoculation*

The method described by Craig and Frederiksen [9] was used with modifications. In brief, plug flats with 40 Square Cells (L × W × H ≈ 5 cm × 5 cm × 7 cm for each cell) (The HC Companies, Twinsburg, OH, USA) were filled with Metro Mix 200 (Sun Gro Horticulture, Agawam, MA, USA). Teliospores of pathotype 5 isolates #27 and #79 were acquired by Louis K. Prom (USDA-ARS Southern Plains Agricultural Research Center). A total of 0.2 g of teliospores was placed in 15 mL of sterile distilled water in a centrifuge tube, shaken into suspension and precipitated by centrifuging at 500 *g* for 10 sec; the water was then decanted [9]. Washed teliospores were suspended in 15 mL of sterile distilled water, and 0.5 mL of the teliospore suspension was added to 50 mL of sucrose agar (3% sucrose, 0.25% agar, *w*/*v*, adjusted to pH 3.8 with lactic acid) in a 250 mL Erlenmeyer flask [9]. The cultures were incubated on a rotary shaker operated at 100 rpm at room temperature [9]. The inoculation for each cultivar were repeated at least 3 times. At day 4 post inoculation, the first leaves of the seedlings were submerged under water in test tubes. The tubes were observed daily and the timing and density of spots, were assumed to represent susceptible symptoms and recorded. We observed and recorded the results up to day 6 post inoculation. As negative controls, PI514279, PI514282, PI514284 and PI514303 were mock inoculated with sucrose agar.

As another treatment, seedlings inoculated in the same fashion as those placed in tubes but left in the individual cells of plug flats were moved to a greenhouse located in College Station, TX, USA at day 4 post inoculation. Each accession was subsequently transferred in 3-gallon (≈11.35 L) pots filled with Metro Mix 200 (ingredients: Canadian sphagnum peat moss, bark, perlite, vermiculite, dolomitic limestone, long-lasting wetting agent, and resilience).

For syringe inoculation and inoculum preparation, the steps described by Prom et al. [3] were followed. In brief, sporidial colonies of pathotype 5 isolates #27 and #79 were incubated in separate flasks filled with potato dextrose broth. The flasks were placed on a rotary shaker set at 150 rpm for 4d under 25 °C. The sporidial suspension was filtered through four layers of cheesecloth. The two suspensions were mixed (*v*:*v*) and adjusted to a concentration of $1 \times 10^5$ spores $mL^{-1}$. The mixed sporidial suspension was used to

inoculate five plants in each replicate by injecting 0.5 to 1.0 mL below the apical meristem of 18 to 20 day-old seedlings using a Precision Glide Needle # 22 G × 1 in. (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) attached to a 30 mL hypodermic syringe.

Pots were placed on benches and plants grown at 25 ± 2 °C with 65% humidity level under a 12-hour photoperiod provided by fluorescent light in a Conviron growth chamber (Conviron, Winnipeg, MB, Canada). These plants were grown to the stage of full panicle development. If the main panicle exhibited no head smut infection, it was cut back and it allowed the plant to produce tillers. When the tillers showed no head smut symptoms, the plant was considered resistant. The treatments were repeated at least three times. For greenhouse inoculation, cultivars with any plants developing symptoms recorded as susceptible.

### *2.3. RNA Extraction and Real-Time qRT-PCR Analysis*

PI514296 and PI514311 were selected based on screening with seedling inoculation methods as PI514296 had low rate of susceptible symptoms shown, while PI514311 was the opposite. Seedlings at day 1 post inoculation (24 h) were used for RNA extraction and Real-Time qRT-PCR analysis as described by Ahn et al. for five defense response related genes [β-1,3-glucanase, chalcone synthase 8 (CHS8), pathogen induced chitinase, flavonoid-3′-hydroxylase and pathogenesis related protein-10 (PR-10)] [13]. RNA extraction and Real-Time qRT-PCR analysis were repeated four times.

### *2.4. Statistical Analysis*

A Student's *t*-test for all possible group comparisons was performed with JMP Pro 14 (SAS Institute, Cary, NC, USA) to compare the timing of conformation for symptoms on the first leaf. Pearson's correlation was tested between three different traits (rate of spot appearance on the first leaf, timing of conformation for symptoms on the first leaf, and infection rate via greenhouse syringe inoculation).

For Real-Time qRT-PCR analysis results, as described by Ahn et al. [13], 2ˆΔΔCts were computed to $\log_2$ (Expression Fold Change) transformation and statistically analyzed by using a two-tailed pooled *t*-test. In all cases, fold values are expressed relative to zero-time control samples [13].

## 3. Results

### *3.1. Phenotypic Variation for Seedling Inoculation*

As Craig and Frederiksen described in their work [9], control mock inoculated accessions did not show any noticeable symptoms such as brown or dark spots. Likewise, Johnsongrass cultivars SH1136 and SH1152 did not show any sign of symptoms as well as they were whether inoculated or not. The 36 accessions showed clear spots when inoculated with *S. reilianum* (Table 1 and Figure 2); infection varied from 28.6% to 100% of the 10 to 32 seedlings of each cultivar tested. Additional sorghum accessions, BTx635 and BTx643, had low infection rate based on appearance of brown or dark spots on the first leaf. The average time for an observation of the symptom fell between 3.4 to 5.8 days. No correlation was found between infection rate and time of appearance of symptoms (Pearson's correlation = −0.08 with *p*-value = 0.66).

All 36 accessions tested for seedling inoculation of pathotype 5 showed symptoms, but the percentage of plants showing symptoms varied. Mean values (days) and standard errors mean (SEM) are shown for timing of the symptom confirmation. Values in a column with the same letter(s) are not significantly different with 95% confidence based on *t*-test for all possible pairs. Total numbers of tested plants are between 10 and 32 for each accession.

**Table 1.** Rate of symptom appearance for seedlings inoculated with Pathotype 5 of *S. reilianum* and placed under water.

| Accession | No. of Inoculated Seedlings | No. of Trials | Disease Incidence | | Average Time (Days) of Detection |
|---|---|---|---|---|---|
| | | | Susceptible (%) | Resistant (%) | |
| PI514279 * | 13 | 3 | 100 | 0 | $5.8 \pm 0.1^{a}$ |
| PI514284 | 16 | 4 | 100 | 0 | $4.3 \pm 0.1^{efghijk}$ |
| PI514287 | 14 | 3 | 100 | 0 | $4.4 \pm 0.3^{efghij}$ |
| PI514289 | 20 | 4 | 100 | 0 | $3.6 \pm 0.1^{lm}$ |
| PI514295 | 14 | 3 | 100 | 0 | $4.5 \pm 0.2^{efghij}$ |
| PI514306 | 12 | 3 | 100 | 0 | $4.3 \pm 0.3^{efghijk}$ |
| PI514308 | 11 | 3 | 100 | 0 | $5.4 \pm 0.2^{abc}$ |
| PI514309 | 11 | 3 | 100 | 0 | $3.9 \pm 0.2^{hijklm}$ |
| PI514311 | 20 | 4 | 100 | 0 | $3.4 \pm 0.1^{m}$ |
| PI514294 | 23 | 5 | 95.7 | 4.3 | $4.4 \pm 0.2^{efghij}$ |
| PI514316 * | 20 | 4 | 95 | 5 | $4.3 \pm 0.2^{efghijk}$ |
| PI514283 | 17 | 4 | 94.1 | 5.9 | $4.4 \pm 0.3^{efghijk}$ |
| PI514301 | 14 | 3 | 92.9 | 7.1 | $4.5 \pm 0.2^{efghij}$ |
| PI514299 | 23 | 5 | 91.3 | 8.7 | $4.0 \pm 0.2^{ijkl}$ |
| PI514313 | 22 | 5 | 90.9 | 9.1 | $4.0 \pm 0.2^{hijkl}$ |
| PI514297 | 10 | 3 | 90 | 10 | $4.9 \pm 0.3^{bcde}$ |
| PI514298 | 29 | 6 | 89.7 | 10.3 | $5.2 \pm 0.2^{bcd}$ |
| PI514314 | 16 | 3 | 87.5 | 12.5 | $4.6 \pm 0.2^{defg}$ |
| PI514302 | 15 | 3 | 86.7 | 13.3 | $4.7 \pm 0.1^{cdefg}$ |
| PI514312 | 23 | 5 | 82.6 | 17.4 | $3.6 \pm 0.2^{lm}$ |
| PI514282 | 11 | 3 | 81.8 | 18.7 | $4.2 \pm 0.3^{efghijkl}$ |
| PI514285 * | 11 | 3 | 81.8 | 18.7 | $4.6 \pm 0.2^{defghij}$ |
| PI514286 | 11 | 3 | 81.8 | 18.7 | $3.7 \pm 0.4^{klm}$ |
| PI514288 | 11 | 3 | 81.8 | 18.7 | $4.9 \pm 0.2^{bcde}$ |
| PI514290 | 11 | 3 | 81.8 | 18.7 | $3.8 \pm 0.3^{jklm}$ |
| PI514300 * | 10 | 3 | 80 | 20 | $4.6 \pm 0.4^{cdefghi}$ |
| PI514303 | 10 | 3 | 80 | 20 | $4.8 \pm 0.3^{bcdefg}$ |
| PI514310 | 14 | 3 | 78.6 | 21.4 | $4.1 \pm 0.3^{ghijkl}$ |
| PI514291 | 32 | 6 | 71.9 | 28.1 | $4.2 \pm 0.2^{ghijk}$ |
| PI514304 | 24 | 5 | 70.8 | 29.2 | $4.8 \pm 0.3^{bcdef}$ |
| PI514292 | 12 | 3 | 58.3 | 41.7 | $4.0 \pm 0.5^{fghijklm}$ |
| PI514307 | 12 | 3 | 58.3 | 41.7 | $4.0 \pm 0.2^{fghijklm}$ |
| PI514305 | 18 | 4 | 55.6 | 44.4 | $3.7 \pm 0.5^{klm}$ |
| PI514280 | 11 | 3 | 54.5 | 45.5 | $4.3 \pm 0.3^{efghijkl}$ |
| PI514293 | 14 | 3 | 50 | 50 | $4.7 \pm 0.4^{bcdefgh}$ |
| PI514296 | 28 | 5 | 28.6 | 71.4 | $5.5 \pm 0.2^{ab}$ |
| SH1136 (JG) | 10 | 3 | 0 | 100 | - |

**Table 1.** *Cont.*

| Accession | No. of Inoculated Seedlings | No. of Trials | Disease Incidence | | Average Time (Days) of Detection |
|---|---|---|---|---|---|
| | | | Susceptible (%) | Resistant (%) | |
| SH1152 (JG) | 10 | 3 | 0 | 100 | - |
| PI514279 Control | 10 | 3 | 0 | 100 | - |
| PI514282 Control | 10 | 3 | 0 | 100 | - |
| PI514284 Control | 10 | 3 | 0 | 100 | - |
| PI514303 Control | 10 | 3 | 0 | 100 | - |
| BTx635 | 10 | 3 | 0 | 100 | - |
| BTx643 | 10 | 3 | 10 | 90 | 4 [bcdefghijklm] |

* Accessions with no symptoms at maturity following inoculation by injection.

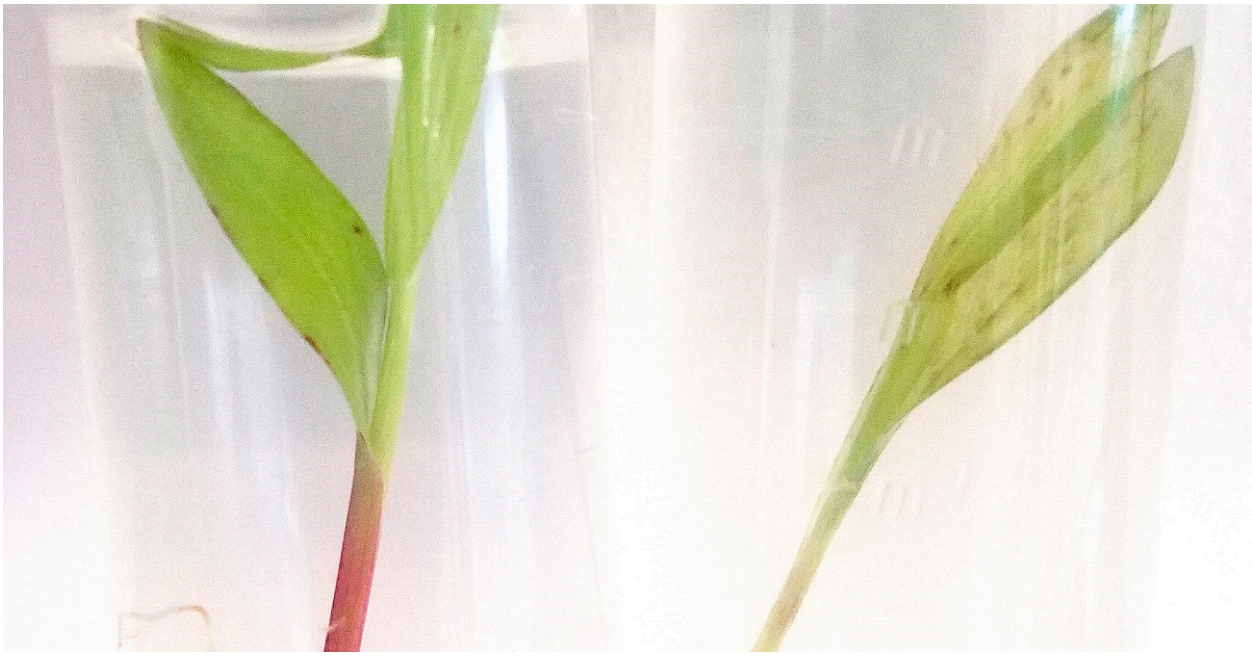

**Figure 2.** Seedling inoculation vs mock inoculation on PI514282. Mock inoculated plants (**left**) did not show symptom, while the pathogen inoculated plants (**right**) cleared showed spots in PI514282 and other accessions.

### *3.2. Seedling Inoculation Activates Chitinase and PR10*

Within 5 tested defense related genes in sorghum, chitinase and PR10 were slightly upregulated in PI514311 (Table 2) compared to 0-time post inoculation. PI514296 slightly upregulated PR10 as well. Expressions of the two genes between two accessions were not significant. Other three tested genes did not show any upregulation at day 1 post inoculation (data not shown).

**Table 2.** Expression of chitinase and PR10 in PI514296 and PI514311 at day 1 post inoculation with Pathotype 5 of *S. reilianum*.

| Accession | Susceptibility (%) | Time (Days) | Fold Change for Chitinase | *p*-Value between Two Cultivars | Fold Change for PR10 | *p*-Value between Two Cultivars |
|---|---|---|---|---|---|---|
| PI514296 | 28.6 | 5.5 ± 0.2 | 0.8 ± 0.4 | 0.15 | 1.7 ± 0.4 | 0.09 |
| PI514311 | 100 | 3.4 ± 0.1 | 2.1 ± 0.7 | | 3.9 ± 1.0 | |

Expression of the two genes is listed in the fold change. *p*-values based on a pooled *t*-test for fold changes and SEM in the two cultivars are shown as well (N = 4). Only PR 10 was somewhat but non-significantly upregulated in both cultivars

### *3.3. Seedling Inoculation Is Not as Effective as Syringe Inoculation for Full Disease Development*

No accessions inoculated and transferred to pots developed any symptoms at the post panicle emergence stage. In contrast, 32 accessions (88.9%) were susceptible to Pathotype 5 of *S. reilianum* with syringe inoculation (Table 3 & Figure 3).

**Table 3.** The results of sorghum plants inoculated with seedling and syringe inoculation methods.

| Accession | Seedling Inoculation (Susceptible/Resistant) | | Hypodermic (Syringe) Inoculation (Susceptible/Resistant) | |
|---|---|---|---|---|
| PI514279 | R | 0% | R | 0% |
| PI514280 | R | 0% | S | 57.1% |
| PI514282 | R | 0% | S | 14.3% |
| PI514283 | R | 0% | S | 20% |
| PI514284 | R | 0% | S | 11.1% |
| PI514285 | R | 0% | R | 0% |
| PI514286 | R | 0% | S | 22.2% |
| PI514287 | R | 0% | S | 8.3% |
| PI514288 | R | 0% | S | 75% |
| PI514289 | R | 0% | S | 10% |
| PI514290 | R | 0% | S | 40% |
| PI514291 | R | 0% | S | 33.3% |
| PI514292 | R | 0% | S | 33.3% |
| PI514293 | R | 0% | S | 11.1% |
| PI514294 | R | 0% | S | 45.5% |
| PI514295 | R | 0% | S | 27.3% |
| PI514296 | R | 0% | S | 22.2% |
| PI514297 | R | 0% | S | 50% |
| PI514298 | R | 0% | S | 28.6% |
| PI514299 | R | 0% | S | 25% |
| PI514300 | R | 0% | S | 25% |
| PI514301 | R | 0% | S | 70% |
| PI514302 | R | 0% | S | 16.5% |
| PI514303 | R | 0% | S | 58% |
| PI514304 | R | 0% | S | 20% |
| PI514305 | R | 0% | S | 30% |
| PI514306 | R | 0% | S | 20% |
| PI514307 | R | 0% | S | 14.3% |
| PI514308 | R | 0% | R | 0% |
| PI514309 | R | 0% | S | 33.3% |
| PI514310 | R | 0% | S | 16.7% |
| PI514311 | R | 0% | S | 55.6% |

**Table 3.** *Cont.*

| Accession | Seedling Inoculation (Susceptible/Resistant) | | Hypodermic (Syringe) Inoculation (Susceptible/Resistant) | |
|---|---|---|---|---|
| PI514312 | R | 0% | S | 80% |
| PI514313 | R | 0% | S | 66.7% |
| PI514314 | R | 0% | S | 11.1% |
| PI514316 | R | 0% | R | 0% |
| SH1136 (JG) | R | 0% | - * | - * |
| SH1152 (JG) | R | 0% | - * | - * |
| PI514279 Control | R | 0% | - | - |
| PI514282 Control | R | 0% | - | - |
| PI514284 Control | R | 0% | - | - |
| PI514303 Control | R | 0% | - | - |
| BTx635 (−) | R | 0% | R | 0% |
| BTx643 (+) | R | 0% | S | 66.7% |

**Figure 3.** Head smut infected panicle on PI514297. 32 accessions showed at least one infected panicle within total 3 trials of syringe inoculation.

Based on Pearson's correlation analysis, no correlation was found between infection rates in seedling gel inoculation and syringe inoculation (correlation = −0.06 with $p$-value = 0.74). Timing for spot appearance and syringe inoculation infection rate was also not supported by statistical analysis (Pearson's correlation = −0.25 with $p$-value = 0.14).

The same accessions tested for seedling inoculation were used. Seedling inoculated plants were moved to a greenhouse and grown to the stage of full panicle exsertion.

Meanwhile, traditional syringe inoculation was conducted on the same accessions. The experiments were repeated at least three times. Along with R/S labeling, raw infection rates were shown. BTx635 is a negative control, and BTx643 is a positive control for syringe inoculation. * = Not applicable as Johnsongrass stem is too thin to apply syringe inoculation. Total number of tested plants for seedling inoculation are between 9 and 12 for each accession. For hypodermic inoculation, 4–11 plants were used for each accession.

## 4. Discussion

Seedlings inoculated with the head smut pathogen in the laboratory and later placed under water led to various infection (showed clear spots) rates of symptom development in terms in the Senegalese accessions; between 28.3% (PI514296) and 100% (PI514279 and 8 other accessions) of inoculated seedlings were affected. However, the presumed symptoms of infection may instead reflect host response signals or even responses unrelated to disease, since none of the matching laboratory inoculated seedlings, later placed on benches and grown to the full panicle emergence stage in the greenhouse showed any signs of infection (all accessions successfully developed normal panicles). As the method is a simple modification of natural infection with higher germination rate of teliospore, this could reflect low rates of successful inoculation. As examples, TAM428 and Tx430 have a 1% natural infection rate when grown in field plots with a history of head smut, while the rates are 44% and 55% under syringe inoculation [9]. A total of 4 of the 36 of accessions were resistant under syringe inoculation; these Senegalese accessions are candidates for resistance against Pathotype 5 of *S. reilianum* especially since some of these lines are also reported to be resistant to Texas isolates of *Colletotrichum sublineola* (Henn.) (equivalent, *C. sublineolum*) [8], Senegalese accessions may be important sources of genetic resistance to other fungal pathogens as well.

BTx643, which is a positive control for syringe inoculation, showed the spots on the first leaf in 1 out of 10 samples in seedling inoculation. This indicates the accession could be reaction class R1 based on Craig and Frederiksen's classification [9]. On the other hand, BTx635 might be reaction class R2 or R3 as the accession did not show noticeable symptoms with all three treatments (seedling inoculation, inoculated seedlings grown to full panicle exsertion stage in the greenhouse and syringe inoculation). Likewise, we can speculate reaction classes of the tested sorghum accessions with the results, but as these accessions were not widely tested to various pathotypes of *S. reilianum*, it is hard to differentiate between R2 and R3. Johnsongrass is known to be susceptible to sorghum anthracnose [14] or vice versa [15]. In this study, however, Johnsongrass did not show any symptom which was expected as Johnsongrass is not reported to be susceptible to *S. reilianum*. Average time for spot appearance differed based on genotypes (3.4 days to 5.8 days). Within the tested accessions, no significant correlation was detected between the two traits. The infection rate for syringe inoculation showed no correlation to the seedling spot appearance rate and its timing for spots among the tested 36 cultivars. The four cultivars' resistance to syringe inoculation had symptom rates of 80% or more in the submerged leaf test. In a similar study, 20 new potential sources of resistance from the SAP lines for P5 and P6 pathotypes of *S. reilianum* [6]. Along with the reported resistant SAP lines, four resistant lines based on syringe inoculation are expected to be used in introgression breeding to develop parental lines and hybrids with head smut resistance [6]. To have a clearer idea, more accessions should be tested in the future. Although not confirmed, based on observations in this study, spots on seedlings under water had a tendency to spread from the lower to the upper stem and the first leaf in many accessions (Figure 4). Further studies are needed to confirm whether spot appearance is directional or random.

**Figure 4.** Spots appeared by seedling inoculation might be directional. Spots on susceptible genotypes might spread from bottom to top under water.

The Real-Time qRT-PCR results suggested that seedling inoculation may activate defense-related genes in aerial parts of sorghum seedlings, but upregulation for chitinase in PI514311 and PR10 in both PI514296 and PI514311 were only minimal at day 1 post inoculation and not statistically significant between two cultivars. Chitinase is capable of degrading fungal cell walls, and it is typically expressed at high levels in sorghum following inoculation with fungal pathogens [16,17]. PR10 is a small acidic protein with potential nuclease activity that is activated in host defense of many species [18]. The minimal upregulations might mean plants need longer time to activate defense related genes as aerial parts of seedling do not have direct contact with the inoculum. Intriguingly, PI514311, the accession with higher infection rate, had higher expressions of chitinase and PR10 without statistical significance when compared with PI514296 (Table 2). It was reported that moderately susceptible Johnsongrass cultivars upregulated defense related genes with *C. sublineola* inoculation compared to a resistant cultivar [12]. In cottons, defense related conserved regions in plant 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) were much more rapidly induced in *Gossypium barbadense* L. (S) than *Gossypium hirsutum* L.

(R) when inoculated with wilt pathogen *Verticillium dahliae* Kleb., (1913) spores [19]. The average time for symptom conformation was 3.4 days in PI514311 when it was 5.5 days in PI514296; it is speculated that defense-related gene expressions might be related with not only infection rate/severity but also various other traits such as timing for the emergence of brown/dark spots. As the genes were not highly activated, other time points and/or other accessions should be tested to have a clearer idea. After all, it is not clear that spot appearance is truly associated with defense against *S. reilianum*.

Further, sorghum seedling contains the preformed cyanogenic glycoside dhurrin, which my play a role in seedling protection [20]. However, smuts are also considered to be 'stealth' pathogens, since they are able to grow through the host without triggering a defense response [21].

In conclusion, Senegalese lines were susceptible to pathotype 5 of *S. reilianum*. Although seedling inoculation did not cause visual infection at panicle exsertion stage, most cultivars showed spots under water. In addition, syringe inoculation successfully infected most of the tested cultivars. Seedling inoculation upregulated a portion of tested defense related genes in aerial parts of sorghum accessions. Since Craig and Frederiksen's seedling inoculation method [9] has not been applied in many studies, this study provides useful information to sorghum plant pathologists as well.

**Author Contributions:** Conceptualization, E.A. and C.M.; methodology, E.A. and L.K.P.; validation, E.A., L.K.P. and C.F.; formal analysis, E.A.; investigation, E.A., L.K.P. and C.F.; resources, C.M.; data curation, E.A., L.K.P. and C.F.; writing—original draft preparation, E.A.; writing—review and editing, L.K.P. and C.M.; supervision, C.M.; project administration, C.M.; funding acquisition, C.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was funded by AFRI, NIFA, USDA (grant number 20156800423492) & by the Feed the Future Innovation Lab for Collaborative Research on Sorghum and Millet, a United States Agency for International Development Cooperative (agreement number AID-OAA-A-13-00047 with title name: Enabling marker assisted selection for sorghum disease resistance in Senegal and Niger).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** No potential conflict of interest relevant to this article was reported.

## References

1. Doggett, H. *Sorghum*, 2nd ed.; Longman Scientific: Essex, UK, 1988; ISBN 0-582-46345-9.
2. Hariprasanna, K.; Rakshit, S. Economic Importance of Sorghum. In *The Sorghum Genome*; Rakshit, S., Wang, Y.-H., Eds.; Springer International Publishing: Cham, Switzerland, 2016; pp. 1–25.
3. Prom, L.K.; Perumal, R.; Erattaimuthu, S.R.; Erpelding, J.E.; Montes, N.; Odvody, G.N.; Greenwald, C.; Jin, Z.; Frederiksen, R.; Magill, C. Virulence and molecular genotyping studies of *Sporisorium reilianum* Isolates in Sorghum. *Plant Dis.* **2011**, *95*, 523–529. [CrossRef] [PubMed]
4. Poloni, A.; Schirawski, J. Host specificity in *Sporisorium reilianum* is determined by distinct mechanisms in maize and sorghum. *Mol. Plant Pathol.* **2016**, *17*, 741–754. [CrossRef] [PubMed]
5. Frederiksen, R.; Odvody, G. *Compendium of Sorghum Diseases*, 2nd ed.; American Phytopathological Society (APS Press): St. Paul, MN, USA, 2000; ISBN 978-0-89054-240-8.
6. Prom, L.K.; Perumal, R.; Isakeit, T.; Erattaimuthu, S.; Magill, C. Response of sorghum accessions against newly documented pathotypes 5 and 6 of head smut pathogen, *Sporisorium reilianum*. *Am. J. Plant Sci.* **2021**, *12*, 432–443. [CrossRef]
7. Cuevas, H.E.; Prom, L.K.; Rosa-Valentin, G. Population structure of the NPGS Senegalese sorghum collection and its evaluation to identify new disease resistant genes. *PLoS ONE* **2018**, *13*, e0191877. [CrossRef] [PubMed]
8. Ahn, E.; Prom, L.K.; Hu, Z.; Odvody, G.; Magill, C. Genome-wide association analysis for response of Senegalese sorghum accessions to Texas isolates of anthracnose. *Plant Genome* **2021**, *14*, e20097. [CrossRef] [PubMed]
9. Craig, J.; Frederiksen, R.A. Comparison of sorghum seedling reactions to Sporisorium reilianum in relation to sorghum head smut resistance classes. *Plant Dis.* **1992**, *76*, 314–318. [CrossRef]
10. Ohadi, S.; Littlejohn, M.; Mesgaran, M.; Rooney, W.; Bagavathiannan, M. Surveying the spatial distribution of feral sorghum (*Sorghum bicolor* L.) and its sympatry with johnsongrass (*S. halepense*) in South Texas. *PLoS ONE* **2018**, *13*, e0195511.

11. Ahn, E.; Prom, L.K.; Odvody, G.; Magill, C. Diseases of Johnsongrass (*Sorghum halepense*): Possible role as a reservoir of pathogens affecting other plants. *Weed Sci.* **2021**, *69*, 393–403. [CrossRef]
12. Ahn, E.; Prom, L.K.; Odvody, G.; Magill, C. Responses of johnsongrass against sorghum anthracnose isolates. *J. Plant Pathol. Microbiol.* **2018**, *9*, 1–6.
13. Ahn, E.; Prom, L.K.; Odvody, G.; Magill, C. Defense responses against the sorghum anthracnose pathogen in leaf blade and midrib tissue of johnsongrass and sorghum. *Physiol. Mol. Plant Pathol.* **2019**, *106*, 81–86. [CrossRef]
14. Ahn, E.; Prom, L.K.; Odvody, G.; Magill, C. Late growth stages of johnsongrass can act as an alternate host of *Colletotrichum sublineola*. *Plant Health Prog.* **2020**, *21*, 60–62. [CrossRef]
15. Xavier, K.V.; Mizubuti, E.S.G.; Queiroz, M.V.; Chopra, S.; Vaillancourt, L. Genotypic and pathogenic diversity of *Colletotrichum sublineola* isolates from sorghum (*Sorghum bicolor*) and johnsongrass (*S. halepense*) in the Southeastern United States. *Plant Dis.* **2018**, *102*, 2341–2351. [CrossRef] [PubMed]
16. Little, C.; Magill, C. Elicitation of defense response genes in sorghum floral tissues infected by *Fusarium thapsinum* and *Curvularia lunata* at anthesis. *Physiol. Mol. Plant Pathol.* **2003**, *63*, 271–279. [CrossRef]
17. Cota, I.E.; Troncoso-Rojas, R.; Sotelo-Mundo, R.; Sánchez, A.; Tiznado-Hernández, M. Chitinase and β-1,3-glucanase enzymatic activities in response to infection by *Alternaria alternata* evaluated in two stages of development in different tomato fruit varieties. *Sci. Hortic.* **2007**, *112*, 42–50. [CrossRef]
18. Jain, D.; Khurana, J.P. Role of pathogenesis-related (PR) proteins in plant defense mechanism. In *Molecular Aspects of Plant-Pathogen Interaction*; Singh, A., Singh, I.K., Eds.; Springer: Singapore, 2018; pp. 265–281.
19. Joost, O.; Bianchini, G.; Bell, A.A.; Benedict, C.R.; Magill, C. Differential induction of 3-hydroxy-3-methylglutaryl CoA reductase in two cotton species following inoculation with Verticillium. *Mol. Plant Microbe Interact.* **1995**, *8*, 880–885. [CrossRef] [PubMed]
20. Nicholson, R.L.; Jamil, F.F.; Snyder, B.A.; Lue, W.L.; Hipskind, J. Phytoalexin synthesis in the juvenile sorghum leaf. *Physiol. Mol. Plant. Pathol.* **1988**, *33*, 271–278. [CrossRef]
21. Cui, Y.; Magill, J.; Frederiksen, R.; Magill, C. Chalcone synthase and phenylalanine ammonia-lyase mRNA levels following exposure of sorghum seedlings to three fungal pathogens. *Physiol. Mol. Plant Pathol.* **1996**, *49*, 187–199. [CrossRef]