Fungal pathogens of *Hyparrhenia rufa* (Nees) Stapf., an invasive weed in Puerto Rico, and their potential as biological control agents[1,2]

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ABSTRACT

Hyparrhenia rufa **(Nees) Stapf., commonly known as jaragüa, faragüa or yaragüa grass, is an aggressive weed that has invaded cropland in Puerto Rico. To reduce the application of herbicides, the use of phytopathogenic fungi has been proposed for its control. Four fungal isolates associated with foliage lesions of *H. rufa* were identified as *Curvularia* sp., *Fusarium* sp., *Phoma sorghina* and *Sphaeropsis* sp. Their pathogenicity was tested under laboratory, greenhouse and field conditions. All fungal species were pathogenic in wounded tissues under laboratory conditions, whereas *P. sorghina* and *Sphaeropsis* sp. were the most virulent species under greenhouse conditions. *Fusarium* sp. was the most virulent in a field assay. All fungal isolates showed low levels of infection (level 1) in the field. Disease severity was estimated at 25% of the experimental plot area. To comply with Koch's postulates, all inoculated fungi were re-isolated on potato dextrose agar after pathogenicity tests conducted under laboratory, greenhouse and field conditions. This is the first report of *Curvularia* sp., *Fusarium* sp., *P. sorghina* and *Sphaeropsis* sp. as foliar pathogens of *H. rufa*.**

Key words: invasive weed, *Hyparrhenia rufa*, jaragüa grass, *Curvularia* sp., *Fusarium* sp., *Phoma sorghina*, *Sphaeropsis* sp., biocontrol

RESUMEN

Hongos patógenos de *Hyparrhenia rufa*, una maleza invasiva en Puerto Rico, y su potencial como agentes de control biológico

Hyparrhenia rufa **(Nees) Stapf., conocida comúnmente como pasto jaragüa, faragüa o yaragüa, es una maleza agresiva que ha invadido terrenos cultivables en Puerto Rico. Para disminuir la aplicación de herbicidas, se ha**

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propuesto el uso de hongos fitopatógenos para su control. Se identificaron cuatro aislados de hongos asociados a lesiones foliares de *H. rufa*: *Curvularia* sp., *Fusarium* sp., *Phoma sorghina* y *Sphaeropsis* sp. Se evaluó su patogenicidad en condiciones de laboratorio, invernadero y campo. Todas las especies de hongos fueron patogénicas al tejido foliar (con herida) en condiciones de laboratorio mientras que *P. sorghina* y *Sphaeropsis* sp. fueron las especies más virulentas en el ensayo de invernadero. *Fusarium* sp. fue la especie más virulenta en el ensayo de campo. Se observaron bajos niveles de infección (nivel 1) en el campo para todos los aislados de hongos evaluados. La severidad de la enfermedad fue estimada en un 25% del área de la parcela experimental. Para completar los postulados de Koch, todos los hongos inoculados fueron re-aislados en agar de papa y dextrosa luego de concluir las pruebas de patogenicidad conducidas en condiciones de laboratorio, invernadero y campo. Este es el primer reporte de *Curvularia* sp., *Fusarium* sp., *P. sorghina* and *Sphaeropsis* sp. como patógenos foliares de *H. rufa*.

Palabras clave: maleza invasiva, *Hyparrhenia rufa*, yerba jaragüa, *Curvularia* sp., *Fusarium* sp., *Phoma sorghina*, *Sphaeropsis* sp., control biológico

INTRODUCTION

Hyparrhenia rufa (Nees) Stapf, commonly known as jaragüa, faragüa or yaragüa, is originally from Africa and belongs to the Poaceae or grass family (Más and Lugo-Torres, 2013). It is widespread and known all over the world as both forage for livestock and as a very aggressive invasive weed (Skerman and Riveros, 1990). In Puerto Rico, it has invaded cropland and is becoming a concern to farmers. It is described as a grass resistant to diseases and insect pests (Skerman and Riveros, 1990). Studies on pathogens of *H. rufa* are scarce. In Kenya, a report of *Hyparrhenia* grass white leaf disease, associated with a 16SrXI phytoplasma, has been published (Obura et al., 2011).

Pathogenic fungi have been used as bioherbicides and incorporated into crop production systems for invasive weed management (Charudattan, 2005). The objectives of this research were to identify fungal pathogens of *H. rufa* and to build a bank of pathogens as potential biological control agents.

MATERIALS AND METHODS

Sample collection

Symptomatic *H. rufa* leaves were collected at the UPR-Agricultural Experiment Station located at Road 101 Km. 8.5, Sabana Yegüas sector of Lajas, Puerto Rico (GPS coordinates: N 18° 0.1092 and W 67° 04.126). Leaves were placed in plastic bags and transferred in a cool box to the Plant Pathology Laboratory, UPR Mayagüez Campus, for processing. At the field plot, disease severity was visually estimated

on the basis of percentage of *H. rufa* plants with leaf symptoms. The soil at this site is classified as Fraternidad, Vertisol order, a fertile soil characterized by a predominant clay composition, high water retention, low hydraulic conductivity and high cationic exchange capacity (Román-Paoli and Sotomayor-Ramírez, 2004).

Fungal isolates

Fungi were isolated from symptomatic foliar tissue. Symptoms were described and five tissue samples (3 mm) from each lesion margin were superficially sterilized with 70% ethyl alcohol and 10% commercial sodium hypochlorite (each treatment was applied for one minute), then rinsed with sterile, deionized, double distilled water. Tissue sections were placed in plates containing acidulated potato dextrose agar (PDA). The plates were incubated at 27° C. After four days of growth, fungal colonies were purified. Colony development and reproductive structures were examined. Fungal identification was based on morphology using taxonomic keys (Barnett and Hunter, 1998; Boerema et al., 2004).

Pathogenicity tests under laboratory and greenhouse conditions

Fungal isolates found associated with leaf symptoms were evaluated under laboratory and greenhouse conditions. For pathogenicity tests in the laboratory, plants of *H. rufa* were selected at random from plots at the UPR-Agricultural Experiment Station, Lajas. Plant height ranged from 60.96 to 195.07 cm. Asymptomatic leaves were detached and brought to the laboratory. Leaves were washed with 10% commercial sodium hypochlorite and rinsed with sterile, deionized, double distilled water. Two leaves were inoculated on the upper surface with and without wounds with one mycelial disk (2 mm) per fungal isolate grown on PDA. Wounds were inflicted with a sterile dissecting needle. Control leaves were treated with PDA disks. After inoculation, leaves were kept in plastic humidity chambers (85 x 40 x 10 cm) at 26° C for eight days on top of lab benches. Inside the plastic boxes, humidity was maintained using a sterile paper towel saturated with sterile, deionized, double distilled water at 25° C. Observed lesions were described and the presence or absence of lesions was recorded.

In the greenhouse, 30 *H. rufa* plants (approximate height 91.22 to 152.4 cm), planted in plastic polyethylene pots (18 cm height and 19.5 cm diameter) containing Pro-mix®[6] as the substrate, were the test

[6]Company or trade names in this publication are used only to provide specific information. Mention of a company or trade name does not constitute an endorsement by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

plants. Every other day, for two weeks, plants were watered and fertilized (20-20-20). Six plants were inoculated per fungal isolate and the control. Two leaves per plant were superficially sterilized with 70% ethyl alcohol and 10% commercial sodium hypochlorite and rinsed with sterile, deionized, double distilled water. Leaves were inoculated on the upper surface as described in the laboratory tests. Inoculated leaves were covered with plastic bags containing a humid cotton ball to maintain moisture for 24 h. Seven and 14 days after inoculation, lesion size was measured with a ruler.

Field assay

Using the four fungal isolates previously mentioned, a field assay was conducted during December 2011. Experimental plots were established at the UPR-Agricultural Experiment Station. The experimental design consisted of a randomized complete block. Each block, triplicated, consisted of five plots equivalent to five treatments (four fungal isolates and the control) for a total of 15 plots (three per treatment). Field plots measured 3.66 x 3.66 m and were separated by 6.07 m. Plant height ranged from 0.92 to 3.96 m. Average temperature and precipitation were 31.6° C and 14.7 mm, respectively, during December 2011.

Twenty-one-day-old fungal cultures were used to prepare the inoculum. For sporulating isolates, a hemocytometer was used to standardized conidial suspension to 10^6 conidia/ml. For non or poorly sporulating isolates a mycelial suspension was used as inoculum. To prepare the mycelial suspension, mycelium was scraped from ten plates per fungal species grown on PDA and homogenized in a blender with 500 ml sterile, deionized, double distilled water. Additional sterile, deionized, double distilled water was added and mixed with a homogenous mycelial suspension to complete 3.8 L of inoculum per fungal isolate.

Inoculum of each fungal species was transported in 7.6 L polypropylene (Nalgene®) containers in a cooler. The inoculum was applied with a portable CO_2 pressurized system of 40 psi (2.81 kg/cm^2) with 1.8 mm nozzle (Valley Industries, Model SG-2200 "Flash" Turbo Spray Gun) connected to an 11.4 L stainless steel tank (R & D Sprayers, Model 107-BG). For each fungal treatment and the control (sterile, deionized, double distilled water), 48 ml of inoculum/s were evenly sprayed to the upper leaf surface of the plants. Each treatment was applied evenly for 60 s. Field applications were carried out during the sunset hours when solar intensity was low, thus enhancing pathogen survival on leaf surfaces.

Data on lesion size (cm) were recorded at 7, 14 and 21 days after inoculation to determine disease severity. Symptoms developed were described. A severity scale was designed to record disease progress on

inoculated plants. Disease severity was estimated based on a scale that ranged from 1 to 4, where: 1 corresponds to 0 to 25 percent; 2 from 26 to 50 percent; 3 from 51 to 75 percent and 4 from 76 to 100 percent of infected plants per plot. Leaves were selected in plants following an X pattern inside each plot. Lesion size was measured with a ruler in five sites at each field plot. Data was statistically analyzed using the ANOVA test and LSD ($p<0.05$). At the end of the experiments, field plots were covered with plastic to reduce inoculum dispersion.

RESULTS

Symptoms at field conditions

Under natural conditions at the UPR-Agricultural Experiment Station, *H. rufa* plants showed diverse symptoms in foliage, stems and inflorescences. Symptoms such as necrotic foliar lesions with chlorotic halos; apical leaf blight; reddish brown lesions in stems and leaves appearing dry; ellipsoidal tan to reddish lesions at leaf margins; foliar long irregular lesions with dark centers; and inflorescence wilt were observed. Overall, disease severity was estimated at 50% in the aerial parts (foliage, stems, and inflorescences) under natural conditions in the area studied.

Fungal identification

From foliar lesions, *Curvularia* sp., *Fusarium* sp., *P. sorghina* and *Sphaeropsis* sp. were isolated in PDA culture media and identified using taxonomic keys (Barnett and Hunter, 1998; Boerema et al., 2004). Identification of *P. sorghina* was confirmed by the CBS-KNAW Fungal Biodiversity Centre[7] (Centraalbureau voor Schimmelcultures) in The Netherlands.

Curvularia sp. was isolated from necrotic, elongated, thin, foliar lesions. *Fusarium* sp. was isolated from apical leaf blight lesions. *Phoma sorghina* was isolated from ellipsoidal tan to reddish lesions close to leaf margins. Lesion centers appeared dry. *Sphaeropsis* sp. was isolated from long reddish lesions of irregular margins and a chlorotic halo. Regardless of lesion type, fungi such as *Aspergillus* sp. and *Penicillium* sp. were also isolated but not considered further. Preliminary reports of these findings have been published elsewhere (Rivera et al., 2011; 2012).

[7]Fungal identification service was mentioned to provide specific information and does not constitute a warranty by the University of Puerto Rico, nor is this mention a statement of preference over other identification services.

Pathogenicity tests under laboratory and greenhouse conditions

Pathogenicity tests conducted under laboratory conditions showed that all fungal isolates evaluated produced foliar lesions in wounded tissues eight days after inoculation (Table 1). *Sphaeropsis* sp. produced lesions in both wounded and unwounded tissues. Lesions were irregular, brownish to reddish-colored with a chlorotic halo. *Curvularia* sp. lesions were irregular, with a chlorotic halo and dark brown centers. *Fusarium* sp. produced small, longitudinal, light tan lesions. *Phoma sorghina* produced round, brown lesions with a wide chlorotic halo and dark brown centers. No lesions were observed in control treatments. Koch's postulates were successfully completed for all fungi evaluated during the assay.

In the greenhouse, the fungi examined were pathogenic, producing lesions in all six plants inoculated, with or without wounds (Figure 1). Foliar lesions caused by *Curvularia* sp. were irregular, with a chlorotic halo and dark brown centers. Lesions ranged from 0.5 to 2.2 cm long and 0.6 to 0.9 cm wide. *Fusarium* sp. produced longitudinal, light tan lesions close to leaf margins measuring 0.3 to 1.5 cm long and 0.3 to 0.4 cm wide. *Phoma sorghina* produced round, cream to brown lesions with a wide chlorotic halo and dark brown centers. Lesions ranged from 2.3 to 9 cm long and 0.4 to 0.9 cm wide. *Sphaeropsis* sp. produced cream, brownish to reddish-colored lesions with a chlorotic halo, measuring 0.5 to 8.0 cm long by 0.5 to 0.7 cm wide. Fourteen days after inoculation, *P. sorghina* and *Sphaeropsis* sp. were found to be the most virulent fungi during these assays. The smallest lesions were caused by *Fusarium* sp. and *Curvularia* sp., considered the least virulent isolates. Control plants did not show symptoms of disease.

Field assay

In the field studies, *Fusarium* sp. was the most virulent fungal isolate, producing an average lesion size of 19.63 cm. Lesions

TABLE 1.—*Disease severity caused by four fungal isolates on* Hyparrhenia rufa *leaves in pathogenicity tests conducted under laboratory conditions (in vitro).*

	In vitro[1]	
Treatment	With incision	Without incision
Control	-	-
Curvularia sp.	+	-
Sphaeropsis sp.	+	+
Fusarium sp.	+	-
Phoma sorghina	+	-

[1]Foliar lesions (+); No foliar lesions (-) developed eight days after inoculation.

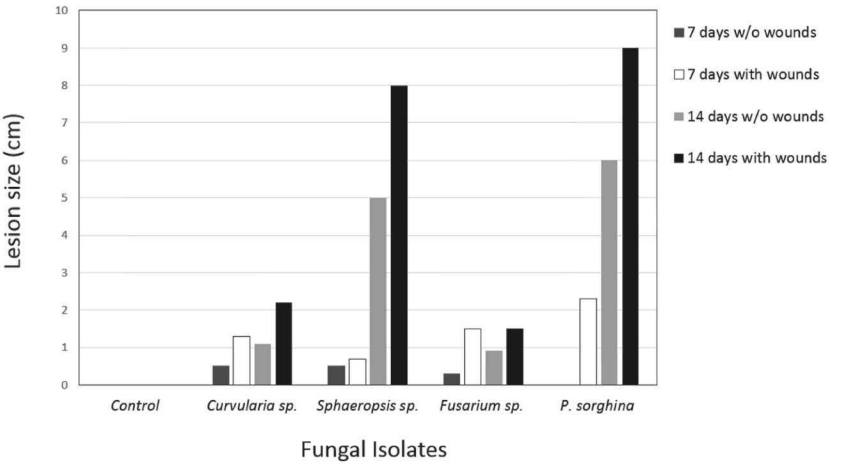

FIGURE 1. Foliar lesion size (cm) caused by four fungal isolates on *Hyparrhenia rufa* during pathogenicity tests conducted under greenhouse conditions. Lesions size is the average of six plants, seven and 14 days after inoculation with and without (w/o) wounds.

ranged from 0.30 to 19.80 cm long and 0.6 to 0.8 cm wide (Figures 2 and 3A). Tan colored lesions, caused by *Fusarium* sp., were observed extending from the tip through the leaf blade (apical leaf blight). *Phoma sorghina* produced lesions with an average size of 8.14 cm. Lesion shape varied from rounded to irregular with cream-colored centers and reddish margins, and wide chlorotic halos (Figures 2 and 3D). *Sphaeropsis* sp. produced irregular long brownish to reddish-colored lesions with a thin chlorotic halo. Average lesion size was 4.28 cm long (Figures 2 and 3B). *Curvularia* sp., the least virulent of all fungi evaluated, produced long, thin, black lesions with a small chlorotic halo. Average lesion size was 2.21 cm long (Figures 2 and 3C).

At field conditions, statistical analysis (ANOVA) detected a significant effect ($P<0.05$) among treatments (Figure 2). Based on the LSD test ($P<0.05$), *Fusarium* sp. was the most virulent isolate producing significantly larger lesions (19.63 cm). *Phoma sorghina* and *Sphaeropsis* sp. were the second most virulent fungal isolates. Control plants showed small levels of natural infection in the field with an average lesion size of 1.13 cm. No fungal re-isolation was attempted from control plants. All fungal isolates inoculated during field trials showed low levels of infection (level 1). Disease severity was estimated at 25% of the experimental plot area. All inoculated fungi were re-isolated on PDA, fulfilling Koch's postulates.

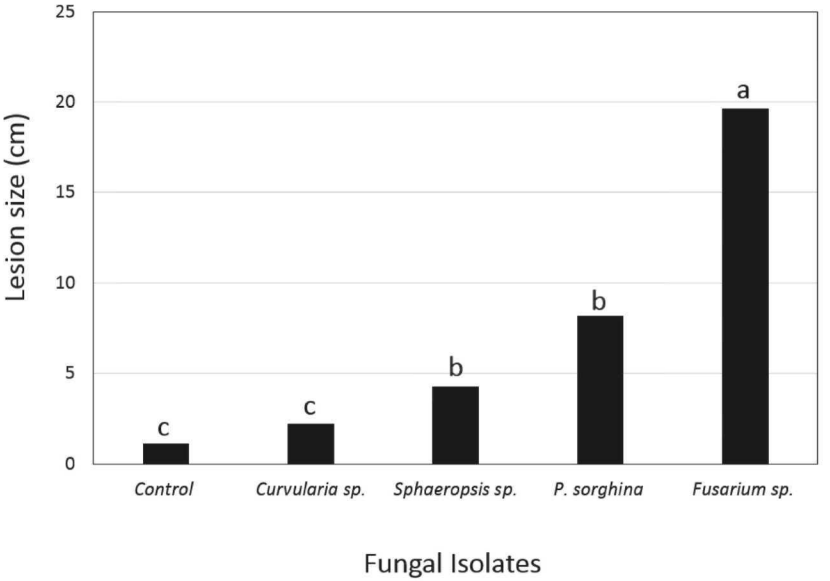

FIGURE 2. Foliar lesion size (cm) caused by four fungal isolates on *Hyparrhenia rufa* during the field assay conducted in December 2011. Lesion size is the average of five plants per treatment seven days after inoculation. Columns with different letters are significantly different ($p < 0.05$).

DISCUSSION

This is the first study of fungal pathogens of *H. rufa* in Puerto Rico and the first report of *Curvularia* sp., *Fusarium* sp., *P. sorghina* and *Sphaeropsis* sp. causing foliar lesions in *H. rufa*. In the laboratory, all fungi were pathogenic to *H. rufa* leaves, while under greenhouse conditions, *P. sorghina* and *Sphaeropsis* sp. were the most virulent isolates. In the field assay, *Fusarium* sp. was the most virulent species producing large ellipsoidal lesions (19.63 cm in average). The genus *Fusarium* comprises a diverse group of plant pathogenic species with a wide range of hosts including plants of economic importance such as corn, sugarcane, sorghum, wheat and rice (Leslie and Summerell, 2006; Tesso et al., 2010). Therefore, *Fusarium* sp. should be carefully studied as a potential biocontrol agent of *H. rufa* due to possible negative impacts to non-target species.

Phoma sorghina and *Sphaeropsis* sp. were the second most virulent isolates in the field trials. *Phoma sorghina* is a common pathogen of tropical and subtropical regions causing diseases in cereals and other grasses commonly used for hay production (Perelló and Moreno, 2005; Pazoutová,

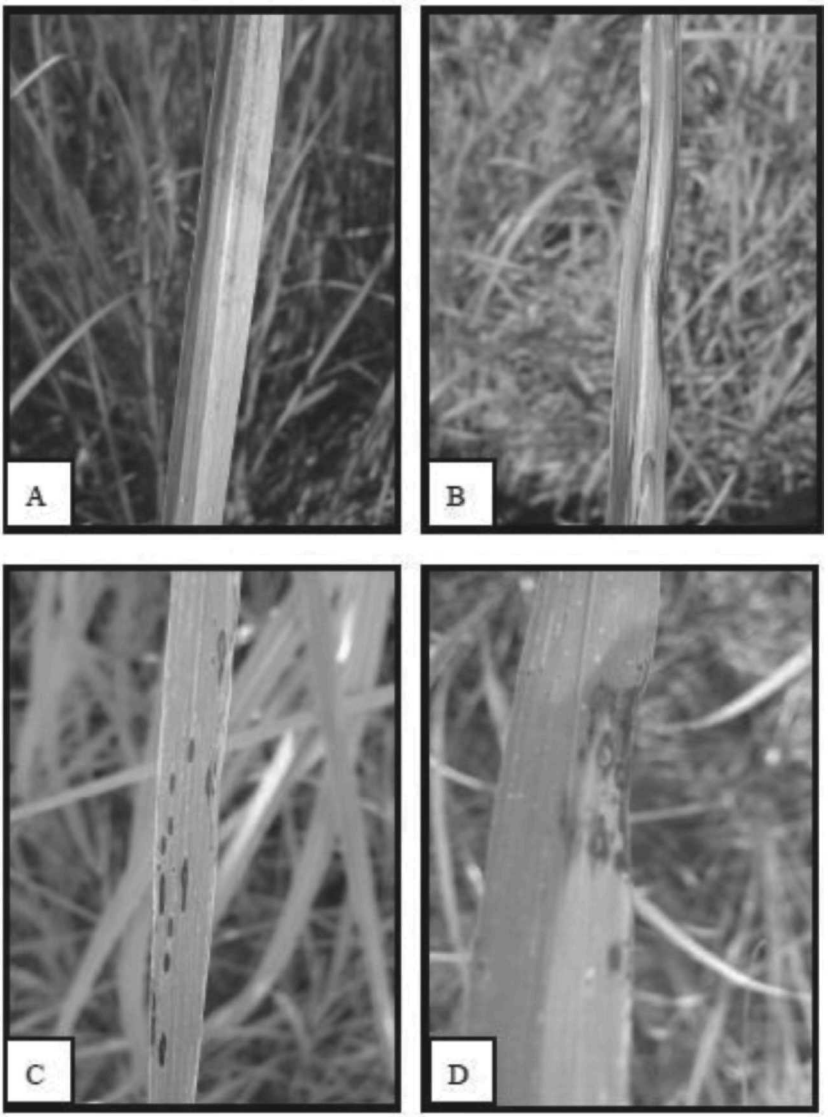

FIGURE 3. Foliar lesions of *H. rufa* caused by (A) *Fusarium* sp.; (B) *Sphaeropsis* sp.; (C) *Curvularia* sp.; and (D) *Phoma sorghina,* during a field assay.

2009). *Phoma sorghin*a has been isolated from leaf spots of the weed, *Phytolacca americana* L., commonly known as pokeweed (Venkatasubbaiah et al., 1992). In Puerto Rico, it has been reported as a weak pathogen of onions

in the southern region of the island (Vélez-Rodríguez and Rivera-Vargas, 2007). *Phoma sorghina*, in association with *Alternaria tenuissima*, *Curvularia lunata* and *Fusarium* spp., causes mold on harvested sorghum grains (Pazoutová, 2009). In Puerto Rico, *Sphaeropsis subglobsa* and *S. tumefaciens* have been reported in bamboo (*Bambusa vulgaris* Schrad. ex J.C. Wendl.), a Poaceae (Stevenson, 1975), and in citron (*Citrus medica* L.) (Rodríguez and Meléndez, 1984), while *Curvularia lunata* has been reported in *Cyperus rotundus* L. (Nieves-Rivera, 1999) and *Sorghum bicolor* (L.) Moench (Cantrell et al., 2006).

The present study demonstrated that phytopathogenic fungi isolated from *H. rufa* can cause damage to weed grasses, with implications for their use as biological control agents. The evaluation of combinations of pathogens might increase their bio herbicidal capacities. In Florida, USA, such enhancement was shown during greenhouse trials in which a mixture of three fungal pathogens was able to control seven grass species, namely crowfoot grass (*Dactyloctenium aegyptium* (L.) Willd.), guinea grass (*Panicum maximum* Jacq.), johnson grass (*Sorghum halepense* L. Pers.), large crabgrass (*Digitaria sanguinalis* L. Scop.), southern sandbur (*Cenchrus echinatus* L.), Texas panicum (*Panicum texanum* Buckl.), and yellow foxtail (*Setaria glauca* (L.) Beauv.). The fungal mixture consisted of *Drechslera gigantea*, *Exserohilum longirostratum*, and *E. rostratum*, and disease severity ranged from 83 to 100% for the different grass species evaluated (Chandramohan and Charudattan, 2001).

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