Screening annual ryegrass for increased tolerance to blast (Pyricularia oryzae)

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Abstract

Annual ryegrass [*Lolium perenne* L. spp. *multiflorum* (Lam.) Husnot] is a high-quality forage utilized heavily in the Southeast for pasture and grazing. Gray Leaf Spot (*Pyricularia oryzae*) is a fungal pathogen that infects gramineous species and is a common problem in perennial and annual ryegrass stands. This study was designed to screen for resistance to *P. oryzae* in the annual ryegrass cultivar Vertyl (PI 619473, FRA) and a heat-tolerant germplasm (HTARG5) derived from Marshall (PI 600770, USA) with intent to use recurrent phenotypic selection to crossbreed the two accessions. Marshall was also included as a susceptible reference cultivar. Two experiments were conducted, the first with 100 plants screened at 12-wk old and the second with 180 plants screened at 6-wk old. Plants were spray inoculated with a spore solution of 1 x 10^5 conidia mL^{-1} and incubated for 72h at 30:25°C (D:N), ≥85% RH. Percentage disease severity (DS) was assessed 3wk post inoculation by dividing the number of diseased leaves by the total number of leaves per plant. Germplasm accession was used as a main effect and was significant in both Exp. 1 (P=0.0341) and Exp. 2 (P=0.0036). For Exp. 1, mean DS was significantly greater in heat-tolerant germplasm (29.3) than Vertyl (19.4) (LSD = 8.1). For Exp. 2, mean DS for Vertyl, Marshall, and the heat-tolerant germplasm were 44.82, 61.02, and 62.55, respectively. Disease severity in Vertyl was significantly lower (LSD = 12.1) than both Marshall and the heat-tolerant germplasm. Mortality was also recorded, however, there was no significant difference between accessions for either experiment. These results confirm that Vertyl has significantly greater resistance to gray leaf spot than Marshall and can be a quality candidate for selection breeding.

Introduction

Annual ryegrass [*Lolium perenne* L. spp. *multiflorum* (Lam.) Husnot] is a cool-season grass that is commonly grown throughout the United States. Anatomically different from close relative perennial ryegrass (*Lolium perenne* L.), *L. multiflorum* has awns protruding from the seed head as well as clasping auricles (Ball, 2015). Native to Europe, annual ryegrass (ARG) has been introduced all over the world for forage production and is also a valued species in the turf industry. In the United States, greater than 90% of ARG acreage is used for winter pasture in the Southeast (Barnes et al., 2003). Throughout the Southeast, ARG is typically planted from the middle of September through October, when the environment can be hot, humid, and wet, making ideal conditions for fungal pathogens such as blast (*Pyricularia oryzae* Cavara).

Annual ryegrass is one of the most nutritious cool-season forages for livestock thanks in part to high digestibility and high crude protein content, which makes it a staple in most ruminant diets (Barnes et al., 2003). During the grazing season in the southern United States, there is a lull in forage productivity when transitioning from fall to winter, as warm-season species begin to senesce, and cool-season species begin to emerge. This fall to winter transition period, along with the corresponding lull between spring and summer are referred to as ‘slumps’ (Adesogan et al., 2009). The potential exists for producers to eliminate the fall slump by planting ARG earlier in the growing season. When established earlier in the season, high-quality stands of ARG can allow producers earlier grazing options, and potential to avoid the increased cost of stored feeds like hay and silage. While there are numerous benefits to establishing ARG earlier in the growing season, the risks associated with higher disease pressure – specifically fungal pathogens – are also increased.

In the southern United States, September and October are historically the driest months, however August, September, and October are typically still hot and humid and can have high rainfall events. This environment fosters a multitude of fungal pathogens that thrive under warm, damp conditions. These factors combined complete the disease triangle (environment, host, pathogen), which is necessary for infection. Aggressive pathogens like blast can also rapidly multiply within a sward and as a result it is common for entire stands of ARG to display infection symptomology. Understanding the conditions these pathogens thrive in is vital for producers when making management decisions like planting ARG.

Methods and Study Site

The purpose of this project was to develop ARG germplasm tolerant to environmental stresses associated with early planting in the southeast (heat) while also exhibiting increased resistance to common

fungal pathogens, specifically *P. oryzae*. For this study, two diploid annual ryegrass accessions (Vertyl and HTARG5, a heat-tolerant selection from Marshall) were used to screen for tolerance to *P. oryzae*. Multiple diploid and tetraploid cultivars (Marshall, OR-34, and Jumbo) were used as check varieties throughout the study. After preliminary data indicated increased pathogen tolerance in Vertyl, a restricted recurrent phenotypic selection breeding program was designed with the goal of combining the tolerant phenotype of Vertyl with the heat-tolerant phenotype of HTARG5.

Three isolates of *P. oryzae* [PLVGLS1, PLVGLS2, and PLVGLS3, collected from perennial ryegrass, tall fescue, and wheat, respectively] were acquired from Rutgers University. After evaluating spore production of these three isolates, PLVGLS3 was the most prolific producer of conidia and was chosen to be used in this study. All subsample transfers for *Pyricularia* isolation and culture were done using aseptic techniques under a laminar flow hood and all equipment was sterilized using a 70% ethanol solution. Subsamples of PLVGLS3 were taken from the original mother plate, transferred to potato dextrose agar (PDA) and left to grow at room temperature for 7 days. Subsamples were transferred from PDA and cultured on V8 vegetable juice agar. Petri dishes containing *P. oryzae* cultures on V8 agar were wrapped in parafilm and placed in a controlled environment chamber at a constant temperature of 27°C under florescent and ultraviolet light. Plates were maintained in this environment for 10 days, after which, parafilm was removed, plates were flipped upside down (side of agar with fungus now facing downward) and returned to the controlled environment chamber for five days. A solution of 20 ml of sterilized deionized water and five drops of Tween20 was combined in a 50 ml falcon tube. Four milliliters of solution were transferred onto each V8 agar dish containing *P. oryzae* spores to bring spores into a solution. Using a small, curved plastic scraper, the solution was pushed across the surface of the agar to release spores from mycelium. Using a 1000 µL pipette, the solution on the V8 plate was transferred into a sterile 50 ml tube and vortexed for two seconds. After vortexing, 10 µL of spore solution was transferred under a glass slide fixed on top of a hemacytometer.

The hemacytometer was placed beneath a microscope under 10x magnification and spores were observed and counted. The four corner squares of the hemacytometer (the 1mm x 1mm corner squares containing 16 square windows each) were used to calculate spore concentration of the stock solution (Figure 1). Individual conidia within each small square were counted. Only conidia fully inside the square were counted, any conidia that overlapped the frame outline were excluded. Total conidia count from each set of 16 squares were added together to create a total sum for each corner square. Totals for each corner square were then added together. That sum was divided by four, then multiplied by 10,000, which represents the volume of the squares on the hemacytometer (Equation 1). Using Equation 2, a dilute working spore solution was prepared with a target concentration of 100,000 spores ml^{-1}.

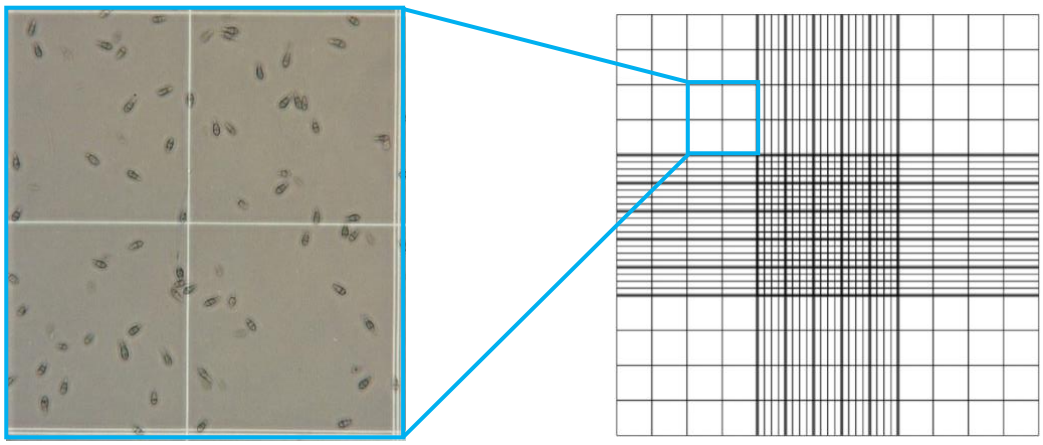

Figure 1. Illustrates conidia counting using the four corner squares (each comprised of 16 smaller squares) of the hemacytometer.

Eq. 1 Corner 1 total + Corner 2 total + Corner 3 total + Corner 4 total = Spore total

383+454+383+402 = 1,622 spores

1,622 spores / 4 = 405.5 spores per mm^2

405.5 x 10,000 = 4,055,000 spores/ml

Eq. 2 $C1 \times V1 = C2 \times V2$

$(4{,}055{,}000)\ (X) = (100{,}000)\ (150)$

X = 3.7 ml of spore solution into 150 ml to achieve a spore concentration of 100,000 spores ml^{-1}

Preliminary Inoculation and Screening

Inoculation tents made of PVC pipe, polyethylene plastic sheeting, and polypropylene shade cloth were assembled inside a greenhouse. Daytime temperatures were maintained between 27 and 32°C. Three humidifiers were placed in each tent to achieve a relative humidity between 90 and 100%. Plants were placed in inoculation tents at high humidity 12hr prior to treatment application to ensure leaf wetness. Plants were removed from tents, placed in large plastic tub, and inoculated by using a CO_2 actuated sprayer to apply the spore solution until visible droplets could be seen on the leaves. Inoculated plants were placed back in the inoculation tent and tents were closed to maintain the desired relative humidity. Plants were kept in this environment for 72h. After that time, plants were transported to a laboratory where 25 leaves were randomly cut from each plant and photographed. These pictures were evaluated using Assess© image analysis software which identifies discolored areas of each individual leaf and calculates a percentage of leaf area displaying symptomology. All percentages were averaged for each plant and all plants within each variety to determine disease severity (DS) among the population of treated plants. Whole plants were returned to a controlled environment chamber after leaf sub samples were taken and were re-assessed for mortality after 21d.

Following collection of preliminary data, two experiments were conducted as described above, the first with 100 plants (50 Vertyl, 50 HTARG5) screened at 6wk old and the second with 180 plants (90 Vertyl, 90 HTARG5) screened at 12wk old. Plants were spray inoculated with a solution of 1 x 10^5 conidia mL^{-1} and incubated in controlled environment chambers for 72h at 30°C (light), 25°C (dark), ≥85% RH. Due to complications with Assess software, DS was rated manually by counting the total number of leaves displaying symptomology on each plant and dividing that number by the total number of leaves on the same plant. Mortality was also recorded, but not included in disease severity index. Disease severity and mortality were assessed using PROC GLM in SAS statistical analysis software with an $\alpha = 0.05$ level of significance. Germplasm accession and replication were considered fixed effects.

Results

Preliminary data from Experiment 1 showed an increased tolerance to blast in Vertyl, as compared to OR34, Jumbo, and Marshall (Figure 2).

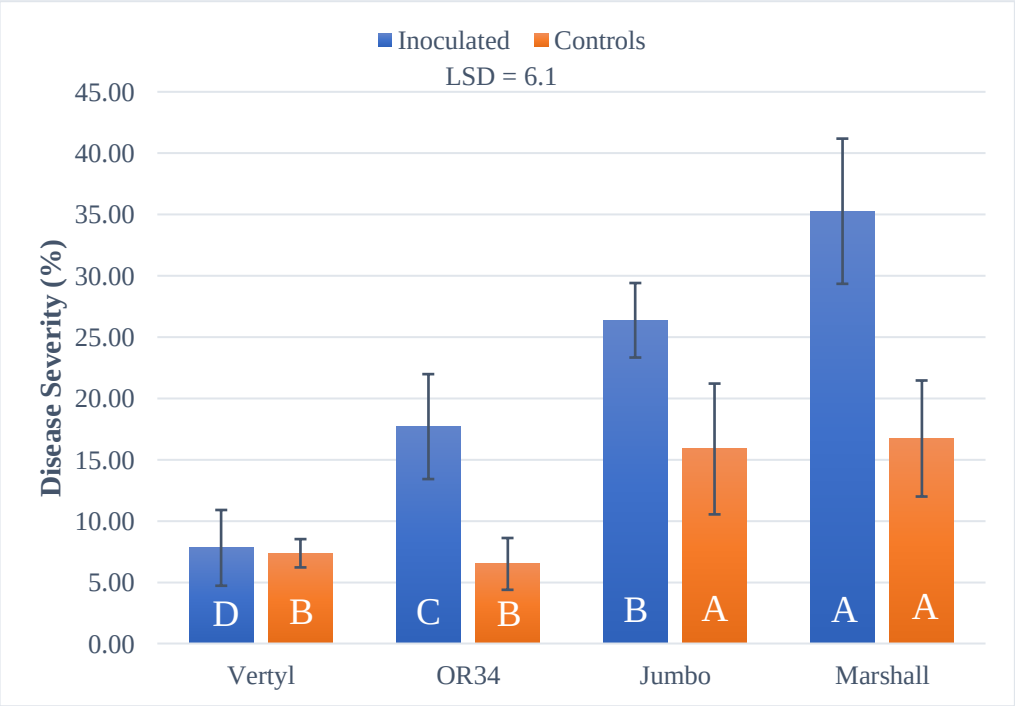

Figure 2. Mean percentage disease severity of four annual ryegrass accessions infested with characteristic *Pyricularia oryzae* lesions 3wk after treatment. Statistical comparisons are within treatment group (across accessions) and columns with different letters are significantly different at $\alpha = 0.05$

There were significant differences in DS between Experiment 2 and 3 (P<0.0001). Results of Experiment 2 (Figure 3, left) revealed a significant difference in DS between germplasm accessions (P=0.0008). Mean DS was significantly greater in Marshall (61.02) and HTARG5 (62.55) than Vertyl (44.82) (LSD = 12.2). Experiment 3 (Figure 3, right) also produced a significant difference in DS due to germplasm accession (P=0.0341). Mean DS for Vertyl, Marshall and HTARG5 were 19.4 and 28.6, and 29.1, respectively. Disease severity in HTARG5 was significantly greater than Vertyl (LSD =8.9).

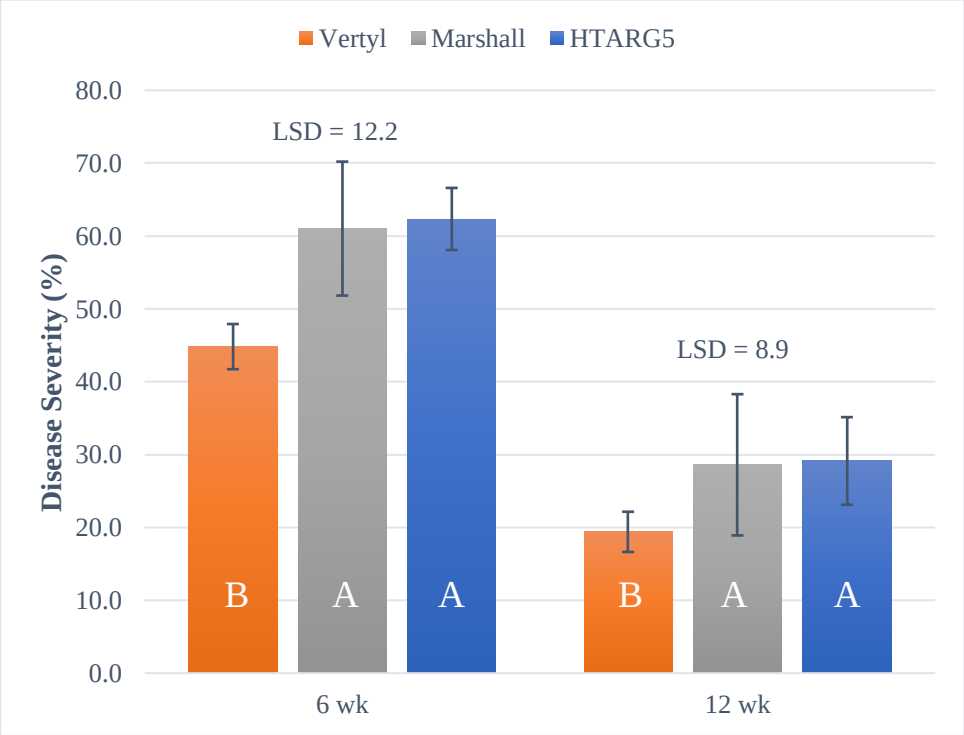

Figure 3. Percentage disease severity in annual ryegrass accessions treated with potato dextrose agar broth containing 1 x 10^5 conidia mL^{-1} of *Pyricularia oryzae* at 6wk (left) and 12wk (right) after seedling germination. Mean comparisons are across accession within treatment group (seedling age) and columns with different letters are significantly different at $\alpha = 0.05$

Conclusions

Preliminary results were similar to those of Makaju et al. (2016), where Vertyl and OR34 showed increased tolerance to *P. oryzae*. Although OR34 showed increased tolerance to the pathogen, the phenotype of OR34 did not display desirable forage-type characteristics but was instead very compact with narrow leaves and considered to be more turf-type. For this reason, OR34 was removed from consideration for further testing. Throughout experiments, Vertyl continued to outperform other accessions, regardless of ploidy level or age of plants at treatment. The results from these experiments confirm that Vertyl has significant resistance to gray leaf spot and can be a quality candidate for selection breeding to improve that characteristic.

Discussion

Significant differences between results of Exp. 1, 2, and 3 are due - at least in part - to age of seedlings at treatment. Treatments were planned to be applied at 6wk after germination in all experiments, however issues with production of viable conidia caused delays in treatment application in Exp. 3. It is widely accepted that older seedlings are inherently more tolerant to pathogenic infection than younger seedlings, similar to results reported by Falloon (1984). Variability in results between experiments is also attributable to broad genetic diversity present within the evaluated germplasm accessions.

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