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Researchers at Rutgers University are evaluating several inoculation techniques to improve selection and breeding for gray leaf spot resistance in perennial ryegrass.

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PURPOSE

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Editor

Jeff Nus, Ph.D.
904 Highland Drive
Lawrence, KS 66044
jnus@usga.org
(785) 832-2300
(785) 832-9265 (fax)

Research Director

Michael P. Kenna, Ph.D.
P.O. Box 2227
Stillwater, OK 74076
mkenna@usga.org
(405) 743-3900
(405) 743-3910 (fax)

Inoculation Techniques for Selection of Gray Leaf Spot Resistance in Perennial Ryegrass

Yuanhong Han, Stacy A. Bonos, Bruce B. Clarke, and William A. Meyer

SUMMARY

In the last decade, gray leaf spot has developed into one of the most devastating diseases of perennial ryegrass. This paper describes two greenhouse and two field inoculation techniques to determine their usefulness as screening techniques for resistance to gray leaf spot disease in perennial ryegrass.

- Effective selection techniques for genetic resistance to gray leaf spot will benefit perennial ryegrass breeding programs.
- Correlations of disease resistance between greenhouse and field evaluations are necessary to effectively select for resistance.
- Two greenhouse and two field inoculation techniques were evaluated to determine their usefulness as screening techniques for resistance to gray leaf spot disease in perennial ryegrass.
- One of the greenhouse and one of the field inoculation techniques yield results similar to those found with natural gray leaf spot infection. These two inoculation techniques should be effective techniques to screen for genetic resistance to gray leaf spot in perennial ryegrass.
- Inheritance studies of resistance are also being conducted to further improve selection techniques to hasten the development of disease resistant cultivars.

Gray leaf spot is a disease of St. Augustinegrass, tall fescue and perennial ryegrass caused by the fungus *Pyricularia grisea*. It was first identified on perennial ryegrass by Dernoeden in 1985 in Maryland (2), but was not officially reported as a pathogen of this host until 1992 (4). In the last decade, gray leaf spot has developed into one of the most devastating diseases of perennial ryegrass, as the geographic range of this disease has increased dramatically (3, 6, 7, 10).

YUANHONG HAN is a graduate research assistant in the Department of Plant Biology and Pathology, Rutgers University. STACY A. BONOS, Ph.D., BRUCE B. CLARKE, Ph.D. and WILLIAM A. MEYER, Ph.D. are assistant professor and professors, respectively, in the Department of Plant Biology and Pathology, Rutgers University, New Brunswick, NJ.

The development of new perennial ryegrass cultivars with improved resistance to gray leaf spot is a major goal of the Rutgers Turfgrass Breeding Program. To assist such breeding efforts, research on improved selection techniques for genetic resistance to gray leaf spot is necessary.

Review of past inoculation methods

It is very important to have reliable techniques for both greenhouse and field inoculation to effectively and consistently select for resistance. Inoculation techniques for gray leaf spot have been evaluated in annual ryegrass, perennial ryegrass, and tall fescue (4, 5, 8). In these experiments, spraying conidial suspensions was the primary inoculation technique. For this method, *P. grisea* is typically grown on V8 juice agar for

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conidia production. Conidia are dislodged from the agar with distilled water to make the conidia suspension. The prepared conidial suspension is sprayed on the surface of grass leaves using a CO_2 pressurized hand sprayer. The application rate varies with experimental design. High temperature and humidity are necessary for incubation after the conidial suspension is applied.

Moss and Trevathan (5) investigated the relationships between inoculation efficiency and temperature, inoculum density, leaf-wetness period, and plant age. They found that with increasing inoculum density, the severity of gray leaf spot increased exponentially with susceptible cultivars. In their studies, the youngest leaf typically had very few lesions, while the older (second, third and fourth) leaves developed many more lesions. The optimum infection age was predicted to be 4.7 weeks old. At 5 C (41 F), no lesions were observed, and the optimum temperature for infection was 26 C (79 F). When the temperature increased to 35 C (95 F), only a few lesions developed. They found that a constant leaf-wetness period is critical for infection and a minimum requirement of 24 hours is needed for an adequate infection.

Landschoot and Hoyland (4) inoculated both 4-week-old and 20-week-old plants and found that the younger plants were more susceptible to gray leaf spot. They also observed that disease was more severe at 29 C (84 F), than at 25 C (77 F).

Tredway (8) inoculated the tall fescue cultivars Coronado, Coyote, Rebel III and Kentucky 31. Plants were kept in the growth chamber for 24 hours at 24 C (77 F) in the darkness immediately after the inoculation. Then conditions in the chamber were changed to 12-hour days at 30 C (86 F) and 75% relative humidity (RH) and 12-hour nights at 24 C (77 F) and 100% RH. Symptoms developed 5 to 6 days after inoculation.

Relationship between greenhouse and field tests

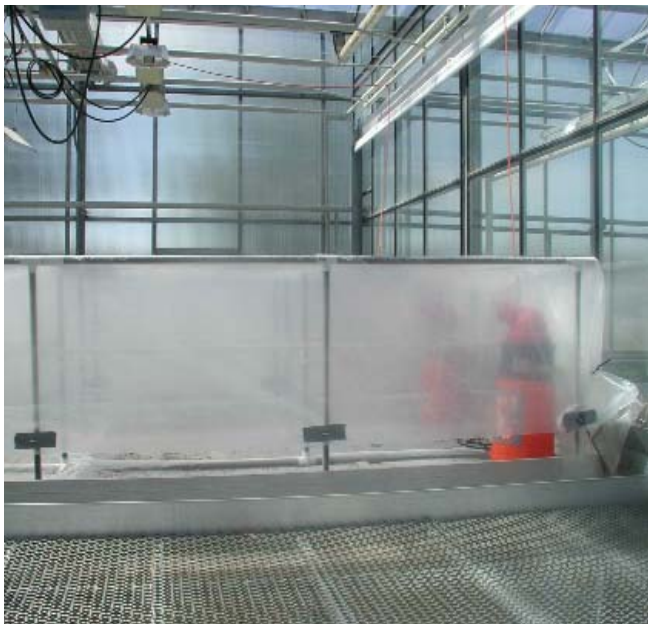
Greenhouse inoculation is an efficient way to screen for resistant sources of germplasm and can be conducted throughout the year. However, when selecting resistant plants, it is important that a similar level of disease resistance is also observed under field conditions. To effectively use greenhouse inoculation as a selection technique it is critical to have good correlation with field performance. Cultivars showing resistance in the greenhouse may be susceptible in the field, or resistant cultivars in the field may be susceptible to the disease under strict greenhouse conditions (1).

Natural outbreaks of gray leaf spot in field turf plots require the presence of the pathogen coupled with warm, humid weather. Natural outbreaks are unpredictable, and may not spread uniformly over the test site. In years with mild summers, or on a site with no history of disease, artificial inoculation techniques may be required to incite gray leaf spot epidemics in field plots. Turf performance after artificial field inoculation also needs to be compared with performance after a natural epidemic.

In our research study, experiments were designed to evaluate four inoculation methods for assessing gray leaf spot in perennial ryegrass (two greenhouse and two field inoculations). All methods were designed to create optimal infection conditions, which include high temperature, high humidity and a prolonged period of leaf wetness. Cultivar performance from these inoculated tests was correlated to cultivar performance from a natural outbreak under field conditions.

Greenhouse inoculation techniques

Two greenhouse inoculation methods utilizing the aforementioned conidial spray technique were tested for their effectiveness in assessing host resistance to gray leaf spot. The conidial suspension was a mixture of conidia from five isolates of *Pyricularia grisea* (RRNJ, RLVA, TFGGA, RSKY2 and RHF2NJ-1) at a concentration of 50,000 conidia per millileter of suspension. Isolates were grown separately and combined in equal concentrations in the conidial suspension.



Greenhouse chamber (greenhouse method 1) used to increase relative humidity and increase incidence of gray leaf spot disease in a greenhouse inoculation technique.

Method 1

In method 1, two white plastic mist chambers (19 ft long X 6 ft wide X 6 ft high) were built on the top of two rectangular benches inside a greenhouse. Four humidifiers were set up at each corner of each chamber. The uncontrolled temperature in the greenhouse ranged from a maximum temperature of 37 C (99 F) during the day to a minimum temperature of 18 C (64 F) during the night. Plants sprayed with the conidial suspension were placed into the chambers and the humidifier was kept on for 24 hours. After the first 24 hours, the humidifiers were turned on and off at four-hour intervals to provide alternating wet and dry cycles.

Several experiments were conducted using this method. Symptoms developed five to six days after inoculation. However, due to a great divergence in results between this method and field trials (data not shown), it appears that this method was not reliable for the greenhouse screening for gray leaf spot resistance.

Method 2

Method 2 was similar to the method described by Tredway (8, 9) for the inoculation of gray leaf spot on tall fescue with some small modifications. A walk-in growth chamber was used in which both temperature and humidity were controlled. Plants were arranged in 48-cell flats, and each flat was put into a plastic container. Each flat of plants was sprayed with 100 ml of the conidial suspension. Right after the inoculation, lids were placed on the containers to obtain 100% RH, and the plants were incubated in the growth chamber for 24 hours at 24 C (77 F) in the darkness. The lids were then removed and plants were subjected to 12-hour days at 30 C (86 F) and 12-hour nights at 24 C (77 F) and 75% relative humidity. At the beginning of each night cycle, water was sprayed on plants and the lids were put back on the containers to keep the leaf surface moist. At the beginning of each day cycle, lids were again removed. Plants were evaluated for gray leaf spot resistance by counting lesions per tiller and/or by estimating percentage of diseased

Growth chamber utilized in greenhouse inoculation method 2. Containers with lids were utilized to maintain leaf wetness. The disease response from this technique correlated well with natural field infection of gray leaf spot.

Plant ID	Mean lesion number in greenhouse[1]	Mean of rating in field trial[2]
9414	14.6	1.0
9440	21.3	1.5
9448	10.1	4.5
9474	16.9	1.0
9536	14.1	5.5
9650	3.1	7.5
9708	3.6	7.5
GLE35	3.3	6.5
RNS18	8.0	7.0

Correlation coefficient = -0.88 (p=0.002)

[1]Mean of 8 replicate clones of perennial ryegrass.

[2]Mean of progenies harvested from perennial ryegrass clone. 1 = more than 90% leaf tissue infected, 9 = free of disease. (Values are means of two rating dates.)

Table 1. The performance of nine perennial ryegrass clones for resistance to gray leaf spot in a growth chamber inoculation test (method 2) compared the performance of their progenies in a natural field epidemic.

leaf tissue 7 days and 14 days after inoculation.

To test the effectiveness of this method and to correlate it with field trials, nine ryegrass plants were selected from a perennial ryegrass nursery in Adelphia, NJ for testing in the greenhouse. These plants were moved into the greenhouse in the winter of 2001. Eight clonal tillers from each plant were transplanted into flats and arranged in a randomized complete block design. The flats were inoculated using method 2 described above.

Lesion number per tiller was counted and the results are shown in Table 1. The same nine plants were harvested from the spaced-plant nursery at Adelphia, NJ in July, 2001. The harvested seed from each plant was planted into a turf plot at Adelphia in September, 2001. A natural epidemic of gray leaf spot occurred approximate four weeks after seeding. The performance of these progeny plots was evaluated twice on a 1-9 scale (where 1 = more than 90% leaf tissue infected and 9 = free of disease). The ratings of these progeny plots are shown in Table 1. In general, plants with the most gray leaf spot resistance in the field had the fewest number of leaf lesions in the growth chamber. The high correlation coefficient (r^2=0.88, p=0.002) indicated that this method was a reliable inoculation technique and estimated field performance of an individual plant's resistance to gray leaf spot.

Field inoculation technique

Two field inoculation methods were evaluated in a perennial ryegrass field test. The study was established at Horticulture Farm II, New Brunswick, New Jersey in August, 2001. Entries were seeded at 93 g/m^2 (2.2 lb seed/1000 ft^2) into 0.75 X 1 m (2 X 3 ft) plots. Plots were arranged in a randomized complete block design with three replications.

Method 1

Two weeks after seeding, a clear plastic wall was built around the whole test to increase the humidity and temperature over the turf plots. The test was inoculated three times at two-week intervals in September with a conidial suspension (mixture of *P. grisea* isolates RRNJ, RHF2NJ-1, RSKY2, RLVA and TFGGA at 28,000 conidia / ml). The test was irrigated lightly twice a day to

Clear plastic walls were erected around the field study to reduce air circulation and increase relative humidity (field method 1). This technique did not significantly enhance disease

enhance leaf wetness and encourage disease development. However, very few gray leaf spot symptoms were observed. The mild weather in September and October in 2001 could be an important reason for the failure in inducing gray leaf spot in this test.

Method 2

In August 2002, one year after establishment, the same field test was inoculated using another method. The test was sprayed at dusk on August 20th with a conidial suspension (mixture of *P. grisea* isolates RRNJ, RHF2NJ-1, RSKY2, RLVA, TFGGA, ANJ01-1 at 42,000 conidia/ml) using a backpack sprayer. Each plot received about 50 ml of the suspension. The test was watered lightly before spraying.

Immediately following inoculation, the test was covered with black plastic film to increase the humidity and retain the moisture on the leaf surface. The cover was removed every morning at 7:30 AM and was put on every evening at 7:30 PM for seven days. During this period, the test was irrigated at 11:00 AM and 3:00 PM for 15 minutes each day and for two minutes just before the cover was placed over the test area each night. Since flutolanil (Prostar 70 WG at 5.8 g product/m^2 or 2.2 oz product/1000 ft^2) was found in previous research (data not shown) not to affect the development of gray leaf spot in the field, it was applied every 14 days to suppress brown patch disease (*Rhizoctonia solani*). Gray leaf spot symptoms appeared six days after the inoculation. The test received a second inoculation one week after the first inoculation. Disease developed into a severe epidemic by the end of the second week. Differences in gray leaf spot were observed between resistant and susceptible selections. Four standard cultivars were included in both this test and the 2001 perennial ryegrass test at Adelphia, which had been attacked by a natural gray leaf spot epidemic in fall 2001 (described previously). A correlation coefficient of 0.87 was

Black plastic covers were placed directly over the top of the turfgrass canopy after inoculation with a conidial suspension (field method 2). This technique incited disease and the results were similar to a natural field infection of gray leaf spot.

Standard cultivars	Mean rating[1] North Brunswick, NJ. *(Field inoculated)*	Mean rating[1] Adelphia, NJ. *(Natural infection)*
Applaud	3.5	4.2
GGH (polycross)	1.6	2.7
Jet	3.9	4.3
Paragon	2.9	3.2

Correlation coefficient = 0.87 (p=0.13)

[1]Using 1-9 scale. 1= more than 90% leaf tissue infected, 9 = free of disease.

Table 2. Gray leaf spot resistance of four standard cultivars in a field test inoculated with gray leaf spot compared to a field test naturally infected with gray leaf spot.

obtained (P = 0.13), indicating that this field inoculation procedure simulated a natural infection of gray leaf spot fairly well.

Discussion

In the greenhouse tests, both method 1 and method 2 successfully induced the symptoms of gray leaf spot. However, the great divergence between these two methods in accurately reflecting field results suggested that subtle changes in incubation conditions significantly affect the experimental results. This also further verified the necessity of testing the correlation between field and greenhouse tests. As a result of this work, we believe that method 2 can be recommended as an effective inoculation technique for the greenhouse screening for gray leaf spot resistance in perennial ryegrass cultivars.

The results of the field experiments indicated that placing a black plastic cover over the turf plots after inoculation (method 2) was an effective way to induce gray leaf spot in the field. Method 1 has been used at the North Brunswick location with varying degrees of success since 1996. There is considerable cost associated with

Significant differences in gray leaf spot resistance between selections of perennial ryegrass indicate inoculation techniques should be effective to screen for gray leaf spot resistance.

the erection of the clear plastic walls; it requires significant labor and it yields inconsistent results. Method 2, however, is inexpensive, less labor intensive and seems to be an efficient method to induce gray leaf spot in field-grown turf plots.

Increasing the inoculum concentration may also enhance gray leaf spot infection. Field inoculation, however, usually involves large turf areas, demanding large amounts of conidia, which is a formidable job. By covering the field with plastic film to induce the disease, reasonably low conidial suspension concentration can be used. According to the performance of the four standard perennial ryegrass cultivars, gray leaf spot disease induced by method 2 was fairly consistent with the natural occurrence observed at Adelphia in 2001.

Ongoing and future research

In an effort to understand the inheritance of gray leaf spot resistance, genetic studies comparing resistant vs. susceptible perennial ryegrass plants are being initiated. Two diallel crosses have been made in 2001 and 2002. Progenies from these crosses were tested in a growth chamber using method 2. Preliminary results indicate that predominant additive gene effects may be involved in gray leaf spot resistance in perennial ryegrass. These results, along with the utilization of the inoculation methods described here, indicate that selection for gray leaf spot resistance in a breeding program is indeed possible.

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