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A Simple *Pythium aphanidermatum* Field Inoculation Technique for Perennial Ryegrass

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***Abstract*. A simple field *Pythium* inoculation technique is needed to be able to assess cultivars for disease resistance and effectiveness of cultural practices or fungicides in the management of *Pythium* blight. We assessed two mixtures as inocula [i.e., an infested tall fescue (*Festuca arundinacea* Schreb.) seed plus wheat (*Triticum aestivum* L.) bran and an infested rye (*Secale cereale* L.) plus barley (*Hordeum vulgare* L.) grain mix], and three covers (black and clear plastic, and a geothermal blanket) for their ease of use and consistency in producing *Pythium* blight epidemics in perennial ryegrass (*Lolium perenne* L.) field plots. Both the fescue seed–wheat bran and rye–barley grain inocula provided good media on which to culture *Pythium aphanidermatum* (Edson) Fitzp. In general, the fescue–wheat bran mix applied at the high level (100 mL/0.9-m^2 plots) produced greater blight ratings in perennial ryegrass than did the low level (50 mL/0.9-m^2 plot). The fescue–wheat bran also was generally more effective than either rate of the rye–barley mix in blighting turf. All covers enhanced blighting, when compared to the uncovered control, by raising the relative humidity. Covering plots with black plastic following inoculation resulted in greater blight ratings than did covering with either clear plastic or the geothermal blanket.**

Pythium blight (cottony blight, grease spot, or spot blight) is a destructive disease of turfgrasses. Six *Pythium* species (i.e., *Pythium aphanidermatum*; *P. graminicola* Subramaniam; *P. myriotylum* Drechsler; *P. torulosum* Coker & Patterson; *P. ultimum* Trow. var. *ultimum*; and *P. vanterpoolii* V. Kouyeas & H. Kouyeas) are known causal agents of *Pythium* blight (Smiley et al., 1992). *Pythium aphanidermatum*, however, is the most common incitant of the disease during periods of high temperature and humidity (Freeman et al., 1963; Hall et al., 1980). At present, *Pythium* blight management on golf courses relies on proper water management and frequent fungicide applications (Smiley et al., 1992).

To develop more effective cultural management techniques and to evaluate new fungicides or biological agents for *Pythium* blight control, a reliable field inoculation technique is needed. Field inoculation of *Pythium* sp. often fails because favorable environmental conditions for disease development are difficult to maintain. Brede (1984) used *P. aphanidermatum*–infested rye-grain as an inoculum and covered creeping bentgrass (*Agrostis palustris* Huds.) field plots following inoculation for several days with a moistened wood-fiber mulch blanket. He reported that the technique was successful in producing a uniform infection in a fungicide evaluation trial in Oklahoma. Cole et al. (1986) used plastic-covered, wood-framed humidity chambers to induce *Pythium* blight in the field in Pennsylvania. Inoculum was prepared by growing virulent isolates of *P. aphanidermatum* on autoclaved rye-grain for ≈7 d. Portable, wood-framed, and clear plastic-covered chambers that had to be elevated each morning were ineffective, and sometimes caused burning of turf foliage in a Maryland study (Dernoeden, unpublished). Sanders and Cole (1986) also developed intermittent misting chambers framed with polyvinyl chloride pipe and covered with translucent plastic covers for field plots. Later, Soika and Sanders (1995) built a polyethylene-covered greenhouse equipped with an intermittent mist system over field plots for their *Pythium* blight fungicide evaluations. While these methods were reliable for producing epidemics in Pennsylvania, they are restrictive because of the small size of the portable misting chambers and greenhouse. In Georgia, Icard (1994) used a mixture of equal parts of oat (*Avena sativa* L.), wheat, barley, and corn (*Zea mays* L.) grains as a medium to culture *P. aphanidermatum*. In field studies, the grain inoculum was covered with porcelain mortars to protect it from feeding birds. While the study areas were irrigated frequently to keep the foliage wet, this inoculation technique failed to produce consistent turf blighting.

Other than building and manipulating portable chambers or greenhouses, there is no simple and reliable field inoculation procedure for evaluating fungicides, cultural practices, biological agents, or disease-resistant cultivars for this disease. Hence, the objective of this project was to develop a simple *P. aphanidermatum* inoculation technique that would consistently blight field-grown turfgrasses. Two different types of inoculum and three different covers were evaluated.

Materials and Methods

Sources of inoculum and experimental sites. Two isolates (MD-P-3 and MD-P-4) of *P. aphanidermatum* were used in the field inoculation studies. The MD-P-3 was isolated from blighted perennial ryegrass from the Bowie Bay Sox Stadium in Bowie, Md., and MD-P-4 was isolated from blighted 'Bardot' colonial bentgrass (*Agrostis tenuis* Sibth.) from the Univ. of Maryland Turfgrass Research and Education Facility (UMTREF) in Silver Spring, Md., in July 1995. They were identified as *P. aphanidermatum* because both isolates produced terminal inflated sporangia, intercalary, broad sac-shaped antheridia, and aplerotic oospores. Established stands of 'Caravelle' perennial ryegrass were used in field studies conducted at the UMTREF.

Preparation of inoculum. Two kinds of inoculum were evaluated, a rye–barley grain mix, and a tall fescue seed plus wheat bran mix. For the grain inoculum, 500 g of seed of each species (50:50 w/w) were placed in a polypropylene tub (152 mm W × 279 mm L × 127 mm H) with 1 L tap water, covered with two layers of aluminum foil, and soaked overnight at room temperature. The second inoculum was prepared by soaking in tap water a 1-L volume of tall fescue seed in a polypropylene tub. The next morning, the fescue seeds were rinsed and drained three times, mixed with 1 L volume of wheat bran and covered with a double layer of aluminum foil. The two inocula were then autoclaved separately three times at 121 °C at 138 kPa for 60 min. The third autoclaving was performed 1 d after the first two. Once cooled, the substrates were separately infested by placing 20 1-cm^2 agar squares from <3-day-old colonies on top of the mix and incubating the inoculum at 29 ± 1 °C. Each inoculum was stirred after 3 d and then incubated at the same temperature for another 4 d. Separate tubs were used for each mix and *P. aphanidermatum* isolate. The infested substrates for both isolates were combined just prior to inoculating plots. One h prior to inoculation, 2 L of tap water were added to 500 g of the grain inoculum, and the mix was macerated in a blender. The fescue–bran mix was not suspended in water or macerated. Clumps of the fescue–bran mix were broken into smaller pieces and were placed on a bench to dry for a few hours just prior to inoculating plots.

Field experiments. Field inoculations were performed on 2 Aug. 1996, and on 30 June, 21 July, 11 Aug., and 19 Aug. 1997 between 1500 and 1600 HR in separate trials. The grain and fescue–bran mixes were applied to plots at three different levels (i.e., 0, 50, and 100 mL vol/plot). Plots were irrigated just prior to and immediately following inoculation. Following inoculation, plots were covered with one of three covers or left uncovered (control). The three covers evaluated were as follows: black or clear plastic (C&A polyethylene film; AT

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Plastics, South Brampton, Ont.), and a gray, geothermal blanket (nonwoven polyester filament cloth, Contech, Middletown, Ohio). The macerated grain or fescue–bran inocula were evenly distributed over separate plots (1.5 × 0.6 m). Plots then were covered overnight and covers were anchored into the soil with metal sod staples. The covers were removed ≈0900 HR the next day. The plots were irrigated at 1100 and 1600 HR. If foliar mycelium did not develop overnight or if the next day were overcast, all plots were re-covered immediately. If sunny, plots remained uncovered during the day. At 1600 HR all plots were re-covered until the next morning. Plots did not have to be covered on any date for more than two nights. The presence of foliar mycelium was an indicator that infection had occurred, but its presence was not used as a measure of blighting. The experiment was arranged in a randomized complete block, split-plot design with three replications. The whole plots were covers and the subplots were the types of inoculum and application levels. Disease was assessed visually for the percentage of plot area blighted using a 0 to 100% linear scale where 0% = no blighting and 100% = entire plot area brown or dead. The field inoculation tests were conducted a total of five times in 1996–97. Blighting was rated several times following inoculation, but only one set of data from each test when maximum blighting was evident were combined into a single analysis (Steele et al., 1997). Data were analyzed using the PROC MIXED procedure in SAS and means were separated by the least significant difference *t* test (LSD) at $P = 0.05$ (Littell et al., 1996).

Environmental measurements (1997 only). Temperature was measured at soil level under all covered and uncovered plots, and relative humidity (RH) was measured in the canopy under one black plastic cover, one gray geothermal blanket, and an uncovered plot. Data were monitored using an AM-416 Multiplexer and a CR-10 datalogger (Campbell Scientific, Logan, Utah) in 1997. The multiplexer allowed more sensors to be connected. The RH under clear plastic was not measured because only three RH sensors were available, and we assumed that the RH under clear plastic would be similar to that under black plastic. The soil temperature sensors were calibrated at 0 °C. The datalogger was programmed to measure all sensors every 15 s and data were averaged every 60 min. There were two temperature sensors for each plot, or a total of six temperature sensors under each type of cover. Data for the turfgrass canopy temperatures under each cover were pooled for the six sensors.

Results and Discussion

Inoculum observations. After 7 d of incubation at 29 ± 1 °C, an abundance of mycelium was produced and oospores were found on both substrates. The rye–barley mix produced much more mycelium than rye or barley alone. The mix created a granular structure with more spaces between grains for mycelia to grow. For example, when only rye was used,

Table 1. Blight ratings elicited by three different inoculum types applied at three levels under three covers in five field inoculation studies conducted in 1996 and 1997.

Inoculum		Plot area blighted (%)			
Type	Level (mL/plot)	Black plastic	Clear plastic	Geothermal blanket	Uncovered control
Tall fescue + wheat-bran	50	32 bc[z]	11 b	15 b	1 a
	100	52 a	22 a	30 a	1 a
Rye + barley	50	15 c	9 b	5 bc	1 a
	100	29 b	6 b	13 b	1 a
No inoculum	---	2 d	5 b	1 c	0 a

[z]Mean separation within columns by LSD *t* test, $P \leq 0.05$. Means represent an average of three replications of each treatment in each of five separate studies.

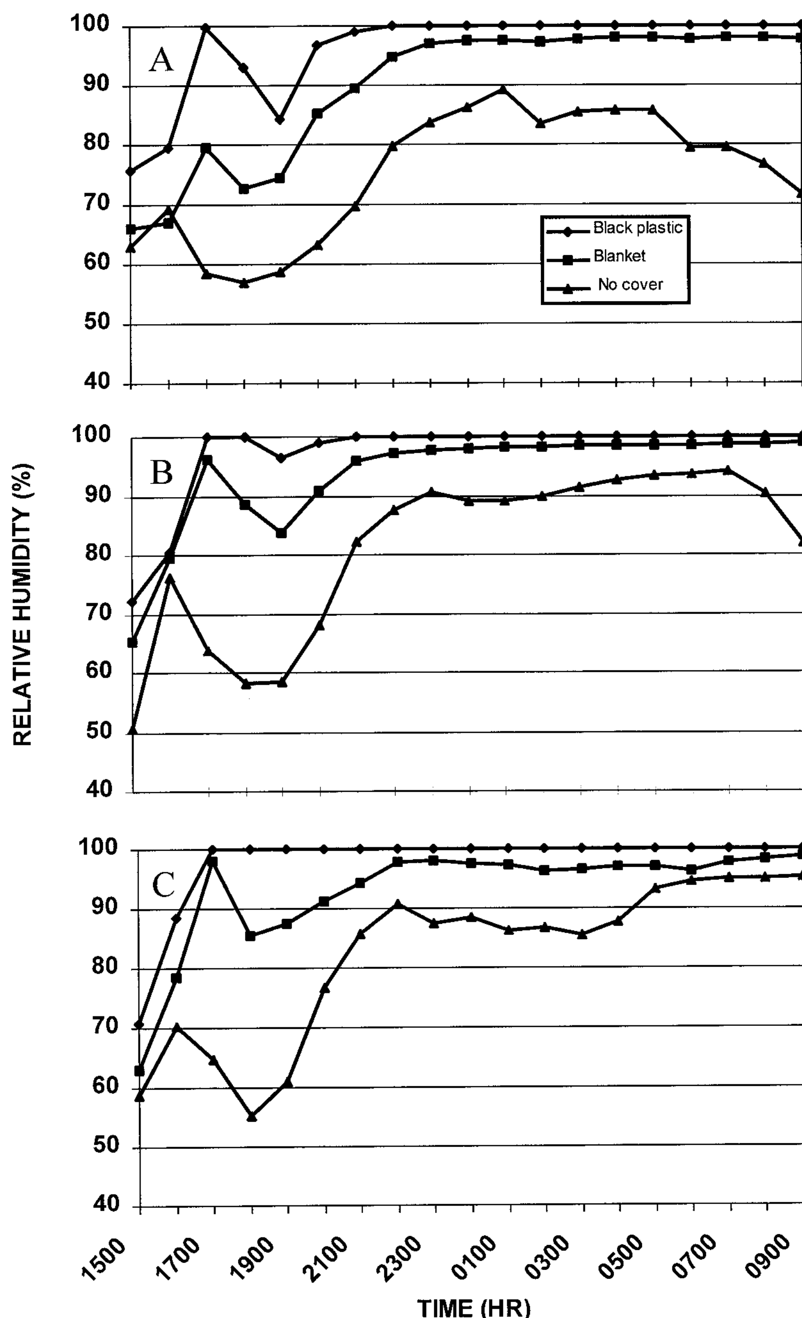

Fig. 1. Relative humidity under covered and uncovered field plots from 1500 to 0900 HR on three dates in 1997 as follows: **A**) 21 to 22 July; **B**) 11 to 12 Aug; and **C**) 19 to 20 Aug.

the grains stuck tightly together after soaking and autoclaving. When a single species of grain was infested, most mycelium grew on the surface of the grains and with little growth between grains. There also was a greater chance for contamination when attempts were made to break the grains into smaller pieces. With the rye–barley grain mix, however, barley grains prevented rye grains from sticking together, resulting in greater space between grains, thus promoting more mycelial growth. For the fescue seed–wheat bran mix, mycelium could be observed on every seed. Evidently, wheat bran was a good source of nutrients and fescue seeds produced a puffy structure that promoted mycelial growth. Hence, both mixes produced a good medium on which to culture *P. aphanidermatum*.

Field experiments. When covers were removed, foliar mycelum, watersoaking of leaves, and blighting were evident. Most tissue collapsed within 3 to 5 d after removal of the covers. Generally, there was little indication of new infections appearing following the removal of covers. The percentage of plot area blighted was dependent on inoculum type and level, as well as cover. Except for the uncovered control, there were significant differences in blighting between the rye–barley mix and the fescue–wheat bran mix at different application levels under all three covers (Table 1). When data were combined over the five trials the fescue–wheat bran mix applied at the high level produced greater blight ratings than did the low level or either level of the rye–barley mix. Except under black plastic, where the high inoculum level caused more blighting, there were no differences in blight ratings among the low and high levels of the rye–barley mix.

Blight ratings differed significantly among the three covers and the uncovered control (data not shown). The average ratings for all inoculum types and levels under the black plastic cover was 26%, which was significantly ($P \leq 0.05$) greater than that under the clear plastic cover (11%), the geothermal blanket (13%), or the uncovered control (1%). Values for the clear plastic and geothermal blanket cover did not differ significantly.

Consistently greater blighting occurred when a black plastic cover and the high level (100 mL vol/plot) of fescue–wheat bran mix were applied (Table 1). Blighting was at times excessive, and the lower level of the fescue–wheat bran inoculum may be preferred if less blighting is desired. In addition, this inoculum was more convenient to use because it did not require macerating in water prior to application. All of the covers were easily managed by two people and they could be cut to fit experimental sites larger than 20 × 20 m.

Environmental conditions. Environmental conditions under selected covers were only measured in 1997. The environmental conditions during the first night following inoculation were critical for infection. Two hours after inoculation, the RH under black plastic reached 100% and generally remained over 90% for 16 h on all three inoculation dates (21 July, 11 Aug., 19 Aug. 1997) (Fig. 1). For the geothermal blanket, a rise in RH above 90% required 1 to 6 h, but once reached was maintained for 11 to 14 h. For the uncovered control, RH rose more slowly in response to a fall in air temperature, but often dropped below 90%. In uncovered plots, RH >90% was sustained for only 5 to 6 h on two of the Aug. 1997 inoculation dates (Fig. 1B and C).

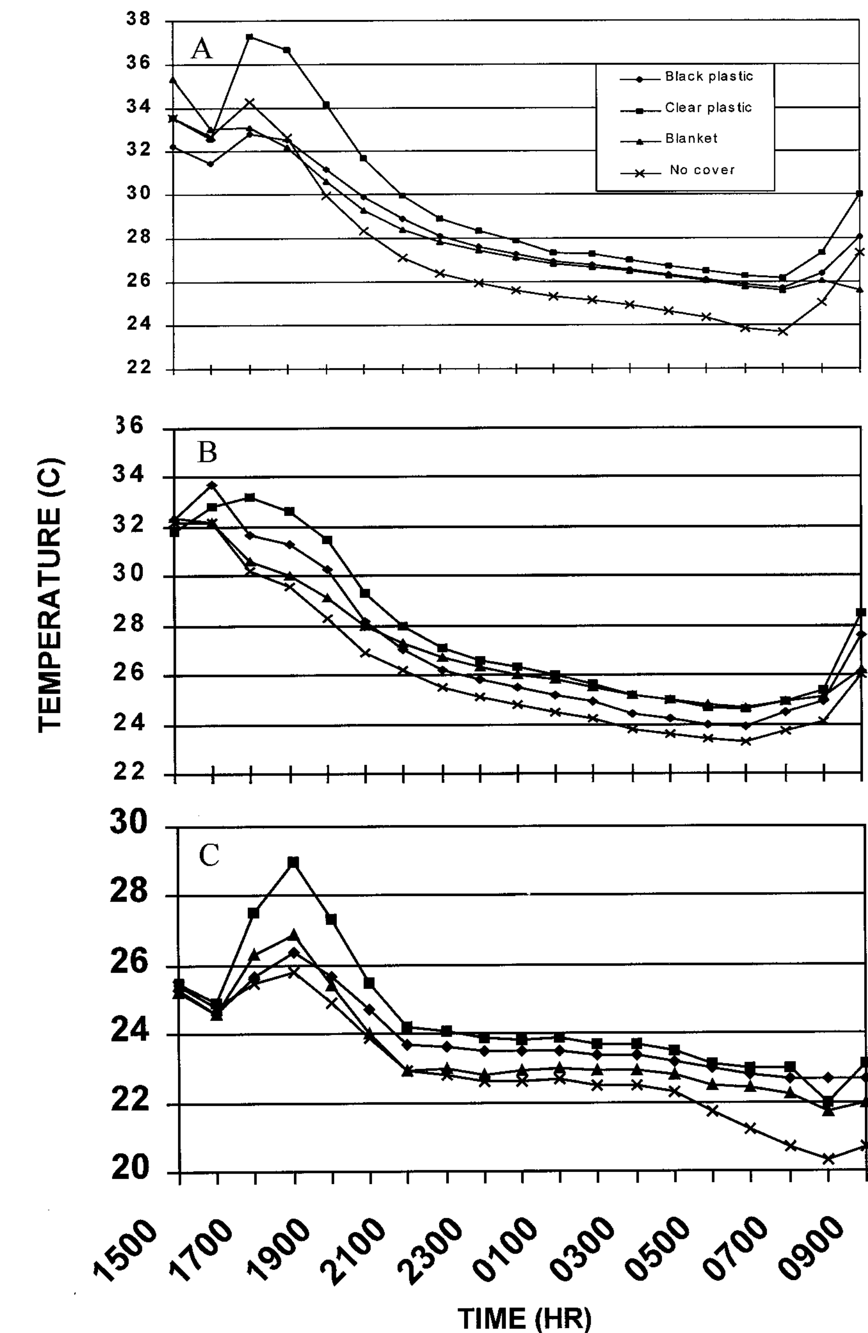

Fig. 2. Canopy temperature under covered and uncovered field plots from 1500 to 0900 HR on three dates in 1997 as follows: **A**) 21 to 22 July; **B**) 11 to 12 Aug.; and **C**) 19 to 20 Aug.

Canopy temperatures rose more rapidly under clear plastic than under the other covers, but there was little difference in canopy temperatures among the covered and uncovered plots, especially after 2100 HR, when the difference was only ≈2 to 3 °C (Fig. 2). Hence, we believe the covers enhanced infection more by promoting long periods at high RH rather than higher temperature. Temperature was probably even less important given that night temperatures in uncovered plots remained >20 °C on all three inoculation dates.

Nutter et al. (1983) reported that the environmental conditions required for a *Pythium* blight outbreak were a maximum daily temperature exceeding 30 °C, minimum temperature not lower than 20 °C, and at least 14 h at a RH >90%. The turfgrass canopy temperature and RH under the black plastic met these requirements. Turf was blighted under black plastic even though the maximum temperature was no higher than 30 °C on 19 Aug. 1997. Disease development under the geothermal blanket was less than that under black plastic. This was probably because the period of high RH (>90%) was shorter under the geothermal

blanket. In uncovered control plots, disease either did not develop or was very limited. This was attributed to low RH, because night temperatures were high enough (i.e., >20 °C) for disease to occur. The temperature under the clear plastic was generally higher than that under the black plastic, but RH was not measured under the clear plastic. Disease ratings under the former, however, were significantly lower than those under the latter. Clear plastic covers caused foliar burning on most inoculation dates, and this injury to the foliage, and possibly to the inoculum itself, may have contributed to lower blight ratings under clear plastic. Furthermore, despite small differences in temperature under covers after 2100 HR, the total accumulation of heat under clear plastic would have been greater, and this also could have reduced blighting by damaging the inoculum. The black plastic covers only occasionally caused foliar burning, which was never as severe as that under clear plastic. In an unrelated study, however, we observed severe foliar burning and death of inoculum when black plastic covers were installed at 1500 HR on a sunny day when air temperature was 31 °C. In another unrelated study, conducted during an overcast and rainy period, black plastic covers were retained for 48 h, but during a 2-h period prior to cover removal at 1600 HR, the sun appeared and the canopy was severely burned. These experiences indicate that plots should be covered on sunny days later than 1600 HR when air temperature is <31 °C. Furthermore, covers should be removed as soon as sunny conditions develop following overcast or rainy weather. The geothermal blanket, which also was less effective than black plastic in enhancing blight, caused no injury to turf on any date.

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