Selective Medium for Isolation and Enumeration of *Phialophora gregata* from Soybean Straw and Soil

ALEMU MENGISTU, Postdoctoral Research Associate, and H. TACHIBANA, Professor, USDA-ARS, Department of Plant Pathology, Iowa State University, Ames 50011; and C. R. GRAU, Professor, Department of Plant Pathology, Russell Laboratories, University of Wisconsin, Madison 53706

ABSTRACT

Mengistu, A., Tachibana, H., and Grau, C. R. 1991. Selective medium for isolation and enumeration of *Phialophora gregata* from soybean straw and soil. Plant Dis. 75:196-199.

Phialophora gregata, the cause of brown stem rot of soybean (*Glycine max*), can be isolated readily from physiologically active host tissue but is difficult to recover from senescent soybean tissue and soil. A selective medium was developed for the direct isolation and quantitative estimation of propagules of *P. gregata* from soybean straw and organic debris from soil. The medium, designated as PGM, contained 20 g of Difco Bacto agar and 129 g of homogenized green beans per liter of distilled water. After autoclaving, 0.8 g of $CuSO_4$ and 10 mg of pentachloronitrobenzene were added, and the medium was adjusted to pH 5.5 with lactic acid. Background colonies of bacteria and other fungi were suppressed significantly on PGM. Colonies of *P. gregata* were identified on PGM after 6 days of incubation. Recovery of *P. gregata* was 8×10^2 colony-forming units (cfu) per gram of wet sieved soil, which was significantly lower than recovery from samples that combined surface straw and debris. Populations of *P. gregata* detected on PGM ranged from 4×10^5 to 1×10^6 cfu/g of air-dried and ground plant debris. The sample of plant debris consisted of surface soybean straw and plant debris recovered from sieved soil sampled directly beneath the soybean straw. A lower detection threshhold was established for PGM than for a modified water agar medium and a selective medium designed for the recovery of the adzuki bean (*Vigna angularis*) strain of *P. gregata*. More propagules of *P. gregata* were recovered from surface straw and soil debris collected from plots continuously cropped to soybean cultivars susceptible to brown stem rot than from soil collected from fields previously planted to a resistant soybean cultivar. Disease severity, based on the percentage of internal stem browning of soybean plants, was highly correlated ($r = 0.93$) with the number of colonies of *P. gregata* recovered from soybean straw and soil. The more aggressive pathotype I was recovered on PGM at a higher frequency than the less aggressive pathotype II.

Phialophora gregata (Allington & Chamberlain) W. Gams (syn. *Cephalosporium gregatum* Allington & Chamberlain), the cause of brown stem rot of soybean (*Glycine max* (L.) Merr.), causes yield losses in many regions of the Midwest (1,2,8–10). The fungus is a vascular parasite in stems and roots (2,8) and survives as a saprophyte in soybean debris (2,4,6). Isolation of the pathogen during the vegetative and reproductive phase of plant growth has been achieved with acidified potato-dextrose agar (8). However, it is difficult to isolate the fungus from soybean straw and soil debris on culture media because of competition from bacteria and other fungi (1,4–6).

Gray (4) recovered *P. gregata* from overwintered soybean straw and straw fragments from soil debris retained on a 140-μm sieve with a modified water agar medium (MWAM) that contained tetracycline hydrochloride and streptomycin sulfate. Although this medium was useful for the detection of *P. gregata*, only six to eight colonies were recovered per 3 g of infested straw, and identification of colonies required microscopic examination. Another selective medium is available for isolation of a specific strain of *P. gregata* that infects adzuki beans (*Vigna angularis* (Willd.) Ohwi & H. Ohashi) (5). The usefulness of this medium has not been determined for the recovery of *P. gregata* from soybean tissue.

Symptoms of brown stem rot are diagnosed readily during the mid to late reproductive stages of growth. However, symptoms of brown stem rot inside stems are not readily apparent once the tissues become senescent. Symptoms of brown stem rot are extremely difficult to identify for overwintered straw. Although selective media for *P. gregata* have been reported (4,5), we have identified a need for a medium that has greater efficiency for recovery of the brown stem rot pathogen. Therefore, the primary objective of this study was to develop a selective medium for the recovery of *P. gregata* from soybean straw and soil debris and to compare the efficiency of this medium to other media developed previously. A second objective was to begin preliminary studies on the application of a selective medium to study the population dynamics of *P. gregata* in agroecosystems.

Present address of first author: University of Wisconsin, Department of Plant Pathology, 1630 Linden Drive, Madison 53706.

Research supported by project 2732 of the Iowa Agricultural Experiment Station and Home Economics, Ames, and the College of Agriculture and Life Sciences, University of Wisconsin, Madison.

Accepted for publication 12 August 1990.

© 1991 The American Phytopathological Society

MATERIALS AND METHODS

Isolation media. A medium developed for the detection of *Cephalosporium gramineum* Nisikado & Ikata (12) was modified for this study. Ingredients for our selective medium for *P. gregata*, thus designated as PGM, were 129 g of green beans (Gerber Products Co., Fremont, MI) and 20 g of Bacto agar (Difco Laboratories, Detroit, MI) in 1,000 ml of distilled water. After autoclaving, 0.8 g of copper sulfate and 10 mg of pentachloronitrobenzene (PCNB) were added, and the medium was adjusted to pH 5.5 with 25% lactic acid.

Other media used for comparison included a 1.5% MWAM amended with streptomycin sulfate and chlorotetracycline hydrochloride at 1 mg/ml (4) and a selective medium (SM) developed for isolation of *P. gregata* from adzuki beans (5). The SM contained glucose (5 g), peptone (5 g), potassium phosphate (1 g), magnesium sulfate (0.5 g), sodium cholate (sodium salt, cholic acid) (0.5 g), PCNB (0.5 g), sodium borate (0.5 g), streptomycin sulfate (0.2 g), tetracycline hydrochloride (0.5 g), and agar (20 g) in 1 L of distilled water. Antimicrobial agents were added after each medium was autoclaved and had cooled to 50 C. Each medium was dispensed at the rate of 20 ml per petri dish (9 cm diameter) and was allowed to solidify for a minimum of 24 hr before use.

Isolation from straw and plant debris. Samples of soybean straw and underlying soil were collected from three fields in early April. Fields were located at the Curtis, Woodruff, and Hinds experimental farms at Iowa State University in Ames and were all within 7 km of each

other. The soil type at the Curtis and Woodruff farms was a Clarion loam; at the Hinds farm, the soil was a Huntsville silt, Spillville loam. The site sampled at the Curtis farm had been planted continuously to the cv. Corsoy 79 for 5 yr. At the Woodruff farm, straw and soil were collected from areas of the field planted to cv. BSR 201 (resistant to brown stem rot) or to Lakota (susceptible). The plots at the Hinds farm had been planted to either BSR 101 (resistant) or Hardin (susceptible). The severity of brown stem rot was high for Hardin and low for BSR 101 the previous year. Straw on the soil surface and soil directly beneath the straw was collected from each plot with a soil auger (1,350 cm^3) to a depth of 30 cm. "Straw" refers to soybean straw that was either lying on the soil surface or partially covered by soil; "debris" refers to plant residue recovered from soil and may or may not be of soybean origin. Four straw and soil samples were collected, mixed, bulked, and kept separate for each of the three sites. The straw and pieces of debris retained on a 16-mesh (1.19-mm-diameter opening) screen were air-dried for 7 days at 22–24 C and were ground with a Wiley mill fitted with a 28-mesh (600-μm-diameter opening) screen. Approximately 60 g of ground straw was recovered per sample from each site. Five 0.1-g subsamples were taken from each sample, diluted 1,000× in distilled water, and assayed for *P. gregata* on the three media.

We previously determined that a dilution of 1×10^{-3} would result in colony numbers that could be easily enumerated. The suspension of ground straw and debris was agitated constantly during dispensing to avoid sedimentation. A 1-ml portion was removed with a Mini-pet (Fisher Scientific, Pittsburgh, PA) repeating pipette fitted with a 16-gauge cannula needle and was distributed on the surface of each medium with a triangular glass rod. Six plates (replications) were used for each selective medium and placed in the dark at 20 C. Colony-forming units of *P. gregata* were counted after 6 days with a dark-field colony counter (Reichert Scientific Instruments, Buffalo, NY). Colonies of *P. gregata* were classified for pathotype based on colony growth rate, morphology, and virulence on a susceptible soybean (3,7). The relationship of colony type and pathotype was validated by inoculating plants of Corsoy 79 with five separate isolates of each putative pathotype of *P. gregata* (7). The percentage of internal stem discoloration and severity of foliar symptoms was measured to determine the pathotype of each isolate tested (3,7).

Isolation from soil. Soil was collected at the Curtis farm from a field naturally infested with *P. gregata*. Soil was wet-sieved through a series of 140- (106-μm opening), 88- (180-μm opening), and 37- (500-μm opening) mesh sieves and a 10-μm Millipore filter. The residue retained on the sieves was discarded, and the soil suspension that passed through each sieve was diluted to 1×10^{-1}, 1×10^{-2}, and 1×10^{-3} with autoclaved distilled water. Soil suspensions were stirred continuously as a 1-ml subsample of diluent was distributed on the surface of the PGM. Six plates (replications) per selective medium were placed at 20 C and colony-forming units were counted after 6 days.

Isolation from soybean straw on soil surface. Resistant cv. BSR 101 and susceptible cv. Hardin were planted the previous season in two fields, each of which had an area with a history of high or low incidence of brown stem rot. Each cultivar was replicated four times at each location. Ten stems, 10–70 cm long, were collected from the soil surface in early April from each of the four replications of each cultivar in each field. Ten stems were bulked as one sample from each replication. Stems were air-dried in paper bags at room temperature (22 C) for 5 days. Stems were ground with a Wiley mill and passed through a 28- (600-μm opening) mesh screen. The mill was thoroughly cleaned between samples with a suction device. The ground stem material was diluted and distributed on the PGM as described earlier. Colonies of *P. gregata* were counted after 6 days of incubation at 20 C.

Statistical analysis. All experiments conducted on the selective media were repeated three times. Data were analyzed with standard analysis of variance and Fisher's least significant difference test for mean comparisons. Results were similar for each repetition; data from the third repetition are presented.

RESULTS

P. gregata was recovered from naturally infested soils and soybean straw on all the media compared in this study (Table 1). However, the PGM was judged to be superior to the MWAM and the SM originally developed for recovery of the strain of *P. gregata* specific to adzuki beans. Colonies of *P. gregata* recovered on PGM were identified after 6 days of incubation. Incubation for 2–3 wk was required before colonies of *P. gregata* were observed on either MWAM or SM. Colonies of *P. gregata* were recognized by morphology and color, which ranged from white to dark brown, with light yellow being most common. Colonies were raised and had either rough or smooth surfaces (Fig. 1). Twenty to 25 times more colony-forming units were recovered on the PGM than on the SM and 50 times more were recovered on the PGM than on the MWAM (Table 1). Numbers of colony-forming units on PGM ranged from 6.4×10^4 to 1.0×10^6 (Table 1). No significant difference was measured between MWAM and SM for the recovery of *P. gregata*.

The two pathotypes of *P. gregata*, types I and II, could be differentiated on PGM (Fig. 1). Colonies of pathotype II grew more rapidly than those of pathotype I. The number of colony-forming units for type I and II isolates from infested fields were 9.64×10^6 and 1.54×10^5 per gram of ground straw, respectively (*data not presented*). Colonies of *P. gregata* were verified to pathotype on cv. Corsoy 79 with the use of percentage of internal stem discoloration and severity of foliar symptoms as a measure of disease severity.

PGM suppressed the growth of most other microorganisms. Growth of other fungi (*Fusarium* spp.) and bacteria (*Pseudomonas* spp.) was rapid and abundant on SM and MWAM, particularly when incubation exceeded 2 wk. Colonies of *Penicillium* spp. were detected on PGM at a low frequency. The *Penicillium* spp. appeared as black colonies on PGM. Frequently, *P. gregata*

Table 1. Colony-forming units of *Phialophora gregata*[a] recovered from 1 g of ground, air-dried soybean straw[b] and plant debris collected from sites planted to one of four soybean cultivars at three field locations

	Plot location and soybean cultivar[d]				
	Curtis	Woodruff		Hinds	
Medium[c]	Corsoy 79	Lakota	BSR 201	Hardin	BSR 101
MWAM	19	3	1	3	1
SM	47	27	22	15	8
PGM	1,012	691	64	451	64
LSD ($P = 0.05$)	85	38	12	51	13

[a]Colony-forming units of *P. gregata* × 10^3 per gram.

[b]Soybean straw was collected from the soil surface. Debris refers to plant residue recovered from soil directly beneath the surface straw and may or may not be of soybean origin. Surface straw and plant debris sieved from soil was combined, air-dried, and ground to form the plant matter used in this study.

[c]Media compared for recovery of *P. gregata* were a modified water agar medium (MWAM) (3), a selective medium (SM) for the adzuki bean strain of *P. gregata* (4), and an experimental medium (PGM) tested for selectivity for *P. gregata*.

[d]The soybean cvs. Corsoy 79 and Lakota are susceptible, and BSR 201 and BSR 101 are resistant to *P. gregata*. Soil and soybean straw were collected in April from sites that had been planted to each cultivar the preceding year.

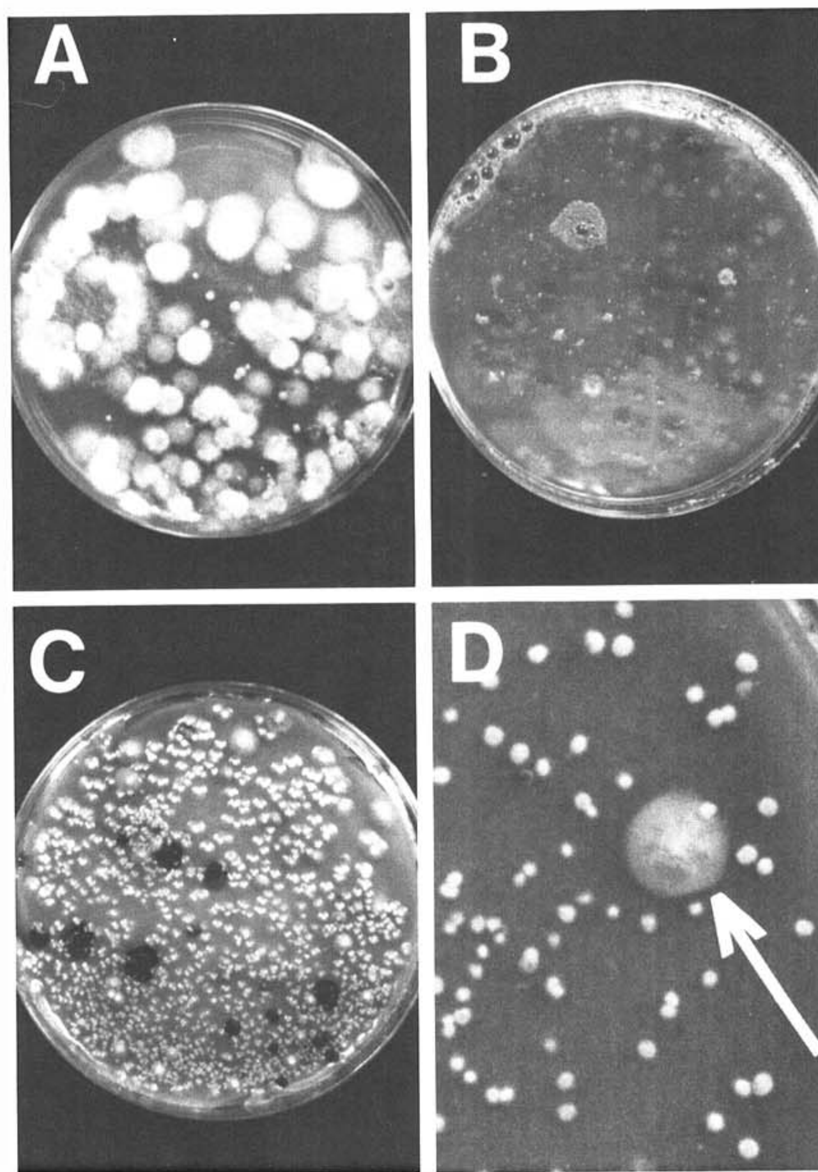

Fig. 1. Comparative selectivity of the *Phialophora gregata* medium (PGM) and other media used for isolation of *P. gregata* from soybean straw and plant debris recovered from soil. Growth of *P. gregata* on **(A)** selective medium for the adzuki strain of *P. gregata* (SM), **(B)** modified water agar medium (MWAM), and **(C)** PGM. **(D)** Colonies of pathotypes I (small colonies) and II (arrow) were differentiated on PGM. *Penicillium* spp. appear as dark colonies on PGM.

Table 2. Colony-forming units (cfu) of *Phialophora gregata* recovered from straw of specific soybean cultivars planted in sites with different severities of brown stem rot (BSR)

	Year		
	1985	1986	1987
Cultivar[a]	Observed incidence of BSR	Disease severity[b]	Cfu of *P. gregata*/g of straw[c]
Hardin	High	79	414
Hardin	Low	42	94
BSR 101	High	23	78
BSR 101	Low	19	36
LSD ($P = 0.05$)		21	108

[a] Hardin is susceptible and BSR 101 is resistant to *P. gregata*.

[b] The severity of brown stem rot was recorded for each cultivar at two sites in 1986. A rating for severity of brown stem rot was based on percentage internal stem discoloration: height of internal stem discoloration divided by total plant height × 100.

[c] Soybean straw of each cultivar was collected in April, air-dried, and ground. Colony-forming units that developed on the *P. gregata* medium were recorded and related to the severity of brown stem rot the previous year. The correlation coefficient was $r = 0.93$ ($P = 0.05$) for the relationship between disease severity the previous year and colony-forming units of *P. gregata* recovered from soybean straw.

was associated with colonies of *Penicillium* spp. Colony size of *Penicillium* spp. was reduced on PGM (Fig. 1). Preliminary studies indicated 0.8–1.0 g/L of copper sulfate was required to allow colonies of *P. gregata* to develop. However, concentrations of copper sulfate above 1 g/L greatly suppressed microbial growth, including *P. gregata*, and concentrations of copper sulfate below 0.5 g/L did not suppress unwanted microorganisms.

Colonies of *P. gregata* on PGM were recovered from the portion of soil that passed through the 37- and 88-mesh sieves, but not from the 140 mesh or the 10-μm Millipore filters. *P. gregata* was not recovered at dilutions lower than 1×10^{-3} from either the 37- or 88-mesh sieve fractions. Colony-forming units of *P. gregata* were 8×10^3, 2.25×10^3, and 2×10^3 from 37 mesh and 2×10^3, 6×10^2, and 5×10^2 from the 88-mesh sieve at dilutions of 1×10^{-1}, 1×10^{-2}, and 1×10^{-3}, respectively.

Cultivar reaction to *P. gregata* influenced the number of colony-forming units recovered on the selective media used in this study. More propagules of *P. gregata* were recovered from plant debris collected within plots continuously cropped to susceptible than from resistant cultivars (Tables 1 and 2). The PGM was superior to the MWAM and SM for the recovery of *P. gregata* (Table 1).

The cvs. Hardin (susceptible) and BSR 101 (resistant) were grown in two plot areas determined to differ in disease potential. Plants of each cultivar were rated for severity of brown stem based on the height of internal stem browning. Cultivars differed for disease severity only in plots with a high incidence of brown stem rot the previous year. Significantly higher numbers of colony-forming units of *P. gregata* were recovered from straw of Hardin plants that had a significantly higher disease rating (Table 2). A positive correlation ($r = 0.93$, $P = 0.05$) was determined between disease severity ratings taken at the end of the previous season and colony-forming units of *P. gregata* recovered from soybean straw the following spring (Table 2).

DISCUSSION

The ability to quantitatively and qualitatively detect *P. gregata* in crop debris would allow predictive estimates of disease incidence and severity and crop loss (8) and aid in better understanding of the biology of this pathogen in different agroecosystems. The PGM was superior to the MWAM and SM media for the recovery of *P. gregata* from soybean straw and soil organic debris. Although *P. gregata* can be detected on the MWAM and SM, pathotypes of *P. gregata* were not differentiated as was possible on the PGM (3–5,7). Another

important feature of the PGM vs. the other media was its superior suppression of unwanted microorganisms. Colonies of *Penicillium* spp, which grew profusely on MWAM and SM, were restricted in radial growth on PGM, thus preventing colonies of *P. gregata* from being masked. It is important to note that colonies of *P. gregata* were found in association with colonies of *Penicillium* spp. The ability of PGM to restrict the growth of other fungi is attributed to the use of copper sulfate. *P. gregata* expressed a tolerance to copper sulfate similar to that expressed by *C. gramineum* (12). An additional advantage of PGM is that it is not a complicated medium to prepare. This contrasts with the SM, which requires 10 ingredients (5).

The PGM was used in our research to study the association of *P. gregata* with soybean straw and organic debris sieved from soil. Colonies of *P. gregata* were recovered in greatest numbers from soil, soil debris, and straw fractions retained on 37- and 88-mesh sieves. The high recovery of *P. gregata* from surface soybean straw and debris beneath it supports the conclusions of other researchers that soybean straw is the primary source of inoculum for *P. gregata* (4–6).

The PGM was used to compare the quantity of inoculum of *P. gregata* associated with soybean cultivars that differ in reaction to the brown stem rot pathogen. The number of colony-forming units recovered from straw of resistant cultivars was about 10-fold less than from susceptible ones. Tachibana et al (11) reported that yield of susceptible soybean lines was 17% greater on land planted to resistant lines for four continuous years when compared with continuous culture with susceptible lines. They concluded that the lower severity of symptoms associated with resistant lines resulted in a reduction in inoculum of *P. gregata.* Results from our current research support the conclusion that soybean cultivars resistant to *P. gregata* can be used to lower inoculum. However, the differences in colony-forming units between resistant and susceptible cultivars were less dramatic when the other selective media were used. Thus, we conclude that the PGM can provide a better means to assess the population dynamics of *P. gregata* in agroecosytems. Future research should employ the PGM as a means to quantify the inoculum of *P. gregata* from soil and soybean straw for the purpose of predicting the incidence and severity of brown stem rot.

LITERATURE CITED

1. Abel, G. H. 1977. Brown stem rot of soybean *Cephalosporium gregatum*. Rev. Plant Pathol. 56:1065-1077.
2. Allington, W. B., and Chamberlain, D. W. 1948. Brown stem rot of soybean. Phytopathology 38:793-802.
3. Gray, L. E. 1971. Variation in pathogenicity of *Cephalosporium gregatum* isolates. Phytopathology 61:1410-1411.
4. Gray, L. E. 1972. Recovery of *Cephalosporium gregatum* from soybean straw. Phytopathology 62:1362-1364.
5. Kobayashi, K., Tanaka, F., Kondo, N., and Ui, T. 1981. A selective medium for isolation of *Cephalosporium gregatum* from soil and populations in adzuki bean field soils estimated with the medium. Ann. Phytopathol. Soc. Jpn. 47:29-34.
6. Lai, P. Y. 1968. Isolation of *Cephalosporium gregatum* from soybean straw. Phytopathology 58:1194-1195.
7. Mengistu, A., and Grau, C. R. 1986. Variation in morphological, cultural, and pathological characteristics of *Phialophora gregata* and *Acremonium* sp. recovered from soybean in Wisconsin. Plant Dis. 70:1005-1009.
8. Mengistu, A., and Grau, C. R. 1987. Seasonal progress of brown stem rot and its impact on soybean productivity. Phytopathology 77:1521-1529.
9. Sinclair, J. B., ed. 1982. Compendium of Soybean Diseases. American Phytopathological Society, St. Paul, MN. 104 pp.
10. Tachibana, H. 1982. Prescribed resistant cultivars for controlling brown stem rot of soybean and managing resistance genes. Plant Dis. 66:271-274.
11. Tachibana, H., Epstein, A. H., and Havlovic, B. J. 1989. Effect of four years of continuous cropping of maturity group II soybeans resistant to brown stem rot on brown stem rot and yield. Plant Dis. 73:846-849.
12. Wiese, M. V., and Ravenscroft, A. V. 1973. Quantitative detection of propagules of *Cephalosporium gramineum* in soil. Phytopathology 63:1198-1201.