

# Molecular Identification of Fungi Associated with Vascular Discoloration of Soybean in the North Central United States

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## ABSTRACT

Harrington, T. C., Steimel, J., Workneh, F., and Yang, X. B. 2000. Molecular identification of fungi associated with vascular discoloration of soybean in the north central United States. *Plant Dis.* 84:83-89.

Brown stem rot is a common but poorly understood vascular wilt disease of soybean. In order to more clearly delimit the causal agent (*Phialophora gregata*) and distinguish it from other morphologically similar fungi from discolored soybean stems, fungi were isolated on a semi-selective medium from discolored and non-discolored soybean stems collected at random across Iowa, Illinois, Minnesota, Missouri, and Ohio. A total of 11 fungi were commonly isolated and characterized based on colony morphology and DNA sequences of the internal transcribed spacer region of the rDNA operon. *Phomopsis longicolla* was the most frequently isolated fungus, but it was isolated more commonly from lightly discolored or non-discolored stems than from discolored stems. *Phialophora gregata* was the next most frequently isolated fungus and was isolated more commonly from discolored stems and more commonly in 1996 than in 1995, which had a warm growing season and relatively little brown stem rot. In inoculation experiments, only *P. gregata* was capable of causing the vascular discoloration and leaf symptoms typical of brown stem rot; none of the seven isolates could be considered non-defoliating. Two other fungi, *Plectosphaerella cucumerina* and *Gliocladium roseum*, were similar in colony morphology to *Phialophora gregata* but were not pathogenic to soybean, and these may be the same species as those referred to by earlier workers as *Acremonium* spp. or the non-defoliating form of *P. gregata*.

Additional keywords: *Cladosporium tenuissimum*, *Diaporthe phaseolorum*, *Fusarium* spp., *Glycine max*, *Macrophomina phaseolina*

Brown stem rot (BSR) is one of the most important diseases of soybean (*Glycine max* (L.) Merr.) in the north-central soybean-production region (10), yet the causal agent of the disease is poorly known. The disease is characterized by a browning of the vascular system and pith, and characteristic chlorosis and necrosis of the foliage develops late in the season, usually in August (2). However, similar vascular discoloration is found late in the season with other diseases of soybean. Some BSR-resistant germ plasm is available (3,24), mostly identified by the extent of vascular browning, but development of resistant cultivars and deployment of resistance have been hampered by imprecise resistance rating, a poor understanding of

the causal agent of the disease, and uncertainty about the variation in aggressiveness in the pathogen (16,24,29,33).

The pathogen was described as *Cephalosporium gregatum* in 1948 by Allington and Chamberlain (2). Unfortunately, the fungus forms rather nondescript conidiophores, phialides, and conidia, and many unrelated species are morphologically similar. Many *Cephalosporium* spp. are now placed in the heterogeneous genus *Acremonium*, but the proper generic placement of *C. gregatum* is still in question. Gams (14) transferred the species to the genus *Phialophora*, though the fungus generally produces non-pigmented conidiophores. Sequences of ribosomal DNA suggest that *Phialophora gregata* is an anamorph of a discomycete (T. C. Harrington, unpublished).

Two or more *Acremonium*-type fungi that are similar to *P. gregata* have been associated with brown stem rot in soybean and adzuki bean (5,18,23), and at least one of these *Acremonium* spp. causes vascular discoloration in soybean (23). Gray (16) reported that the disease in soybean is caused by either of two forms of *P. gregata*, a defoliating form (type I) and a non-defoliating form (type II), with the latter causing vascular discoloration but no foliar symptoms. Gray and Hepburn (18) did not

distinguish type I and type II isolates of *P. gregata*. Phillips (29) found at least three virulence genotypes of *P. gregata* based on vascular discoloration, and Willmot et al. (33) distinguished type I and type II isolates based on foliar symptoms. Mengistu and Grau (23) also recognized type II isolates of *P. gregata* and isolates of an unidentified *Acremonium* sp. that caused vascular discoloration but limited foliar symptoms. Later, Mengistu et al. (25) characterized type II isolates as growing faster than type I isolates, while the opposite was reported by Gray (16), and their (25) illustration of type II colonies show them to be atypical of *P. gregata*. Although it is not clear if type II isolates (sensu Gray, 16; Willmot et al., 33; and Mengistu et al., 25) are *P. gregata* or another *Acremonium*-type sp., we examined isolates of the *Acremonium* spp. of Mengistu and Grau (23) and found them to be *Plectosphaerella cucumerina* (Lindf.) Kleb. (anamorph = *Plectosporium tabacinum* (van Beyma) M. E. Palm), a common pyrenomycete in soybean and other plants that may be pathogenic to some hosts (6,27,28). Because its anamorph is similar in morphology to *Phialophora gregata*, it is possible that *Plectosphaerella cucumerina* was misidentified as the so-called "non-defoliating" form of *Phialophora gregata* in earlier studies (16,25). Gray and Hepburn (18) also found that some adzuki bean isolates identified as *P. gregata* were likely an unrelated *Acremonium* sp., and conidia and conidiophores of their fungus look similar to the anamorph of *Plectosphaerella cucumerina*. The brown stem rot pathogen in adzuki has recently been designated as a distinct specialized form, *Phialophora gregata* f. sp. *adzukicola* (21).

With the advent of polymerase chain reaction (PCR), inexpensive DNA sequencing, and a relatively large databank of ribosomal DNA sequences, it is now possible to more objectively characterize and identify fungal species. We applied these techniques to fungi associated with brown stem rot of soybeans. Our objectives were to clarify the etiology of this disease, more clearly characterize the causal agent, and delineate the pathogen from similar fungi isolated from vascular discoloration in soybean, using a larger number of isolates and sampling a wider geographic area than had been utilized in earlier studies.

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This project was supported in part by the Iowa Soybean Promotion Board. Journal Paper No. J-18309 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3226, and supported by Hatch Act and State of Iowa funds.

Accepted for publication 29 September 1999.

Publication no. D-1999-1115-02R  
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## MATERIALS AND METHODS

We used the semi-selective medium of Mengistu and Grau (25) to isolate from stems collected from randomly selected soybean fields in the Midwest at the end of the 1995 and 1996 field seasons. The isolated fungi were tentatively categorized to species by colony morphology and restriction fragment length polymorphisms (RFLP) of the internal transcribed spacer region (ITS) of the nuclear ribosomal DNA. The complete DNA sequences of the ITS region were used to aid identification. Nine of these fungi were inoculated into soybean in order to determine which are capable of causing the symptomatology characteristic of brown stem rot.

**Isolations.** In a separate study (34), 1,462 soybean fields in Iowa, Illinois, Minnesota, Ohio, and Missouri were randomly sampled in 1995 and 1996, based on stratification of counties by soybean acreage. The fields were systematically sampled from late September through early November, usually after leaf senescence, without regard to foliar or stem symptoms. In each field, 20 stems pieces, approx. 20 cm long, were collected at just above ground line, and these samples were evaluated for the stem discoloration typical of brown stem rot (34). A random subsample of those field samples was used for fungal isolations in our study. In 1995, 48 subsamples from Iowa and 20 subsamples from each of the other states were used for isolations. In 1996, 50 and 25 subsamples, respectively, were used. In 1995, the subsample selection for isolations was completely random, while the 1996 subsampling was random but restricted to only one subsample per county to increase the geographic distribution of the subsamples from each state. The stem pieces from each field were stored at 4°C until isolations, which were completed within 6 months of field collection.

Of the 20 stems randomly collected from each of the subsampled fields, 2 of the most discolored and 2 of the least discolored stems were used for isolation. In a few of the field samples, each of the 20 stems was heavily discolored or each was

only lightly discolored, and isolations were attempted from only 2 stems from those fields. Stem pieces were surface sterilized in 0.5% NaOCl solution for 2 to 3 min, and four small slivers of the inner stem were excised with a sterile scalpel and placed into *P. gregata* medium (PGM) in a plastic Petri dish (90 mm in diameter). The PGM (25) is somewhat selective for *Phialophora gregata*, though other fungi will grow on it at a relatively slow rate. The plates were incubated in the dark for at least 2 weeks at 15°C before colonies growing from the stem segments were characterized.

**Identifications.** Colonies growing from the individual stem pieces were classified into unique mycelial phenotypes based on pigmentation, texture, and relative growth rates. Each of these mycelial phenotypes was given a RFLP-ITS characterization based on *HaeIII* digests of the ITS region, which was amplified using primers ITS-1F (15) and ITS-4 (31). Template DNA for the PCR was obtained by scraping the colony on the PGM isolation medium lightly with a pipette tip (19). No DNA was extracted.

The PCR reaction mixture (100 µl) included 2.5 units of *Taq* polymerase (Promega Corp., Madison, WI) per reaction, the buffer supplied with the enzyme, 4 mM MgCl<sub>2</sub>, 200 µM of dNTPs, and 0.5 µM each primer. The thermocycler (MJ Research, Inc., Watertown, MA) conditions were an initial denaturation at 95°C for 95 sec, followed by 35 cycles of 52°C for 40 sec (annealing), 72°C for 2 min (elongation), and 94°C for 30 sec (denaturation). A final elongation was allowed for 10 min at 72°C to ensure a double-stranded amplification product. The amplified DNA was not purified before restriction enzyme digestion. After PCR, the restriction enzyme *HaeIII* (5 units in 0.5 µl, Promega Corp.) was added directly to the PCR reaction mix (17.5 µl), along with 2.0 µl of 10× buffer (supplied with the restriction enzyme). The digestion was allowed to proceed for 1 to 16 h at 37°C. Both the amplified DNA and the restriction enzyme fragments of these products were electrophoresed separately in 2% agarose gels in 1× Tris-borate-EDTA (TBE) buffer

(89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0), along with 100 base pair (bp) ladders (Gibco BRL, Inc., Gaithersburg, MD), to determine the size of the amplification and restriction products. The gels were stained with ethidium bromide and visualized using UV light. A digital image of the gel was analyzed using Gel-Reader 2.0.5 (National Center for Supercomputing Applications, University of Illinois, Urbana-Champaign).

For each mycelial/RFLP phenotype found, the ITS region of at least one isolate was sequenced using the ITS-1F and ITS-4 primers. PCR products were purified using Microcon-100 Microconcentrators (Amicon, Inc., Beverly, MA) and sequenced with the ABI PRISM 377 genetic analyzer (Perkin-Elmer Corp., Norwalk, CT) in the DNA Synthesis and Sequencing Facility at Iowa State University. Sequences of representative isolates were deposited in GenBank (Table 1) and used for searching for similar sequences in various databanks using the BLAST program (version 2.0, National Center for Biotechnology Information, United States National Institutes of Health, Bethesda, MD).

Representative cultures of each mycelial/RFLP phenotype were examined microscopically and compared to descriptions of relatives identified through ITS sequence comparisons. *Fusarium* spp. were cultured on carnation leaf agar, while potato dextrose agar (PDA) and malt extract agar (MEA) were used for culturing the other species.

**Pathogenicity tests.** Representative isolates of the nine most common mycelial/RFLP phenotypes in 1995 were used in two duplicate experiments. The susceptible soybean cv. Kenwood was inoculated by placing spores and hyphae into the stem (below the cotyledons, at 1 to 2 cm above the soil line) of 15-day-old seedlings between the V1 and V2 growth stages (12). In addition to the inoculations of cv. Kenwood, the seven *P. gregata* isolates were also inoculated into cv. BSR101, which is resistant to brown stem rot. Seeds were planted in sterile potting mix (1:2:2 soil:peat:perlite) in pots (15 cm in diame-

**Table 1.** Sizes of the amplified internal transcribed spacer region (ITS) product and *HaeIII* restriction fragments, and the isolate and accession numbers for the ITS sequence of a representative of each of 11 fungal species identified from soybean stems

Fungal species	PCR product (bp) <sup>1</sup>	Size of <i>HaeIII</i> restriction fragments (bp)	Isolate sequenced	Accession number <sup>2</sup>
<i>Phialophora gregata</i>	655	295, 190, 160	P63	AF132804
<i>Plectosphaerella cucumerina</i>	595	390, 155	P8	AF132805
<i>Phomopsis longicolla</i>	630	270, 135, 100, 65	P167	AF132796
<i>Macrophomina phaseolina</i>	630	475, 135	P207	AF132795
<i>Cladosporium tenuissimum</i>	600	605	P196	AF132797
<i>Gliocladium roseum</i>	615	425, 130	P195	AF132803
<i>Fusarium solani</i>	615	240, 130, 120, 90	P185	AF132801
<i>F. oxysporum</i> 1	595	350, 150, 90	P179	AF132799
<i>F. oxysporum</i> 2	605	265, 150, 90, 75	P405	AF132800
<i>F. graminearum</i>	595	350, 150, 90	P183	AF132798
<i>F. acuminatum</i>	610	365, 150, 90	P323	AF132802

<sup>1</sup> PCR = polymerase chain reaction and bp = base pairs.

<sup>2</sup> Sequence deposited in GenBank.

ter, two pots per cultivar/isolate in each of the two experiments) and placed in a growth chamber at 21°C and 14 h of light (330 to 430  $\mu\text{Em}^{-2} \text{s}^{-1}$ ). The plants were thinned to three per pot immediately before inoculations.

Inoculum was obtained from isolates grown on PG- medium (non-acidified PGM without copper sulfate or pentachloronitrobenzene) at 18°C for 3 weeks. The agar surface was scraped with a spatula, and a small bit of this slurry was forced into the stem of the soybean seedling with a sterile syringe needle (22 gauge) at 1 to 2 cm above the soil line. Using a haemocytometer, we determined that a range of  $2.25 \times 10^4$  to  $8.0 \times 10^4$  spores of *P. gregata* were picked up by the needle. Control plants were similarly treated but with scrapes of sterile PG- agar medium. After inoculation, the temperature of the growth chamber was set to 17°C, and the plants were fertilized twice (at 1 and 3 weeks after inoculation) with 1 g/liter of 20-10-20 fertilizer.

At 5 weeks after inoculation, the plants were harvested and rated for incidence of chlorotic and necrotic leaflets, and the extent of vascular browning above the inoculation point was measured after cutting into the stem. The entire experiment was repeated, and the mean percent of symptomatic leaflets and linear extent of vascular discoloration for each isolate were determined from the 12 inoculated plants. An arcsine transformation of the percent leaflets symptomatic and a  $\log_{10}$  transformation of the extent of vascular discoloration were used to normalize the data for analyses comparing the nine fungal species. Analysis of variance (ANOVA) was used to determine if there was variation among the isolates of the nine species in inducing symptoms. The treatment means were separated with the Duncan multiple range test. For comparisons of the *P. gregata* isolates on cvs. Kenwood and BSR101, a two-way ANOVA of non-transformed data was used. All statistical analyses were conducted with the use of SAS software (SAS Institute Inc., Cary, NC).

## RESULTS

**Fungal identification.** Five putative species of *Fusarium* and six other fungal species were commonly isolated from discolored and non-discolored soybean stems (Tables 1 and 2). Two of the *Fusarium* spp. had identical *Hae*III restriction patterns of the ITS amplification product (Fig. 1, Table 1) but had differing ITS sequences. Each of the other fungi had unique RFLPs and distinct mycelial morphology. In general, all colonies emanating from the soybean stems that were suspected to be *P. gregata* or *Plectosphaerella cucumerina* were checked for their ITS-RFLP pattern. Other colonies were tested to establish the consistency of the pattern for each species and to resolve ambiguous groupings based

on mycelial morphology. More than 500 colonies were tested for ITS restriction patterns. Representatives of each of the mycelial/RFLP phenotypes were identified to species using microscopic characters and complete DNA sequences of the ITS region.

Three of the isolated species had relatively slow growth rates and only lightly pigmented mycelia on PGM, and microscopic examination showed that they formed *Acremonium*-like conidiophores (unpigmented and poorly differentiated) and small, unpigmented conidia produced from phialides. Based on these conidiophores and conidia, one of the species was thought to be *Phialophora gregata* (23). Many of the isolates of the second species formed perithecia and ascospores that matched those described for *Plectosphaerella cucumerina* (28). The third species formed branching conidiophores with wet droplets of conidia at the branch tips, typical for *Gliocladium* spp. The RFLPs and ITS sequences of representa-

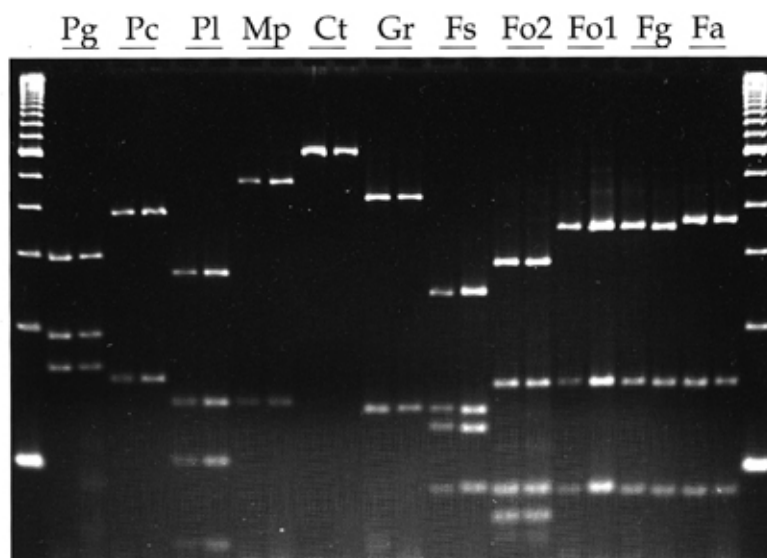
tives of these three taxa (Table 1) differed substantially, thus confirming that they were three separate species.

A search of similar ITS sequences using the BLAST program confirmed the morphological identification of *Phialophora gregata* and *Plectosphaerella cucumerina*. The ITS sequences (ITS1, 5.8S rDNA gene, and ITS2) of six of our *Phialophora gregata* isolates were identical (100% match of the 523 bp) to each other and to that of accessions U66727 and U66729, the ITS sequence of two other *P. gregata* isolates (5). The ITS sequences (ITS1, 5.8S, and ITS2 = 466 bp) of five soybean isolates of *Plectosphaerella cucumerina* were identical to each other (though one isolate from a *Nicotiana* sp. had 9 bp differences from the other five), and the more common ITS sequence was identical to accessions L36640 and U66732 of *P. cucumerina* (5,27). The *Gliocladium* sp. (isolate P195) had an ITS sequence closest (99% match, 477 of 481 bp) to *G. catenulatum* Gilm. (AF048737) and *G. penicillioides*

**Table 2.** Percentage of discolored (in vascular system and pith) and non-discolored soybean stems yielding fungal species in isolations on semi-selective medium for *Phialophora gregata* in 1995 and 1996

Fungal species	1995		1996	
	Non-discolored (n = 256) <sup>z</sup>	Discolored (n = 250)	Non-discolored (n = 288)	Discolored (n = 290)
<i>Phialophora gregata</i>	7.8	26.8	13.5	58.0
<i>Phomopsis longicolla</i>	69.9	52.4	74.3	40.0
<i>Macrophomina phaseolina</i>	7.0	10.8	3.1	3.4
<i>Cladosporium tenuissimum</i>	2.0	2.8	3.5	6.6
<i>Gliocladium roseum</i>	7.8	2.0	1.4	2.1
<i>Plectosphaerella cucumerina</i>	1.6	4.4	1.7	2.4
<i>Fusarium</i> spp.	5.8	7.2	2.8	7.6
Unidentified species	2.3	4.8	5.2	4.8

<sup>z</sup> n = number of stems from which isolations were attempted.



**Fig. 1.** *Hae*III digests of internal transcribed spacer region amplification products of the fungi isolated from discolored soybean stems. Representative products of two isolates of each species are shown. A 100 base pair ladder is shown in the first and last lanes. The fungi are *Phialophora gregata* (Pg), *Plectosphaerella cucumerina* (Pc), *Phomopsis longicolla* (Pl), *Macrophomina phaseolina* (Mp), *Cladosporium tenuissimum* (Ct), *Gliocladium roseum* (Gr), *Fusarium solani* (Fs), *F. oxysporum* (Fo) 2 and 1, *F. graminearum* (Fg), and *F. acuminatum* (Fa).

(AF048733). Our *Gliocladium* sp. formed *Verticillium*-like and penicillate conidiophores and fit descriptions of *G. roseum* Banier (4,9), though no ITS sequence of *G. roseum* was available for comparison.

Among the *Fusarium* spp. isolated, four RFLP patterns were found (Table 1, Fig. 1), but two mycelial phenotypes (*F. oxysporum* sp. 1 and a second species with red colony color on PDA) were found to have the same RFLP pattern. Although the two species had the same RFLP pattern, they had differing ITS sequences. The five unique *Fusarium* spp. ITS sequences were compared in BLAST searches. The *Fusarium* sp. with the RFLP pattern of *F. oxysporum* 1 but a red pigment on PDA had a unique ITS sequence (P183) that matched exactly (457 of 457 bp) to AF006342 of *F. culmorum* (W. G. Sm.) Sacc. and U34578 of *Gibberella zeae* (Schwein.) Petch, and morphologically this species was identified as the anamorph of *G. zeae*, *F. graminearum* Schwabe. Two species identified as *F. oxysporum* Schlechtend:Fr. (species 1 and 2) based on microscopic characters on carnation agar had differing RFLPs but identical ITS1 sequences, which proved closest to those known for *F. oxysporum*. However, the ITS2 sequences of these two *F. oxysporum* phenotypes differed, as has been noted for other isolates of *F. oxysporum* (26). When the entire ITS sequences were used in BLAST searches, isolate P179 (*F. oxysporum* 1) proved closest (455 of 456 bp) to *F. oxysporum* (U34571, U34566, and X93902), and isolate P405 (*F. oxysporum* 2) proved closest (469 of 470 bp) to an unidentified species of *Fusarium* (U34567). A fourth species, represented by isolate P323, was morphologically identified as *F. acuminatum* Ellis & Everh. but had an ITS sequence that matched closest (474 of 475 bp) to *F. sambucinum* Fuckel (U38279). A fifth species was identified as *F. solani* (Mart.) Sacc. based on conidiophore and conidium morphology, and isolate P185 had an ITS sequence nearest to *Neocosmospora vasinfecta* E. F. Smith

(L36627, 468 of 478 bp) and *Nectria haematococca* (Berk. & Broome) mating population VI (L36620, 464 of 478 bp) (27).

Three other isolated species were easily distinguished from *Phialophora gregata* and other soybean isolates by pigmented mycelium or pigmented spores, and each of these three species had unique ITS-RFLP patterns (Table 1, Fig. 1). One RFLP pattern was particularly common, and its ITS sequence (isolate P167) matched precisely to accession U97658 (492 of 492 bp matching) of *Phomopsis longicolla* T. W. Hobbs (35). Several morphological variants were observed among isolates with this RFLP pattern, and these likely represent more than one species in the *Diaporthe phaseolorum/Phomopsis longicolla* complex (13). Many of the isolates, including isolates P167 and P172, morphologically matched *P. longicolla* according to the criteria of Hobbs et al. (20). Isolate P196 of the *Cladosporium* sp. had an ITS sequence that matched most closely (459 of 463 bp) to that of *C. tenuissimum* (Y15966), and it morphologically matched Ellis' (11) description of *C. tenuissimum*. It also matched morphologically to the description of *C. cladosporioides* (Fres.) de Vries given by Carris and Glawe (4) but not to the *C. cladosporioides* description by Domsch et al. (9), and the ITS sequence of our *Cladosporium* sp. was closer to that of *C. tenuissimum* than it was to the ITS sequence of *C. cladosporioides* (L25429; 7). Isolates of the third pigmented species formed microsclerotia typical for *Macrophomina phaseolina* Petr., and isolate P207 had an ITS sequence closest (484 of 495 bp) to *M. phaseolina* (U97333).

**Isolation frequencies.** *Phomopsis longicolla* was the most commonly isolated fungus from soybean stems in both 1995 and 1996 (Table 2). However, it was isolated more frequently from stems with little or no internal discoloration than from stems with the brown discoloration typical for brown stem rot. In contrast, *Phialophora gregata*, the second most frequently

isolated fungus, was isolated more than three times as frequently from discolored stems than from non-discolored stems. Also, the weather was more conducive (cooler mid-season) and brown stem rot more prevalent in 1996 than in 1995 (F. Workneh and X. B. Yang, unpublished), and *P. gregata* was isolated much more frequently in 1996 than in 1995 (Tables 2 and 3). *Macrophomina phaseolina* was isolated more frequently in 1995 than in 1996 (Table 2) and was isolated more frequently from samples collected in more southerly sites (Missouri, Ohio, and Illinois) than from more northerly sites (Iowa and Minnesota). *Plectosphaerella cucumerina*, the five *Fusarium* spp., *Cladosporium tenuissimum*, *Gliocladium roseum*, and isolates that could not be identified were each isolated from fewer than 10% of the discolored stems (Table 2).

*Phialophora gregata* was isolated from 50% of the fields sampled in Iowa in 1995 and from 84% of the Iowa fields in 1996, and similar frequencies were found for Minnesota and Illinois fields (Table 3). Fewer of the fields in Ohio and Missouri yielded *P. gregata*. In 1996, when brown stem rot was particularly prevalent, 77% of the discolored stems from Iowa yielded *P. gregata*, but only 33 and 26% of the discolored stems from Ohio and Missouri, respectively, yielded *P. gregata* (Table 3).

**Pathogenicity tests.** When soybean plants were inoculated with representatives of nine species of fungi isolated from soybean stems, only the seven tested *P. gregata* isolates produced the foliar and vascular symptoms of brown stem rot. In both experiments, none of the soybean plants died after 5 weeks, but all of the *P. gregata*-inoculated plants showed vascular discoloration and leaves with chlorosis and necrosis. Isolations on PGM from the inoculated stems yielded the inoculated fungal species 5 weeks after inoculation in all cases, showing that viable inoculum of each of the species was placed into the vascular system.

**Table 3.** Isolation of *Phialophora gregata* from non-discolored and discolored soybean stems in 1995 and 1996 from states of the North Central region

State, year	Fields		Non-discolored stems		Discolored stems	
	Fields sampled (no.)	<i>P. gregata</i> (%)	Stems sampled (no.)	<i>P. gregata</i> (%)	Stems sampled (no.)	<i>P. gregata</i> (%)
Iowa						
1995	48	50.0	96	14.6	90	35.6
1996	50	84.0	96	22.9	97	77.3
Illinois						
1995	20	25.0	40	0	40	15.0
1996	25	84.0	50	10	49	69.4
Minnesota						
1995	20	45.0	40	7.5	40	32.5
1996	25	84.0	49	18.4	49	63.3
Ohio						
1995	20	35.0	40	5.0	40	27.5
1996	25	52.0	47	2.1	49	32.7
Missouri						
1995	20	15.0	40	2.5	40	12.5
1996	25	32.0	46	4.3	46	26.1

The ANOVA for data on percent leaflets chlorotic or necrotic on cv. Kenwood showed significant variation among the isolates ( $P = 0.0001$ ), between the two experiments ( $P = 0.0007$ ), and a significant isolate  $\times$  experiment interaction ( $P = 0.0001$ ). We found significantly less symptom development in the *P. gregata*-inoculated plants during the second experiment, apparently due to a brief period of elevated temperature in the growth chamber. A comparison of mean values using Duncan's multiple range test showed that the *P. gregata* isolates caused the greatest leaflet symptoms; the percentage of chlorotic or necrotic leaflets with inoculations with *Plectosphaerella cucumerina*, *M. phaseolina*, *C. tenuissimum*, *F. oxysporum* 1, and *G. roseum* did not differ from that with control inoculations; and inoculations with *F. oxysporum* 2, *F. solani*, and *Phomopsis longicolla* produced an intermediate level of leaflet chlorosis or necrosis (Table 4).

The amount of stem discoloration varied also, with the ANOVA showing a significant variation among isolates ( $P = 0.0001$ ) and an isolate  $\times$  experiment interaction ( $P = 0.0055$ ). Only the *Phialophora gregata*-inoculated plants showed the linear extent of discoloration in the stem to be significantly greater than that found in the control inoculations (Table 4). The mean extent of vascular discoloration in the susceptible cv. Kenwood after inoculation with the seven *P. gregata* isolates ranged from 67 to 117 mm (Table 4).

In comparably inoculated plants of cv. BSR101, which carries resistance to brown stem rot, all plants showed symptoms of brown stem rot after inoculation with the *P. gregata* isolates. The mean extent of vascular discoloration in BSR101 of the seven *P. gregata* isolates ranged from 74 to 89 mm, and the mean percent of leaflets showing symptoms ranged from 30 to 52% (Table 4). The two-way ANOVA (Table 5) showed significant variation among iso-

lates, cultivars, and the two experiments, and significant cultivar  $\times$  isolate interactions for percent leaflets symptomatic. Also, ANOVA found significant variation in the linear extent of stem discoloration among isolates and between the two experiments, and there was significant experiment  $\times$  isolate interaction (Table 5). Although the cultivars did not differ overall in the extent of linear discoloration of the stem, there was significant isolate  $\times$  cultivar interaction. Isolations from the inoculated stems of both cvs. Kenwood and BSR101 yielded *P. gregata*.

## DISCUSSION

The use of rDNA sequences allowed unequivocal delimitation of fungal species associated with discolored stems of soybeans. *P. gregata* was isolated commonly from soybean stems showing brown discoloration of the vascular system. Of nine tested species of fungi from soybean stems, only the seven tested *P. gregata* isolates produced the vascular discoloration and leaf symptoms typical of brown stem rot. Symptoms were produced in both the susceptible (Kenwood) and the resistant (BSR101) soybean cultivars, with slightly less discoloration of stems and leaves in the more resistant cultivar. *P. gregata* was reisolated from the inoculated soybean plants, thus completing Koch's postulates.

*Phomopsis longicolla* (the *Diaporthe phaseolorum* complex) was also frequently isolated from soybean stems, but it was more frequently isolated from lightly or non-discolored stems than from discolored stems. Members of this species complex are very common on soybeans, colonizing the plants late in the season as they senesce (30), and our isolations were made from stems late in the season, generally after leaf senescence and sometimes after harvest. Aside from *P. longicolla* and *Phialophora gregata*, the other fungal species were isolated much less frequently, and there was generally little difference in the

frequency of isolation between discolored and non-discolored stems. Under our test conditions, none of these species could induce the vascular browning and leaf symptoms typical of brown stem rot (17). *Phomopsis longicolla* causes seed decay and may cause a stem blight (30), and *M. phaseolina* causes charcoal rot of stressed plants (32), but few symptoms were produced in our inoculations, perhaps because the inoculated seedlings were harvested only 5 weeks after inoculations. It should also be noted that these two species did not produce conidia on the agar medium used to harvest inoculum for our study, but the species did become established in the inoculated plants because they were successfully reisolated after 5 weeks.

*Fusarium* spp. have been associated with disease of soybean (8), but none of the *Fusarium* spp. encountered proved to be effective vascular wilt fungi in our inoculations. A form of *F. solani* with a unique ITS sequence (27) causes sudden death syndrome of soybean, but the sudden death syndrome pathogen does not grow on PGM and our isolates of *F. solani* had a different RFLP pattern and ITS sequence than the sudden death syndrome pathogen. *Plectosphaerella cucumerina* has been confused with the *F. solani* associated with sudden death syndrome of soybean (1,27) and has been isolated from cysts of the soybean cyst nematode (4). It has been isolated from many plant species (28) and may be a weak pathogen on some (6), though it does not appear to be a pathogen on soybean.

In Iowa, Illinois, and Minnesota, 84% of the randomly sampled soybean fields and more than 63% of the discolored stems yielded *Phialophora gregata* in 1996, a year with a high incidence of brown stem rot (F. Workneh and X. B. Yang, unpublished). Incidences of *P. gregata* were lower in Ohio and Missouri, especially in 1995, a relatively warm growing season. Only 3 of the 20 sampled fields from Mis-

**Table 4.** Percent leaflets symptomatic and linear extent of vascular discoloration in inoculated stems of the soybean cvs. Kenwood and BSR101<sup>2</sup>

Species	Isolate no.	Kenwood		BSR101	
		Leaflets (%)	Discoloration (mm)	Leaflets (%)	Discoloration (mm)
<i>Phialophora gregata</i>	P19	59 a	98 a	37 b	88 a
<i>P. gregata</i>	P113	52 ab	97 a	36 b	81 a
<i>P. gregata</i>	P129	51 ab	92 a	39 b	74 a
<i>P. gregata</i>	P126	46 b	117 a	38 b	89 a
<i>P. gregata</i>	P21	46 b	96 a	52 a	81 a
<i>P. gregata</i>	P115	27 c	90 a	30 b	87 a
<i>P. gregata</i>	P109	26 c	67 b	32 b	83 a
<i>Fusarium oxysporum</i> type 2	P175	12 d	10 cd	ND	ND
<i>Fusarium oxysporum</i> type 1	P188	5 def	8 cd	ND	ND
<i>Fusarium solani</i>	P185	9 de	3 cd	ND	ND
<i>Phomopsis longicolla</i>	P172	10 de	0 d	ND	ND
<i>Cladosporium tenuissimum</i>	P199	5 def	0 d	ND	ND
<i>Gliocladium roseum</i>	P191	2 ef	7 cd	ND	ND
<i>Macrophomina phaseolina</i>	P203	1 f	0 d	ND	ND
<i>Plectosphaerella cucumerina</i>	P156	0 f	0 d	ND	ND
<i>P. cucumerina</i>	P161	0 f	0 d	ND	ND
Control	...	0 f	0 d	ND	ND

<sup>2</sup> Means within a column followed by the same letter not significantly different ( $P = 0.05$ ) based on Duncan's Multiple Range Test. ND = not determined.

souri yielded *P. gregata* in 1995. The lower incidence of the fungus from the more southerly locations, and the lower incidence in 1995 compared to 1996, may be due to the low temperature requirements of *P. gregata* (23). Nonetheless, the recovery of *P. gregata* was generally high, and brown stem rot appears to be one of the more common of the major diseases of soybean in the upper Midwest (10).

In this study, we relied heavily on the restriction fragments of the ITS region and on ITS sequences to classify and tentatively identify isolates, and the *Hae*III-ITS patterns proved to be an objective criterion for identifying *P. gregata* and similar species from discolored soybean stems. Because the ITS region could be amplified directly from scrapes of mycelium on the isolation medium, and because the *Hae*III restrictions could be done in the PCR reaction buffer, it was relatively easy to determine the RFLP pattern of 50 isolates in a single day. The ITS product was readily obtained with most of the species, although *Phomopsis longicolla* proved more difficult than the rest. The taxonomically difficult genus *Fusarium* was relatively easy to characterize by ITS-RFLPs, but the ITS sequences were only partially successful in identifying these species. Other fungi could be identified to species, or at least closely related species were suggested, through ITS sequences. *Phialophora gregata* and *Plectosphaerella cucumerina*, the most difficult species to distinguish based on morphology, were readily amenable to ITS amplification from mycelial scrapes. These two species commonly grow together in mixed culture and, when ITS amplification was attempted from scrapes of such mixed mycelia, the ITS product of *P. cucumerina* was most frequently produced.

With our isolation and identification techniques, and the subsequent patho-

genicity tests, we were able to clarify that only *Phialophora gregata* is capable of causing brown stem rot. The earlier association of other *Acremonium*-like fungi with brown stem rot (5,18,23) may be due to the presence of *Plectosphaerella cucumerina*, *Gliocladium roseum*, or other *Acremonium*-type fungi not encountered in our study. Neither *P. cucumerina* nor *G. roseum* were capable of causing brown stem rot in our inoculations, though an unidentified *Acremonium* sp. did cause vascular discoloration in an earlier study (23). The conflicting reports (16,23,25,33) of defoliating and non-defoliating forms of *Phialophora gregata* on soybean are difficult to reconcile without examination of the so-called type II (non-defoliating) isolates, the strains that induced vascular discoloration but not foliar symptoms in inoculation tests (16,23,33). It is possible that some reports of the non-defoliating form are actually *Plectosphaerella cucumerina*, and type II colonies illustrated by Mengistu et al. (25) look like those of *P. cucumerina*. We have inadvertently inoculated soybeans with mixed cultures of *P. cucumerina* and *Phialophora gregata* and found erratic production of foliar symptoms with limited vascular discoloration. Alternatively, the non-defoliating isolates may represent a less aggressive form of *P. gregata*.

The *P. gregata* *Hae*III-ITS pattern was diagnostic for the species because it was not found in any of the other fungal species isolated from discolored stems of soybean. We sequenced the ITS region of eight isolates of *P. gregata* and found the sequences to be identical, and the unique *Hae*III digestion pattern was found with the more than 300 isolates tested to date. However, we did see variation in aggressiveness among the seven tested isolates of *P. gregata* on both cvs. Kenwood and BSR101, and significant isolate × cultivar interac-

tion in leaflet symptoms was seen, with isolates P109 and P115 causing significantly less leaflet discoloration on Kenwood than did the other isolates of *P. gregata*. However, each of the seedlings inoculated with these isolates of *P. gregata* caused some foliar symptoms, and none could be considered type II isolates sensu Gray (16), Mengistu and Grau (23), or Willmot et al. (33). Because of such ambiguity, we propose that the terms “non-defoliating” and “type II” not be applied to *P. gregata*. Variation in aggressiveness among *P. gregata* strains is under investigation, and preliminary work shows that variation in the degree of symptoms caused by Midwestern strains of *P. gregata* is high, as has been found with the adzuki bean pathogen, *P. gregata* f. sp. *adzukicola* (22), but all soybean isolates of *P. gregata* appear capable of causing foliar symptoms, even in the resistant cv. BSR101.

#### ACKNOWLEDGMENTS

We thank G. Munkvold for his help in identifying the *Fusarium* spp. and reviewing the manuscript, J. Mihail for identifying *Macrophomina phaseolina* isolates, M. Palm for providing isolates of *Plectosphaerella cucumerina*, and C. Grau for supplying isolates of *Phialophora gregata* and *Plectosphaerella cucumerina* from Wisconsin for comparisons.

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**Table 5.** Two-way analysis of variance of percent leaflets symptomatic and linear extent of xylem discoloration in soybean plants (cvs. Kenwood and BSR101) inoculated with seven isolates of *Phialophora gregata*

Symptom, source of variation	df <sup>2</sup>	Mean square	F value	P > F
Leaflets symptomatic				
Isolates	6	1,767.1	10.0	0.0001
Cultivars	1	1,552.1	8.78	0.0036
Cultivar × isolate	6	779.4	4.41	0.0004
Experiments	1	13,945.6	78.89	0.0001
Isolate × experiment	6	92.0	0.52	0.7922
Cultivar × experiment	1	539.1	3.05	0.0831
Cultivar × isolate × experiment	6	138.4	0.78	0.5847
Replicates (experiment)	10	199.8	1.13	0.3447
Vascular discoloration				
Isolates	6	33,009.1	2.59	0.0208
Cultivars	1	9,143.8	0.72	0.3981
Cultivar × isolate	6	32,667.7	2.57	0.0220
Experiments	1	263,557.3	20.72	0.0001
Isolate × experiment	6	42,421.4	3.33	0.0044
Cultivar × experiment	1	28.6	0.0	0.9622
Cultivar × isolate × experiment	6	22,797.8	1.79	0.1056
Replicates (experiment)	10	7,715.3	0.61	0.8061

<sup>2</sup> Degree of freedom.

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