Variation in Morphological, Cultural, and Pathological Characteristics of *Phialophora gregata* and *Acremonium* sp. Recovered from Soybean in Wisconsin

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ABSTRACT

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Isolates of *Phialophora gregata* recovered from soybean stems were classified into two pathotypes based on their ability (type I) or inability (type II) to cause chlorosis, necrosis, and wilt of foliage. Both pathotypes caused a similar degree of internal stem discoloration, but type I isolates caused a greater amount of plant height reduction. Type I isolates constituted 72% of all *P. gregata* isolates evaluated for pathogenicity. *Acremonium* was isolated at a lower frequency than *P. gregata*. *Acremonium* isolates caused low to moderate degrees of vascular and pith discoloration but caused no foliar symptoms and only a slight reduction in plant height. Maximum growth rate was measured at 20 and 24–28 C for *P. gregata* and *Acremonium*, respectively. Isolates of *P. gregata*, unlike *Acremonium*, did not sporulate on acidified potato-dextrose agar but did sporulate on green bean agar. Electrophoretic studies revealed isozyme differences between *P. gregata* and *Acremonium*. *Fusarium oxysporum* and *Verticillium dahliae* were not isolated from field-grown soybean plants that showed vascular discoloration or foliar symptoms.

Brown stem rot of soybean (*Glycine max* (L.) Merrill) is a prominent disease in North America (1–3,26). Variation within *Phialophora gregata* (Allington & Chamberlain) Gams (9), the cause of brown stem rot, has been studied for factors that could conceivably influence symptom expression and soybean productivity. Phillips (20) evaluated isolates from Illinois, Iowa, North Carolina, and Georgia and found no difference in their ability to cause discoloration of vascular and pith tissues, and no isolates caused foliar symptoms. In contrast, Gray (10) identified two forms of the pathogen; all isolates caused browning of internal stem tissues, but isolates differed in their ability to cause foliar symptoms. Type I isolates caused chlorosis, necrosis, and wilt of foliage, but type II isolates caused no foliar symptoms. Gray (11) showed the importance of foliar symptoms; Wayne soybeans, inoculated with type I isolates, yielded 38% less seed than plants inoculated with type II isolates.

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Discoloration of vascular and pith tissues is a commonly accepted and characteristic symptom of brown stem rot and is influenced by several factors (4,5,12,14,16,18–20,22,28,30). However, less agreement is associated with the occurrence and significance of foliar symptoms associated with the disease. Allington and Chamberlain (2) described foliar symptoms in their first report, yet many authors do not mention foliar symptoms and only refer to internal stem symptoms. Foliar symptoms are influenced by host genotype (14), air temperatures (12), plant age (12), soil moisture (14), and pathogen variability (10,11). The use of foliar symptoms alone and as a supplement to stem symptoms can be used to differentiate between soybean genotypes resistant or to *P. gregata* (24,25). However, pathotypes of *P. gregata* used in artificial inoculations and present in naturally occurring populations in disease nurseries must be characterized to better predict expected symptom expression.

The cause of vascular and pith discoloration must be ascertained for the purposes of diagnosis and development of brown stem rot-resistant cultivars. Although *P. gregata* is considered the cause of brown stem rot, fungi such as *Acremonium* sp. (14), *Verticillium dahliae* (27), *Fusarium oxysporum* (8), and *Stilbella* sp. (9,15) can be isolated from soybean plants showing discoloration of internal stem tissues. Misidentification is unlikely between *P. gregata* and *F. oxysporum* or *V. dahliae* but is conceivable between *P. gregata* and *Acremonium* sp. or *Stilbella* sp.

The purposes of this study were to determine the types and frequencies of fungi recovered from soybean stems showing discoloration of vascular and pith tissues in Wisconsin and to determine the variability in virulence based on expression of foliar symptoms for isolates of *P. gregata* and other fungi recovered from soybean stems.

MATERIALS AND METHODS

Diseased soybean plants showing stem and foliar symptoms typical of brown stem rot were collected from soybean fields in seven Wisconsin counties: Waushara, Iowa, Dane, Green, Rock, Jefferson, and Racine. Soybean plants were sampled at growth stages R5–R7 (7), and 30–120 plants were collected per field. Two pieces each of stem and root tissue (2 × 5 mm) were excised from each plant about 5 cm above and below the soil line. Excised stem and root tissues were immersed in 0.25% NaOCl for 60 sec, blotted, plated on acidified potato-dextrose agar (APDA), and incubated for 10 days at 20 C.

Isolates were cultured in green bean broth (GBB) for taxonomic studies and inoculum production. Ninety-eight grams of strained green bean baby food (Gerber) was added to 1 L of distilled water and strained through three layers of cheesecloth to remove fibrous materials. The medium was dispensed into 50-ml Erlenmeyer flasks and autoclaved for 25 min. Flasks were seeded with mycelial plugs cut from colony margins. Isolates were incubated at 22 C for 10 days on a rotary shaker at 70 rpm.

An additional group of isolates was obtained from soybean stems collected at the R5–R7 growth stages from 10 locations in southern Wisconsin (Dane and Rock counties). Isolates were recovered and handled in a manner like that described above except isolates were from stems only and were grown on GBB agar. This group of isolates was used in temperature-pathogenicity studies.

Cultural characteristics and morphology of isolates. Thirty monoconidial *Phialophora-Acremonium*-like isolates were selected and examined for morphol-

ogic and cultural characteristics. Each isolate was examined for sporulation, dimensions of conidia, and presence or absence of distinct swollen phialidic and collarate formations when cultured in GBB or on APDA. Radial growth on APDA at 12, 16, 24, 28 and 32 C was measured after 10 days of incubation.

Isozyme patterns of isolates evaluated. The 30 isolates were incubated in GBB for 8 days at 22 C in preparation for electrophoretic studies. Cultures were vacuum-filtered with Whatman No. 1 filter paper. A small portion of mycelium retained on the filter paper was removed and placed in a precooled 25-ml vial. Mycelium was homogenized with a Con-Torque power grinder (Eberbach Corporation). The procedures for the extraction, electrophoresis, and staining of isolates were as described by O'Malley et al (17). Seven isozymes assayed in this study were buffered in aminocitrate (pH 6.1) or tris-borate EDTA (pH 8.0) and replicated twice. The position of isozyme bands in each acrylamide gel was measured and a ratio was calculated for the distance an isozyme band moved to the distance moved by a bromphenol dye reference front. This ratio was designated as the R^f value (21). The relative color intensity of isozyme bands in a gel was visually graded as no color, light, or dark.

Variation in pathogenicity. Plants of Hodgson 78, a susceptible group I maturity soybean cultivar, were grown in tapered pots 4 × 20 cm containing a mixture of soil, peat, and sand (1:1:1). The tapered pots were placed in trays that were set in plastic containers (22 × 9 × 30 cm) half-filled with tap water to maintain a soil water potential in the range of 0 to 20 centibars. Soybean seedlings were grown under a 13-hr photoperiod (light intensity ranging from 320 to 430 μE m^{-2} s^{-1}) at 24–26 C during the day and 22 C at night. Thirty *Phialophora-Acremonium*-like isolates were cultured in GBB to produce inoculum for pathogenicity tests. Cultures were vacuum-filtered through Whatman No. 1 filter paper to separate mycelium and conidia. Inoculum was standardized to 3×10^7 conidia per milliliter. Each isolate was replicated three times with seven plants inoculated per replicate. Twelve-day-old plants were inoculated by pipetting 8 ml of inoculum into the periphery of the taproot 3 cm below the soil line. Control plants received filtrate of GBB alone. Plants were incubated in the greenhouse as described previously.

Four weeks after inoculation, plants were assessed for 1) severity of foliar symptoms based on the Horsfall-Barrett scale of 0–11, where 0 = no foliar symptoms present, 1 = 0–3%, 2 = 3–6%, 3 = 6–12%, 4 = 12–25%, 5 = 25–50%, 6 = 50–75%, 7 = 75–87%, 8 = 87–94%, 9 = 94–97%, 10 = 97–100%, and 11 = 100% of the leaves necrotic; 2) proportion of internal stem discoloration to total plant height (both pith and vascular tissues included in measurement of internal stem symptoms); and 3) plant height. These measurements were used to determine the relative virulence of each isolate.

Effect of temperature on virulence of *P. gregata*. Forty-six mass isolates of *P. gregata* were grown on GBB for 2 wk at 20 C. Conidia were harvested by washing the colony surface with sterile distilled water. Inoculum concentration was adjusted to 1×10^6 conidia per milliliter for each isolate. Three 2-wk-old-seedlings of Chippewa 64 soybean were inoculated by injecting 0.1 ml of inoculum suspension into stems at the soil line with a syringe and 20-gauge needle. Uninoculated plants were injected with sterile distilled water. Plants were incubated for 4 wk in greenhouse environments of 20 and 28 C, and each group of plants was supplemented with 16 hr of fluorescent illumination (light intensity of 320–430 μE m^{-2} s^{-1}).

Plants were evaluated for extent of vascular and pith discoloration of internal stem tissues and severity of foliar symptoms. The following disease severity classes were used to measure the virulence of each isolate: 0 = no symptoms; 1 = discoloration of vascular and pith tissues only; 2 = discoloration of vascular and pith tissue and chlorosis of lower leaves; 3 = discoloration of vascular and pith tissues and chlorosis, necrosis, and wilt of lower leaves; 4 = discoloration of vascular and pith tissues, chlorosis, necrosis, and wilt of lower and midplant leaves; and 5 = vascular discoloration, wilt, and necrosis of all leaves. Severity of foliar symptoms was evaluated first, then stems were split longitudinally and examined for discoloration of pith and vascular tissues.

RESULTS

Cultural characteristics and morphology of isolates. Based on cultural and morphological characteristics, isolates were classified into two major groups (Table 1, Fig. 1). The mycelium of the first group ranged from white to dark brown, with light tan being most common. Colony surfaces were raised and either rough or smooth. Conidia were absent on APDA but were abundant in GBB and were hyaline, and mean width and length ranged 2.1–3.3 and 3.0–5.1 μm, respectively. This group of isolates was further characterized by conidiophores that possessed swollen, thick-walled, phialidic structures. Phialides were barrel-shaped, and each had a distinct collarate formation. These isolates were identified as *P. gregata* based on the criteria described by Gams (9). The second group of isolates had light gray mycelia and an appressed and slimy mycelial surface. Some isolates had white tufts of twisted aerial hyphae. Isolates of the second group sporulated abundantly on both APDA and GBB. Conidia also were hyaline and measured 1.5–2.8 μm wide and 2.1–3.9 μm long. Conidiophores were slender and morphologically inseparable from the phialidic structures. Phialidic walls were slightly thicker than walls of vegetative hyphae. These isolates were identified as belonging to the genus *Acremonium* but were not speciated.

Frequency of isolation. *P. gregata* was readily isolated from soybean plants collected from seven fields at a frequency of 90 and 66% from stems and roots, respectively. *Acremonium* sp. was isolated from stems and roots at frequencies of 3 and 2%, respectively. *F. oxysporum* and *V. dahliae* were not isolated from soybean plants that showed

Table 1. Taxonomic characteristics and virulence of isolates of *Phialophora gregata* and *Acremonium* sp. recovered from soybean plants showing symptoms of brown stem rot

Fungus[a]	No. of isolates	Conidial size (μm) $\bar{X}$ length	Range	Standard error	$\bar{X}$ width	Range	Standard error	Foliar symptom severity[b] (%) $\bar{X}$	Range	Internal stem discoloration (%) $\bar{X}$	Range	Plant height[c] (cm)
P. gregata	4	4.0	2.7–5.8	0.11	2.6	1.7–3.4	0.08	87	70–98	80	56–100	57.8
	8	3.9	3.1–6.8	0.13	2.8	2.1–3.6	0.08	36	20–50	52	16–79	62.6
	5	4.0	3.0–7.1	0.13	2.7	2.1–3.6	0.07	10	7–15	46	24–75	62.7
	7	3.9	2.8–6.6	0.13	2.8	2.1–3.4	0.06	0	0–0	50	48–83	76.0
Acremonium sp.	5	2.9	2.3–3.5	0.05	2.0	1.5–2.3	0.04	0	0–0	21	7–38	94.1

[a] Isolates obtained from plants sampled from Waushara, Dane, Iowa, Green, Rock, Jefferson, and Racine counties and identified as *P. gregata* or *Acremonium* sp.

[b] Severity was expressed as a percentage of foliar tissue that showed chlorosis and necrosis ($LSD_{0.05}$ = 15.5) and the height of internal stem discoloration expressed as a percentage of the total stem length ($LSD_{0.05}$ = 16).

[c] Height of uninoculated plants was 103.7 cm ($LSD_{0.05}$ = 14.6).

internal stem discoloration and foliar symptoms characteristic of brown stem rot.

Isozyme patterns of isolates. Isozyme patterns of the 30 isolates examined in this study are presented in Table 2. The electrophoretic patterns (R^f values) and intensity of three of the seven enzymes evaluated (phosphoglucose isomerase, phosphogluconic dehydrogenase, and malate dehydrogenase) separated the isolates into two major groupings. Twenty-five of the 30 isolates had the same R^f value for each of the three enzymes but different from the remaining five isolates. The 25 isolates with similar bands and R^f values were previously identified as *P. gregata*, whereas the other five isolates were identified as *Acremonium* spp. according to criteria proposed by Gams (9). Migration of bands and color intensity was greater for 6-phosphogluconic dehydrogenase and phosphoglucose isomerase than for malate dehydrogenase. There was no difference for band intensity between the two buffers.

Variation in virulence among isolates. Criteria for evaluation of virulence were based on the severity of foliar symptoms, percent internal stem discoloration, and reduction in plant height. Results from pathogenicity tests (Table 1) provided the basis to divide the 25 isolates of *P. gregata* into two pathotypes as previously proposed by Gray (10). Eighteen (type I) of 25 isolates of *P. gregata* caused foliar symptoms and discoloration of vascular and pith tissues, and seven isolates (type II) only caused internal stem discoloration (Table 1). Type I isolates of *P. gregata* differed in their ability to cause foliar symptoms. Isolates of *P. gregata* can be classified into two pathotypes primarily based on the severity of foliar symptoms but supplemented by their effects on plant height. Type I isolates, as a group, reduced plant height more than type II isolates and consequently were considered more virulent. There were significant relationships between plant height and severity of foliar symptoms ($R^2 = 0.26$, $P = 0.003$) and extent of internal stem discoloration ($R^2 = 0.34$, $P = 0.0006$). In addition, the severity of foliar symptoms increased as the height of internal stem discoloration increased ($R^2 = 0.34$, $P = 0.0007$). Plant height was reduced more by isolates that caused both severe stem and foliar symptoms. *Acremonium* caused limited discoloration of vascular and pith tissues and no foliar symptoms and did not reduce plant height. *Acremonium* was recovered from soybean stems, but isolates were relatively poor pathogens of soybean.

Effect of temperature on virulence of *P. gregata*. Temperature influenced disease severity caused by isolates of *P. gregata* and thus influenced the designation of isolate types. Thirty-one of the 46 isolates evaluated caused greater severity of foliar symptoms when incubated at 20 than at 28 C (Fig. 2). Incubation at 28 C resulted in an aggregation of isolates in the middle severity classes (2 and 3), and only 13% of the isolates fell in classes 4 and 5. In contrast, 59% of the isolates caused severe foliar symptoms (classes 4 and 5) when incubated at 20 C. In general, *P. gregata* caused greater severity of foliar symptoms at lower air temperatures, but six of 46 isolates did cause greater disease severity, and nine caused equal severity at 28 C compared with their reactions at 20 C. Overall mean disease severities were 3.65 and 2.52 for isolates evaluated at 20 and 28 C, respectively. Based on criteria proposed by Gray (10), 36 of 46 isolates were classified as type I (classes 3–5) when incubated at 20 C, and 24 of 46 when incubated at 28 C. The height of vascular discoloration was not significantly different among isolates and was not influenced by air temperature.

Influence of temperature on radial

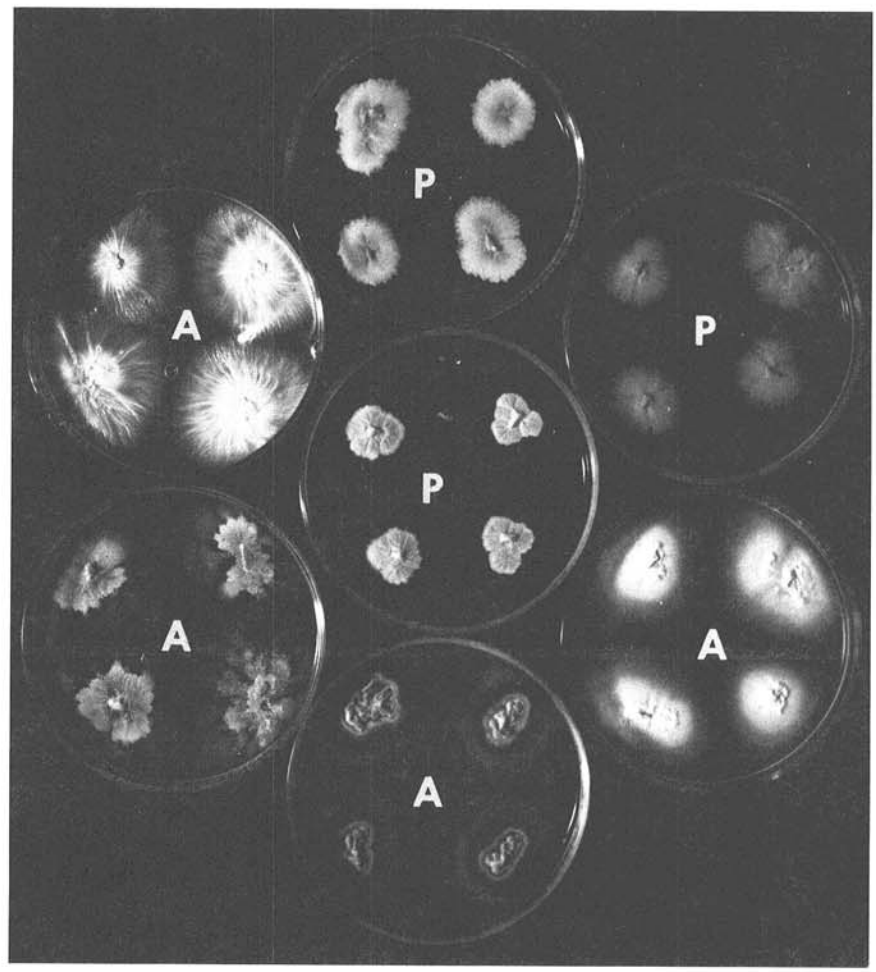

Fig. 1. Comparison of colony types of *Phialophora gregata* (P) and *Acremonium* (A) isolated from soybean stems.

Table 2. List of enzymes with the Enzyme Commission (EC) numbers and electrophoretic R^f values measured for studies on isolates of *Phialophora gregata* (Pg) and *Acremonium* sp. recovered from soybean stems[a]

Enzyme	EC number	Mean R^f value[b]		
		Pg Type I	Pg Type II	Acremonium
Aconitase	4.2.1.3	0.00	0.00	0.00
Esterase	3.1.1.1	0.00	0.00	0.00
Fumarase	4.2.1.2	0.00	0.00	0.00
Lactate dehydrogenase	1.1.1.27	0.00	0.00	0.00
Malate dehydrogenase	1.1.1.37	0.30	0.30	0.20
6-Phosphogluconate dehydrogenase	1.1.1.44	0.40	0.40	0.30
Phosphoglucose isomerase	5.3.1.9	0.52	0.54	0.30

[a] Twenty isolates of *P. gregata* and five isolates of *Acremonium* sp. were used.
[b] Buffers used were A = aminocitrate, pH 6.1, and B = tris-borate EDTA, pH 8.0.

growth of *P. gregata* and *Acremonium* sp. A marked difference in radial growth was observed within isolates of *P. gregata* and between isolates of *P. gregata* and *Acremonium* spp. (Fig. 3). Radial growth of *Acremonium* isolates was greater than growth of isolates of *P. gregata* at all temperatures tested, but differences were even more magnified at temperatures higher than 20 C. Radial growth of *P. gregata* was not dramatically different between 12 and 24 C but was less at temperatures higher than 20 C and ceased at 32 C. Mean radial growth of *Acremonium* isolates was 27 mm at 24–28 C and slightly less at 32 C. The optimum temperature for radial growth of *P. gregata* was 20 C. Radial growth of type I isolates was also greater than growth of type II isolates when incubated at or below 20 C. However, there was no significant difference in radial growth between pathotypes above 24 C.

DISCUSSION

P. gregata was the predominant fungus isolated from soybean plants showing discoloration of internal stem tissues and foliar symptoms of wilt, interveinal chlorosis, and necrosis—all characteristic of brown stem rot (2). *Acremonium* sp. was consistently isolated but at a much lower frequency than *P. gregata*. The classification of the two vascular inhabitants, *P. gregata* and *Acremonium* spp., was based on morphological characteristics described by Gams (9), and classical taxonomic criteria were verified by electrophoretic techniques (17,21) that identified isozyme bands that were different for each genus. Conidial size alone could not be used to distinguish *P. gregata* from *Acremonium*. Although conidia of *P. gregata* were slightly larger, there was an overlap in conidial size of *P. gregata* and *Acremonium* sp. *P. gregata* was readily distinguished from *Acremonium* by its characteristic bottle-shaped phialides and collarate formations. In addition, *P. gregata* grew very slowly and sporulation was very sparse on APDA in contrast to the rapid growth and extensive sporulation of *Acremonium* on APDA. Allington and Chamberlain (2) also reported minimal sporulation of *P. gregata* on PDA. We encourage researchers to carefully examine isolates identified as *P. gregata* that sporulate profusely on PDA. Sporulating and nonsporulating isolates of *P. gregata* have been reported (13,20,23), but confusion between *Phialophora* and *Acremonium* could lead to this conclusion. We are concerned about the lack of published reports on the isolation of *Acremonium* from the vascular system of soybean (3,24). We commonly isolate *Acremonium* from soybean stems in combination with *P. gregata*. Unless single-spored, many *P. gregata* cultures are contaminated with *Acremonium*. The latter fungus often becomes dominant in time, especially if cultures are incubated above 25 C. Although *Acremonium* was readily recovered from stems and roots of soybean plants showing symptoms of brown stem rot, this inhabitant of the vascular system did not cause a significant pathological effect when evaluated in a controlled environment. Based on radial growth studies, this fungus has the potential to be more active than *P. gregata* at higher air temperatures. The relationship between *P. gregata* and *Acremonium* under different environments and host genotypes has not been investigated but could be an important area of research to enhance our understanding of brown stem rot. For example, can *Acremonium* suppress the pathological activity of *P. gregata*, especially at higher air temperatures? How do crop rotation and crop sequences influence the population and activity of *Acremonium* in relation to *P. gregata* (5)?

Variation in virulence among isolates of *P. gregata* was detected when the severity of foliar symptoms was used to classify *P. gregata* into type I or II isolates as proposed by Gray (10). Foliar

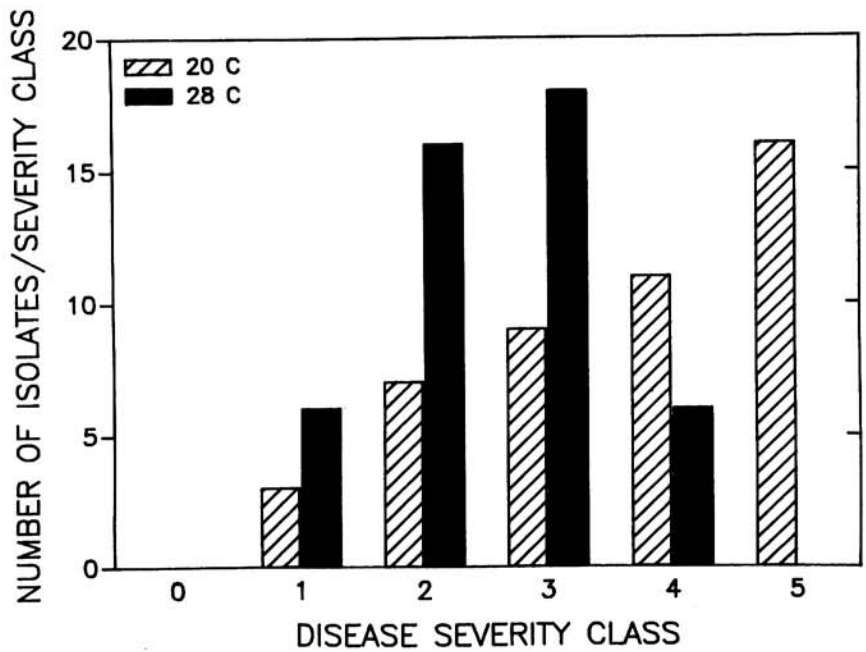

Fig. 2. Effect of temperature on virulence of isolates of *Phialophora gregata* on Chippewa 64 soybeans incubated at 20 and 28 C. Disease severity classes were as follows: 0 = no symptoms; 1 = vascular and pith discoloration only; 2 = vascular and pith discoloration and chlorosis of unifoliate leaves; 3 = vascular and pith discoloration, wilt, chlorosis, and necrosis of lower trifoliolate leaves; 4 = vascular and pith discoloration, wilt, chlorosis, and necrosis of lower and midplant leaves; and 5 = vascular and pith discoloration, wilt, chlorosis, and necrosis of all leaves.

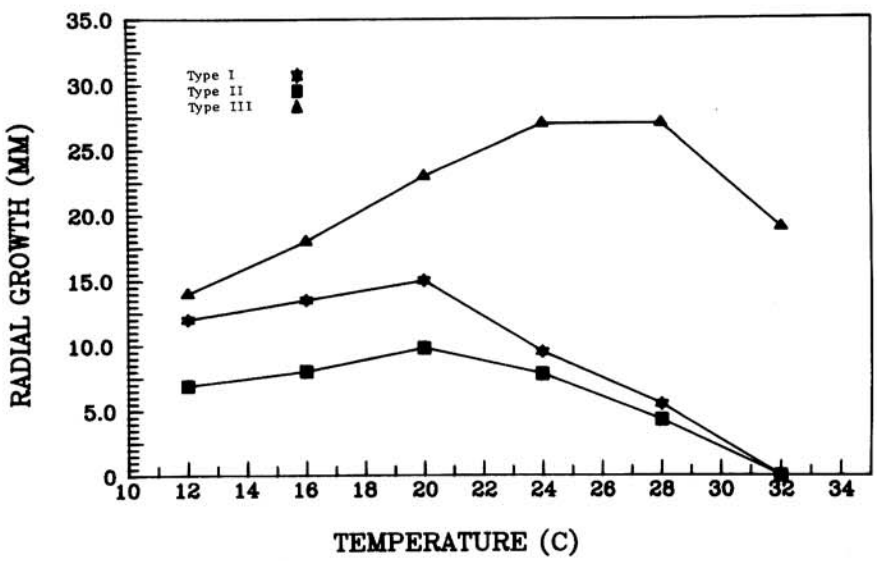

Fig. 3. Mean radial growth of 25 isolates of *Phialophora gregata* (types I and II) and five isolates of *Acremonium* sp. (type III) incubated on acidified potato-dextrose agar at six temperatures for 10 days.

symptoms also were important to distinguish isolates of *P. gregata* from *Acremonium* spp. The expression of foliar symptoms, compared with stem symptoms, was influenced by temperature, and subsequently, the classification of isolates was different at 20 and 28 C. Phillips (18) reported isolates of *P. gregata* caused no difference in severity of internal stem symptoms between 15 and 27 C. However, severity of stem symptoms caused by all isolates was less when plants were incubated above 30 C. Gray (12) reported small differences in severity of stem symptoms at 22 and 28 C but no differences in severity of leaf symptoms. Our data agree for stem symptoms but not for foliar symptoms. Other factors, such as soil moisture (14) and photoperiod (19), may be partial explanations for this contrast in results.

Sebastian et al (22,23) have successfully used the severity of foliar symptoms to identify soybean genotypes resistant to *P. gregata*. Heritability estimates for resistance to *P. gregata* were greater when foliar symptoms were used as a measure of disease rather than internal stem symptoms (25). However, the proper pathotype of *P. gregata* and incubation environment is needed if foliar symptoms are used to select plants resistant to brown stem rot. Internal stem symptoms have been used successfully in breeding for resistance to *P. gregata* (6,28–31). However, the use of foliar symptoms provides a nondestructive means for selecting resistant plants and evaluating family lines.

Results from this study suggest that significant variability for virulence exists within *P. gregata* and needs to be considered when selecting isolates for research on brown stem rot. Although variation in disease reactions of soybean genotypes is also influenced by environmental factors, future efforts in cultivar improvement for resistance to brown stem rot need to focus on the use of highly virulent isolates of *P. gregata*.

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