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ETIOLOGY OF CEPHALOSPORIUM GREGATUM IN SOYBEAN

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Etiology of Cephalosporium gregatum in Soybean

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ABSTRACT

Cephalosporium gregatum was isolated from taproots of soybeans within 7 weeks after planting in field plots infested with the pathogen. The fungus was detected in the tops (ninth node) of plants at the early pod-filling stage within 1 day after artificial inoculation of hypocotyls, and within 2 days after a fungal suspension was added to the nutrient solution in which the plants were growing. Conidia are appa⁻ tly the principal means of spread within the plant. In both greenhouse and field experiments, more stem browning developed in plants inoculated at 4-6 weeks than in those inoculated 8-12 weeks after planting. Infected plants exposed to low temperatures (18-24 C) for 4 weeks had significantly less stem browning than those exposed for 10 and 12 weeks.

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Additional key words: Glycine max, brown stem rot, symptom development.

Brown stem rot of soybean (*Glycine max* [L.] Merr.) caused by *Cephalosporium gregatum* Allington & Chamberlain, has been reported from the United States (1, 16), Canada (7), and Mexico (10). Internal stem browning usually begins at or below the soil line, and moves upward throughout the season (1). The fungus presumably enters the host through the roots, and spreads slowly through the stem (2). Weber et al. (16) postulated that, to significantly reduce yield, *C. gregatum* must be functioning in the plant before symptoms appear. Gray et al. (6) showed that plants with browning throughout the stem had significantly lower yields than those with up to 6 inches of browning.

There is a disagreement on the influence of temperature on brown stem rot development. Chamberlain & Allington (3) and Chamberlain & McAlister (4) reported that internal browning appeared within 3 weeks after inoculation at 15 C, whereas plants grown at 21 and 27 C had few or no symptoms. These results are somewhat correlated with the behavior of the pathogen in culture, with optimum temperatures for growth and sporulation between 15 and 20 C (2). Phillips (11), however, observed that the most extensive stem browning occurred at 27 C, and was not completely inhibited at 32 C. Kunkel (9) and Tachibana (15) reported that there is no relationship between temperature and internal stem browning.

The effects of physiological plant age on disease development also have been disputed. Chamberlain & McAlister (4) stated that internal browning progressed more rapidly in greenhouse and field plants beyond the pod-filling stage than in younger plants, irrespective of temperature. They attributed this to a complex of factors (4). Dunleavy (5) reported that the percentage of infected field grown plants of each of eight varieties with different maturity groups was not related to time of bloom or to plant maturity.

This report further elucidates the etiology of *C. gregatum* in soybean by showing: (i) time of root infection in the field; (ii) the speed with which the fungus spreads within the soybean plant; (iii) the relationship between plant age at time of inoculation and development of internal stem browning; and (iv) the effect of exposure time to low temperatures (18-24 C) on internal stem browning. A preliminary report of this work has been published (13).

MATERIALS AND METHODS.–An Illinois isolate of *C. gregatum*, used throughout this study, was maintained on soybean seed agar (SSA) or in liquid soybean seed extract (SSE) shake cultures at room temperature (23-25 C). SSE was prepared by

boiling 35 g soybean seed in 500 ml distilled water for 30 min. The broth was decanted, and the volume made to 1 liter with distilled water. Twenty g of agar were added to 1 liter of SSE to prepare SSA. Conidial suspensions were prepared by adding sterile distilled water to 2-week-old plate cultures of *C. gregatum*, and dislodging the conidia with a sterile dissecting needle. Suspensions were decanted through two layers of sterile cheesecloth and stored at 5 C. Inoculum was never stored for more than 4 hr. Conidial suspensions were centrifuged for 20 min at 1,100 *g*, the supernatant was discarded, and the centrifugant diluted to the desired concentration with sterile distilled water. Unless otherwise indicated, Beeson soybeans were used in all experiments conducted in a growth chamber at 24 C, 70% relative humidity, and 12-hr light of 8,000 ft-c.

Infection of taproot.–Clark-63 was seeded in a field plot known to be infested with *C. gregatum*, and which had been planted to soybeans for 2 successive years. Beginning when plants were 5 weeks old, 12 plants were removed randomly at weekly intervals, and the lateral roots were removed. Taproots were immersed in a 0.525% sodium hypochlorite solution for 10 sec, and split longitudinally. Small sections of vascular tissue were plated on SSA containing 100 ppm streptomycin and 100 ppm tetracycline. After 5 days at 21 C, plates were examined for *C. gregatum*.

Spread of C. gregatum.–Two experiments determined the pathogen's rate of spread through the stem. Surface-sterilized seed were germinated in sterile, moist vermiculite. After 1 week, two seedlings were transferred to each of 14 sterile 500-ml Erlenmeyer flasks containing ca. 550 ml sterilized, half-strength Hoagland's solution (8).

Each hypocotyl was wrapped with glass wool and inserted in a 9-mm hole in a No. 25 cork. Two additional holes permitted the insertion of an aerator into each flask, and provided a vent for escaping air. All flasks were wrapped with two layers of aluminum foil to exclude light. Flasks were filled with sterile distilled water and nutrient solution at 2-day and weekly intervals, respectively. Inoculum was prepared by shredding 3-week-old shake cultures of *C. gregatum* in a Waring Blendor for 5 sec. The resulting slurry was centrifuged for 10 min at 600 *g*, and 10 ml of the centrifugant were diluted to 25 ml with sterile distilled water. At early pod-filling stage (7 weeks), aerators were removed. Twenty-five-ml samples of either inoculum or sterile distilled water were added to each flask. Isolations of *C. gregatum* were attempted from the roots and from each node of four inoculated and one control plant for each of 7 consecutive days. All leaves, petioles, and lateral roots were removed, and each stem was immersed in 10% ethyl alcohol for 2 min and rinsed in sterile distilled water for 1 min. The taproot and each node of each plant were aseptically cut, and the internal tissues from each section were plated on individual culture plates containing acidified SSA (5 ml 25% lactic acid/liter media).

Resulting fungal colonies were examined after 7-10 days' incubation at room temperature (23 C), and the occurrence of *C. gregatum* was noted.

For the second experiment, two seeds were planted in each of several 16-oz Styrofoam cups containing sterile moist vermiculite. At weekly intervals, all cups were irrigated with Hoagland's solution (8). When in early pod-filling stage, 20 plants were removed from the vermiculite by cutting about 4 cm below the crown so that some roots remained. Hypocotyls and roots were immersed in 10% ethyl alcohol for 2 min, then in sterile distilled water. Each plant was cut at a 45-degree angle just above the crown, and the roots were discarded. The cut ends of hypocotyls from 16 plants were inoculated by immediately immersing them into 6-inch culture tubes containing 10 ml of a conidial suspension (ca. 25,000/ml water). Four additional plants were placed into tubes containing 10 ml sterile distilled water as a control. After inoculation, the cut stems were incubated at 24 C, 50% relative humidity, and 24-hr light of 8,000 ft-c. To promote water uptake, a 2-mm section was cut daily from the lower end of each hypocotyl with a sterile scalpel. Isolation attempts as previously described were made from each node of four inoculated and one control stem for each of 4 consecutive days.

Effect of plant age on symptom development.- Surface-sterilized seeds were germinated in sterile moist vermiculite in a greenhouse at 18-24 C. After 1 week, seedlings of uniform height were planted four/6-inch clay plot each containing about 1.5 kg of field soil autoclaved 3 hr at 121 C. Six weeks after seeding, and at three biweekly intervals thereafter, plants were inoculated by the basal stem inoculation method (BSI) using a 22-gauge needle and syringe. At each inoculation date, 40 plants were inoculated with ca. 0.15 ml of a 32,000 conidia/ml suspension. Eight plants were injected similarly, with sterile distilled water as controls. Pots were completely randomized. Isolations were attempted periodically throughout the experiment. At 16 weeks after seeding, all plants were rated for percentage of continuous internal stem browning. Our data have shown that the best criterion for symptom measurement is the percentage of the stem browned, rather than height of browning or number of nodes browned.

In a similar experiment conducted in the field, seed were planted on 19 May 1970 in four 20- X 20-ft plots in 30-inch rows, six seed/ft, in an area not previously planted to soybeans for at least 10 years. Beginning 6 weeks after planting, one row was selected randomly from each of the four plots and ca. 80% of the plants were inoculated by the BSI method, the remaining 20% of the plants in each row were left noninoculated as controls. Four biweekly inoculations were made. Twenty weeks after planting, all plants were rated for continuous internal stem browning.

Effect of cool temperature on internal stem browning.–Five-week-old soybeans were inoculated in the greenhouse by the BSI method. At inoculation and at four biweekly intervals thereafter, 40 inoculated and eight control plants were transferred

TABLE 1. Recovery of *Cephalosporium gregatum* from roots and nodes of soybean plants in the early pod-filling stage

Days after inoculation	Roots	Nodes in ascending order 1	2	3	4	5	6	7	8	9
		Inoculation by addition of fungal suspension to plant growth medium								
1	+[a]	0[b]	0	0	0	0	0	0	0	0
2	+	0	+	+	0	+	0	+	0	+
3	+	+	+	0	0	+	+	0	0	+
4	+	+	+	0	0	+	+	0	0	+
Control	0	0	0	0	0	0	0	0	0	0
		Inoculation by dipping severed hypocotyls in conidial suspension								
1	–[c]	+	0	0	+	+	+	0	+	+
2	–	+	+	0	0	+	0	+	+	0
3	–	+	+	+	0	0	0	0	0	+
4	–	+	+	+	+	+	0	+	0	+
Control	–	0	0	0	0	0	0	0	0	0

[a] + = successful isolation of *C. gregatum*.
[b] 0 = no *C. gregatum* was isolated.
[c] – = isolations were not made from roots.

into a greenhouse cool chamber maintained at 18-24 C. One group of plants remained in the greenhouse throughout the experiment. Pots were completely randomized both in and out of the cool chamber. All plants were rated for continuous internal stem browning 17 weeks after planting.

RESULTS. *Movement of C. gregatum in soybean plants*. During the 7th through the 11th week after soybean seeds were planted in a field infested with *C. gregatum*, the pathogen was isolated from the taproots of 42, 50, 58, 92, and 100% of the plants, respectively. The fungus was isolated from the ninth nodes (tops) of plants at the early pod-filling stage within 2 days after inoculum had been added to the nutrient solution (Table 1), and from the interior of taproots within 1 day. The fungus was also isolated from the tops of plants within 1 day after their hypocotyls had been dipped in a conidial suspension (Table 1).

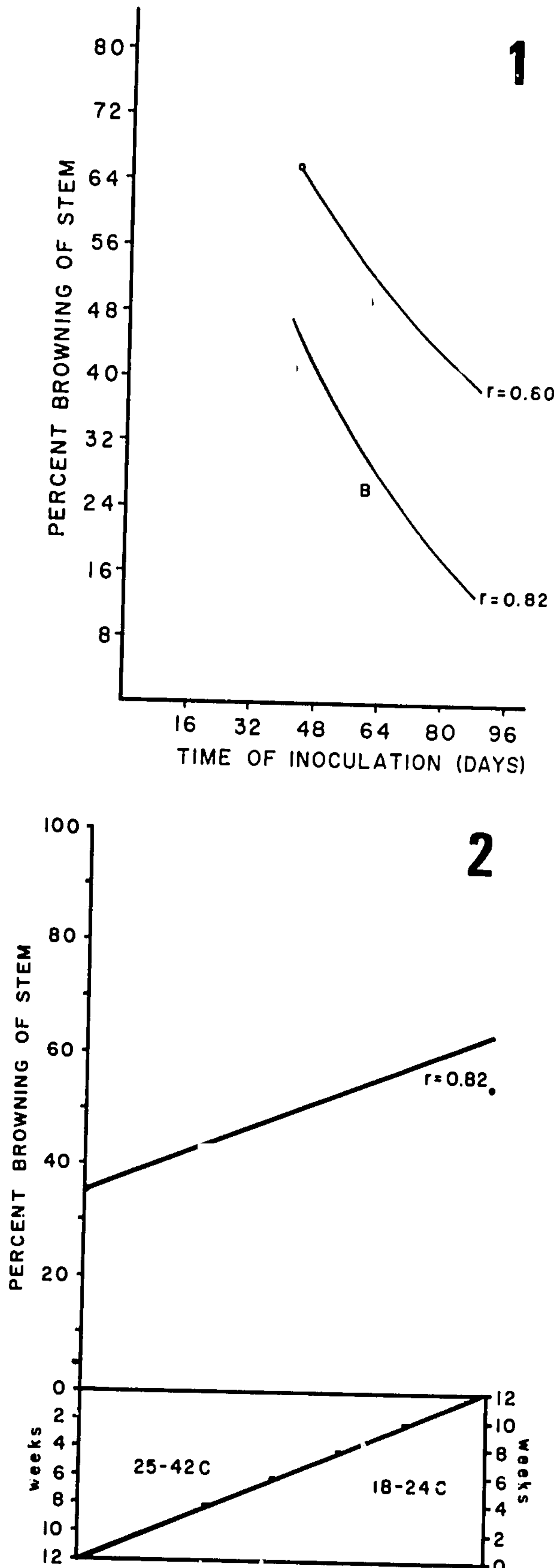
Effects of plant age at time of inoculation and low temperatures on development of internal stem browning. Percentage of internal stem browning and age of plant at time of inoculation were inversely related (Fig. 1). Internal browning in greenhouse plants inoculated at 6, 8, 10, and 12 weeks and rated 16 weeks after planting was 65, 61, 35, and 46%, respectively. This inverse relationship was also observed in the field (Fig. 1): 41, 40, 35, and 5% stem browning at 6, 8, 10, and 12 weeks, respectively. The 12-week inoculation was not significantly different from the control at the 5% level, according to Duncan's multiple range test (DMR) (14). Isolations from four of six infected plants 6 weeks after inoculation yielded *C. gregatum* from the ninth and tenth nodes, while browning had progressed only to the second node.

Time of exposure of infected plants to low temperatures and extent of internal browning were directly related (Fig. 2). Plants grown at temperatures of 25-42 C throughout the experiment (12 weeks) had the least browning (35%). Plants exposed to low temperatures (18-24 C) for 4, 6, 8, 10, or 12 weeks had 42, 46, 62, 65, and 54% browning, respectively. There was no browning in the control plants. All differences were significant at the 5% level (DMR) (14) except those between 8- and 10-week exposures.

DISCUSSION. *Cephalosporium gregatum* enters the taproots of relatively young soybeans (7 weeks). It can spread from the roots to the top nodes of the plant within 2 days after artificial inoculation, suggesting that conidia are the principal means of spread within the plant, and providing evidence for the conidial transport theory of Allington & Chamberlain (2). Similar results and conclusions were reported by Presley et al. (12) using *Verticillium albo-atrum* in cotton. The hypothesis by Weber et al. (16) that *C. gregatum* must be functioning in the plant before browning occurs is confirmed by the isolation of the pathogen well in advance of the browning.

The direct relationship of length of exposure to low temperatures and inverse relationship of plant age at time of inoculation to extent of internal stem browning suggest that if maximal symptom development is to occur in the field, young plants must be infected and be exposed to cool temperatures.

A revised concept of the etiology of *C. gregatum* in soybean is proposed. After the fungus has entered the roots, it grows slowly through the plant, producing typical stem-browning symptoms. If there is a brief period of low temperatures, optimal for sporulation in culture, conidia will be produced, spread throughout the plant, germinate, and produce the discontinuous internal stem-browning symptoms frequently observed in the field. Warm temperatures,



optimal for vegetative growth in culture, after a cool period could stimulate renewed mycelial growth from the conidia which have spread to the tops of the plants. Such temperature conditions, cool weather followed by a warm, dry period at the end of the growing season, are always prevalent when the most advanced symptom, withering and browning of leaves, is commonly observed (4). This symptom is thought to be caused by a water deficit in the leaves as a result of xylem plugging by the fungus (4).

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Fig. 1-2. **1)** Effect of plant age at time of inoculation with *Cephalosporium gregatum* on stem browning in soybean; A = greenhouse plants rated at 16 weeks; B = field plants rated at 20 weeks; r values are significant at the 5% level. **2)** Effect of cool temperatures (18-24 C) after exposure to warm temperatures (25-42 C) on stem browning in soybeans inoculated with *C. gregatum*. Ratings were made 12 weeks after inoculation. Points on diagonal axis indicate weeks at warm and cool temperatures; r value is significant at the 5% level.

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