Test Tube Method of Bioassay for Thielaviopsis basicola Root Rot of Soybean

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ABSTRACT

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Polystyrene test tubes (100 × 17 mm) containing 16 g of soil infested with endoconidia or chlamydospores of *Thielaviopsis basicola* were found to be suitable experimental units for assessing infection of soybean by this fungus. Seedlings germinated for three days on moist paper toweling were transplanted singly into the tubes and incubated at 20 C. Increasing inoculum densities of endoconidia or chlamydospores from 10^1 to 10^5 per gram soil resulted in correspondingly increased symptom severity. At 10^4 per gram, symptom expression in plants grown in the tubes was well developed within 10 days, whereas comparable symptom severity of plants in pots, with other conditions the same required 20 days. The method has been used for assessing virulence of isolates of the pathogen, screening soybean cultivars for resistance, and for assaying soils for inoculum potentials of the pathogen. The advantages of the method are its economies in terms of soil, inoculum, and space, and the relatively short time required for disease development.

Thielaviopsis basicola commonly causes a root rot disease of soybeans in southern Michigan (2). There are no known resistant cultivars and other control methods have not been developed. Good symptom development in naturally or artificially infested soil requires about three weeks in the greenhouse. For pathogenicity tests, and for evaluation of cultivar resistance, biological control methods, and inoculum potentials in natural soils, we desired a method that would allow development of adequate symptoms earlier and with greater economy of space and inoculum. We here report such a method.

MATERIALS AND METHODS

Preparation of endoconidia and chlamydospores.—Isolates 157, 170, 171, and 172 of *Thielaviopsis basicola* (Berk. & Br.) Ferr. were isolated from diseased soybeans from various fields in southern Michigan by use of carrot tissue (1), and were maintained on Czapek's agar containing 5 g of yeast extract per liter. Endoconidia were obtained from 6-day-old cultures by washing the agar surface with sterile distilled water. Chlamydospores were prepared from 4-week-old cultures by first using the method of Papavizas and Adams (4), then removing the remaining endoconidia by 6-10 centrifugations for 20 seconds each at 1,500 rpm. Chlamydospore chains were broken by grinding a concentrated chlamydospore suspension in a glass tissue homogenizer. The ground suspension was passed through four layers of cheesecloth to remove residual hyphae. The concentrations of endoconidia or chlamydospores in the suspensions were determined by hemacytometer counts.

Disease tests.—Soybean [*Glycine max* (L.) Merr.] plants were grown in 100 × 17 mm polystyrene test tubes or in 14 × 11.5 cm waxed paper cups. For each treatment, one seedling was grown in 16 g of soil in 10 test tubes, or ten plants were grown in 1 kg soil in duplicate paper cups. Unless otherwise indicated, the soil used for these tests was natural Conover loam (5).

Seeds were surface-sterilized by soaking for 15 minutes in 1,000 μg/ml (1,000 ppm) sodium hypochlorite (1/50 dilution of commercial bleach), and then in aerated distilled water for about four hours. This sequence was repeated. Seeds then were placed on wet paper towels in trays covered with a polyethylene sheet and kept on a laboratory bench. Surface sterilization prevented development of contaminants on the seedlings, and soaking enhanced uniformity of germination. After three days, germinating seeds with radicles about 4 cm long were selected for disease tests.

The soil was infested by adding a *T. basicola* spore suspension of known concentration to a known weight of sieved (2 mm) soil, bringing it to 50% of water holding capacity and mixing thoroughly. Soil was placed in test tubes, and the seedlings were planted into the tubes. The surface of the soil then was moistened with 1 ml water. The tubes were placed randomly in metal racks. Unless stated otherwise, the seedlings were kept in a growth chamber maintained at 20 C, with a photoperiod of 15 hours per day and a light intensity of 9,595 lux (950 foot-candles). The seedlings were watered when the soil surface became dry. After 10 days the seedlings were removed and disease symptoms were rated on a scale of 0-6 based on the presence or absence of lesions or the length of the girdled section of the tap root, as follows: 0, no lesion present; 1, lesions present, but not coalescing to girdle the tap root; 2, a girdling lesion 1-5 mm long; 3, a girdling lesion 6-20 mm long; 4, a girdling lesion 21-40 mm long; 5, a girdling lesion 41-60 mm long; 6, a girdling lesion greater than 60 mm long (Fig. 1).

Seeds for disease tests in paper cups were sown in infested soil in these cups and kept on the greenhouse bench for three days, when the cups were transferred to soil temperature tanks at 20 C. The seedlings remained in the tanks for 18 days when they were removed and disease symptoms rated on a subjective scale from 0 to 6, with 0 = no disease, 1-2 = mild disease, 3-4 = moderate disease, and 5-6 = severe disease.

All experiments were done two or more times with reproducible results.

RESULTS

Polystyrene test tubes were found to be suitable receptacles for soil and plants in the study of *T. basicola* infection of soybean seedlings. Seedlings grew uniformly in the test tubes (Fig. 2), and the roots of diseased plants showed necrotic lesions characteristic of *T. basicola* infection (Fig. 1).

Effect of inoculum concentration and time on disease development.—To establish a satisfactory inoculum density and a suitable time for evaluating disease development in test tubes, Conover loam was infested with five concentrations of endoconidia of *T. basicola* isolate 170 in a 10-fold series from 16 to 1.6×10^5 spores per gram oven dry soil. On successive days from 5 through 15 days after transplanting seedlings, disease was assessed in 10 plants for each inoculum concentration.

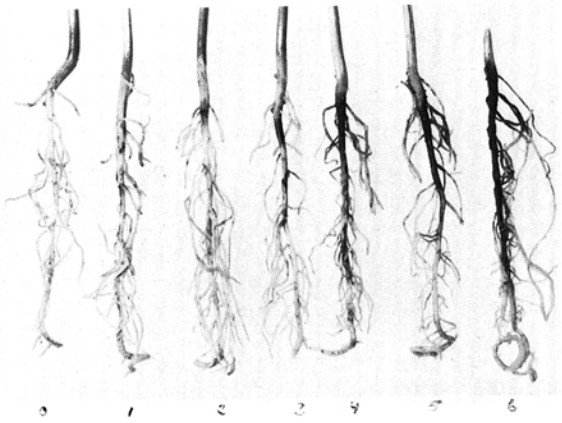

Fig. 1. 'Hark' soybean seedlings with symptoms of root rot caused by *Thielaviopsis basicola*. Disease index categories from 0 (healthy) to 6 (severe) are illustrated.

Fig. 2. Ten-day-old 'Hark' soybean seedlings growing in polystyrene test tubes (100 × 17 mm) arranged in a rack.

Disease increased as inoculum concentrations were increased, and with time (Fig. 3). Symptoms were observed as early as five days after transplanting but disease severity sufficient for discriminating easily among treatments required 8 to 13 days. In all tests with test tubes, disease ratings were made 10 days after seedlings were transplanted.

By contrast, disease developed more slowly in paper cups in a temperature tank at 20 C, with other conditions the same. Such plants were also larger than plants of comparable age grown in test tubes. Although root lesions could be seen in plants grown in paper cups 11 days after seeding, tap roots were not girdled until 16-19 days. Disease index of plants in paper cups 21 days after seeding was 2.0.

Effect of method of watering.—Because watering the seedlings when the soil surface was dry might result in sufficient variation in soil moisture to affect disease development, we compared disease development under two moisture conditions. In one treatment test tubes each with two holes (1 mm in diameter), one at the bottom and one midway up the side of the tube, were immersed to a depth of 9 cm in a pan containing sterile sand saturated with distilled water. In this way uniform moisture was maintained. In the other treatment test tubes without holes were watered when the soil surface became dry. Soil was infested with endoconidia of isolate 170 at 10^4 per g soil. Disease in each treatment was evaluated after 10 days.

There was no difference in the disease severity of soybeans grown under the two conditions of water maintanance. Both had disease indices of 3.0; standard

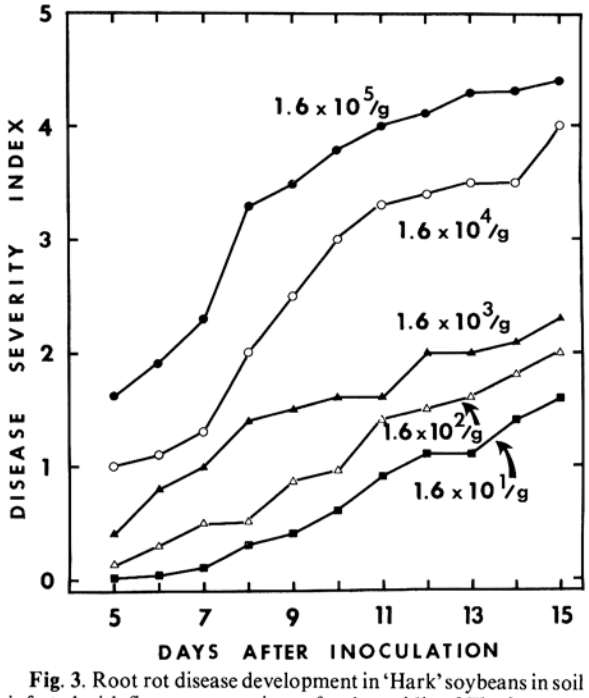

Fig. 3. Root rot disease development in 'Hark' soybeans in soil infested with five concentrations of endoconidia of *Thielaviopsis basicola* isolate 170.

deviations were 0.47 for continuous watering and 0.51 for intermittent watering.

Disease development in steamed vs. natural soil.—Disease development was compared in autoclaved (1 hour) soil mix, autoclaved Conover loam, and natural Conover loam in test tubes. The inoculum was endoconidia of *T. basicola* isolate 157 at a concentration of 10^4 per g soil. The experiment was done in the growth chamber and in soil temperature tanks at 20 C in the greenhouse.

In soil temperature tanks, disease development was greater in autoclaved soil mix (disease index = 3.4) and in autoclaved loam (disease index = 3.3) than in natural loam (disease index = 2.3). However, in the growth chamber disease severity was similar in all three soils; viz., 3.6, 3.5, and 3.3, respectively.

Comparison of pathogenicity of endoconidia and chlamydospores.—The pathogenicity of endoconidia and chlamydospores of *T. basicola* isolate 157 on soybean cultivar Hark was compared at inoculum densities of 10^4, 10^3, 10^2, and 10 propagules per g of soil in test tubes.

Respective disease indices were 3.3, 1.7, 0.4, and 0.1 for endoconidial inoculum, and 3.2, 2.0, 0.6, and 0.1 for chlamydospore inoculum. Values for endoconidia and chlamydospores at a given inoculum concentration did not differ in paired 't' tests.

Virulence of various isolates.—Virulence of isolates 157, 170, 171, and 172 of *T. basicola* on soybean cultivar Hark was compared at five concentrations of an endoconidia-chlamydospore mixture (10^5, 10^4, 10^3, 10^2, and 10 propagules per g soil) in test tubes.

All isolates were pathogenic at the concentrations tested, and infectivity increased as the inoculum concentration increased. Mean disease index for isolates 157 and 171 was 2.3, and these isolates were more virulent than isolate 170 (mean disease index = 1.9) and isolate 172 (mean disease index = 1.5). Isolate 170 was significantly more virulent than isolate 172. Least significant range (LSR) by Tukey's 'w' procedure was 0.22 ($P = 0.05$). Respective disease indices for the highest inoculum level were 4.9, 4.7, 3.8 and 2.8, and the LSR ($P = 0.05$) was 0.4.

Relative susceptibility of soybean cultivars.—Thirty-one soybean cultivars were screened for resistance to *T. basicola*. The inoculum consisted of endoconidia of isolate 170 applied at a concentration of 10^4 per g soil.

All 31 cultivars were susceptible to the pathogen; however, different levels of susceptibility were observed (Table 1). Disease indices ranged from 1.9 for cultivar Beeson, the most resistant, to 4.4 for cultivar Wilkin, the most susceptible.

In a subsequent greenhouse experiment, the comparative susceptibilities of five cultivars, representing a range of susceptibility, were evaluated. Ten seedlings of each cultivar were grown in duplicate paper cups in soil infested to contain 10^4 endoconidia of isolate 170 per gram. Disease was assessed after 21 days. The same order of susceptibility was obtained as in the test tube assays; i.e., in order of increasing susceptibility, it was Beeson, Altona, Wayne, Harosoy, and Amsoy-71. Their respective disease indices were 2.4, 4.2, 5.2, 5.3, and 5.7. The least significant range ($P = 0.05$) by Tukey's 'w' procedure was 0.5.

To determine whether different isolates of *T. basicola* affected different soybean cultivars similarly, the pathogenicity of isolates 157, 170, 171, and 172 was tested on the same five cultivars. The inoculum for each isolate consisted of endoconidia applied at a concentration of 10^4 per g soil. All four isolates affected the soybean cultivars in a similar manner, the cultivar × isolate interaction being nonsignificant ($P = 0.05$) (Fig. 4). The order of susceptibility was the same as that found previously.

Inoculum potentials in naturally-infested soils.—Seven soil samples were collected from various soybean fields in southern Michigan, each with a history of *T. basicola* root rot. Disease indices of seedlings grown in these soil samples were 0.8, 1.0, 1.1, 1.1, 1.4, 1.4, and 3.3.

TABLE 1. Root rot disease development in soybean cultivars in test tubes containing soil infested with endoconidia of *Thielaviopsis basicola* isolate 170

Cultivar	Disease index[a]	Cultivar	Disease index[a]
Beeson	1.95	Clay	3.25
Woodworth	2.45	Harwood	3.25
Anoka	2.50	Lincoln	3.25
Clark-63	2.50	Corsoy	3.30
Dunn	2.65	Kent	3.30
Altona	2.75	Swift	3.30
Chippewa	2.80	Vansoy	3.30
Williams	2.80	Wirth	3.40
Rampage	2.85	Amsoy	3.45
Traverse	2.85	Blackhawk	3.50
Hodgson	2.90	Harosoy-63	3.50
Wells	2.90	Steele	3.60
Wayne	3.00	Amsoy-71	3.80
Harosoy	3.05	Calland	3.85
Hark	3.10	Wilkin	4.40
Chippewa-64	3.15		

[a]Mean of 10 replications each with a single seedling. Disease index was based on a scale of 0-6. Least significant range ($P = 0.05$) by Tukey's 'w' procedure was 0.37.

DISCUSSION

The test tube method for assay of *T. basicola* infection

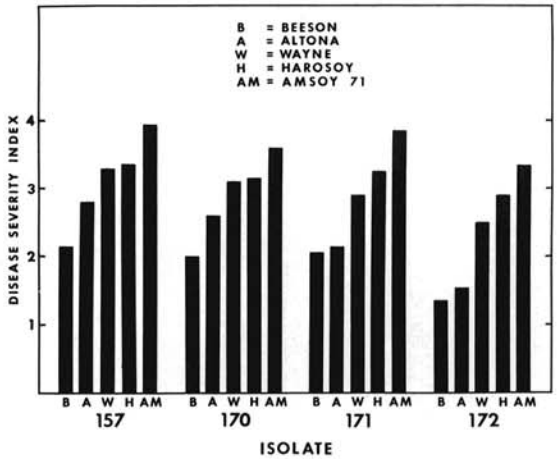

Fig. 4. Root rot disease development in soybean cultivars Beeson, Altona, Wayne, Harosoy, and Amsoy-71 grown in test tubes containing soil infested with each of four isolates of *Thielaviopsis basicola*. Least significant range by Tukey's 'w' procedure was 0.25 ($P = 0.05$).

of soybean is suitable for quantitative work involving this disease. It was used successfully for (i) assessment of virulence of isolates of the pathogen, (ii) screening of cultivars for resistance to the disease, and (iii) assaying field soils for inoculum potentials. Of 31 varieties screened in the test tube method, 'Beeson' was the most resistant. This result was confirmed in a more conventional greenhouse test. Since a spectrum of disease susceptibility was shown, further screening may yield cultivars with even greater resistance.

The lower disease indices obtained with naturally infested soils as compared with artificially infested soil indicated that inoculum densities in soybean fields are lower than those used to infest soil in these experiments. This was confirmed by counts made on dilution plates using a modified selective medium, and which showed that populations of *T. basicola* ranged from about 10 - 1,000 per gram (3).

The test tube method has several advantages over the use of clay pots or similar large containers for disease assessment: (i) the quantity of soil needed (16 g per plant) is small compared to the quantity (100 g per plant) required in 14 × 11.5 cm containers, (ii) the quantity of inoculum required is correspondingly small. Thus, when inoculum is a limiting factor, as in the case of chlamydospores of *T. basicola* free of endoconidia, more soil can be infested with the test tube method, (iii) the amount of space needed in the growth chamber or greenhouse is reduced, and (iv) a shorter time was required for the appearance of measurable disease.

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