

Pathogenesis of *Thielaviopsis basicola* in Nonsterile Soil

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ABSTRACT

Early stages of pathogenesis by *Thielaviopsis basicola*, from chlamyospore germination to lesion development, were studied on cotton seedlings inoculated with field soil infested with spores of the pathogen. Chlamyospores, conidia, and hyphal fragments germinated within 20 hr and the germ tubes penetrated epidermal cells or root hairs directly within 24 hr. Propagules germinated only in the immediate vicinity of the host. The disease progressed as hyphae grew within the tissues and upon the root surface. The surface growth gave rise to new infection loci. New conidia were produced within 36 hr and new chlamyospores after 3-4 days.

When field soil, naturally or artificially infested

with *T. basicola* chlamyospores, was kept dry prior to its use in inoculation of cotton seedlings, very few chlamyospores germinated and little or no disease occurred. When the same soil was kept moist prior to inoculation, more chlamyospores germinated and severe disease occurred. Greenhouse inoculations of cotton, cowpea, and Pinto bean with clones of the pathogen from cotton, tobacco, bean, cherry, and citrus showed clonal variation from highly virulent to nearly avirulent. Laboratory studies on cotton seedlings confirmed these results and showed that chlamyospore germination and hyphal development, in and on the root, of the nearly avirulent clones was markedly restricted.

Thielaviopsis basicola (Berk. & Br.) Ferr. produces endoconidia and dark, thick-walled chlamyospores in diseased tissue. Chlamyospores are the main survival propagules (8, 11) and are, therefore, the primary and major source of infection of plants. Yet most studies of this fungus have dealt primarily with conidia or with mixtures of conidia, chlamyospore chains, and hyphal fragments. Recently, Linderman and Toussoun (4) reported techniques for separating conidia and hyphal fragments from chlamyospores so that pure preparations of the latter could be added to soil for study. These techniques made possible the present paper which presents details of pre- and post-penetration behavior of chlamyospores and of conidia in nonsterile soil in contact with roots of growing seedlings. Evidence is also presented on the role of soil moisture in disease initiation and on the pathogenic variability of the fungus.

MATERIALS AND METHODS.—A method similar to that of Toussoun and Snyder (10) was used to follow the stages leading to parasitism, except that the confined soil inoculum units were placed on intact growing seedlings. Cotton (Acala 4-42) (*Gossypium hirsutum* L.) seedlings were grown in flats of U.C. Mix (1) for 3-4 days at 32 C in greenhouse constant temperature control rooms. Uniform seedlings were then removed from the flats and placed in inoculation chambers (four seedlings per chamber) (Fig. 1) constructed of hinged plastic petri dishes notched on one side to accommodate the seedling hypocotyls. Confined inoculum units of field soil, amended with the fungus (4) and wet enough to be cohesive, were placed on the roots of hypocotyls of the cotton seedlings. When hypocotyls were inoculated, the inoculation chambers were placed in 1-gal covered plastic freezer containers to maintain a high humidity.

To follow the behavior of the pathogen from germination to penetration, portions of the inoculum were

removed and examined as soil smears stained with cotton blue diluted with water. Penetration was observed after inoculated tissue was washed in a stream of water and stained with 0.02% acid fuchsin in lactic acid. Chlamyospores or conidia whose germ tubes had penetrated the root cells were not washed away. Roots were observed as whole roots or as longitudinal hand sections consisting of the epidermis and several cells of the underlying cortex.

Isolates of *T. basicola* were maintained on potato-dextrose agar (PDA) slants as single conidium cultures.

Greenhouse inoculations were made with equally concentrated conidial suspensions of each clone by pipetting 10 ml of the suspension around the base of each plant (1 week old). The plants were harvested and experiments repeated by planting seeds in the potting medium now containing both conidia and chlamyospores produced on diseased roots of plants from the previous experiments. Although the inoculum levels in the second method were unequal due to the variation in previous infection, the initial inoculum density was sufficiently high to carry over to the second experiment. The results of the two methods of inoculation were comparable. The results of all inoculations were determined after 1 week. The disease ratings used ranged from 0 for no infection to 10 for maximum infection based on the extent of root and hypocotyl discoloration and lesion development.

The field soils used in laboratory inoculations were obtained from cotton fields in the San Joaquin Valley, California. The soil artificially infested with *T. basicola* was a brown, coarse, sandy loam (pH 6.6) containing no natural infestation, and the other soil was a dark brown loam (pH 6.9) naturally highly infested with *T. basicola*.

RESULTS.—*Stages in development of the pathogen leading to pathogenesis.*—The major propagule responsible for the long time survival of *T. basicola* in nature

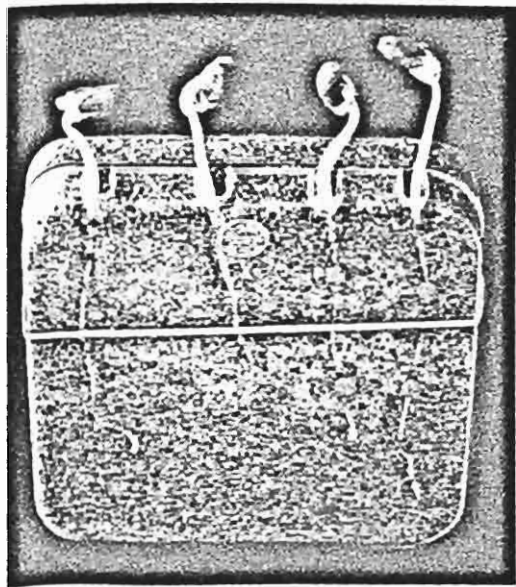


Fig. 1. Plastic inoculation box technique showing cotton seedlings which were grown 3-4 days in the greenhouse and then transplanted into plastic boxes notched to accommodate the hypocotyls. The two seedlings on the right have been inoculated at two sites with small units of chlamydospore-amended field soil.

is the chlamydospore (8, 11), but conidia (4), and possibly hyphal fragments, may also be important in secondary infections. A study was therefore made with clone C (isolated from cotton [*Gossypium hirsutum* L.]) to determine the efficacy of these structures (i.e., chlamydospores, conidia, and hyphal fragments) in initiating disease on roots of cotton seedlings. Major emphasis, however, was placed on the behavior of chlamydospores added to nonsterile soil. Small units of this fungus-amended soil (where conidia were used, the soil was infested immediately before inoculation) were placed in contact with the host tissue. Longitudinal hand sections of roots and smears of the soil inoculum were made 12, 20, 24, 36, and 48 hr after inoculation. Chlamydospores, conidia, and hyphal fragments were germinating within 20 hr after inoculation of roots. Chlamydospores and conidia germinated in low numbers and only when in contact with the root. Germination increased when inoculum was near a wound. Germ tubes penetrated epidermal cells and root hairs after 24 hr (Fig. 2-a, b). Penetration was direct without formation of any special structure, except for a slight enlargement of the tip of the germ tube from which a tapered peg penetrated the host cell. All epidermal cells of the root and hypocotyl appeared to be equally susceptible. Once inside the host cell, the fungus developed the characteristic beaded hyphae (Fig. 2-c). Subsequent proliferation of the fungus occurred by intra- and intercellular hyphal growth. Of much greater interest, however, was the rapid development of hyphae on the host surface. These hyphae originated when the germ tube of a spore, having penetrated a cell, initiated side branches on the surface

which then successively penetrated other epidermal cells (Fig. 2-e, f). Such surface hyphae also originated from hyphae growing in the host. In either case, these surface or runner hyphae were not beaded, and they ramified in all directions without following any host cell pattern. Thirty-six hours after inoculation new conidia were produced from phialides borne on surface hyphae or on hyphae which emerged through the epidermis.

Lesion development, as evidenced by a yellow-brown tissue discoloration, progressed from a fleck stage at 36-48 hr (flecks being small lesions caused by single chlamydospores) to a coalesced stage where flecks merged after 3-4 days. New chlamydospores appeared in or on severe lesions after 3-4 days. These new chlamydospores were either imbedded in the cortical tissue or projected out from surface hyphae. According to Mathre et al. (6), invasion progressed to the endodermis within 5 days, but the endodermis was a barrier except in very severe infections when new chlamydospores were seen in the xylem vessels. Others (2, 5) have also claimed occasional invasion of vascular tissue. Still other anatomical studies, however, have shown a distinct absence of the fungus in the vascular cylinder due to pericycle resistance (3, 7, 9). In the present studies chlamydospore production was observed mainly in the cortex or on the epidermis except where the cortex was split, thereby exposing the endodermis and vascular cylinder. Large masses of chlamydospores in these tissues were observed under such conditions (Fig. 3).

Effects of prewetting soil inoculum on disease incidence.—Chlamydospores of *T. basicola* maintained for several months in air-dried field soil germinated very poorly if at all (4), whereas germination was greatly increased when the same soil was kept moist for several days prior to testing. Experiments were conducted, therefore, with clone C to determine if wetting the inoculum soil several days prior to inoculation of cotton roots would increase the incidence of disease. Seedlings were inoculated with small units of field soil which had been amended with pure preparations of chlamydospores (4) of clone C. Both hypocotyl and root inoculation experiments were made. Half the seedlings were inoculated with soil that had been kept air-dry for several months; the other half were inoculated with similar soil kept moist 5-9 days prior to use. Disease severity ratings were recorded after 4-7 days. Little or no disease occurred when soil that was dry prior to inoculation was used, but severe disease occurred when previously wet soil was used (Fig. 4).

To determine why no disease occurred when previously dry chlamydospore-amended soil was used, soil smears and longitudinal hand sections of roots were made and examined after 24 and 48 hr. Few chlamydospores had germinated.

Further experiments were made with a naturally infested field soil known to contain a high population of *T. basicola*. A portion of this soil which was air-dried for several months was moistened 4-5 days before

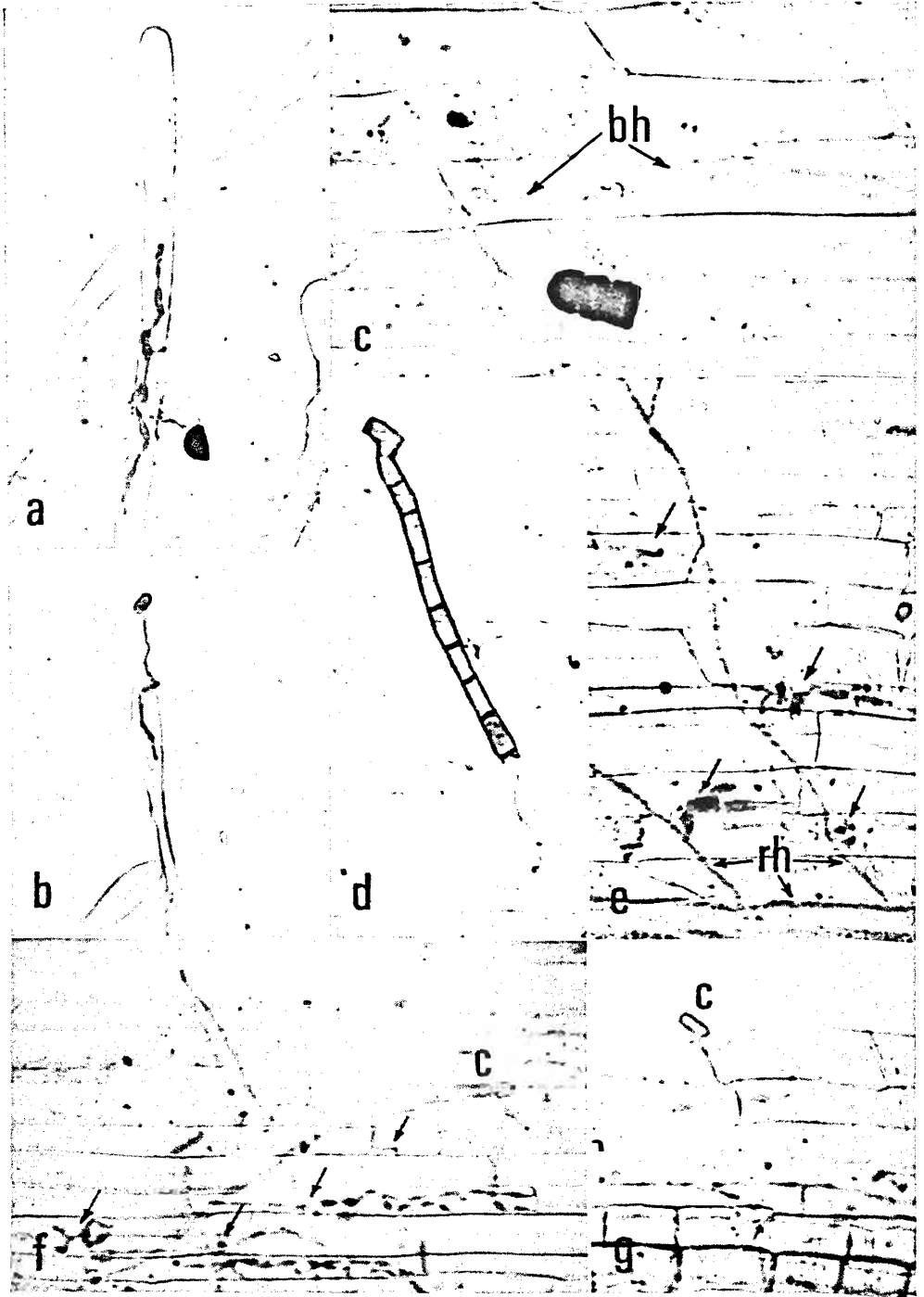


Fig. 2. Penetration and lesion formation on cotton roots by *Thielaviopsis basicola*: a) Chlamydsore penetration of root hair. b) Conidial penetration of root hair. c) Germinated chlamydsore and epidermal cell filled with typical beaded hyphae (bh). d) Resistant cell of hyphal fragment germinating and penetrating cotton epidermis. e) and f) Multiple infections (arrows) on cotton roots from *T. basicola* runner or ramifying hyphae (rh)-(e) and conidium (c)-(f). g) Penetration of epidermal cells by conidial germ tube of weakly pathogenic clone CH. Note that proliferation by this clone, once inside the host cells, is quite limited. Such cells penetrated by virulent clone C would be filled with beaded hyphae (see Fig. 2-c).

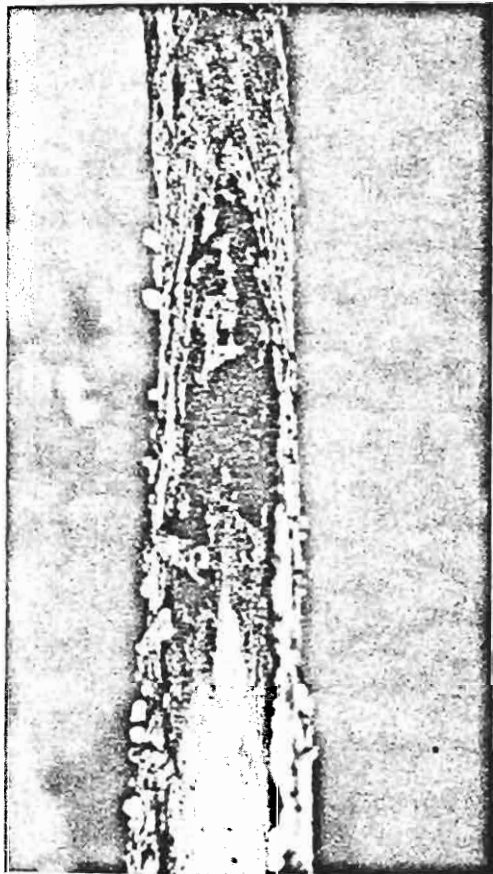


Fig. 3. Masses of chlamydospore chains of *Thielaviopsis basicola* produced on cotton seedling where the cortex had split.

use. Cotton was then seeded in these two soil lots maintained at the two moisture regimes and seedlings were examined 1 week later. Root rot was much more severe on seedlings grown in the soil that was wet several days prior to planting. The same soil samples were also used as inoculum units placed on cotton roots in the plastic inoculation chambers. The resulting lesions were rated (0-10 scale) for severity after 7 days. The average ratings for three experiments, each with 40 inoculations per treatment, were 1.7 for the previously dry and 4.6 for the previously wet field soil.

These studies clearly showed that soil moisture was important as a prepenetration factor in preparing chlamydospores of *T. basicola* directly, indirectly, or both, for germination and pathogenesis on cotton.

Variation in virulence of clones.—Laboratory experiments with other clones of *T. basicola* suggested that pathogenic variation might exist. Accordingly, clones of the fungus from California, Canada, and Mexico isolated from cotton (C), Pinto bean (*Phaseolus vulgaris* L.) (B and B₂), soybean (*Glycine max* L.) (SB), Garbanzo bean (*Cicer arietinum* L.) (G), cherry (*Prunus* sp.) (CH), citrus (*Citrus* spp.) (T₉₃, T₂₃), and tobacco (*Nicotiana tabacum* L.) (T₁, T₂) were tested on cotton, Pinto bean, and cowpea (*Vigna*

sinensis [Torner] Savi) grown in U.C. Mix (1) in the greenhouse. Disease ratings (0-10 scale) were made 1 week after inoculation and the results of all experiments averaged (Fig. 5). These data show that *T. basicola* exists as clones ranging from highly virulent to nearly avirulent on the hosts selected. Two of the nearly avirulent clones (CH, T₉₃) were selected for further comparative laboratory studies with the virulent clone C. Chlamydospores of these clones were added to field soil which was moistened several days prior to inoculation of seedlings in the plastic inoculation chambers. Lesion severity ratings, recorded after 1 week, confirmed the greenhouse experiments which had indicated the near avirulence of clones CH and T₉₃. Root sections prepared 24 and 48 hr after inoculation were made to determine the reason for the low level of disease obtained with these two clones. These preparations revealed that few chlamydospores had germinated, as compared to clone C, and that the extent of hyphal proliferation within the root and on its surface was restricted. Thus it appeared that these weakly pathogenic clones responded little to the presence of cotton roots.

DISCUSSION.—Information about the behavior of soil fungus propagules in natural soil immediately prior to host penetration is limited. In these studies we have followed the behavior of conidia and chlamydospores of *T. basicola* in natural soil from germination to penetration of cotton roots. These studies were possible largely because of the inoculation technique that allowed the placement of small units of field soil containing a high number of propagules of the test fungus in contact with host root or hypocotyl tissue. This soil and the host tissue beneath could be examined at intervals to determine the behavior and progress of the fungus in establishing a parasitic relationship with the host.

It was found that few spores, particularly chlamydospores, germinated, and only if in contact with the root surface. This is probably due to the limited distance root exudates move from the root into the soil. While the initial penetration was accomplished directly into the host tissues, unlike *Fusarium solani* f. sp. *phaseoli*, as shown by Toussoun and Snyder (10), a thallus was subsequently formed, mostly by means of surface runner hyphae which established new infection loci. This behavior is similar to that of *F. solani* f. sp. *phaseoli* as described by Weinke (12), and may be more common of soil-borne pathogens than is realized. Such a surface thallus produces, in the case of *T. basicola*, new conidia and chlamydospores in abundance. These propagules are readily sloughed off into the soil to increase the inoculum potential of this fungus and its chances for survival.

The increased incidence of disease with artificially or naturally infested field soil that was kept moist prior to inoculation suggests that this increase is due in part to an increase in the germinability of chlamydospores. This agrees with our earlier findings (4). Speculation at that time (4) concerning the possible significance of high soil moisture in disease epidemiology under arid

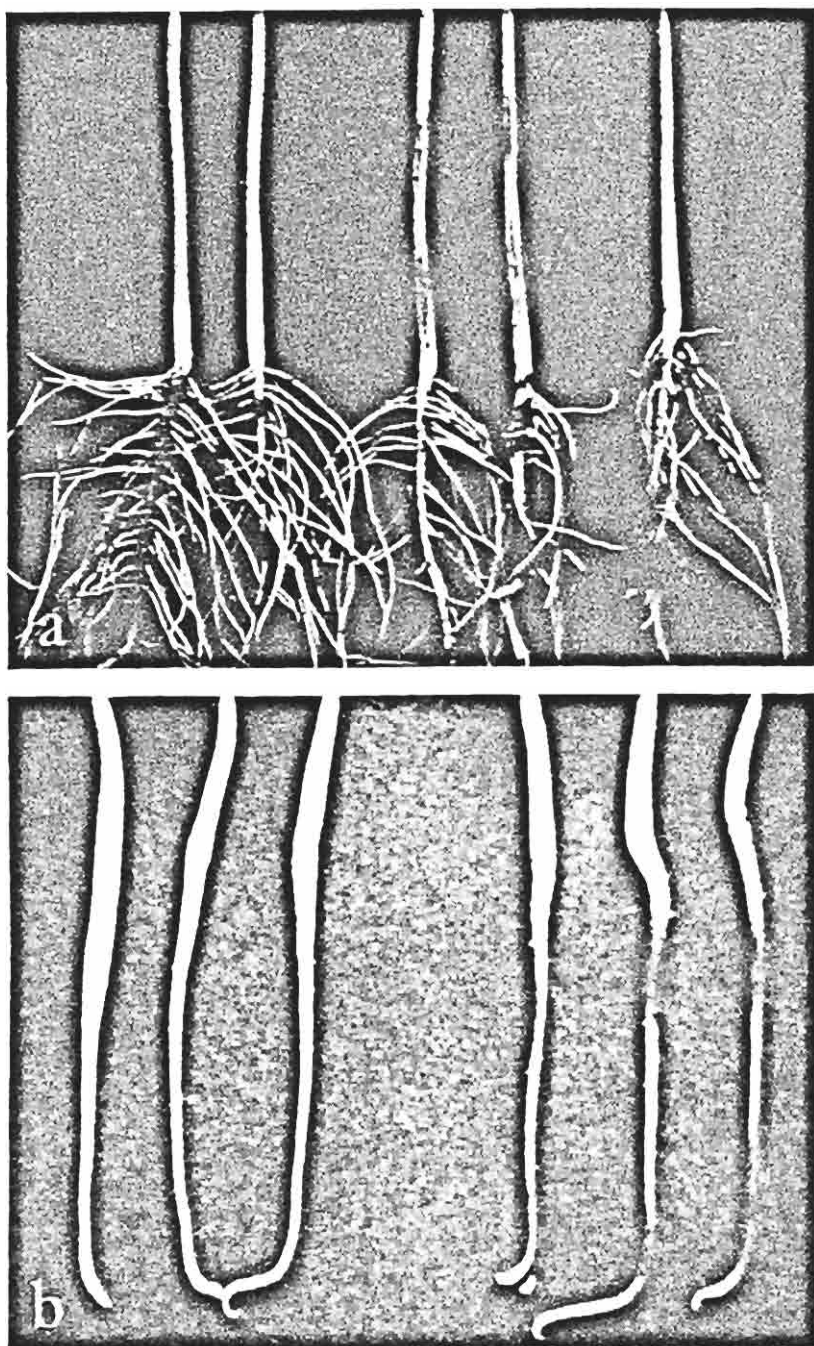


Fig. 4. Cotton seedlings inoculated with *Thielaviopsis basicola*. **a)** Hypocotyl inoculations: The two seedlings on the left were inoculated with chlamydospore-amended field soil which was dry prior to inoculation and show no visible signs of infection. The middle two seedlings were inoculated with the same soil which had been wet several days prior to inoculation and show severe lesions. The seedling on the right was not inoculated. **b)** Root inoculations with previously dry field soil (left, two seedlings) and previously wet field soil (right, two seedlings).

conditions is supported by the present findings. High soil moisture due to rain or irrigation may thus, in addition to affecting the host plant, affect the chlamydospores of *T. basicola* by increasing their germinability, thereby increasing the probability of infection. Differences in the microbial activities in the soils may also play a part in this phenomenon.

Reports in the literature on host specialization in *T. basicola* have generally been conflicting, possibly due to the use of too few clones from different hosts, or localities, or both. Our studies, dealing with a wide selection of clones, demonstrated a rather complete range of clonal virulence. Only the behavior of the nearly avirulent clones CH and T₉₃ suggested some

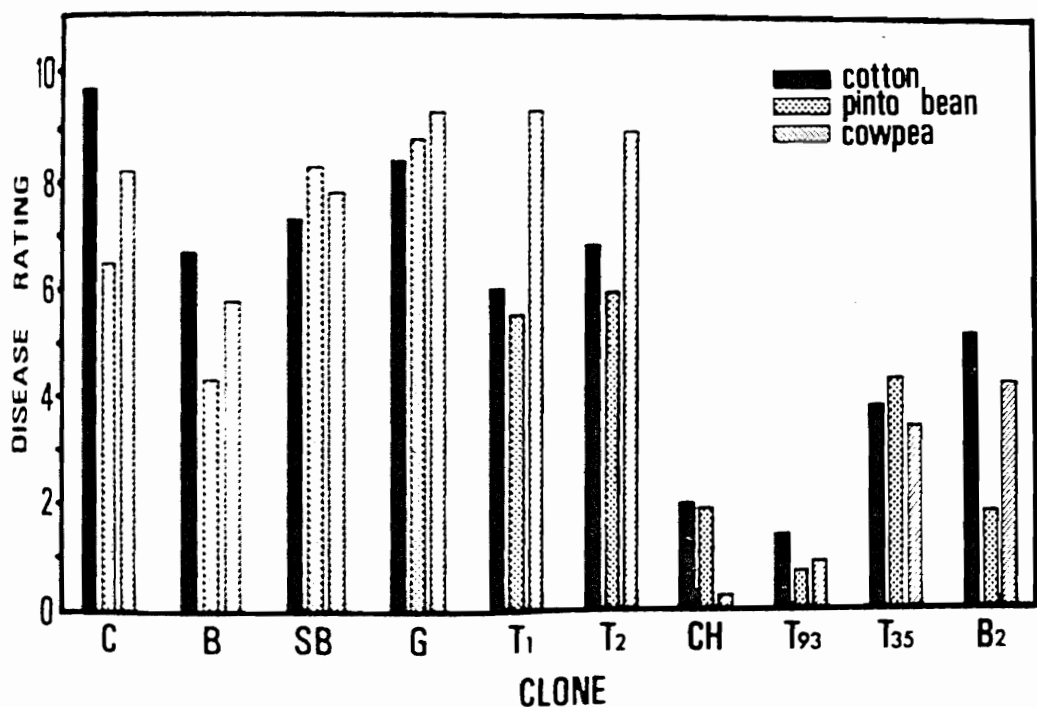


Fig. 5. Pathogenic variation of 10 clones of *Thielaviopsis basicola* on cotton, cowpea, and Pinto bean. These results are the averages of the disease ratings from 3-4 experiments. The ratings, based on the amount of root and hypocotyl infection as indicated by chlamydospore production and tissue discoloration, ranged from 0 for no infection to 10 for the most severe infection. Origin of isolates: C = cotton, B and B₂ = Pinto bean, SB = soybean, G = Garbanzo bean, T₁ and T₂ = tobacco, CH = cherry, T₉₃ and T₃₅ = *Citrus* spp.

degree of host specialization. Clones nearly avirulent to cotton did not respond to cotton roots as did virulent clones. Their chlamydospore germination was relatively low and the host greatly restricted their development. If it is true that germination of resting structures is triggered by root exudates, then a differential reaction to these exudates appears to exist, in that chlamydospores of virulent clones germinate more readily on cotton roots than weakly pathogenic clones. Yet all clones germinate readily in soil when 10% carrot juice is added (*unpublished data*). Insight into this phenomenon may help us to understand some of the mechanisms of host root resistance.

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