Importance of Chlamydospores as Primary Inoculum for *Alternaria solani*, Incitant of Collar Rot and Early Blight on Tomato

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ABSTRACT

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Chlamydospores were produced in hyphal and conidial cells of *Alternaria solani* on a basal salts agar medium and in infected tissues of several tomato cultivars. Conidia required desiccation as a prerequisite to chlamydospore transformation of cells. In contrast, chlamydospore production in hyphal cells was inhibited by desiccation. Collar rot developed on tomatoes grown in soil contaminated with chlamydospore inoculum or fresh hyphal cultures of the pathogen. Moreover, these inocula persisted in soil for 12 mo. Chlamydospores placed in depths of 0, 4, 8, and 12 cm in soil initiated infections and incited collar rot on tomato. A root rot developed when the inoculum was placed from 0 to 20 cm deep and chlamydospores were present in roots of plants infected by *A. solani*. Thus, chlamydospores were introduced into soil by tomato residue infected by *A. solani*, were responsible for long-term survival of the fungus, and, therefore, were the primary soilborne inocula for the pathogen.

Alternaria solani Sorauer, incitant of collar rot and early blight (19,25) on tomato (*Lycopersicon esculentum* Mill.), overwinters on alternate crop and weed hosts (11,17,19,25), contaminated seed (6,11, 19,25), and infected host debris in soil (2,8–11,17,19,25). Because of frost and killing freezes, overwintering of the pathogen on alternate solanaceous hosts was unlikely in Oklahoma. Also, there was no substantial evidence to verify dissemination of *A. solani* by contaminated seed or infected tomato transplants (C. L. Patterson, *personal observations*). In some areas of Oklahoma, however, a high incidence of collar rot was often observed in fields repeatedly cropped with tomatoes infected by *A. solani*. In addition, early blight epidemics usually were first evident and most severe in these fields. Further, soil fumigation reduced the incidence of collar rot (3,8). Thus, the life cycle of *A. solani* included a soilborne stage (2,8–11,17,19,25), and inoculum persisted in fields for several months (2,17) in the absence of a susceptible solanaceous host (11,17,19,25).

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Conidia and hyphae of *A. solani* are resistant to unfavorable environmental conditions (17,19,25). Rands (17) demonstrated that conidia overwintered in infected potato leaves buried in soil and suggested they were a source of primary inoculum for the next crop. Overwintering hyphae within potato debris infected by *A. solani* was also suspected (17), but conclusive evidence of long-term persistence was lacking.

Production of chlamydospores has been reported for some pathogenic *Alternaria* species (1,2,14,16,22), including *A. solani* (2,14). Atkinson (1) believed the prolonged survival (5 yr) of *A. raphani* Groves & Skolko in dry soil cultures was attributable to chlamydospores. Basu (2) observed chlamydospores in tomato tissues infected by *A. solani* and in cultures of the fungus on agar media. He also described germination of the propagules in vitro. In addition, collar rot developed on tomato plantings in fields artificially contaminated with chlamydospores in tomato residue infected by *A. solani* or cells of conidia produced in vitro (2). Basu (2) further established that the propagules persisted in soil for 7 mo or longer. Patterson and Powell (14) also observed chlamydospores in tomato tissues infected by *A. solani* and cultures of hyphal cells. Their investigation concluded that the propagules could initiate foliar infections and incite early blight symptoms.

A recent review (19) of tomato early blight included chlamydospores as a source of primary inoculum for *A. solani*, but their importance relative to other infective propagules (8–11,17) has not been determined. Thus, delineation of soilborne inocula was pertinent to the biology (2,6,11,14,17), epidemiology (7,9,10,15), and control (3,4,7,8,13,15, 19,25) of collar rot and early blight epidemics. There were several deficiencies in data, however, that required investigation before elucidating the importance of chlamydospore inoculum. The deficiencies were as follows: 1) association of chlamydospores with a larger cross section of *A. solani* isolates than previously studied (2,14); 2) the influence of host genotype and pathogen interactions on production of chlamydospores in infected tomato tissues; and 3) ecological and pathological comparisons of inocula in soil.

This project was initiated for the following reasons: 1) to determine the affinities of in vitro and in situ chlamydospore production within a representative population of *A. solani*; 2) to investigate the influence of host-pathogen interactions on chlamydospore development in infected tomato tissues; 3) to assess virulence and longevity of several soilborne inocula (2,11,14,17) by multiple comparison (20); and 4) to ascertain the relation of chlamydospore proximity in the rhizosphere, infection of roots, and development of collar rot.

MATERIALS AND METHODS

Isolates of *A. solani* were obtained from excised early blight lesions on tomato leaves. Hyphal tips from young colonies were subcultured on potato-dextrose agar (PDA) acidified to pH 4.5 with 25% lactic acid (APDA) (5). Sporulation was induced by growing the fungus on 2% Difco cornmeal agar for 48 hr at 27 C in darkness and subsequently transferring the fungus to the S-medium of Shahin and Shepard (18). Conidia were seeded on APDA and single colonies were transferred to PDA slants. Cultures were maintained by periodic monthly transfer, inoculation

and reisolation from tomato, and storage at 7 C.

Tomato cv. Sunny was employed in all tests except for cultivar evaluations. Surface-disinfested (60 sec in 2% NaOCl) seed was planted to produce disease-free tomatoes. Friable, moist, unsterile field soil collected in an area previously planted with grassland pasture was used as a potting medium. Tomatoes were maintained in a greenhouse at about 30 C. Flood irrigation of potted plants was applied as required to maintain moisture, prevent soil dispersion, and decrease the potential for cross-contamination of inoculum treatments. Plants were observed for early blight and collar rot during a 14- and 45-day period, respectively. Infected plants were incubated in plastic bags for 24 hr to induce sporulation, tissues were microscopically observed for conidia and chlamydospores, and isolations were prepared from tissues to confirm infection by *A. solani*.

Experimental designs were completely random. Single or multifactorial analysis of variance (ANOVA or MANOVA, respectively) were applied to determine sources of variation (20). Scheffé's test for multiple comparisons was implemented to derive values (Ss_Q) required to identify significant mean differences at the 99% ($P = 0.01$) confidence level. Data resulting from experiments addressing longevity of inoculum in soil were defined by nonlinear (curvilinear) regression analysis. Homogeneity of regression coefficients was determined by multifactorial analysis of covariance (MANOCO). Significant differences in treatment means at each observation interval were identified by Scheffé's test ($P = 0.01$). All data were reported as mean percentages of the factors appraised.

Association of chlamydospores with isolates of *A. solani*. Chlamydospore production was determined by evaluating 20 isolates (designated Ast1–Ast20) of *A. solani*. An actively growing, nonsporulating, 4-day-old hyphal colony (4 mm diameter) from each isolate was transferred to 15 ml of a basal salts agar medium (BSAM) in 9-cm-diameter petri dishes. The medium contained (per liter of distilled water): 680 mg of K_2HPO_4, 180 mg of $MgSO_4$, 149 mg of KCl, 14.2 mg of $ZnSO_4$, 4.0 mg of $CuSO_4$, 4.0 mg of $FeCl_3$, 500 mg of Difco yeast extract, 10 g of Difco Noble agar, and L-glutamine (equivalent of 0.2% actual N) as the nitrogen source. Four replicate cultures of each isolate were incubated at 27 C and 12 hr diurnal light and observed for chlamydospore production during a 28-day assessment period. The test was repeated five times.

Initiation of infection by chlamydospores and development of collar rot. Chlamydospores produced by hyphae of each *A. solani* isolate were desiccated 24 hr at 30 C under forced air, pulverized in a 40-mesh Wiley mill, suspended in sterile distilled water, and passed through four layers of cheesecloth to remove agar debris. The suspensions were adjusted to 10^4 chlamydospores per milliliter. Unsterile field soil contained in 10-cm-diameter × 15-cm-deep (1,178 cc) pots was contaminated with 10 ml of the inoculum. Tomatoes were direct-seeded into the soil and thinned to one plant per pot. Plants with four to six true leaves were inoculated to saturation (about 1 ml) with 10^3 conidia per milliliter of the same *A. solani* isolate contained in the contaminated potting medium. Inoculum from each isolate was represented by 10 pots. A control included 10 uninoculated plants grown in noninfested soil. The test was repeated five times.

Influence of cultivar genotype and *A. solani* isolate interactions on chlamydospore production. Fresh-market tomato cultivars All Star, Big Set, Bingo, Carnival, Celebrity, Duke, Floramerica, Floridade, Freedom, Independence, Jet Star, Liberty, Mountain Pride, Pacific, Pick-Red, Revolution, Spring Giant, Sunny, Valerie, and Willhite 101 were planted in about 1,178 cc of potted soil and thinned to one plant per pot. Ten milliliters of chlamydospore inoculum (10^4 per milliliter) derived from three *A. solani* isolates (Ast2, Ast7, and Ast19) was added to 10 individual pots of soil per cultivar. Leaves were inoculated with conidia of the same isolate in contaminated soil. A control included 10 uninoculated tomato plants (cv. Sunny) grown in noninfested soil. The test was repeated two times.

Comparative assessment of soilborne inoculum. Infected tomato residue, chlamydospores, fresh or desiccated conidia, and fresh or desiccated hyphal fragments from three isolates of *A. solani* (Ast2, Ast7, and Ast19) were evaluated for the ability to initiate infection and incite collar rot. Chlamydospore inoculum was prepared as previously described. Conidia were produced on S-medium (16), collected in a washing of sterile distilled water, and observed for chlamydospores. Additional conidia were produced, desiccated 12 hr at 24 C under forced air on Gelman 0.45-μm membrane filters, washed from the membrane as needed, and observed for chlamydospores. Hyphae that did not produce conidia or chlamydospores were grown on BSAM. The cultures were homogenized in sterile distilled water in a Waring blender and passed through four layers of cheesecloth to remove agar debris. Additional hyphae were grown on BSAM, desiccated 24 hr at 24 C under forced air before chlamydospore production, pulverized in a 40-mesh Wiley mill, suspended in sterile distilled water, and passed through four layers of cheesecloth. Hyphal cells were observed for chlamydospores and each fragment was considered to represent an infective propagule. Tomato leaves 50–75% infected by one of each *A. solani* isolate were desiccated (30 C, 48 hr under forced air) and pulverized in a 40-mesh Wiley mill to obtain a fine leaf powder containing hyphae, conidia, and chlamydospores of the fungus. The estimated number of chlamydospores, conidia, and hyphal fragments in water suspensions per milliliter was determined by hemacytometer counts, added to soil for a final concentration of about 10^4 propagules per gram, and compared with 0.1 g of tomato residue infected by *A. solani* per gram of soil. Tomatoes were planted in contaminated and uncontaminated soil contained in 1,178-cc pots. Individual inoculum treatments were represented by 10 pots per five replications. The test was repeated two times. Factors included in MANOVA were *A. solani* isolate, inoculum source, and number of experiments.

Longevity of inocula in soil. Soil was contaminated with inoculum sources (described earlier) from one isolate of *A. solani* (Ast19). Contaminated and uncontaminated soils were placed in plastic flats 37 × 25 × 5 cm (length × width × depth = 4,625 cc). Tomato seed were planted in four replicate flats of each soil treatment, placed in a greenhouse, and thinned to 50 plants per flat, or the flats of soil were placed in a fallow field previously cropped with bermuda grass (*Cynodon dactylon* (L.) Pers.) pasture on 1 January. The flats were separated 5 m to prevent cross-contamination of inoculum treatments. Four replicates per treatment were retrieved at 12 monthly intervals and direct-seeded with tomatoes.

Inoculum longevity was determined by the incidence of collar rot. Factors included in regression analysis and MANOCO were inoculum source and percentage of collar rot at each monthly assessment interval.

Soil placement of chlamydospores, infection of tomato, and incidence of collar rot. Inoculum of one *A. solani* isolate (Ast19) was prepared by thoroughly comminuting chlamydospores and unsterile field soil resulting in a final concentration of 10^4 propagules per gram. Polyvinylchloride (PVC) cylinders 10 cm in diameter were longitudinally bisected and reassembled by securing the seams with adhesive material. The cylinders were filled with unsterile field soil to the desired level of inoculum placement, and 10 g of the chlamydospore per soil concoction was horizontally distributed in the soil profile at depths of 0, 4, 8, 12, 16, or 20 cm. Individual inoculum depths were represented by four replications of five PVC cylinders. Tomato seeds were placed on the soil surface and covered with about 0.5 cm of additional soil. Seedlings were thinned to one plant per cylinder.

For assessment of disease incidence,

the cylinders were opened lengthwise. Roots were extracted from the region of inoculum placement, separated from the soil, washed in a running water bath for 12 hr, and examined for root rot. Fifty 5-mm root segments from each inoculum depth were surface-disinfested 60 sec in 2% NaOCl, rinsed in sterile distilled water, and incubated on 15 ml of APDA in 9-cm-diameter petri dishes (five segments per dish) at 27 C. Root infection by *A. solani* was confirmed by preparing hyphal tips from colonies resembling the pathogen for sporulation and identification on S-medium (16). The test was repeated two times.

Factors assessed during MANOVA were depth of inoculum placement and the number of experiments conducted. Parameters evaluated were percentage of root rot, collar rot, and *A. solani* infections.

RESULTS

Chlamydospore production. Numerous chlamydospores were evident in all cultures of *A. solani* on BSAM 14–21 days after incubation at 27 C. The propagules were produced from differential swelling of individual hyphal cells (Fig. 1A) in the curly mycelium (11) and were dark brown, single-celled, thick-walled, 8–15 μm in diameter (mean = 11 μm), and occurred singly, in chains, or in clusters (Fig. 1B). After 28 days of incubation, nearly all hyphal cells differentiated into chlamydospores.

Infection and development of collar rot. Chlamydospores of all *A. solani* isolates infected tomato roots and hypocotyls and incited collar rot. Early blight symptoms also developed on leaves inoculated with conidia. In addition, chlamydospores were invariably observed in necrotic tomato leaf, epicotyl, hypocotyl, and root tissues infected by *A. solani*.

Host genotype and pathogen interactions. There were no apparent differences in susceptibility of tomato cultivars to infection by chlamydospores or conidia of *A. solani*. Collar rot developed on all cultivars grown in soil contaminated with chlamydospores, and early blight lesions were produced on leaves inoculated with conidia. Chlamydospores were always observed in tissues infected by *A. solani*.

Comparison of inoculum sources. All tomato plants grown in soil contaminated with chlamydospores developed collar rot. Symptoms occurred on 90% (average of three isolates) of the plants grown in soil contaminated with tomato residue infected by *A. solani*. Collar rot was observed on 96% of the plants grown in soil contaminated with fresh hyphae before cell differentiation and formation of chlamydospores. Chlamydospores were not evident in cells of desiccated hyphae, and collar rot developed on only 8% of the plants grown in soil contaminated with the inoculum. The propagules were not detected in cells of fresh conidia but were produced by about 60% of the cells in desiccated conidia. Disease incidence on tomatoes grown in soil contaminated with these inocula was 6% and 92%, respectively. Collar rot did not develop on plants grown in soil contaminated with disease-free tomato residue or in uncontaminated control soils.

Disease incidence in treatments including soil contaminated with chlamydospores, infected residue, fresh hyphae, or desiccated conidia did not significantly ($P = 0.01$) differ but was greater than in soil contaminated with desiccated hyphae, fresh conidia, or the controls.

Inoculum longevity in soil. Collar rot developed on 100% of the tomatoes immediately planted into soil contaminated with chlamydospores of *A. solani* (Fig. 2). The infection rate varied between 98% and 100% during six monthly planting intervals and decreased thereafter at each interval to 56% in soil planted 12 mo after contamination.

Collar rot occurred on 96% of the plants immediately planted into soil contaminated with tomato residue infected by *A. solani*. Disease incidence decreased to 88% in plantings 5 mo after soil contamination. The decreasing trend continued with each planting interval thereafter, and incidence of collar rot on plants grown in soil 12 mo after contamination was 42%.

About 58% of the cells in desiccated conidia produced chlamydospores, and collar rot developed on 88% of the tomatoes immediately planted in soil contaminated with the inoculum. Disease incidence declined to 76% in plantings 4 mo after contamination. The decreasing trend in disease incidence continued, and 32% of the plants developed collar rot in soil 12 mo after contamination. In contrast, chlamydospores were not evident in fresh conidia. Only 12% of the tomatoes immediately planted in soil contaminated with fresh conidia developed collar rot. Disease incidence decreased to 4% in soil planted

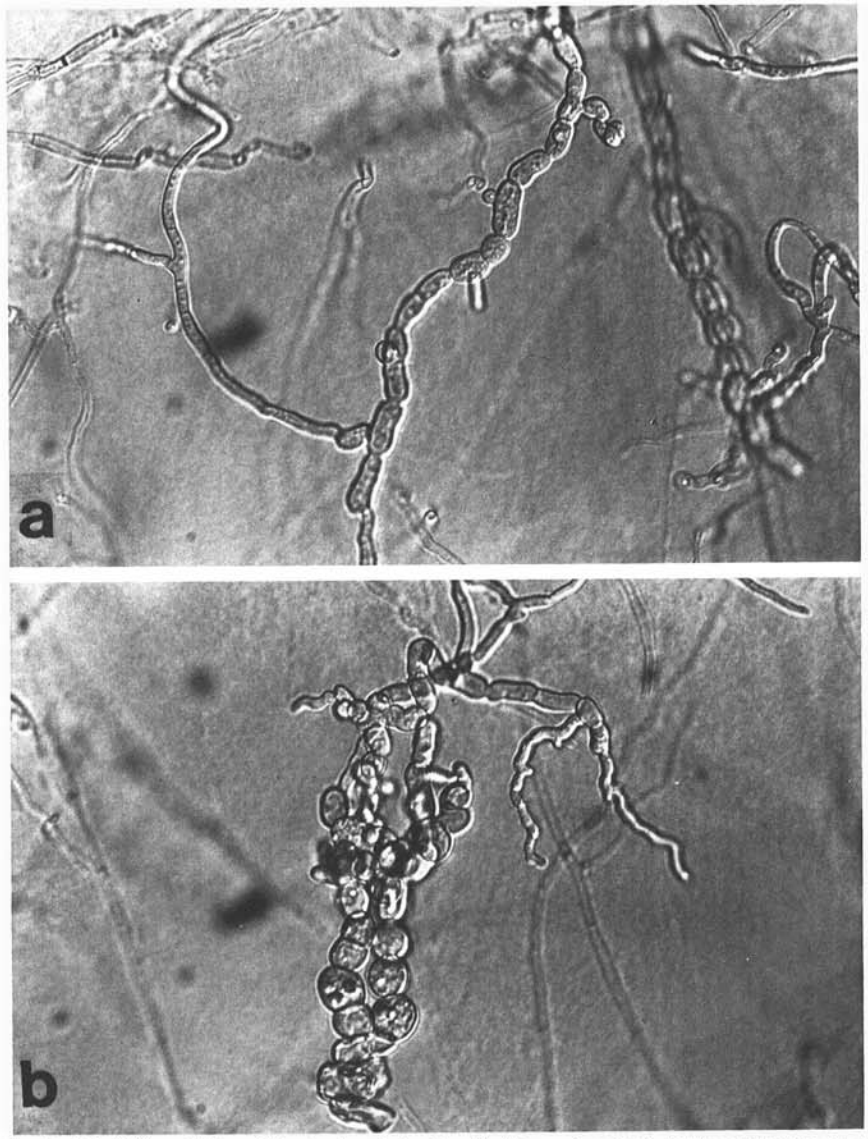

Fig. 1. (A) Differential swelling by hyphal cells of *Alternaria solani* during chlamydospore formation. (×450) **(B)** Chlamydospores of *A. solani* produced on basal salts agar medium. (×450)

1 mo later and was not evident in plantings thereafter.

In soil planted immediately after contamination with fresh hyphae, 94% of the tomatoes developed collar rot. There was a slight but nonsignificant ($P = 0.01$) increase in disease incidence 1 mo after contamination. Disease incidence decreased thereafter, and collar rot was evident on 50% of the tomatoes planted 12 mo after soil contamination. Collar rot occurred on 6% of the tomatoes immediately planted into soil contaminated with desiccated hyphae. Disease incidence decreased to 0.5% 1 mo after contamination, and collar rot did not develop in plantings thereafter.

The decline in chlamydospore and fresh hyphae inocula (Fig. 2) was homogeneous within treatment regressions and collectively described by the function of a third-degree (cubic: $E(-Y) = B_0 + B_1X + B_2X^2 + B_3X^3 = 0.88$) polynomial (20). Rapid decline in desiccated hyphae and fresh conidia was not amenable to regression analysis. Treatment differences in incidence of collar rot varied with each planting interval. After 12 mo, disease incidence on tomatoes in soil contaminated with chlamydospores and fresh hyphae did not significantly differ ($P = 0.01$), nor did treatments of fresh hyphae or infected residue. Further, infected residue or desiccated conidia treatments did not differ. These four treatments did, however, significantly differ ($P = 0.01$) from soil contaminated with fresh conidia, desiccated hyphae, or noninfected residue and uncontaminated control soils.

Depth of chlamydospore placement in soil. All plants grown in soil contaminated with chlamydospores at depths of 0 and 4 cm developed collar rot (Table 1), but disease incidence significantly decreased ($P = 0.01$) with increasing inoculum depth. Collar rot did not develop when inoculum was placed at 16 or 20 cm. Root rot, however, developed on all plants regardless of inoculum depth. *A. solani* was isolated from 86 to 94% of all root segments cultured, and chlamydospores were evident in infected tissues.

DISCUSSION

Data presented in this report further supported the association of early blight epidemics with monocropped tomato culture (10,17) and incorporation of debris infected by *A. solani* into soil (2,9–11,17). Research also verified previous reports (2,14) of persistence of chlamydospores in soil (2) and their function as a source of inoculum for the pathogen (2,14). Although not included in the results section, the propagules survived adverse soil conditions, including temperatures ranging from −5 to 33 C, 75 cm of rainfall, and two storms resulting in 7 and 10 cm of snow and ice lasting 4 or 6 days, respectively. The inoculum was identical to that previously described for *A. solani* (2,14) and other pathogenic *Alternaria* spp. (1,14,16,22) and similar in appearance to chlamydospores produced by pathogenic species of *Fusarium* (12,21).

These studies further demonstrated that chlamydospores were common in the diverse population of *A. solani* isolates observed. In addition, there were no significant differences in infection, disease incidence, or development of the propagules in infected tissues attributable to genotype of the tomato host. Prerequisites for initiation of chlamydospore formation by conidia and hyphae did, however, differ. Conidia required desiccation before chlamydospore transformation of cells, whereas desiccation was inhibitory to production of the propagules by hyphal cells. Moreover, conidia void of chlamydospores did not survive in soil for any significant length of time. Although the propagules were not evident in fresh hyphae, perseverance during adverse soil conditions likely was attributable to chlamydospore production after soil incorporation of inoculum (2). Nevertheless, multiple comparisons concluded that long-term persistence of *A. solani* in soil was achieved primarily by sources of chlamydospore inoculum.

Mechanisms of infection by chlamydospores were not determined. Inoculum may infect tomato roots, hypocotyls, or crowns, incite collar rot and, thus, provide a focus of conidia (2) for regional dissemination when other sources of *A. solani* inoculum (6,11,17) are nil. It is also probable that chlamydospores accumulate on foliage in soil dispersed by splashing water from rain or sprinkler irrigation, infect leaves directly, and incite early blight (14), and sporulating lesions provide conidia that spread to neighboring tomato fields. The latter method of infection could, in part, account for initial development of early blight on lower leaves in contact with or near the soil surface (14,19,25). In areas where climate inhibits overwintering of alternate hosts infected by *A. solani* (11,17,19,25) it is, therefore, reasonable that chlamydospores are the most important means of survival and primary soilborne inoculum for the

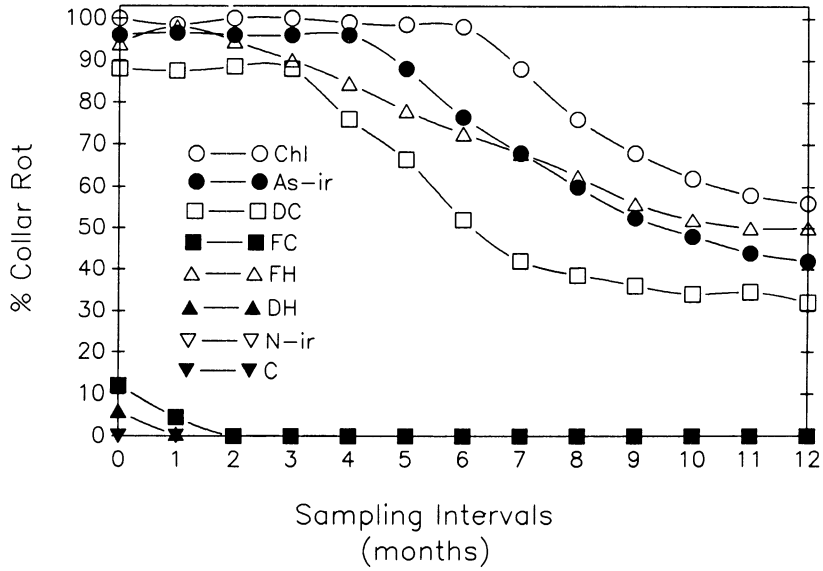

Fig. 2. Longevity of *Alternaria solani* inocula in soil. Chl = chlamydospores, As-ir = residue infected by *A. solani*, DC = desiccated conidia, FC = fresh conidia, FH = fresh hyphae, DH = desiccated hyphae, N-ir = noninfected residue, C = uncontaminated control. $-Y = 0.88$; $Ss_Q(0.01) = 11.13$.

Table 1. Association of collar rot and root rot incidence on tomato cv. Sunny, depth of *Alternaria solani* chlamydospores in soil, and frequency of reisolation of the pathogen from infected root, hypocotyl, and epicotyl tissues

Depth of inoculum (cm)	Collar rot (%)[a,b]	Root rot (%)[a]	*A. solani* isolated (%)[a]
0	100	100[c]	88[c]
4	100	100	92
8	65	100	92
12	10	100	90
16	0	100	86
20	0	100	94

[a] Results are reported as an average of two experiments including four replications of five pots per treatment.
[b] $Ss_Q(0.01) = 28.51$.
[c] No significant differences between treatment means.

pathogen.

This investigation provided additional information pertinent to the collar rot/early blight epidemiology complex. Although collar rot did not develop from chlamydospore infections below 14 cm in soil, *A. solani* incited a root rot that was not previously described on tomato. The symptoms resembled collar rot, but there were no apparent aboveground differences in plant growth. Root infections are likely a significant means of maintaining soilborne chlamydospore inoculum when extended periods of drought or other adverse conditions (7,15) do not favor the disease cycle of *A. solani* and development of collar rot or early blight epidemics.

Delineation of chlamydospore inoculum provides an opportunity to assess potential methods for collar rot and early blight control. Treatment of soil with registered (4) and experimental fungicides, identification and evaluation of biological control agents, removal of infected debris from fields (10), and plowing to invert and bury residue more than 20 cm are strategies applicable for management of soilborne diseases. The potential for recontamination of soil with *A. solani* crop and weed host (17) residue infected by conidia, however, is high. When applicable chemical, biological, or sanitary practices for collar rot and early blight likely would need repeating with each cropping period.

Vanderplank (23) considered the entire pathogen population, all sources of inoculum, environmental conditions, and host-pathogen genotype interactions as influential factors affecting the regional occurrence of foliar disease epidemics. In light of previous research (2,14) and results presented here, the soilborne cycle of *A. solani* must, therefore, be considered when planning seasonal methods for control of diseases caused by the pathogen. Development (7,24) and utilization (15) of forecast systems to predict early blight epidemics and initiate foliar fungicide (4) applications should include chlamydospore inoculum in the epidemic model. Enhancement of the collar rot/early blight disease control spectrum also requires additional ecological investigations of soilborne chlamydospore inoculum. Thus, future research needs include soil moisture and temperature effects on formation, germination, and infection of roots by chlamydospores, and further assessment of potential *A. solani* associations with roots of alternate (17,19) and potential host plants that may result in maintanence or increases in soilborne inoculum.

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