

Colonization of Peanut Seed by *Cylindrocladium crotalariae*

D. M. Porter, F. S. Wright, R. A. Taber, and D. H. Smith

Research plant pathologist and agricultural engineer, U.S. Department of Agriculture, Agricultural Research Service, Tidewater Agricultural Experiment Station, Suffolk, VA 23437; and professors, Department of Plant Pathology and Microbiology, Texas A&M University, Texas Agricultural Experiment Station, College Station, 77995.
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ABSTRACT

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Cylindrocladium crotalariae, the causal agent of *Cylindrocladium* black rot (CBR) of peanut (*Arachis hypogaea*), was isolated at a much higher frequency from peanut seed (retained by a screen 6.4 × 25.4 mm) shortly after curing than 7 mo after initial isolation assay, when the frequency decreased approximately 87%. The frequency of *C. crotalariae* in seed of the cultivars Florigiant, VA 81B, and NC 6 was 15.4, 23.4, and 21.8%, respectively, before storage, and 1.6, 4.0, and 5.6%, respectively, after storage at ambient conditions for 7 mo in an unheated building. Under similar disease pressure, seed of NC 8C, a peanut cultivar with resistance to *C. crotalariae*, was colonized less frequently than seed of cultivars

Florigiant, VA 81B, and NC 6. Seed colonization was directly correlated to incidence of CBR in the field. Seed colonization increased as disease incidence increased. Significant differences were noted among cultivars of Florigiant, VA 81B, and NC 6 in the isolation frequency of *C. crotalariae*. Colonization by *C. crotalariae* was similar in seed stored at ambient and refrigerated (5 C) conditions. The fungus was not isolated from peanut seed treated with seed protectants. Hyphae of *C. crotalariae* ramified both intracellularly and intercellularly throughout the testae of discolored seed. In seed with dark brown testae, hyphae were observed in the cotyledonary tissues.

Additional keywords: seed transmission, seed treatment.

Peanut (*Arachis hypogaea* L.) pods (fruit) are produced in the top 5-cm soil layer, an area of intense microbial activity. Although the shell of the peanut pod (2) and the testa of the peanut seed (8) serve as barriers against some microbial invasion, fungal pathogens often invade peanut seed, including *Cylindrocladium crotalariae* (C. A. Loos) D. K. Bell & Sobers, first described in Georgia in 1965 (1). Infected peanut pods, roots, and pegs (gynophores) become discolored and turn black. Although initially observed on only one farm in Virginia in 1972 (3), *Cylindrocladium* black rot (CBR) now occurs throughout the peanut production areas of Virginia and North Carolina and is a serious threat to the peanut industry (9,10,11,12). Other states (Alabama, Oklahoma, and Texas) and countries (Australia, Japan, China, and India) are also experiencing serious difficulties with CBR. However, the exact mechanisms responsible for the spread of *C. crotalariae* from one location to another are not known. Birds (5), farm implements (7), movement of hay (*personal observation*), dispersal in roots (12), and seed transmission (6,10) have been implicated. Our study was undertaken to determine the effects of time of storage, peanut cultivars, and seed protectants on percent colonization of peanut seed by *C. crotalariae*.

MATERIALS AND METHODS

Peanut seed was obtained from four grower-owned peanut fields in which plants exhibited severe symptoms of CBR. Disease incidence (percentage of plants in 300–400 m of row exhibiting CBR symptoms) in cultivars NC 6, Florigiant, and VA 81B at the Hare farm was 35, 45, and 45%, respectively. Disease incidence at the Griffin (cultivar VA 81B), Holland (cultivar NC 8C), and Faulk farms (cultivar NC 6) was 25, 35, and 80%, respectively. During October 5–10, plants at all four locations were dug, windrowed, and left to dry in the field for 3–6 days. Pods (approximately 220 kg of each cultivar from each location) from diseased areas (3 × 300–400 m) were mechanically picked

with a grower-owned combine. Pods were placed in burlap bags and artificially cured by forced air in drying trailers to 9–10% moisture content by cooperating growers. Pods of each variety from each grower were stored at ambient conditions in an unheated building. Mean temperatures during this time fluctuated between –8 and 34 C.

Foreign material and loose-shelled seed were removed before shelling with a small, commercial-type peanut sheller during the last week of October. Only seed retained by a 6.4 × 25.4-cm screen were used after hand-picking to remove those seed with dark brown testae. Each seed lot was divided into 900-g subsamples, and each subsample was placed in a numbered, nylon mesh (15.2 × 30.5-cm) bag. Twenty-eight bags of each seed lot were prepared per cultivar from the Hare farm, and 14 bags each were prepared from the Griffin, Faulk, and Holland farms (126 subsamples). Seed from the Hare farm was placed in storage either in an unheated building (14 subsamples per cultivar = 42 subsamples) or in a cold seed storage facility maintained at 5 C (14 subsamples per cultivar = 42 subsamples). Seed from the Faulk, Griffin, and Holland farms (14 subsample bags from each location = 42 subsamples) was stored at the same ambient conditions. At monthly intervals beginning in November and ending in May, two subsample bags from each farm site and each cultivar were selected for seed colonization analysis.

The frequency of *C. crotalariae* from each seed lot was determined by plating seed (five per dish) on a sucrose QT agar medium (4) in sterile petri dishes, 90 mm in diameter. To insure better seed/agar contact, seed was cut into two equal apical and basal halves (10). The cut end of each seed was placed, without surface-disinfestation, in contact with the agar surface. At monthly intervals (November through May), 250 apical seed halves of each cultivar from nine test sites were plated and incubated for 12 days at 30 C. After incubation, each seed half was observed for the presence of fungal colonies typical of *C. crotalariae*. Data presented are the averages of two trials, with five replicates of 50 seed per trial. Data were analyzed statistically by regression and correlation procedures in SAS (14), using completely randomized block experimental designs. Effects in the model included temperature, cultivars, and quadratic changes across months. Correlations between seed colonization and time stored for each disease incidence in the field were performed.

Seed protectants and application rates were as follows: captan + DCNA (60:20% of product) at 2.5 g/kg; carboxin + captan + DCNA (18.75:45:15% of product) at 2.6 g/kg; captan + maneb + PCNB + etridiazole (18:18.75:10:2.5% of product) at 2.5 g/kg; thiram + PCNB (42:30% of product) at 5.6 g/kg; and carboxin + thiram + DCNA (30:42:30% of product) at 7.5 g/kg. Fungicides

were applied in a small, rotating, laboratory seed treader. Two weeks after treatment, seed was washed in distilled water to remove traces of fungicide adhering to the testa. Seed with intact testae was plated on sucrose QT agar medium without surface-disinfestation. After 12 days of incubation at 30 C, seed were observed for fungal colonies typical of *C. crotalariae*. Untreated

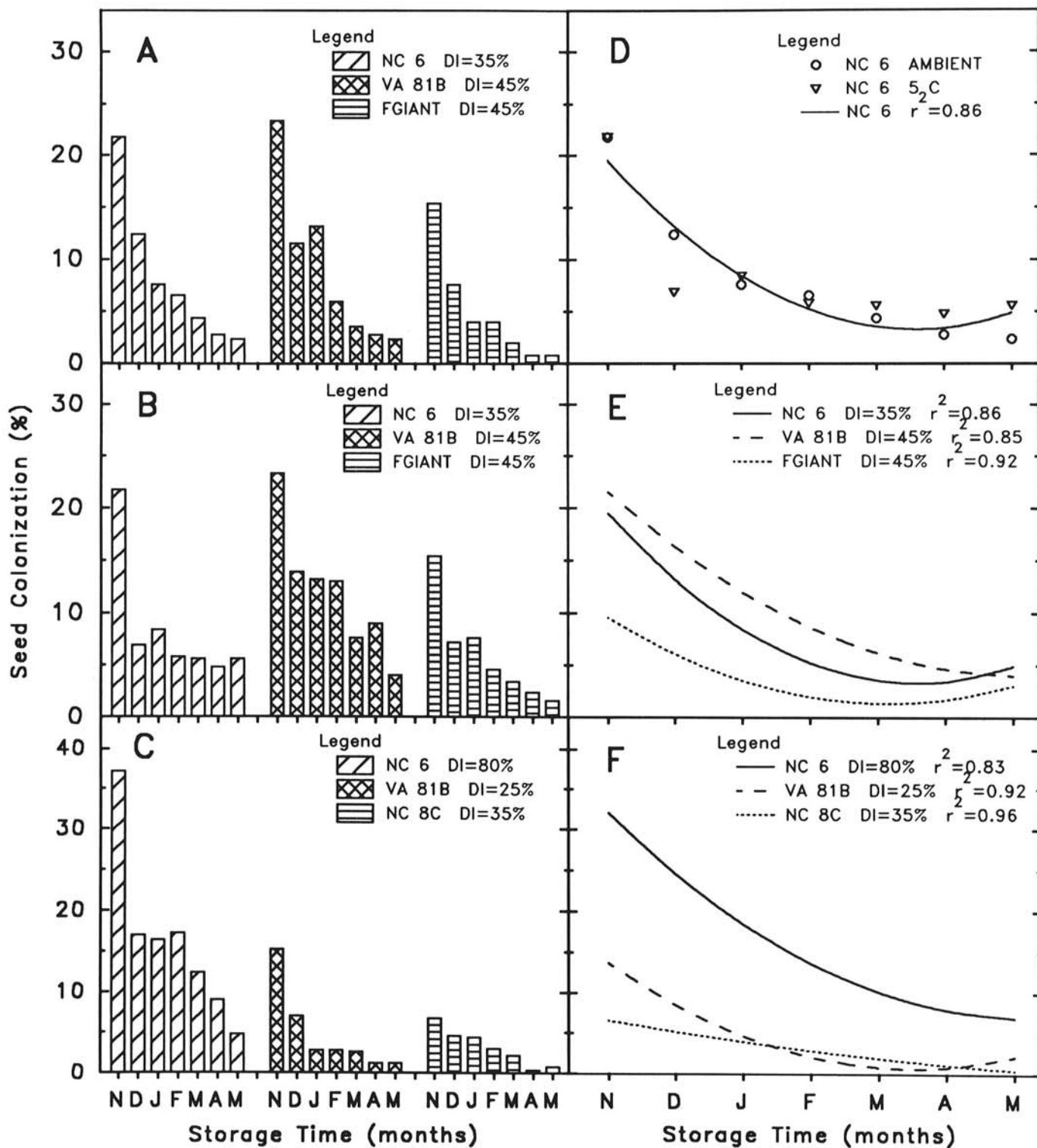


Fig. 1. Colonization of peanut seed (%) (A-C) by *Cylindrocladium crotalariae* over storage time (months). Disease incidence (DI) is equal to percentage of plants exhibiting foliar symptoms of *Cylindrocladium* black rot. A and B, Seed from three farms of cultivars NC 6, VA 81B, and Florigiant (Fgiant) stored under ambient and 5 C refrigerated conditions, respectively. C, Seed of cultivars from three other farms stored under ambient conditions. Correlation analysis (D-F) of colonization of peanut seed by *C. crotalariae* over storage time (month). D, Regression line and data for NC 6 cultivar stored at ambient and 5 C refrigerated conditions. E, Regression lines for cultivars stored at ambient and 5 C refrigerated conditions. F, Regression lines for cultivars, NC 6, VA 81B, and NC 8C stored at ambient conditions. (Regression equation fitted to data, $Y = C_0 + C_1X + C_2X^2$.)

seed were plated similarly. This experiment was conducted twice, with five replicates of 50 seed for each fungicide formulation.

Tissue-staining techniques were used to detect hyphae of *C. crotalariae* on and in peanut seed testa. Seed with discolored testae was cleared by immersion in a 5% KOH solution until desired testa color prevailed. Slide mounts, prepared by placing pieces of testa in a droplet of lactophenol plus acid fuchsin, were microscopically observed.

Histological studies were conducted to locate hyphae of *C. crotalariae* in peanut seed. Seed were fixed in formaldehyde, acetic acid, and alcohol for 48 h, dehydrated in a tertiary butyl alcohol series, and embedded in paraffin. Sections were stained in fast green and safranin and observed (120X). Thick sections were also cut from specimens embedded in epoxy resin and stained in methylene blue.

RESULTS AND DISCUSSION

According to Russell (13), a large number of seed is required to accurately assess the frequency of fungal colonization of seed, especially when colonization frequency is low. Previously, we showed (10) that *C. crotalariae* was isolated at a low frequency from peanut seed from plants growing in areas exhibiting severe disease. In the current study, the frequency of *C. crotalariae* ranged from 6.8% (NC 8C) to 37.2% (NC 6) during the first month of storage but declined rapidly during storage. After 7 mo of storage, the percent colonization of *C. crotalariae* in all cultivars and field sites averaged approximately 3%. Anticipating a low isolation frequency of *C. crotalariae* in peanut seed during parts of this study, we assayed large numbers of seed (31,500) to more accurately reflect colonization percentages.

Seed obtained from peanuts from plants exhibiting a high incidence of CBR was colonized readily by *C. crotalariae* (Fig. 1A-C). After 1 mo of storage (November), the frequency of *C. crotalariae* in seed (seed retained by a 6.4- × 25.4-cm screen and with seed having dark brown testae removed by hand) averaged 20.0% but declined steadily during storage. The average percent colonization of three peanut cultivars in the two storage conditions dropped to 2.8% after 7 mo of storage, with the greatest reduction in percent colonization of *C. crotalariae* occurring during the first month (Fig. 1A and B). Seed colonization by *C. crotalariae* decreased monthly with time regardless of storage condition (Fig. 1A and B). Colonization was most frequent from

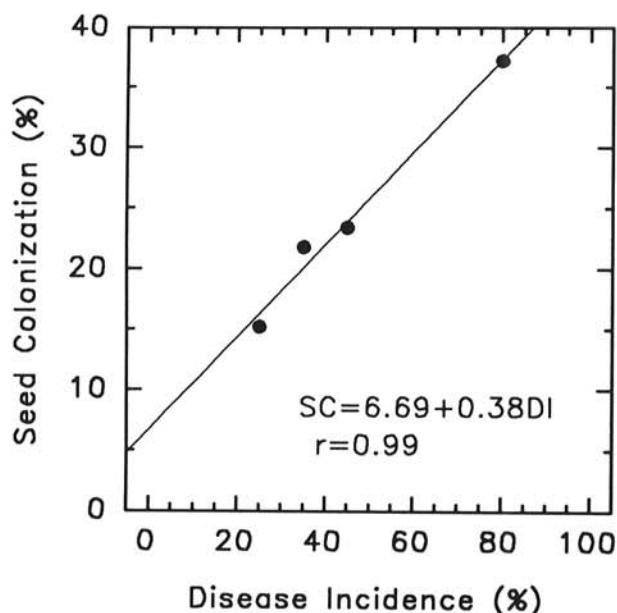


Fig. 2. Correlation analysis of peanut seed colonization (SC) for two cultivars (NC 6 and VA 81B) by *Cylindrocladium crotalariae* and the disease incidence (DI), defined as the percentage of plants exhibiting foliar symptoms of *Cylindrocladium* black rot.

seed maintained at 5 C (Fig. 1B) than from seed maintained under ambient (Fig. 1A) conditions. However, differences between storage conditions and seed colonization were not significant ($P = 0.5$). The correlation between time and colonization of seed by *C. crotalariae* can be best demonstrated by a regression line. For example, Fig. 1D was prepared showing the data and regression line representing seed colonization of NC 6 cultivar stored under ambient and refrigerated conditions.

C. crotalariae was isolated more frequently ($P = 0.05$) from seed of cultivars NC 6 and VA 81B, each highly susceptible to the fungus, than from Florigiant (Fig. 1A and B). Because no differences ($P = 0.05$) in colonization frequency of *C. crotalariae* were shown for storage at ambient and refrigerated (5 C)

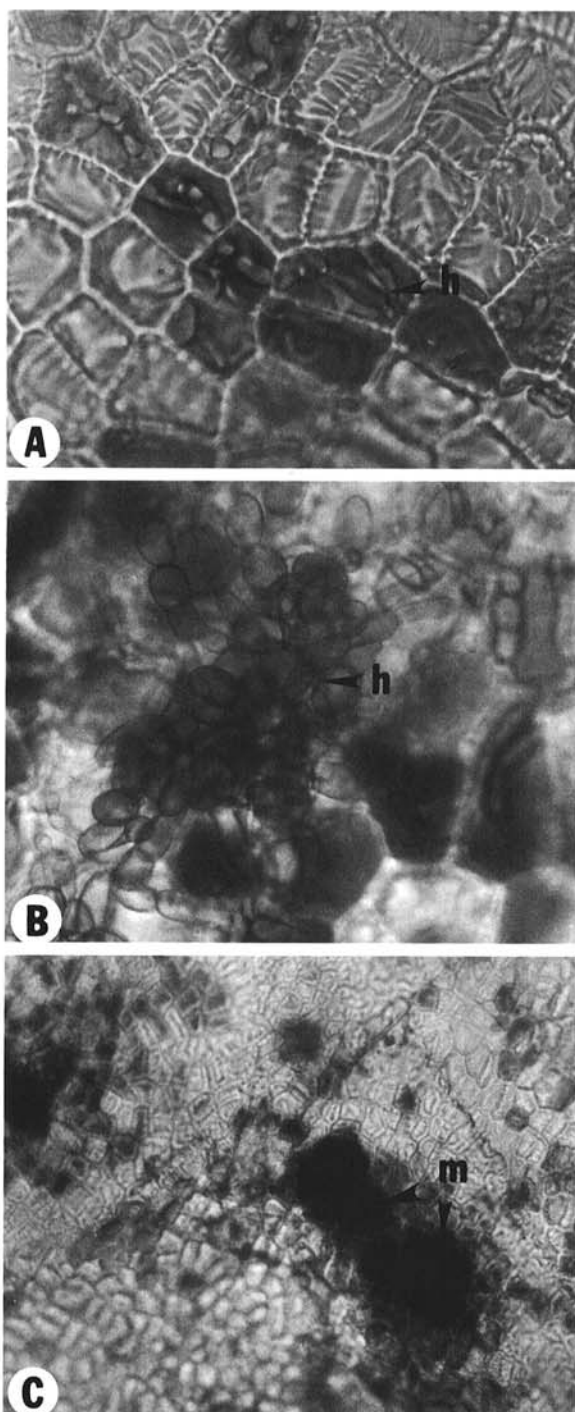


Fig. 3. Colonization of peanut testa by *Cylindrocladium crotalariae*. A, Surface view of cleared testa showing sclerenchyma cells and hyphae (h). B, Proliferation of hyphae below the sclerenchyma cells. C, Microsclerotia (m) in the testa.

conditions, data from Fig. 1A and Fig. 1B were combined, and regression lines for each cultivar during the 7-mo storage are given in Fig. 1E. Additional cultivar differences in colonization of seed by *C. crotalariae* are shown in Fig. 1C. The relationship of seed colonization and storage time are shown in Fig. 1F. Seed of NC 8C, a cultivar with resistance to *C. crotalariae*, were colonized less frequently than seed from susceptible cultivars (NC 6 and VA 81B). After 7 mo of storage, seed colonization of NC 8C was less than 1%.

Colonization of peanut seed by *C. crotalariae* showed a positive linear relationship, with a high degree of correlation to disease severity in the field for cultivars NC 6 and VA 81B (Fig. 2).

For example, colonization of NC 6 seed over the 7-mo period was 8.2% in a field (Fig. 1A) with a disease incidence of 35%, and 16.3% in a field (Fig. 1C) with a disease incidence of 80%. At other sites, VA 81B seed from plants exhibiting 45% disease incidence (Fig. 1B) were colonized at 12.0% (mean of 7-mo study), whereas seed from plants exhibiting 25% disease (Fig. 1C) incidence were colonized at 4.7%. Colonization of seed of NC 8C, a resistant cultivar, was about one half that of a susceptible cultivar, VA 81B, even when field incidence levels in the susceptible and resistant cultivars were 25 and 35%, respectively (Fig. 1A and C). The higher the frequency of *C. crotalariae* in seed at initiation of storage, the higher the frequency of seed

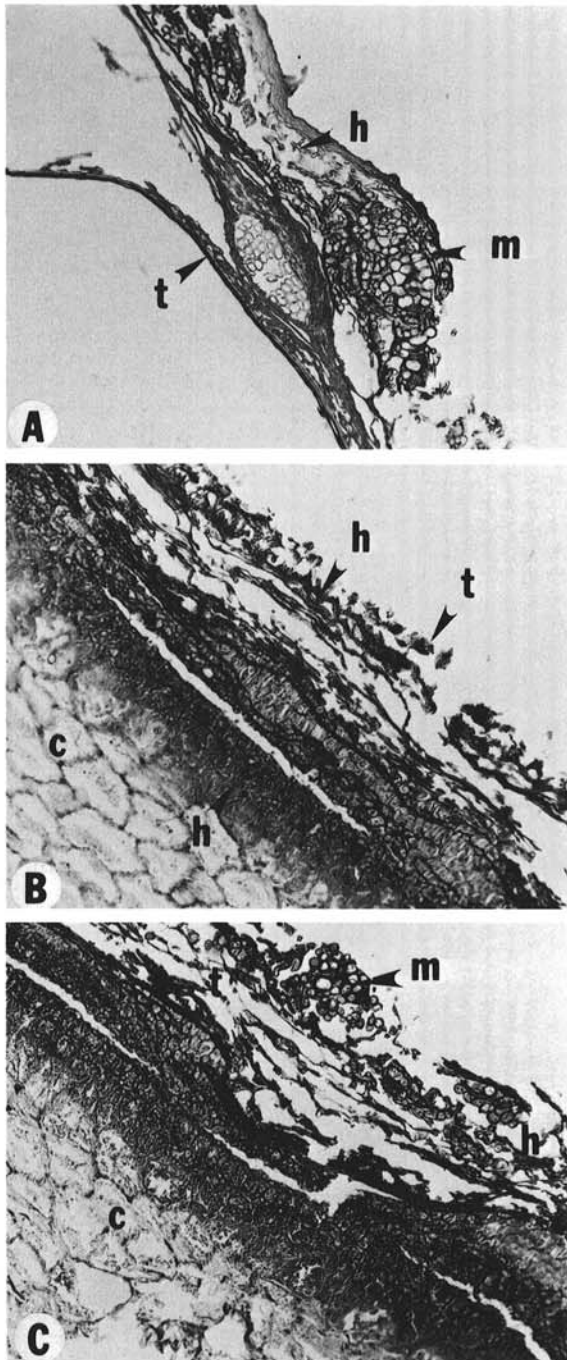


Fig. 4. Hyphae (h) and microscerotia (m) of *Cylindrocladium crotalariae* in testa (t) and cotyledons (c) of peanut seed. A, Hyphae and microscerotium below sclerenchyma cells of testa. B, Hyphae in testa and cotyledonary cells. C, Disruption of testa cells caused by the formation of a microscerotium.

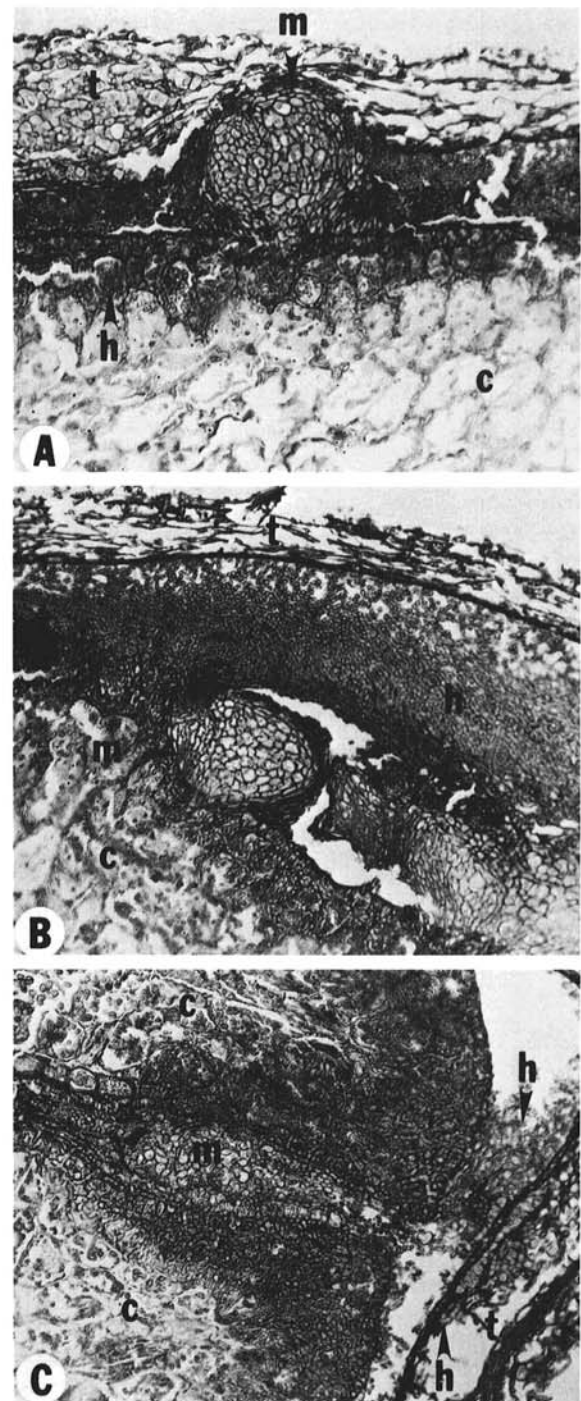


Fig. 5. Hyphae (h) and microscerotia (m) of *Cylindrocladium crotalariae*. A, In the testa (t); B, In the cotyledon (c); C, Developing between the cotyledons.

infection at the end of storage, regardless of temperature (Fig. 1A-C).

In histological studies of seed having dark brown testae (seed normally removed during the sorting process), we observed that testae and cotyledonary tissues were readily colonized by ramifying intercellular and intracellular hyphae. In seed with light brown testae (those not removed in the sorting process), hyphae of *C. crotalariae* were localized mainly in testa cells. Hyphae permeated areas between the sclerenchyma cells of the testa (Figs. 3 and 4) and proliferated profusely to form microsclerotia (Fig. 5), demonstrating the importance of removing discolored seed from the seed chain to reduce seed dissemination of the pathogen to uninfested areas. This is the first description of the colonization of peanut seed by *C. crotalariae*.

According to Bell (D. K. Bell, *personal communication*), *C. crotalariae* has not been isolated from commercial peanut seed lots treated with seed protectant fungicides. In our seed-treatment study, approximately 2.5% of untreated seed yielded *C. crotalariae*, whereas the fungus was not isolated from peanut seed treated with any of five seed protectants, including both broad spectrum fungicides (e.g., captan) or systemic fungicides (e.g., carboxin). Indeed, the effectiveness of seed protectants against *C. crotalariae* (10) may be due to fungal propagules (hyphae and microsclerotia) being localized on or in the testa (Fig. 5). With time, however, cotyledons might be invaded. Testa of such invaded seed would become discolored and would be removed during the sorting process. Johnson (6) has also indicated that *C. crotalariae* could be carried as a surface contaminant on peanut seed. The continued use of seed protectants to treat seed with superficial contaminants in conjunction with improvements in the sorting process to remove peanut seed with discolored testae from the seed chain could dramatically decrease the potential of seed dispersal of *C. crotalariae*.

Although the mechanisms of long-distance spread of *C. crotalariae* are unknown, transmission of the pathogen by infested seed from one area to another has been implicated. In this study, we demonstrated that the probability of transmitting *C. crotalariae* by seed is remote, provided seed are 1) properly screened to eliminate shriveled, diseased seed; 2) are sorted (by hand or electric eye) to remove seed with discolored testae; and 3) are treated with a protectant fungicide before planting.

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