Histopathology of Soybean Seeds Infected with *Alternaria alternata*

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ABSTRACT

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Mature and immature soybean (*Glycine max*) seeds infected with *Alternaria alternata* were small and shrunken with light to dark brown lesions. The fungus was recovered from 100% of surface-sterilized seeds exhibiting symptoms. Hyphae and conidia of *A. alternata* were concentrated near the hilum region on the surface of seed coats of infected seeds. In histopathological studies, hyphae of the fungus were observed in all layers of the seed coat and endosperm. In moderately to heavily infected seeds, hyphal mats obliterated the endodermis and endosperm. A brown discoloration was observed in unstained seed coat, endosperm, and cotyledonary tissues of moderately to heavily infected seeds.

Alternaria spp. are found worldwide and cause Alternaria leafspot and are seedborne on soybeans (*Glycine max* (L.) Merr.) (9,15). The pathogen causes damping-off of soybean seedlings, but usually occurs on maturing plants. Because the disease usually occurs late in the growing season, yield loss is minimal (15). Infection of leaves, pods and seeds increases with delay in harvest. Symptoms of the disease include irregular to circular necrotic brown lesions with concentric rings on leaves and pods (15). These may coalesce to form large affected areas. Infected leaves senesce prematurely. Seeds infected with *Alternaria* spp. are shrunken and green to brown. Heavily infected seeds do not germinate (I. K. Kunwar, *unpublished*). High levels of seedborne *Alternaria* spp. have been associated with wet years (5), frost injury (19), and stink bug and bean leaf beetle damage (6,14). *Alternaria tenuissima* (Kunze:Fr.) Wiltshire is the most frequently associated with soybean seeds (11,14,15).

The seed coat of a soybean is covered with a cuticle and consists of three distinct layers, epidermis (one-cell-thick palisade layer), hypodermis (one-cell-thick hourglass cell layer), and endodermis (six-to-eight-cells-thick parenchymatous cell layer) (1).

In 1984, we consistently isolated *Alternaria alternata* (Fr.:Fr.) Keissler from naturally infected, surface sterilized soybean seeds showing symptoms of infection by *Alternaria* spp. Information on histopathology of soybean seeds infested with *A. alternata* is lacking. We report on histopathological studies of symptomatic soybean seeds naturally infected with *A. alternata*.

MATERIALS AND METHODS

In 1984, both asymptomatic and symptomatic soybean seeds were harvested from cultivars Corsoy and Cumberland plants in an experimental field plot at the Cruse Farm, University of Illinois Urbana-Champaign, Urbana. There were 24 treatments with three replications for each treatment in a randomized complete block. Each experimental unit was 12 × 90 m. Over all treatments, the average mean percentage for recovery of *Alternaria* sp. from Corsoy was 9.8 and from Cumberland was 31.0. The difference was significant ($P = 0.05$, LSD = 5.2). For our study, 100 symptomatic and asymptomatic cultivar Cumberland seeds each were selected at random from seeds representing all treatments and replications. Each symptomatic and asymptomatic seed lot was surface sterilized separately for 5 min with 0.5% NaOCl (10% Clorox) and then washed in two changes of sterilized deionized distilled water. The seeds were plated on 9-cm-diameter culture plates containing potato-dextrose agar (Difco Laboratories, Detroit, MI) acidified (pH 4.5) with lactic acid at five seeds per plate and incubated under 12-hr alternating cool fluorescent light (800 μEin/m^2/sec) for 5 days at 25 ± 2 C. Any fungus growing from the seeds was isolated and identified.

Component plating. One hundred asymptomatic and symptomatic seeds were soaked separately with one seed per test tube for 30 min in sterilized distilled deionized water. Soaked seeds were dissected aseptically into three components: seed coat and endosperm, cotyledons, and hypocotyl-radicle axis. Each component was surface sterilized as described previously and washed individually in sterilized deionized water. The components of individual seeds were plated on 9-cm-diameter culture plates containing three layers of moist filter paper (Whatman No. 2) and incubated as described previously.

Cleared whole mounts. Twenty-five asymptomatic and symptomatic seeds each were cleared and stained by boiling in lactophenol and 1% aqueous solution of trypan blue (20:1, v/v) for 10 min. Cleared seeds were dissected into seed coat, hilum region, endosperm, cotyledons, and hypocotyl-radicle axis components. Seed coats were dissected further into two components: epidermis plus hypodermis, and endodermis.

Histopathology. Asymptomatic and symptomatic seeds (25 each) were softened by soaking them separately in sterilized deionized water for 2 hr at 60 C, then fixed in formalin-acetic acid-50% ethanol (5:5:90, v/v) for 48 hr (4). Fixed seeds were dehydrated in a tertiary butyl alcohol series and embedded in paraffin (Paraplast, Sherwood Medical Industries, Inc., St. Louis) (4). Serial microtome sections 10–20 μm thick were cut and adhered to microscope slides with Haupt's adhesive (4). Sections were stained with safranin followed by one of the following: light green, toluidine blue O (13), or thionine and orange G (12). Some sections were mounted unstained. All sections were mounted in Canada balsam or Permount (Fisher Scientific Co., Fair Lawn, NJ) and studied by using bright-field microscopy.

RESULTS

Soybean seeds showing symptoms of infection by *A. alternata* were shrunken, with circular to irregular, light to dark brown discolored areas covering portions of the seed. Many immature infected seeds were small, shrunken, and green with scattered brown discolored areas (Fig. 1).

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The recovery of *A. alternata* from surface-sterilized asymptomatic and symptomatic seeds was 4 and 100%, respectively (Fig. 2). Other fungi were found infrequently.

Component plating. *A. alternata* was recovered from 100% of the seed coats, 30% of the cotyledons, but not from the hypocotyl-radicle axis of symptomatic seeds and from only 4% of the seed coats of asymptomatic seeds.

Cleared whole mounts. Hyphae and conidia of *A. alternata* were concentrated near the hilum region when present on the surface of seed coats (Fig. 3). *A. alternata* produced characteristic muriform conidia borne in long, often branched chains (Fig. 2). Conidia were 20–63 μm long, 9–18 μm thick at the base. Immature hyphae were hyaline to pale brown and mature hyphae were light to dark brown. Hyphal width ranged from 3.0 to 6.1 μm.

Hyphae were found within the hilum region and intracellularly in the hilar tracheid. Hyphae were sparse in the palisade cell layer, but abundant in the hourglass cell layer (Fig. 4). Occasionally hyphae were seen growing out of the lumen of hourglass cells. Hyphae were abundant in the endodermis, in the aleurone layer (Fig. 5) and in the parenchyma cell layer. In all cell layers, hyphae grew in a random fashion, while on the surface of the aleurone layer, they branched in a fanlike manner (Fig. 5). Hyphae were not observed on the surface or within the embryo. The seed coat and embryonic tissues in moderately to heavily infected seeds had a brown discoloration. No hyphae or discoloration was observed in asymptomatic seeds.

Histopathology. Hyphae of *A. alternata* were ecto- and endophytic in the seed coat and endosperm. Few hyphae were observed in the palisade cell layer. Hyphae were more abundant in the hourglass cell layer and were inter- and intracellular. Hyphae grew randomly, throughout this layer (Fig. 6). Hyphae also were present in the endodermis and endosperm. In moderately to heavily infected seeds, the endodermis and endosperm could not be distinguished because of the formation of a compact hyphal mat (Figs. 7 and 8). Hyphae were observed outside and inside the hilum, in the sclerenchyma, in the parenchyma, and within the hilar tracheid. Hyphae were not observed in any of the embryo tissues.

A brown discoloration was observed in the endodermis and endosperm tissues in unstained sections of lightly infected seeds (Fig. 7). In moderately to heavily infected seeds, the discoloration was light to dark brown and occurred in the seed coat, endosperm, and upper layers of the cotyledonary tissues (Fig. 8). No hyphae or discoloration was observed in sections of asymptomatic seeds.

DISCUSSION

The fungal hyphae of *A. alternata* could be distinguished from those of other fungi found in soybean seed coat tissues based on hyphal width and reaction to stains (8). The hyphal width of *Cercospora sojina* ranges from 0.8 to 1.6 μm, that of *Colletotrichum truncatum* from 3 to 11 μm, and that of *Phomopsis* from 3.8 to 8.7 μm. Other identifying characteristics of *C. sojina*, *C. truncatum*, and *Phomopsis* sp. have been published (8). Immature hyphae of *C. sojina* are light green when stained with safranin and light green and blue with trypan blue. Mature hyphal cells of *C. sojina* appear dark brown without staining and the cytoplasm in hyphal cells occasionally stains light green when stained with safranin and light green. Mature hyphae of *C. sojina* do not take trypan blue stain. Mature hyphae of *C. truncatum* were brown without staining, immature hyphae appeared green when stained with safranin and light green or blue with trypan blue, and both contained prominent oil globules. Immature and mature hyphae of *Phomopsis* were hyaline; they stained red or green when stained with safranin and light green and blue with trypan blue and lacked oil globules.

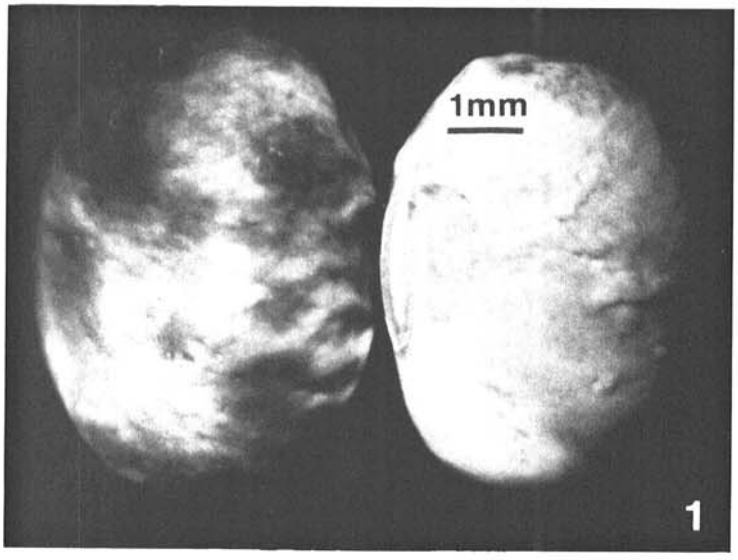
Hyphae of *A. alternata* were present ecto- and endophytically in soybean seed coat and endosperm, the latter being inter- and intracellular. The conidia were of the same shape and size as described previously (2). *A. alternata* was recorded in 30% of the cotyledons but in 0% of the hypocotyl-radicle axes of symptomatic seeds. Component plating of seed tissue allows for direct observation of the pathogen, but contamination during dissection may result in recording a higher incidence than actually occurs. Similar observations were made by Singh et al (17) studying the penetration of *A. sesamicola* in sesame seeds.

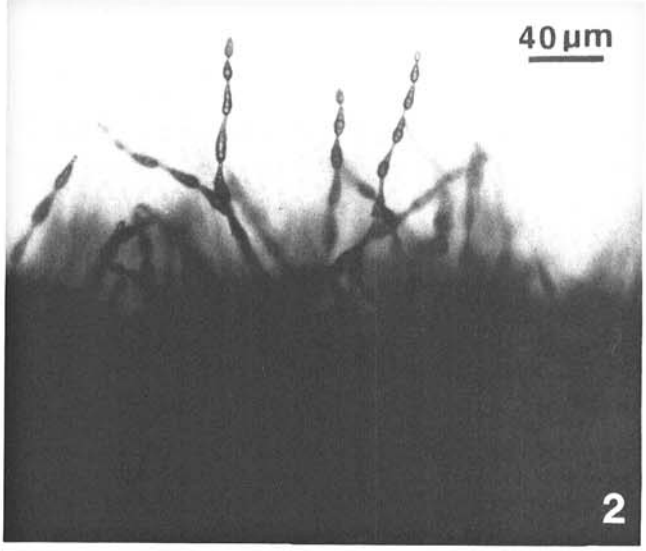
The aggregation of hyphae in the hilar region suggested that the fungus may have entered developing seeds through the funiculus and spread into the hypodermis. The fungus may penetrate seeds through epidermal pores (3) or cracks in the seed coat (20).

The anatomy of infected seeds differed little from that of noninfected seeds, suggesting that infection took place late in seed development. If infection took place early in seed development, tissue distortion would be expected. The extent of hyphal penetration may be related to seed moisture content.

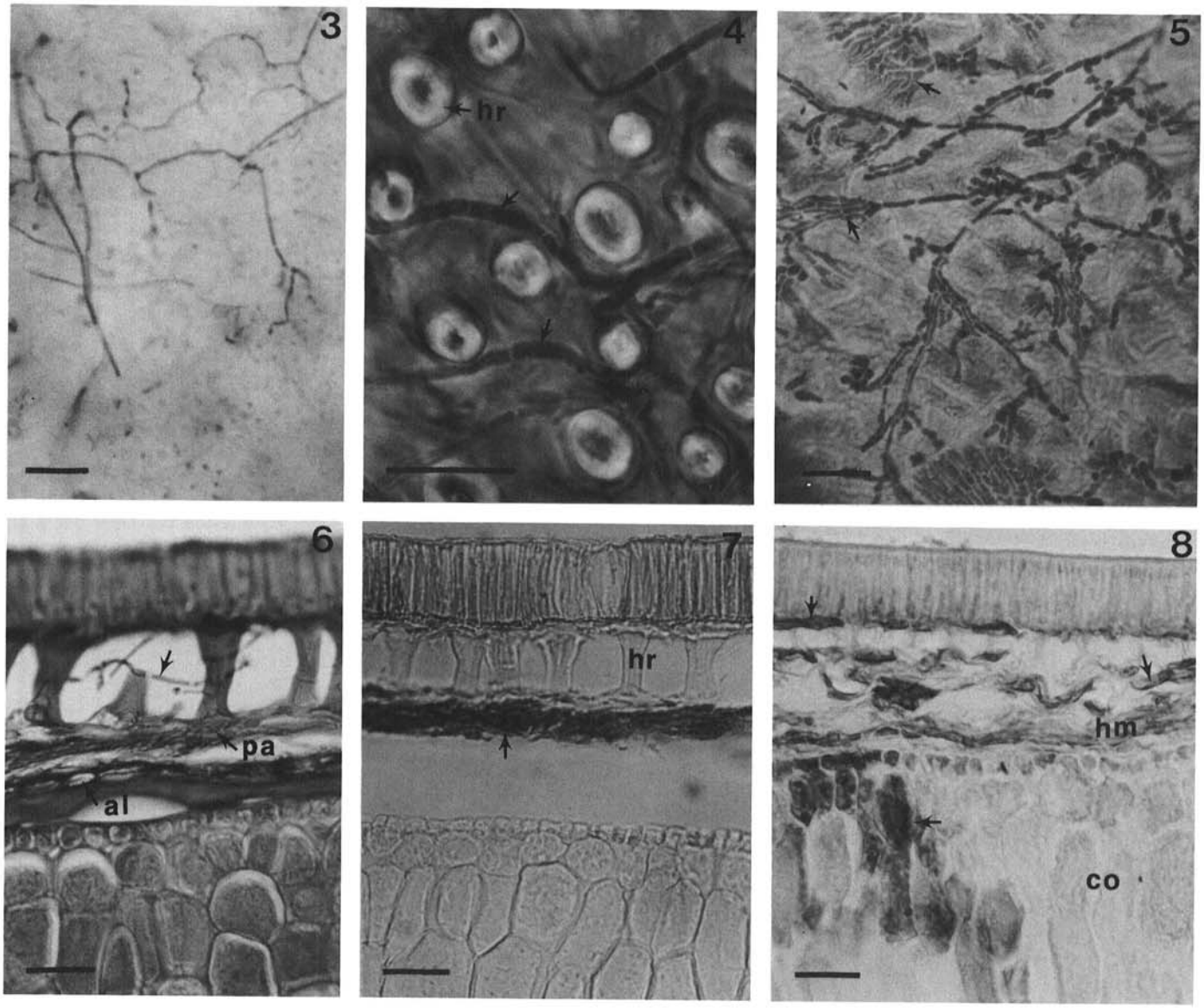
Internal browning of the seed coat and of a few cell layers of the cotyledons were consistently observed in infected seeds. The extent of the discoloration was related to hyphal penetration, with heavily colonized tissues having the greatest amount of discoloration. Tenuazonic acid produced by *A. alternata* is reported to cause necrotic lesions on leaves of various plants (7,10,18) and may be responsible for the discoloration in infected soybean seeds.

Hyphae of *A. alternata* were not observed in the embryo in either cleared whole mounts or in histopathological sections. Singh et al (17) in their studies on penetration of sesame seeds by *A. sesamicola*





Figs. 1–2. Soybean seeds infected with *Alternaria alternata*: **1,** brown discoloration of seeds; **2,** conidial chains growing from a surface-sterilized symptomatic seed.



Figs. 3–8. Photomicrographs of soybean seeds infected with *Alternaria alternata*. **Figs. 3–5,** cleared whole mounts: **3,** hilum region showing hyphae; **4,** seed coat with the hourglass layer on top in surface view showing hyphae (arrows) traversing among hourglass (hr) cells; and **5,** endosperm showing fanlike branching (arrows) of hyphae. **Figs. 6–8,** transverse sections of seeds: **6,** stained section of lightly infected seed, showing hyphae (arrows) in hourglass layer of the seed coat (note that parenchymatous [pa] and aleurone layers [al] are distinct); **7,** unstained section of moderately infected seed showing brown discoloration and hyphal mat (arrow) below the hourglass layer (hr); **8,** unstained section of severely infected seed with brown discoloration (arrows) in seed coat, endosperm, and cotyledon (co). Note hyphal mat (hm) in indistinguishable endodermis and endosperm. Scale bar = 40 μm.

Kawa., and penetration of sunflower seeds by *A. tenuis* (16) observed that the thick cuticle of the endosperm acted as a barrier to the penetration of the embryo by the pathogen. We did not see any similar morphological barrier in soybean seed embryos. This suggests that *A. alternata* was restricted from colonizing the embryo for some undetermined reason.

A. alternata was observed and isolated from 4% of asymptomatic seeds. Thus, the fungus can be seedborne without causing symptoms. We have shown for the first time that *A. alternata* can be seedborne in symptomatic and asymptomatic soybean seeds and that when the fungus is present, no other fungus was isolated or observed in symptomatic infected seeds.

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