

Histopathology of Mixed Infections by *Colletotrichum truncatum* and *Phomopsis* spp. or *Cercospora sojina* in Soybean Seeds

Indra K. Kunwar, Tribhuwan Singh, and J. B. Sinclair

Visiting research associate, former visiting assistant professor of botany, and professor, respectively, Department of Plant Pathology, University of Illinois at Urbana-Champaign, 1102 S. Goodwin Avenue, Urbana 61801. Present address of T. Singh: Department of Botany, University of Rajasthan, Jaipur, India 302004.

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ABSTRACT

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Histopathologic examinations of mixed infections of *Colletotrichum truncatum* and either *Phomopsis* spp. or *Cercospora sojina* in field-grown seeds of soybean (*Glycine max*) were made by using bright-field microscopy. *Phomopsis* spp. colonized seed coat and embryo tissues, and *C.*

sojina and *C. truncatum* were confined to seed coat tissues. *Phomopsis* spp. was restricted in its colonization of seed coat tissues when *C. truncatum* was present. There was an additive effect on deterioration of seed coat tissues when both *C. truncatum* and *Phomopsis* spp. were present.

Additional key words: *Alternaria* spp., *Cercospora kikuchii*, *Colletotrichum gloeosporioides*, *Fusarium* spp.

Cercospora sojina Hara causes frogeye leafspot of soybeans [*Glycine max* (L.) Merr.]; *Colletotrichum truncatum* (Schw.) Andrus & Moore causes soybean anthracnose; and the soybean *Phomopsis* spp. complex consisting of *Diaporthe phaseolorum* (Cke. & Ell.) var. *sojiae* Wehm., *D. phaseolorum* (Cke. & Ell.) var. *caulivora* Athow & Caldwell, their anamorphs of *Phomopsis*, and an unidentified *Phomopsis* spp., cause pod and stem blight, stem canker, and *Phomopsis* seed decay, respectively (13). All of these fungi are seedborne in soybeans, occur worldwide and reduce yields and seed quality (6,11,13,16). Seeds colonized by *C. sojina* are discolored light to dark gray and usually are brown near the hilum and crack perpendicularly to the hilum. Concentric rings of light and dark areas occur. Some seeds are papillate as described by Sherwin and Kreitlow (12). Seeds colonized by *C. truncatum* show small brown speckled areas that are moldy, dark, and shriveled; those colonized by the *Phomopsis* spp. complex are elongated, flattened, fissured, and are wholly or partially covered with mycelium (10,13). Seeds may be white or chalky and do not germinate. All of these fungi may infect soybean seeds without causing symptoms (13).

Histopathological studies on soybean seeds infected with *C. truncatum* show that this fungus usually colonizes seed coat tissues, but rarely embryo tissues, and forms acervuli in seed coat tissues (10,11). The more aggressive *Phomopsis* spp. penetrate seed coat and embryo tissues (6,10).

Field studies have shown that seedborne *Cercospora kikuchii* (T. Matsu & Tomoyasu) Gardner was antagonistic to seedborne *Fusarium* spp. and *Phomopsis* spp. in soybeans (5). Hepperly et al (4) showed that inoculating soybean pods with *C. truncatum* and *C. gloeosporioides* Peng. reduced *Phomopsis sojiae* in soybean seeds. However, no histological studies were presented in these reports. We used histopathological methods to study the colonization and penetration of soybean seeds by *C. truncatum* and *C. sojina* or *Phomopsis* spp. in mixed infections, and the nature of the antagonism between these fungi within soybean seeds.

MATERIALS AND METHODS

For the study of mixed infection of *C. truncatum* and *Phomopsis* spp., seeds of soybean cultivar Hobbitt (provided by Illinois Foundation Seeds, Inc., Tolono) were used. Some seeds showed symptoms caused by *C. truncatum*, some showed symptoms caused by *Phomopsis* spp., and some showed symptoms caused by both fungi. Many seeds were symptomless.

For the study of mixed infection by *C. sojina* and *C. truncatum*, seeds were used from soybean plants of an unknown cultivar that had been either uninoculated or inoculated with a conidial suspension to runoff in the field with isolate TN-140 of *C. sojina* (16) and showing symptoms of *C. sojina*.

One hundred symptomatic and asymptomatic seeds each were surface-sterilized with 0.5% NaOCl (10% Clorox) for 5 min and washed in two changes of sterile deionized water before they were plated on acidified (pH 4) potato-dextrose agar (APDA) (Difco). All fungi were isolated from the seeds and identified by cultural characteristics and fruiting structures.

Studies of whole tissues. Twenty-five seeds each of asymptomatic and symptomatic seeds from either uninoculated plants or plants inoculated separately with *C. sojina* were used. The seeds were boiled in deionized distilled water for 2–3 hr and then dissected into tissue groups of seedcoats, endosperms (aleurone layer), cotyledons, and hypocotyl-radicle axes. Each tissue group was cleared and stained separately by boiling in lactophenol containing 1% trypan blue (5:1, v/v) for 5–10 min in a test tube. Seedcoats, aleurone layers, and hypocotyl-radicle axes were mounted separately on microscope slides, cotyledons were squashed under a coverslip, and all were observed under a bright-field microscope.

Histopathological studies. For microtome sectioning of cultivar Hobbitt seeds, separate samples of 25–30 symptomatic and asymptomatic seeds were boiled in deionized distilled water for 2 hr. For seeds of the unknown cultivar, 35 seeds each of symptomatic (naturally infected) and asymptomatic seeds from uninoculated plants and symptomatic seeds from inoculated plants were boiled for 2 hr in deionized distilled water. One or two transverse incisions were made into the seeds to ensure dehydration, infiltration, and embedment. All seeds were fixed in 70% ethanol for 48 hr, dehydrated through a tertiary butyl alcohol series, and embedded in Paraplast (Sherwood Medical Industries, Inc., St. Louis, MO) (7). After solidification, the paraffin blocks were cut to expose one

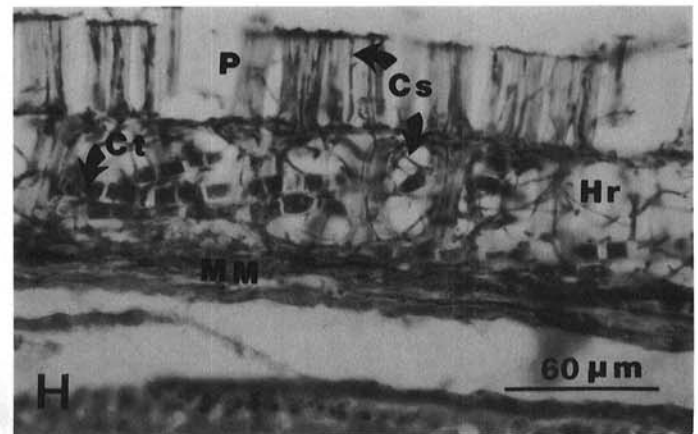
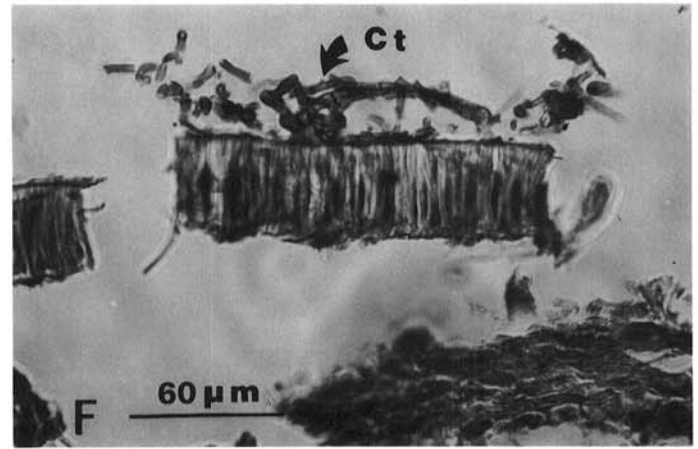
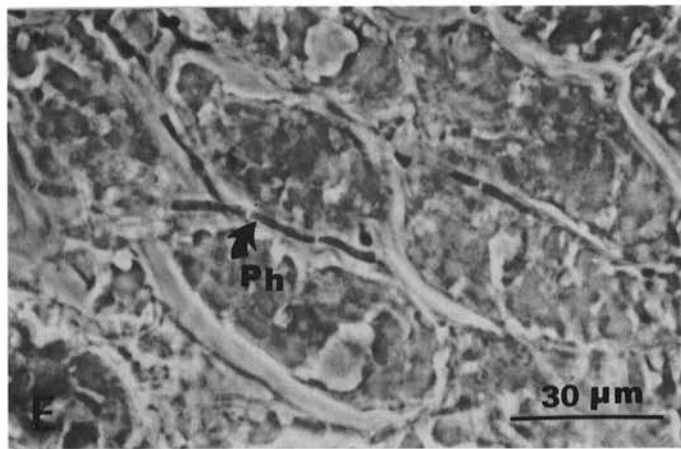
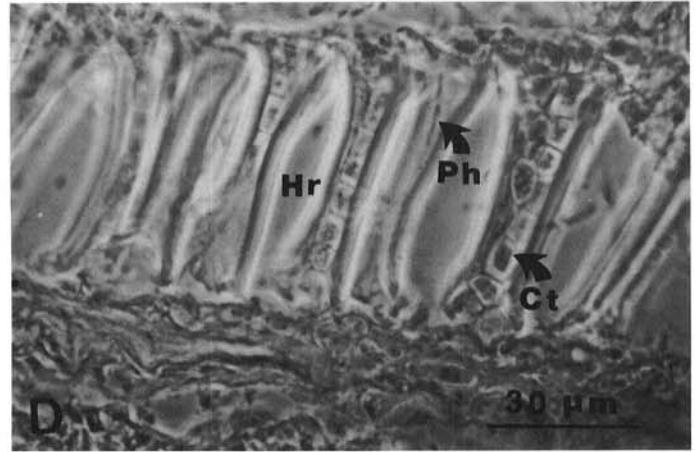
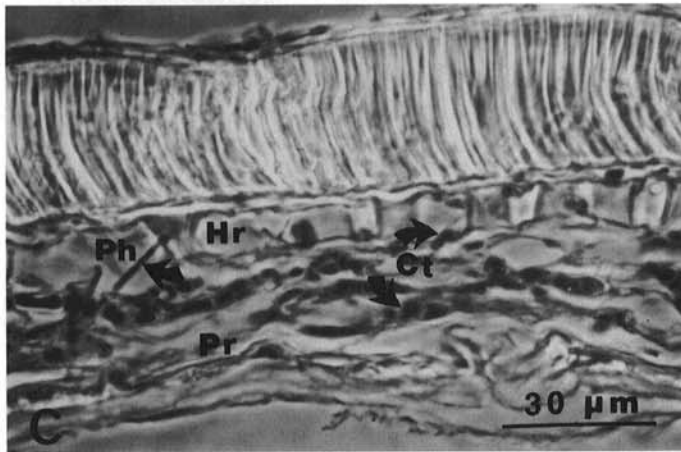
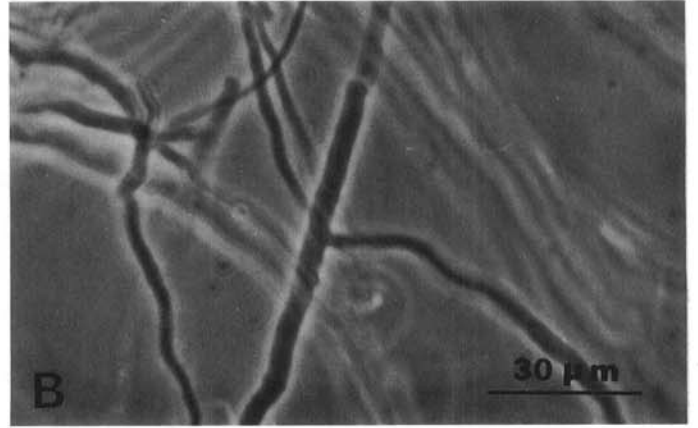
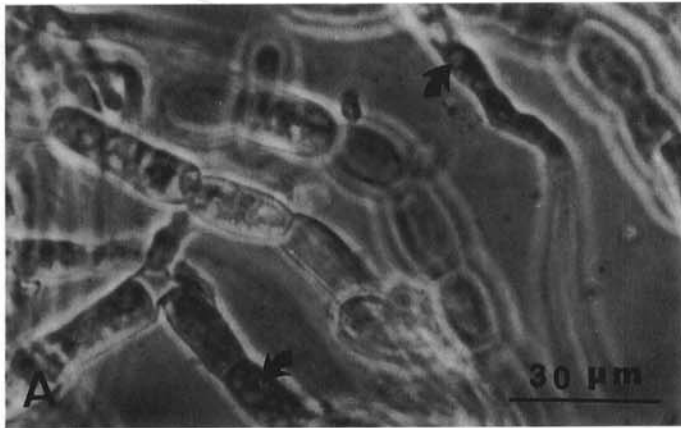


Fig. 1. Photomicrographs of **A**, mycelium of *Colletotrichum truncatum* showing prominent oil globules (arrows); **B**, mycelium of *Phomopsis* spp. without distinct oil globules. **C-H**, Transverse sections of soybean seeds showing mixed infections: **C**, seedcoat showing hyphae of *C. truncatum* (Ct) and *Phomopsis* spp. (Ph) in hypodermis (Hr) and parenchyma (Pr); **D**, intracellular hyphae of *C. truncatum* (Ct) and *Phomopsis* spp. (Ph) in hypodermal cells (Hr); **E**, intercellular hyphae of *Phomopsis* spp. in cotyledonary cells; **F**, dark mycelium of *C. truncatum* (Ct) above palisade layer; **G**, endocarp of the pod wall attached to the seedcoat showing acervuli (Ac) within it (Pe), intact palisade (P) and hypodermis (Hr), mycelial mat in the endodermis and endosperm (MM), indistinguishable cotyledonary cells (CC); **H**, mycelium of *C. sojae* (Cs) in the palisade (P), and *C. sojae* (Cs) and *C. truncatum* (Ct) in the hypodermis (Hr), and mycelial mat (MM) in the endodermis and endosperm.

side of the seed tissues. The blocks were softened by immersion in aqueous 1% sodium lauryl sulphate for 24 hr, then washed in deionized distilled water and transferred to a mixture of glycerol and glacial acetic acid (1:1) for 7 days (14). Serial microtome sections were cut at 10–20 μm , deparaffinized, stained with safranin and light green, and mounted in Canada balsam (7).

RESULTS AND DISCUSSION

Studies on whole tissues. Soybean seeds consist of a seed coat, endosperm (aleurone layer and parenchymatous cells), and an embryo made up of two large, fleshy cotyledons, a plumule, and a hypocotyl-radicle axis. The seed coat contains three distinct layers; epidermis (palisade cell layer), hypodermis (hourglass cell layer), and endodermis (parenchyma) (2,9). No mycelium was observed in any tissues of asymptomatic seeds. Seeds with symptoms of infection by *C. sojae*, *C. truncatum*, or *Phomopsis* spp. alone showed the presence of the respective hyphae in the seed tissues similar to that described previously (6,10).

These fungal hyphae could be distinguished from those of other fungi found in soybean seed coat tissues based on hyphal width and reaction to stains (6,10,14). The hyphal width of *C. sojae* ranges from 0.8 to 1.6 μm , that of *C. truncatum* from 3 to 11 μm , and that of *Phomopsis* from 3.8 to 8.7 μm . Other identifying characteristics of *C. truncatum* and *Phomopsis* spp. were recorded from the trypan blue mounts from the fresh APDA cultures (Fig. 1A and B). Immature hyphae of *C. sojae* are light green when stained with safranin and light green and blue with trypan blue. Mature hyphal cells of *C. sojae* 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. sojae* 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* and of *Fusarium* 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 *Alternaria* and *Fusarium* were not found in any of the tissues studied. In other studies, mature and immature hyphae of *Alternaria* were dark brown without staining.

***Colletotrichum truncatum* and *Phomopsis* spp.** Seeds showing symptoms of mixed infection by *C. truncatum* and *Phomopsis* spp. had intercellular hyphae of both fungi in the three seed coat layers. Hyphae were abundant in the hypodermis, where large intercellular spaces are present (Fig. 1C). The large intercellular spaces allow for more growth and easier observation than do the closely packed palisade cell layer and endodermis. Hyphae of both fungi occurred intracellularly in the hypodermis (Fig. 1D). In the palisade cell layer, hyphae grew parallel to the cell walls, while in the other two cell layers, the hyphae grew randomly. The hyphae of *C. truncatum* were more abundant than those of *Phomopsis* spp. throughout the three cell layers. Hyphae of *Phomopsis* spp. were found in the intercellular spaces of the cotyledons, and the contents of cells in proximity to hyphae had coagulated and their protoplasts had ruptured (Fig. 1E). Some cells had prominent nuclei and large vacuoles. Mycelial mats were formed by both fungi in the endodermis and endosperm (Fig. 1G). Mycelial mats stained green with fast green.

Neither fungus was observed in the hilum or stellate parenchyma. This indicates that the fungi may have penetrated the seeds through epidermal pores or cracks in the seed coat. Wolf and Baker (15)

suggested that breaks in soybean seed coats, the occurrence of which is genetically controlled (8), provide penetration sites. Schneider et al (11) showed that *C. truncatum* was localized in wounds in the soybean seed coat and suggested that other fungi could enter through these wounds. Occasionally the dark mature mycelia of *C. truncatum* were found on the surface (Fig. 1F) and in the palisade and parenchyma cells of the seed coat.

In the endosperm, cells in close proximity to hyphae of *C. truncatum* were empty and collapsed, suggesting that these cells might have been enzymatically dissolved by the fungus (1,3,10). Mycelia of *C. truncatum* were observed occasionally on the abaxial side of the cotyledons and stained green, but were never observed in the intercellular spaces near the abaxial side of the cotyledons. Some seeds were shrivelled with portions of the endocarp of the pod wall still attached. The endocarp of the pod wall on such seeds was covered with acervuli of *C. truncatum* (Fig. 1G) in the seed. Although the mycelia of *C. truncatum* were abundant in the palisade, hourglass, and parenchyma of the seed coat, the palisade and hypodermis remained intact while the cotyledons were shrivelled to the extent that individual cells were indistinguishable. However, hyphae of *Phomopsis* spp. could be seen in what remained of the cotyledons.

***Cercospora sojae* and *Colletotrichum truncatum*.** The various seed components (seedcoat, aleurone layer, cotyledon, and hypocotyl-radicle axis) of the whole-mount preparations separated easily. Hyphae of both fungi were observed in the seedcoat and aleurone layer. No hyphae were observed in asymptomatic seeds. In microtome sections, the hyphae of *C. truncatum* and *C. sojae* in a mixed infection were present outside the seedcoat and were both inter- and intracellular in the palisade cell layer and hypodermis (Fig. 1H). The hyphae grew parallel to the cell walls in the palisade cell layer and at random in the hypodermis. The hyphae of both the fungi were intercellular in the endodermis. Hyphae of *C. sojae* were more abundant than those of *C. truncatum*. Mycelial mats were formed by both fungi in the endodermis and endosperm.

Phomopsis spp. penetrated more of the seed tissues than either *C. sojae* or *C. truncatum*, which agrees with the results of others (10,11). The mycelium of *C. truncatum* may not compete well with that of *Phomopsis* spp. during penetration, but the abundance of hyphae of the former fungus found within the seed coat tissues suggests that it competes in colonization of the seed coat. Hepperly et al (4) found that as the quantity of *C. truncatum* increases there is a concomitant decrease in *Phomopsis* spp. Our results confirm these observations. In mixed fungal infections of soybean seedcoats by *C. sojae* and *C. truncatum*, hyphae of *C. sojae* were more abundant than those of *C. truncatum*, suggesting an antagonistic effect between them.

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