Erwinia-Induced Internal Necrosis of Immature Cotton Bolls

L. J. Ashworth, Jr., D. C. Hildebrand, and M. N. Schroth

Plant Pathologist, Crops Research Division, ARS, USDA, U.S. Cotton Research Station, Shafter, California 93263; and Plant Pathologists, Department of Plant Pathology, University of California, Berkeley 94720, respectively.

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ABSTRACT

Internal necrosis of immature cotton bolls was observed in the San Joaquin Valley of California in 1965. The necrotic tissue was reddish brown in color, and affected locules had a soft, slimy consistency. Affected seed coats also were discolored, and the seed contents often were completely decayed. Lint of infected mature open bolls was tan in color, and the locules were compact instead of having the white and fluffy consistency of healthy mature bolls. A bacterium, probably a strain of *Erwinia herbicola*, was associated with the disease. It induced typical symptoms of the disease when flowers and bolls were inoculated. In absence of insect transmission, locular necrosis appeared to depend on occurrence of supernumerary carpels. Development of this secondary internal boll splits the placentae and results in an opening to the outside of the bolls and in intertwining of the fibers of affected locules. This allows the bacterium to enter the boll, where it spreads from the interplacentae space into locules. Phytopathology 60:602-607.

Xanthomonas malvacearum is the only pathogenic bacterium generally accepted to gain entrance to carpellary tissue through stomata of developing cotton bolls (*Gossypium hirsutum* L.). *Bacillus subtilis*, however, was reported to directly penetrate the pericarp of the boll (5). Less aggressive species of bacteria also are reported to cause boll decay. *Bacillus gossypina* (28) was described as a cause of internal rot of bolls that have a small opening to the outside as a result of imperfect joining of carpel tips. Galleries of lepidopterous insects provide an avenue of entry for *Erwinia aroideae*, *Bacillus pumilus*, and *Aerobacter aerogenes*, according to Cognee & Frinking (12). Morrill (23) suggested that *Chlorochroa ligata* transmitted *B. gossypina*. Other hemipterous insects were suggested as possible vectors of unidentified bacteria (11, 24, 25). Still other workers have attributed boll rot diseases to bacteria without describing either the bacteria or the methods by which they gain entry to the cotton boll (6, 7, 9, 15, 18, 29).

This paper reports on an *Erwinia* sp., similar to *E. herbicola* (Duggeli) Dye, that infects the placentae of normal cotton bolls and causes severe intra- and interlocular necrosis.

MATERIALS AND METHODS.—Observations on internal necrosis of uninjured immature cotton bolls were made in 1965 at six locations in the southern San Joaquin Valley of California. Seven hundred to 1,600 bolls (2-4 weeks old) were collected at each location, split longitudinally, and examined for necrosis of placentae and locule contents. Normal bolls were separated from bolls having supernumerary carpels so that the amount of necrosis occurring in the two types of bolls could be determined. Supernumerary carpels are small bolls that sometimes develop between the placentae at the base of outwardly normal-appearing bolls. Supernumerary carpels often abort, but sometimes produce one to several seeds (8, 22). They usually split the placentae, causing intertwining of fibers (hair cells of the seed coat epidermis) of affected locules. Low temperatures have been associated with occurrence of supernumerary carpels of *G. hirsutum* (8, 10, 22) and *G. herbaceum* (8), although Beckett (8) and McMichael (*personal communication*) suspected that the condition is governed by a hereditary character whose expression is markedly influenced by environmental conditions.

Thin hand sections and fresh, frozen sections (20-30 μ thick) were prepared from necrotic placental tissue and discolored seeds from field specimens. Fibers were mounted in water for observation. The slimy material coating the fibers and seeds of necrotic locules also was mounted in water for observation.

Isolations were made from tissue of 200 bolls collected from a field in which about 4% of the bolls were affected with locular necrosis. The bolls were surface-disinfested by soaking them for about 1 hr in a 0.5% sodium hypochlorite solution. While wet, the bolls were split longitudinally, and pieces of necrotic placental tissue were aseptically transferred to potato-dextrose agar (PDA) plates. Discolored seeds and pieces of discolored fibers were aseptically transferred to tubes containing a small amount of sterile water, then mashed with a sterile glass rod. The mashed tissue was serially diluted in melted PDA. Single colony transfers were made from these plates after 3-5 days. Single colonies of bacteria from placental tissue were obtained by serially diluting colonies developing on agar plates. The number of bacteria on the surface of bolls was determined by shaking five sets of five bolls in new plastic bags containing 10 ml of sterile water. Two 1-ml samples from each bag were diluted 1:1,000 in PDA. The number of colonies developing in these plates was the basis for estimating the number of bacteria on the surface of bolls.

Flowers and bolls in situ were inoculated in the field, but only bolls were inoculated in greenhouse experiments. Current-day, 1-day-old, and 2-day-old flowers were atomized with a dense water suspension of bacteria. The inoculations were made in the late afternoon to delay desiccation of inoculum. Bolls from the flowers were examined for internal necrosis after 3 weeks. Three hundred 3-week-old bolls were used, instead of flowers, in a similar test. One hundred bolls sprayed

with water and covered for 4 days, as above, served as a control for the experiment. Two hundred bolls were atomized with a suspension of bacteria; 100 bolls were not covered and 100 were covered with glycine bags for 4 days. One isolate that necrosed locules when it was injected into bolls was used in the tests described above. Observations for internal necrosis were made after 3 weeks. All other boll inoculations were made by injecting a droplet of a dilute water suspension of bacteria into one or more locules/boll by a syringe with a 26-gauge needle. Most bolls were examined for infection after 10-14 days, but some bolls were allowed to mature.

Morphological and physiological comparisons were made among 11 pathogenic isolates of the *Erwinia* sp. from cotton and several other *Erwinia* species. The Hugh-Leifson peptone basal medium (19) was used for sugar tests, and other physiological tests were made according to standard methods (27), except for β-glucosidase (17). In a pathological comparison among *Erwinia* species, 10 bolls were inoculated in situ with each bacterium, and their pathogenic potentials were determined 10 days after inoculation. In this test, 12 isolates of the *Erwinia* from cotton bolls were compared with isolates of other *Erwinia* species.

RESULTS.—*Observations on occurrence of internal necrosis of immature cotton bolls.*—Internal necrosis of immature bolls was brought to our attention in August 1965 by J. L. McGurk. The bolls were free of obvious mechanical injuries, such as caused by insect larvae, which might provide an avenue of entry for microorganisms. The range of symptoms observed is illustrated in Fig. 1. Damage, in most cases, was confined to the central part of the placental column where the placentae meet. Necrosis of the placental column always was from the stylar end of the boll. It often was confined to 2-4 mm below the tip of the boll, where a certain amount of darkened tissue is an apparently normal result of stylar dehiscence. Necrosis could occasionally, however, extend to near the base of the placental column (Fig. 1-A). Locules of affected bolls (Fig. 1-A, D) were reddish brown in color, soft, and slimy. Seed coats in affected locules also were discolored, and the seed contents often were completely decayed (Fig. 1-D). Fibers of infected mature open bolls are tan in color, and the locules are compact or "tight" (3) instead of white and fluffy like locules of healthy mature bolls (Fig. 1-C). These fibers, however, appear to be intact. Tight locules of affected bolls do not adhere to the dry carpel walls at maturity, but may fall to the ground (Fig. 1-C).

About 7,000 bolls from six locations were examined for internal necrosis. Bolls with obvious mechanical penetrations of the carpels were excluded from the study. The amounts of placental column- and locule-necrosis observed are in Table 1. Placental column necrosis was considerably more common than locule necrosis. Locule necrosis was not observed in one of six fields. We observed a positive relationship between bolls with supernumerary carpels and occurrence of locule necrosis. It is not an exclusive relationship because a mean of 0.4% of normal-appearing bolls had locule necrosis. In contrast, however, a mean of 21.6% of bolls with supernumerary carpels had locule necrosis. There also was more placental column necrosis in these bolls (26.7%) than in normal-appearing bolls (15.3%) (Table 2). Necrosis of normal-appearing bolls usually was confined to one locule, while all locules usually were affected in bolls with supernumerary carpels where the locule contents intertwined (Fig. 1-B).

Microorganisms associated with internal necrosis and results of pathogenicity tests.—Examinations of sections of placental tissue revealed that fungal mycelia (usually coenocytic) were common in the central portion of necrotic placental columns. Bacterial cells also were common in the canal-like void of necrosed placental columns (Fig. 1-A). Only bacteria, however, were observed in decayed seeds and in the slime surrounding fibers of affected locules (Fig. 1-B, D). The lumen of fibers of affected locules appeared to be free of microorganisms.

Species of *Rhizopus, Mucor,* and *Cladosporium* were isolated from centrally necrosed placental columns. Three culturally dissimilar bacteria were isolated from placentae, intralocular slime, and from decayed seeds, but one type that produced white colonies on PDA predominated. Only bacteria were tested for pathogenicity, since fungi were not detected in locular material. Lint of water-injected control bolls was damaged, turning a yellow brown color, when injected with more than 0.2 ml of water (26).

A bacterium producing white colonies on PDA regularly induced the reddish-brown internal decay observed in the field. Five isolates of each of two less common bacteria were found to be nonpathogenic. When injected into locules, 12 out of 15 isolates of the white colony-type multiplied rapidly, inducing widespread discoloration and decay within 5 days. But as will be shown later, not all single colony reisolates were pathogenic. Bolls to about 25 days of age were highly susceptible to necrosis by the bacterium. Near-mature bolls (about 45 days of age) were much less susceptible. A significant amount of infection occurred in water-injected bolls in field tests (Table 3). We do not know whether the bacteria were carried into the bolls when they were injected with water, or whether they entered via puncture wounds in water films made by dew. Either avenue was possible, because dew is present on bolls for about 5 hr daily in the San Joaquin Valley (4). No infection of control bolls occurred in a greenhouse test (Table 3). The bacterium was detected on the surface of bolls in the field. We found, with five samples of bolls, about 10^4 cells of the bacterium/boll. Ten white colony-type isolates from the surface of bolls were tested for pathogenicity; seven isolates were pathogenic.

Mode of infection.—We sought to determine how infection occurs in normal bolls and in bolls with supernumerary carpels. Greater amounts of placental infection were induced when 1-day-old flowers (31.7% of 148 bolls) and 2-day-old flowers (30.0% of 93 bolls)

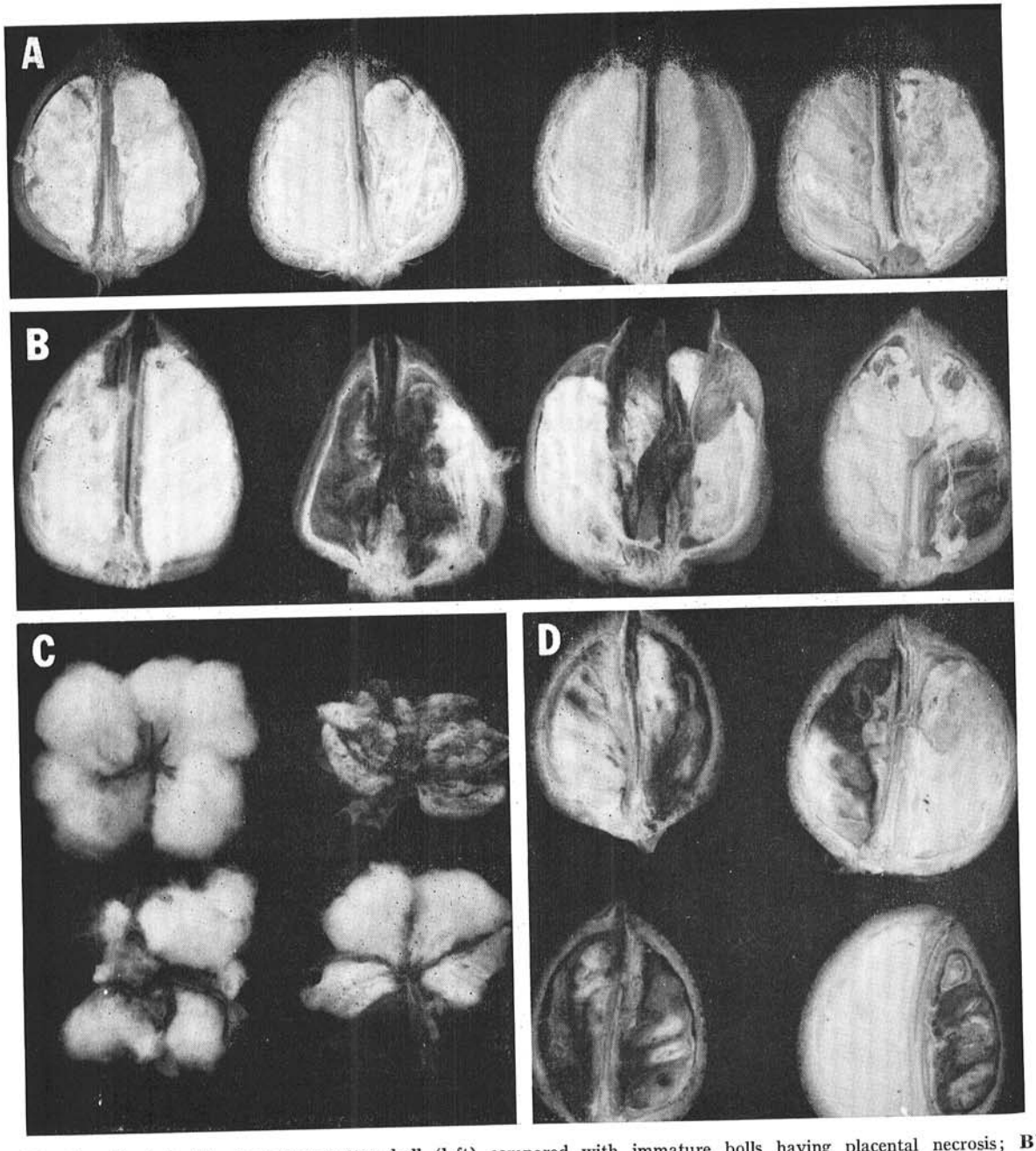

Fig. 1. A) A healthy immature cotton boll (left) compared with immature bolls having placental necrosis; **B)** natural-occurring locular necrosis of immature bolls, and bolls with supernumerary carpels (center) compared with normal bolls; **C)** mature normal cotton boll (upper left) compared with a mature infected supernumerary carpel boll (upper right) and mature infected normal bolls (bottom); an infected locule has dehisced from the lower right hand boll; **D)** necrosis induced by *Erwinia herbicola* from cotton bolls when injected into locules of immature bolls.

were sprayed with suspensions of the bacteria than when current-day flowers (2.1% of 188 bolls) were inoculated. Infection was limited to the placental column in this test in which no bolls with supernumerary carpels occurred. These results indicate that infection does not proceed from the central portion of the placental column into locules unless the placental column separating locules is ruptured by a supernumerary carpel. In another experiment, the amount of placental necrosis was not increased over the amount already present when 3-week-old bolls were sprayed with a suspension of bacteria and covered for 4 days with glycine bags. Therefore, placental infection may occur during (but not after) an approximate 48-hr period when flowers senesce and dehisce from young bolls. Data indicate that bolls with supernumerary car-

TABLE 1. Occurrence of internal necrosis of normal-appearing immature cotton bolls in the southern San Joaquin Valley of California in 1965

Dates of collection	Bolls observed	Internal necrosis		
		Placental column	Placentae and locules	Total
	No.	%	%	%
17 Aug.	1,462	10.1	0.9	11.0
27 Aug.	1,610	17.1	1.7	18.8
8 Sept.	1,445	21.9	1.0	22.9
22 Sept.	878	15.4	0.1	15.5
23 Sept.	696	10.8	0.3	11.1
28 Sept.	822	9.1	0	9.1
Mean		14.1	0.7	14.7

TABLE 2. Occurrence of internal necrosis of immature cotton bolls with normal carpels and with supernumerary carpels

Dates of collection	Internal necrosis of bolls[a]			
	Bolls with normal carpels		Bolls with supernumerary carpels	
	Placental column	Placentae and locules	Placental column	Placentae and locules
	%	%	%	%
27 Aug.	16.5	0.9	33.2	19.7
8 Sept.	21.9	1.0	33.7	21.6
22 Sept.	15.4	0.1	0	0
23 Sept.	13.8	0.1	66.6	66.6
28 Sept.	9.1	0	0	0
Mean	15.3	0.4	26.7	21.6

[a] Results based upon examination of a total of 5,431 bolls collected on the dates shown above.

TABLE 3. Results of inoculations of bolls with the cotton pathogen. The amount of necrotic locules observed 10-14 days after bolls were injected with sterile water or with water suspensions of one isolate of the bacterium

Boll age when inoculated	Water control		*Erwinia*	
	Bolls inoculated	Locule necrosis	Bolls inoculated	Locule necrosis
Days	*No.*	%	*No.*	%
Original single colony isolate[a], inoculations in the field				
7-10	37	24.3	34	83.3
15-25	106	23.6	116	54.3
A single colony reisolate[b], inoculations in the field				
15-25	84	10.7	170	70.5
A single colony reisolate[b], inoculations in the greenhouse				
7-10			80	95.0
15-25	20	0	80	90.0
35-50			80	20.0

[a] One of 33 pathogenic isolates from naturally infected bolls.

[b] A pathogenic single colony reisolate obtained during initial pathogenicity study.

pels are more prone than normal bolls to locular infection (Table 2).

The cotton pathogen.—An examination of the organism isolated from bolls suggested that it belonged in the genus *Erwinia,* as it was gram-negative, rod-shaped, peritrichous, and fermented glucose anaerobically. An investigation was initiated to compare this organism with other *Erwinia* species. Eleven isolates of the cotton pathogen were used in the tests. Other species tested were *E. amylovora* (two strains), *E. quercina* Hildebrand & Schroth (one strain), *E. chrysanthemi* (one strain), *E. carotovora* (four strains), and a strain of *Erwinia* isolated from insects (30) which we consider to be *E. herbicola,* based upon the discussion of Graham & Hodgkiss (16).

Results of our tests suggest that the cotton pathogen probably is a strain of *E. herbicola*; it and the strain of *E. herbicola* with which it was compared were alike in 12 of 15 physiological tests. On the other hand, the cotton pathogen had but 3, 5, and 7 similar reactions, respectively, with *E. amylovora, E. quercina,* and the soft-rotting *Erwinia* species (Table 4). The cotton pathogen, like *E. herbicola,* produced a yellow water insoluble pigment on Hugh-Leifson substrate (19), a characteristic which distinguishes *E. herbicola* from other *Erwinia* species. The cotton pathogen lacked β-glucosidase; it occurred in the other species tested (Table 4). This characteristic is more variable in *E. herbicola* than in other *Erwinia* species, with the exception of *E. amylovora* (D. W. Dye, *unpublished data*).

The cotton *Erwinia, E. carotovora, E. chrysanthemi,* and *E. aroideae* induced identical locule necrosis of cotton bolls. Unlike the other *Erwinia* species that necrosed locules, none of the isolates from cotton bolls necrosed carpel tissue (Table 5, Fig. 2).

DISCUSSION.—Bolls with supernumerary carpels have been associated with fungal-induced boll rot disease in Arkansas (14) and with a boll rot of unknown cause in Mississippi. There, Carns & Dick (10) reported that crop damage ranged from negligible to very heavy, with up to 56% of the bolls having supernumerary carpels. Obviously, *Erwinia* infection could present a serious problem under the conditions they described. Results of our observations indicate that bolls with supernumerary carpels are more prone to infection than are normal bolls. We observed 21.6% infection of abnormal bolls versus 0.4% infection of normal bolls in the San Joaquin Valley. But we observed an incidence of only 2.4% of bolls with supernumerary carpels, much less than observed in Mississippi (10). Necrosis of normal locules is attributed to insect transmission of the bacterium. The brown stinkbug, *Euchistus impictiventris* (Stal.), transmits the bacterium, resulting in up to 70% infection of immature bolls in the Imperial Valley of California (2). While the 0.4% infection of normal bolls observed in the San Joaquin Valley may have been the result of insect transmission, the status of transmission by the species indigenous to the San Joaquin Valley, *E. conspersus,* has not been determined.

We stated earlier that the cotton *Erwinia* was not observed within the fiber lumen, and that fibers of in-

TABLE 4. Physiological comparison of the cotton pathogen with several other *Erwinia* species

Medium or test	Cotton pathogen	*E. herbicola*	*E. amylovora*	*E. quercina*	*E. carotovora*	*E. chrysanthemi*
Lactose	+[a]	+	−	−	+	+
Maltose	−	+	−	−	+	+
Inositol	+	+	−	−	+	+
Inulin	+	+	−	+	+	+
Xylose	+	+	+	−	+	+
Sorbitol	+	+	v	−	+	+
Starch hydrolysis	+	+	−	−	+	+
Citrate utilization	+	+	−	−	+	−
Malonate utilization	+	−	−	−	v	−
Gelatin liquefaction	−	−	v	−	v	−
Nitrate reduction	−	−	−	−	+	+
Voges-Proskauer	+	+	−	+	+	+
Yellow pigment	+	+	−	−	v	−
Carrot soft rot	−	−	−	−	+	+
β-Glucosidase	−	+	+	+	+	+

[a] Plus = positive reaction; minus = negative; v = variable.

fected bolls appear to be intact at maturity. However, immature fibers of affected bolls are reddish brown, and mature fibers are tan rather than white when bolls open. This discoloration seems likely to be due to degradation of substances present in or on the primary wall of fibers. The thin primary wall, containing pectic substances as well as cellulose, envelopes the cell during the first 15-20 days of fiber development (1). Besides the pectic component of the primary cell wall, water soluble sugars and nitrogenous substances are associated with the surface of the wall (*unpublished data,* R. H. Garber, J. L. McMeans, & V. T. Walhood, U.S. Cotton Research Station, Shafter, Calif.). Thus, the primary wall appears to offer readily available substrates for growth of bacteria and other microorganisms. This probably explains why relatively young bolls suffer greater damage than bolls nearing maturity. For in near-mature and mature bolls, the primary wall of fibers occurs only as a thin, cuticlelike layer on the surface of secondary thickenings, which are essentially pure cellulose in commercially grown cotton varieties (21). Therefore, complete disorganization of fibers would be expected only if the organism were strongly cellulolytic.

TABLE 5. Necrosis of locule contents and of carpel walls of 15- to 25-day-old cotton bolls by *Erwinia* species

Erwinia species	Isolates tested	Necrosis	
		Locules	Carpel walls
	No.	%	
Cotton *Erwinia*	4	0	None
	8	20-100	None
Erwinia carotovora	5	20-100	None
	2	80-100	Moderate[a]
E. atroseptica	1	0	None
E. chrysanthemi	1	100	Severe[b]
	1	100	Moderate
E. aroideae	2	80-100	Severe

[a] Carpel necrosis not accompanied by mummification of the boll.

[b] Carpels severely necrosed, mummified, opening within 3-5 days after inoculation.

The type of boll rot we observed is similar to the disease reported by Stedman in 1894, and attributed to *B. gossypina* (28). He reported that the disease may or may not have external symptoms and that it affected up to 35% of the crop. He also reported that near-mature bolls were much less severely affected than young bolls by the disease, and that severe rotting occurred in bolls slightly open at the tip because of incomplete fusion of the carpels. According to Earle (13), Baker was unable to confirm the work of Stedman, but Eyles (15) listed *B. gossypina* as a cause of

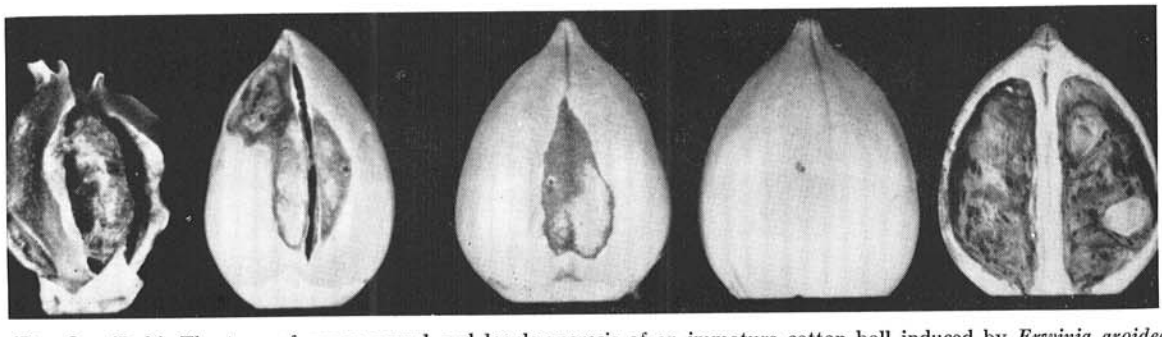

Fig. 2. (Left) The type of severe carpel and locule necrosis of an immature cotton boll induced by *Erwinia aroideae* and by a strain of *E. chrysanthemi*; moderate carpel necrosis induced by certain strains of *E. carotovora* and *E. chrysanthemi* (second and third bolls from left); necrosis-free carpels and necrotic locules of bolls inoculated with *E. herbicola* from cotton bolls (second from right); and certain strains of *E. carotovora* (right).

internal soft rot of cotton bolls in Rhodesia. The California *Erwinia* differs from *B. gossypina* in that it does not induce carpel necrosis. But this also was the case with five of seven isolates of *E. carotovora* tested in this study. Carpel necrosis is caused by *E. aroideae* as a field problem in Oklahoma (20). A strain of this bacterium received from R. E. Hunter, Oklahoma State University, Stillwater, in other tests (2), was transmitted to immature cotton bolls to which it induced carpel necrosis. But it is not known whether *E. aroideae* is associated with California cotton.

Our tests and others (12, 20, 28) suggest that internal necrosis with or without carpel necrosis can be caused by several *Erwinia* species. The formation of pectolytic enzymes, as indicated by ability to rot carrot root tissue, is not required for locule necrosis, since the organism found in affected bolls in California does not rot carrot. Other tests indicate that it belongs to the *E. herbicola* group rather than to the soft-rotting *E. carotovora* group of the genus. It compared favorably with *E. herbicola* in 12 of 15 physiological tests. Also like *E. herbicola*, it produced a yellow water-insoluble pigment that distinguishes the *E. herbicola* group from other members of the genus.

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