Structural Integrity of the Cotton Fruit and Infection by Microorganisms

L. J. Ashworth, Jr., and R. B. Hine

Departments of Plant Pathology, University of California, Berkeley 94720, and University of Arizona, Tucson 85721.

ABSTRACT

Crystal violet stain was absorbed in the interplacental cavity, but not in locules of immature bolls having intact subcarpellary bracts. However, stain was readily absorbed into the pithlike subcarpellary tissue and into 5-10% of the locules of bolls whose carpellary bracts were broken off. Stain appeared to be absorbed through the broken edges of bracts. Benomyl, a systemic fungicide, was detected in carpel tissue of immature bolls in 2 days, and in lint after 10 days, but not in seed coat nor embryo tissue, indicating that benomyl is absorbed and diffused slowly within boll tissue. Our results support observations on the ontogeny and anatomy of the cotton fruit which indicate that immature bolls are closed systems that are waterproof and sterile unless the integrity of either the carpel wall or placentae is destroyed, barring direct penetration by local or systemic invaders. Phytopathology 61: 1245-1248.

Additional key words: boll rots; fungal diseases; bacterial diseases; fruit anatomy and disease.

This report represents an attempt to reconcile apparent differences between ontogenetic and anatomic understandings of the immature cotton (*Gossypium hirsutum* L.) fruit (boll) and proliferating lists of possible or implied pathogens of the boll (7, 9, 10, 15, 19). The structural integrity of the immature boll must be a prime factor in considering the 75 or more species of microorganisms reported to attack bolls (19). Plant-pathological literature has been pervaded with the idea that immature bolls lack structural integrity since 1894, when Stedman (22) suggested that openings at the tips of bolls having imperfectly joined carpels provided access to locules for *Bacillus gossypina*. Cauquil & Mildner (9) and Cauquil & Ranney (10) interpreted results of their tests to support Stedman's idea, many years after ontogenetic and anatomic studies (11, 13) provided evidence that his position was untenable. Their differences with anatomists have not been reconciled.

Gore (13) showed that the 3-5 carpels which make up a boll arise separately from a common cone-shaped meristem. The carpels are at first crescent-shaped, with the margins curved inward toward the center of the developing boll. The carpels enlarge circumferentially from a dorsal meristem consisting of a line of light colored cells at the center of the developing carpel; growth soon brings the outer edges of carpels together and they fuse. From this point, the two incurving edges of carpels grow inward where they meet and fuse (13). Opposing carpels, therefore, are separated by a double wall or septum. The inner edges of individual carpels grow together to become the placentae from which arise the ovule-bearing placental ridges (11, 13). Therefore, individual carpels are distinct from one another except at their common point of origin. Doak (11) showed that the apices of young carpels are held together by pressure as they expand against the surrounding staminal column and, unlike developing carpels (11, 13), the individual segments of the staminal column are fused together early in their development. Furthermore, individual carpels, although originating from a common basal meristem, do not necessarily meet in the center of the boll, except perhaps near the top where they press against the staminal column prior to fertilization and dehiscence of the flower (11). The central core of the boll, then, is hollow, and the adjacent tissue is called the placental column (11). Carpels are slightly indented along the line of the dorsal meristem, which is referred to as the dorsal suture. The halves of the individual carpels separate along this meristem at maturity, thus accounting for loculicidal dehiscence of the fruit (13).

Results of ontogenetic and anatomic studies, therefore, indicate that locular contents of individual carpels of immature bolls are closed systems, being waterproof and sterile except when the integrity of either the carpel wall or placentae is destroyed (barring direct penetration by local or systemic invaders). This paper reports results of experiments on absorption of crystal violet and benomyl; the results are used to interpret structural integrity of immature bolls as it relates to entrance of potentially parasitic microorganisms.

Integrity of boll surfaces as determined by absorption of crystal violet.—Apparently injury-free unopened bolls with intact, subtending bracts and with bracts broken off, with peduncles cut off close to the base of bolls, were immersed for 2 and 24 hr in 0.75% aqueous crystal violet solution. This solution, according to Amsden & Lewis (1), has wetting characteristics similar to water (60 dynes/cm^2 compared to 73 dynes/cm^2 for water). After immersion, bolls were examined first to determine whether separation of carpels occurred during immersion, and those in which separation had occurred were discarded. This was necessary because it was found in earlier inoculation tests (*unpublished data*) that 1-4% of unopened carpels separated along the dorsal meristem when immersed only a short time in water or aqueous solution of disinfestant. We observed that initial separation of the halves of a carpel was not always readily evident to the eye; however, separation was revealed by application of slight pressure adjacent to the dorsal meristem which caused movement of one side of the carpel away from

TABLE 1. Absorption of crystal violet stain by detached immature cotton bolls

Stain absorption sites	Bolls without bracts		Bolls with bracts
	2 hr	24 hr	2 hr
Placental column, apex	%	%	%
Downward, 1-5 mm	12.0	67.7	5.9
Interplacental void	1.9	0.0	2.0
Nectaries	0.0	0.0	0.0
Subcarpellary tissue	100.0	100.0	1.0
Locules	4.6	9.7	0.0
Total bolls in test	108.0	31.0	101.0

the other which remained motionless. Bolls still intact after immersion in stain solution were examined to determine whether stain was absorbed.

Absorption of stain was similar at most sites, whether bolls had intact bracts or whether bracts were broken off before bolls were immersed in stain solution (Table 1). Stain was absorbed downward 1-5 mm from the boll apex into wall tissue of locules in 6-12% of the bolls within 2 hr, and in 68% of the bolls within 24 hr. The outer surfaces of the locule walls exposed in the interplacental void (canal) were generally free from stain, as were the nectaries where stain did not penetrate below the layer of papilliform cells which make up the nectary proper. Stain was preferentially absorbed into subcarpellary tissue and by lint of locules of bolls whose bracts were broken off before bolls were immersed in stain. Evidently, the stain was absorbed through the broken edges of bracts and then diffused quickly throughout the pithlike tissue basal to locules. In most instances, however, locules were free from stain after 2 and 24 hr. Little stain appeared to be absorbed by intact bracts (Table 1). Also, stain failed to move more than 1-2 mm from the cut surface of peduncles.

Absorption of benomyl by bolls.—In an initial experiment, immature bolls with bracts broken off were covered with water or water containing 500 ppm benomyl (Benlate, 50% WP, E. I. du Pont de Nemours & Co., Wilmington, Del.) in 0.5-pint jars which were covered and then shaken continuously (at 30 C) to keep benomyl in suspension. The wet bolls (25 bolls/treatment) were transferred to plastic bags after 48 hr, then incubated at 30 C for 8 additional days, making a total of 10 days when bolls were treated. Bolls free from cracks developing during treatment were washed twice with chloroform to remove external deposits of benomyl. A spore suspension of *Aspergillus flavus* (our isolate No. 606) then was injected into bolls, and the bolls were incubated in plastic bags at 30 C. After 5 days, the bolls were examined for infections.

Five of 25 and nine of 25 bolls cracked during treatment before inoculation in the water control and benomyl treatments, respectively, and were discarded. All locules of bolls in the water control (94 of 94) were infected with *A. flavus,* while only 4 of 68 locules (6%) were infected by the fungus in the benomyl

treatment, indicating that benomyl can be absorbed by intact bolls.

In a second test, 40 bolls each with bracts and with bracts broken off were treated with water and with benomyl as described above. In a third treatment, 25 bolls with bracts were immersed in a standing benomyl suspension which was allowed to settle overnight before receiving the bolls. Bolls were kept from contacting the precipitate by resting them on a porous plate suspended in the desiccator jar in which the bolls were treated for 10 days at 30 C. The bolls were washed with chloroform, as before, then air-dried at 30 C at the end of the treatment periods. When dry, the bolls were separated into carpel tissue, lint, seed coats (acid-delinted seed), and embryo tissues. These materials were ground to pass a 20-mesh screen, and 5-g samples of each were extracted by shaking in 200 ml of chloroform for 1 hr. The crude extract was filtered and, except for residues of embryo tissue, dried over a steam bath. Embryo residues failed to dry to less than about 2 ml-volume, due to oil in the extracts. Extract residues were diluted with chloroform (0.5-10 ml), then bioassayed for benomyl content. In each case, three replicates for each residue were tested as follows. One-, 3-, 5-, 7-, or 9-µliter portions of diluted extract residues were pipetted into 10-mm diam wells cut, with a cork borer, in the center of the agar surface of petri plates poured with 20 ml of potato-dextrose agar containing streptomycin sulfate (100 mg/liter). After the chloroform evaporated, the wells were filled with warm agar and the plates incubated overnight to allow diffusion of the toxicant. Then plates were seeded with 1 ml of a spore suspension of *A. flavus* and incubated for 48 hr at 30 C, after which zones of inhibition were measured. These zones of inhibition were compared with zones caused by known amounts of benomyl. The standard curve was prepared by measuring zones of inhibition caused by triplicate samples of 1-, 3-, 5-, 7-, and 9-µg of technical grade benomyl, in wells of agar plates as described above. It was similar to the semilogarithmic dosage response curve reported by Peterson & Edgington (18).

In this test, as in others, some bolls cracked during the treatment period, and were discarded before bioassays were made for presence of benomyl.

Results of bioassays of carpel tissue, lint, seed coat tissue, and embryo tissue for presence of benomyl were negative when bolls were immersed in a precipitated suspension of benomyl. Benomyl was detected in boll tissues from experiments in which benomyl was kept in suspension by constant agitation. Benomyl was detected in carpel tissue and lint, but not in seed coat and embryo tissue (Table 2). More benomyl was detected in bolls with intact bracts than in bolls whose bracts were broken off, indicating that bracts were an important absorbing organ. The data indicate that benomyl is absorbed slowly by bolls, with somewhat more being absorbed after 10 days than after 2 days.

DISCUSSION.—Results of these experiments support observations of Gore (13) and Doak (11) which show that locules of immature bolls are closed systems. Both

TABLE 2. Absorption of benomyl by detached immature cotton bolls

Bolls	Length of treatment	
	2 days	10 days
	μg/g[a]	μg/g
With bracts		
Carpel tissue	65	90
Lint	ND[b]	38
Seed coat tissue	ND	ND
Embryo tissue	ND	ND
Without bracts		
Carpel tissue	22	65
Lint	ND	TR[c]
Seed coat tissue	ND	ND
Embryo tissue	ND	ND

[a] μg benomyl/g air-dry tissue.
[b] Benomyl not detected.
[c] Benomyl detected but not measurable on standard curve.

crystal violet stain and benomyl were absorbed into, and diffused slowly within, immature bolls having intact bracts. But when bracts were broken, the stain was absorbed quickly through their broken edges and diffused into the pithlike tissue basal to carpels. This observation is not surprising in light of studies of Gore (13). His ontogenetic studies show that the first evidence of the cotton flower is the appearance of three bract primordia on the floral meristem. When these three bracts subtending the rest of the floral meristem are fully developed, they represent the uppermost structures on the peduncle that receive major vascular bundles to serve the calyx but, above this point, there is much anastomosing of small divergent bundles; a distinct pith, as in the peduncle, is absent. His observations also account for the conclusions of Cauquil & Ranney (10), who used bolls with bracts broken off in their tests to determine whether bolls are waterproof. In our tests, benomyl did not absorb and diffuse from broken edges of bracts, as well as crystal violet, because more benomyl was detected in tissues of bolls with intact bracts than of bolls with bracts broken off.

Our data do not agree with reports (9, 10, 21, 22) which conclude that minute openings in carpel walls of immature bolls are important avenues of entry for microorganisms which are not able to penetrate undamaged carpel walls. An internal void or canal often occurs along the longitudinal axis of bolls, according to results of ontogenetic studies (11, 13). Microorganisms appear to regularly invade and persist in this canal, which often is open to the outside of the boll (2, 11). The canal probably is the source of many of the microorganisms isolated by several workers (7, 9, 10, 15, 21). It also may be the source of the microorganisms that decay surface-disinfested bolls within 2-5 weeks after they are stored over sterile water in closed jars (10). But the void appears to have no pathological significance, with two exceptions: (i) The septa of locules exposed to the void could be a site of infection for organisms having the innate ability to penetrate bolls directly. In this case, however, the walls simply would be another potential site of infection; (ii) loss of integrity of septal walls of locules and placentae, as occurs when a supernumerary capsule forms at the basal carpellary meristem (8, 16), offers ready access to internally exposed lint by organisms incapable of directly penetrating the boll. Erwinia herbicola is an example of this type of organism (2).

Unquestionably, numerous fungi may be detected on and in mature lint and seed of cotton (4, 7, 10, 14, 15). These fungi closely parallel those in the lists reported by Bagga (7), Cauquil & Ranney (10), and Pinckard & Chilton (19). They can invade and discolor lint (6) and seed (4, 14) if given an avenue of entry to locules. Similarly, the observation of Cauquil & Ranney (10) that fungi invade and sporulate on petals of senescent flowers is not questioned. It is doubtful, however, that the flowers have any more pathological significance for most fungi than for E. herbicola, as reported by Ashworth et al. (2).

The combined results of anatomical studies and studies reported here suggest a basis for grouping boll pathogens according to their parasitic potential, with the anatomy of the boll being an important consideration.

The structural integrity of the cotton boll is unimportant to a group of boll pathogens typified by Glomerella gossypii and Xanthomonas malvacearum, for they invade directly uninjured carpel tissue (10). A second group of microorganisms, including Erwinia herbicola (2, 3) and Nematospora spp. (17), are introduced into locules on the mouthparts of sucking insects. In such cases, the structural integrity of the boll is not lost but simply violated, since the wounds caused by mouthpart insertion are not avenues of entry for the organisms (3, 17). The structural integrity of the boll must be lost, however, before a third group of microorganisms can attack locules. Such pathogens include the A. flavus group of fungi, which are unable to infect either thrifty or senescent carpel tissue (5). Another possible member of this group is Rhizopus sp. It does not infect through the carpel surface of thrifty unopened bolls, but can invade excised discs cut from carpels of senescent bolls, according to Garber et al. (12). They did not, however, demonstrate that the fungus goes on to penetrate locules.

Pathogens in the latter group, then, require a mechanical opening as an avenue of entry to locules in order to invade lint. This type of opening typically occurs at fruit maturity, when individual carpels separate at the carpellary meristem (5). Premature carpel separation occurring when immature bolls suffer sunburn damage is of minor importance. Insect larvae also induce avenues of entry to locules when they tunnel through walls of carpels; the tunnels not only provide direct access to locules for fungi such as A. flavus, but also to numerous other microorganisms (6, 9). Following invasion of locules by this route, at least two fungi (A. niger and Rhizopus sp.) can go on to invade carpel walls from the inside. The resulting necrosis of the carpel walls induces premature separation of carpels which further increases the amount of bolls prone to infection by A. flavus and other microorganisms (6).

These fungi infect thrifty carpel tissue, once established on lint, presumably because of the presence of a substrate favorable for inducing parasitism, as reported by Purdy (20) for *Sclerotinia sclerotiorum.*

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