A Comparison of Nematode and Bacteria-Colonized Galls Induced by *Anguina agrostis* in *Lolium rigidum*

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ABSTRACT

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Morphological differences between seed galls containing nematodes and those colonized by bacteria are described. The two types of galls can be readily recognized; the former are dark brown and the latter are yellow. Sectioning and viewing under Nomarski optics showed that the walls of galls containing nematodes are about twice the thickness of the walls of those containing bacteria. Scanning electron microscopy showed the orderly manner in which both nematodes and bacteria are packed in the anhydrobiotic galls. Transmission electron microscopy of freeze-etch replicas and ultrathin sections of both types of galls revealed the presence of particles in the galls containing bacteria. These particles are 25–30 nm in diameter, occur in close association with the bacteria and their capsules, and are found in large numbers in the walls of the galls. The possible nature of these structures, and their relationship to toxin production in the galls that contain bacteria, are discussed.

Additional key word: ultrastructure.

Galls induced by the nematode *Anguina agrostis* (Steinbuch, 1799) Filipjev, 1936 (9) in the place of seeds of rye grass (*Lolium rigidum*) become toxic to animals when further colonized by *Corynebacterium rathayi* (11).

Although all of these galls are initiated by *A. agrostis,* they may become colonized predominantly either by bacteria or by nematodes when mature (7,11). The factors that determine whether nematode reproduction or bacterial colonization will occur are not clearly known. The two types of mature galls, although of somewhat similar size, differ completely in color, content, and toxicity. The toxin is associated with galls containing bacteria and resides predominantly in the wall of the gall rather than in the bacterial component (11).

We investigated the wall morphology and contents of these two types of galls to determine whether a relationship exists between the observed toxicity of the bacterial gall wall and its ultrastructure.

MATERIALS AND METHODS

Mature galls containing either nematodes or bacteria were obtained from field-infected *L. rigidum* plants collected near Katanning in Western Australia and Manoora in South Australia. Both types of galls were freed of lemma and palea and treated as follows, depending on whether they were to be examined in the dry or hydrated states.

Fixation of dry galls. Dry galls were placed in 4% paraformaldehyde in pure glycerol at 24 C for several days. The preparation and use of this non aqueous fixative is described in detail elsewhere (12). In our experiments the method was slightly modified by placing 2 g of paraformaldehyde in 50 ml of glycerol in a closed container and holding it at 60 C for several hours until it went into solution.

The fixed material was either transferred to pure glycerol prior to freeze-etching or, if it was to be sectioned, was washed several times in phosphate buffer prior to fixation in osmium tetroxide which was followed by dehydration and embedding.

Fixation of hydrated galls. Both types of galls were hydrated by placing them between damp filter papers in a petri dish at 5 C overnight. They were transferred to a small drop of 0.115 M phosphate buffer (pH 7.3) in a covered solid watch glass at 5 C for several hours. Then this drop was flooded with cold 4% paraformaldehyde in the same buffer and at the same temperature and left in that fixative at 5 C for periods of up to 1 wk. The gall material was cut into several portions with a sharp scalpel while in the fixative, then washed in phosphate buffer, and finally washed in water. Gall material to be freeze-etched was placed in 25% glycerol; material to be sectioned was further fixed in phosphate-buffered 1% osmium tetroxide (pH 7.3), washed in buffer, then in water, and finally taken through an ascending ethanol series into Araldite (Ladd Research Industries, Inc., Burlington, VT 05401) by using propylene oxide as an intermediary (3).

Culture technique. Bacterial cultures were obtained by immersing bacterial galls in a 1:1,000 mercuric chloride solution for 3 min, washing them several times in sterile distilled water, and plating them on a previously described medium (2).

Light microscopy. Araldite-embedded sections 1–2 μm thick were cut with glass knives held in an LKB Ultratome (LKB Producter AB, Stockholm-Bromma, Sweden), and placed in small drops of distilled water on glass slides which were placed in an oven at 60 C. The heat caused the sections to expand and as the water evaporated they adhered to the slides. These sections were examined dry by using Nomarski differential interference contrast optics.

Scanning electron microscopy. The osmium-bridging properties of thiocarbohydrazide were utilized prior to observations with the scanning electron microscope (SEM). This procedure (4), which proved to be very useful in our examination of the internal structures of the two types of galls, was carried out as follows: after aldehyde fixation the galls were rinsed in buffer, postfixed in 1% osmium tetroxide, rinsed several times in distilled water over a 15-min period, washed 20–30 min in freshly made 1% thiocarbohydrazide, and then several times in distilled water. This sequence was repeated three times. After the last rinse in water, the material was dehydrated in a graded acetone series and critical-point dried. These galls were then cut lengthwise with a razor blade and affixed to an aluminum stub with the freshly cut surface uppermost. This material was examined in a Philips PSEM 500

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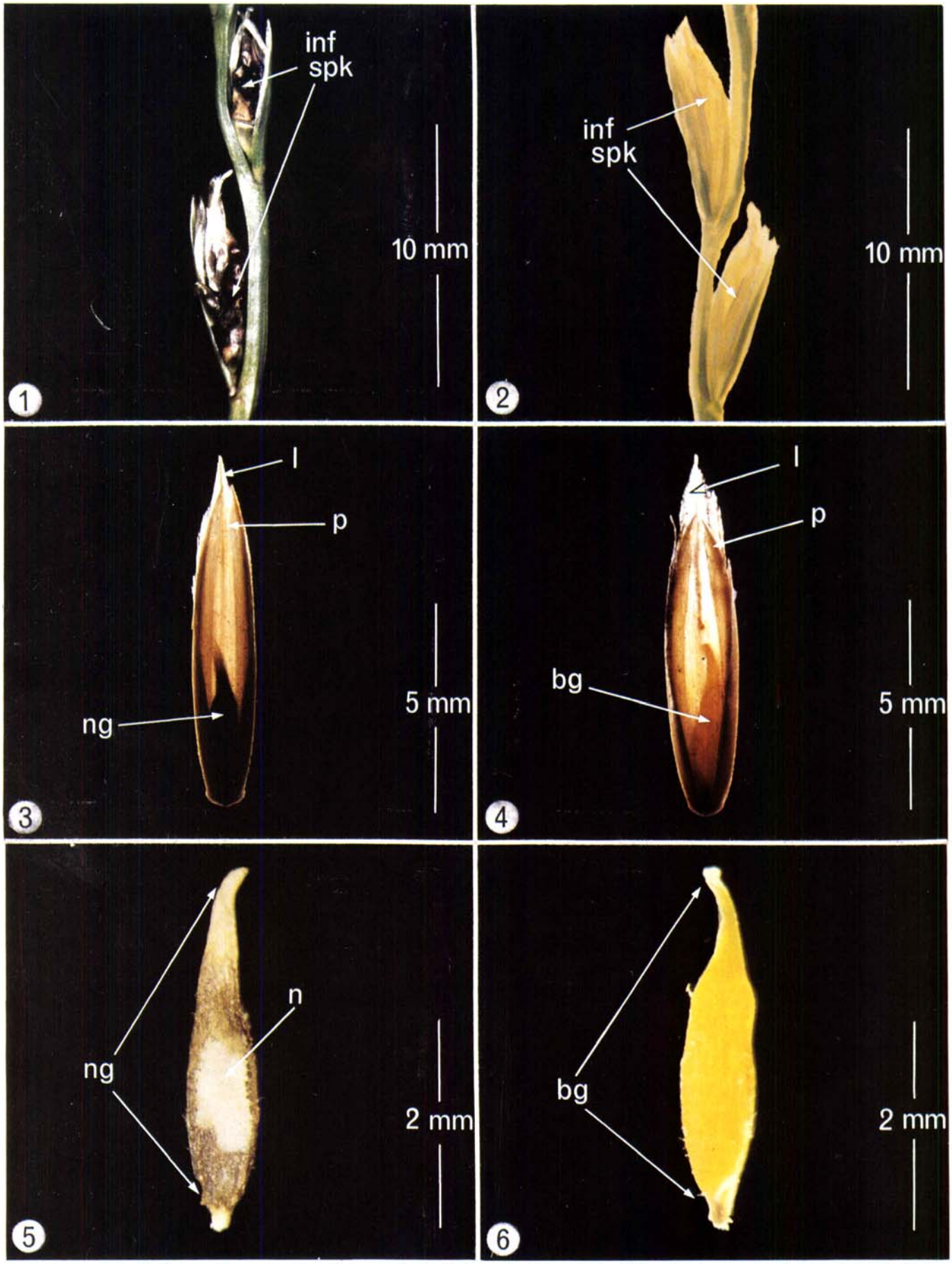

Fig. 1-6. Comparison of galls containing nematodes with those colonized by bacteria. **1,** Two spikelets infected with nematodes. inf spk = infected spikelets. **2,** Two spikelets infected with bacteria. inf spk = infected spikelets. **3,** Nematode seed gall enveloped by the lemma and palea. ng = nematode gall, l = lemma, p = palea. **4,** Seed gall colonized by bacteria enveloped by the lemma and palea. bg = gall containing bacteria, l = lemma, p = palea. **5,** Nematode seed gall with part of gall wall cut away to reveal nematodes within. ng = nematode gall, n = nematodes. **6,** Gall colonized by bacteria = bg.

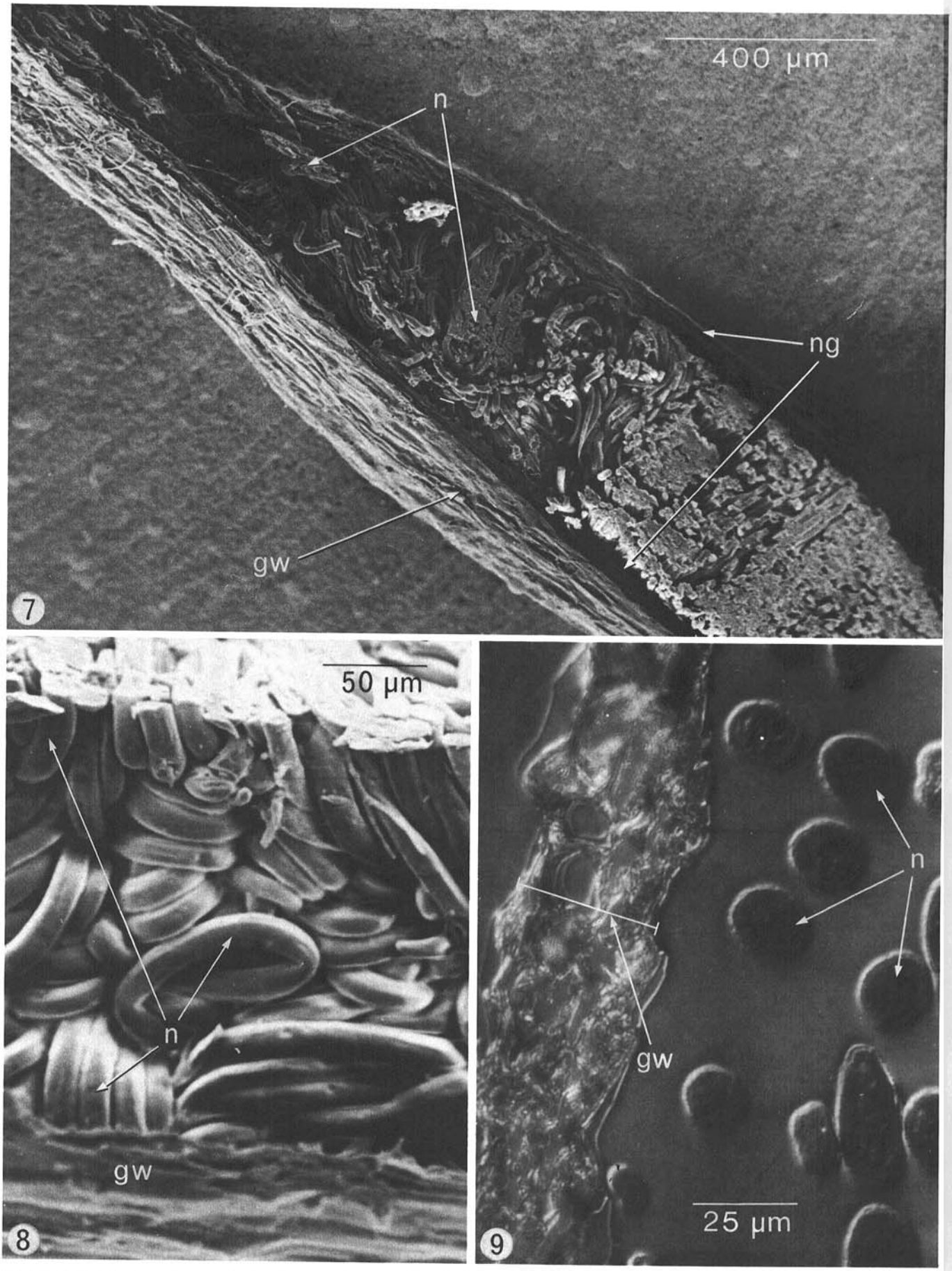

Fig. 7–9. Scanning electron micrographs showing parts of a nematode-colonized *Lolium rigidum* seed gall. **7,** Cut area of gall. ng = nematode gall, gw = gall wall, n = nematodes. **8,** Enlarged part of cut area of gall showing the regular packing of the infective larvae. n = nematodes, gw = gall wall. **9,** Nomarski interference contrast photograph of a section through a nematode gall. gw = gall wall, n = nematodes.

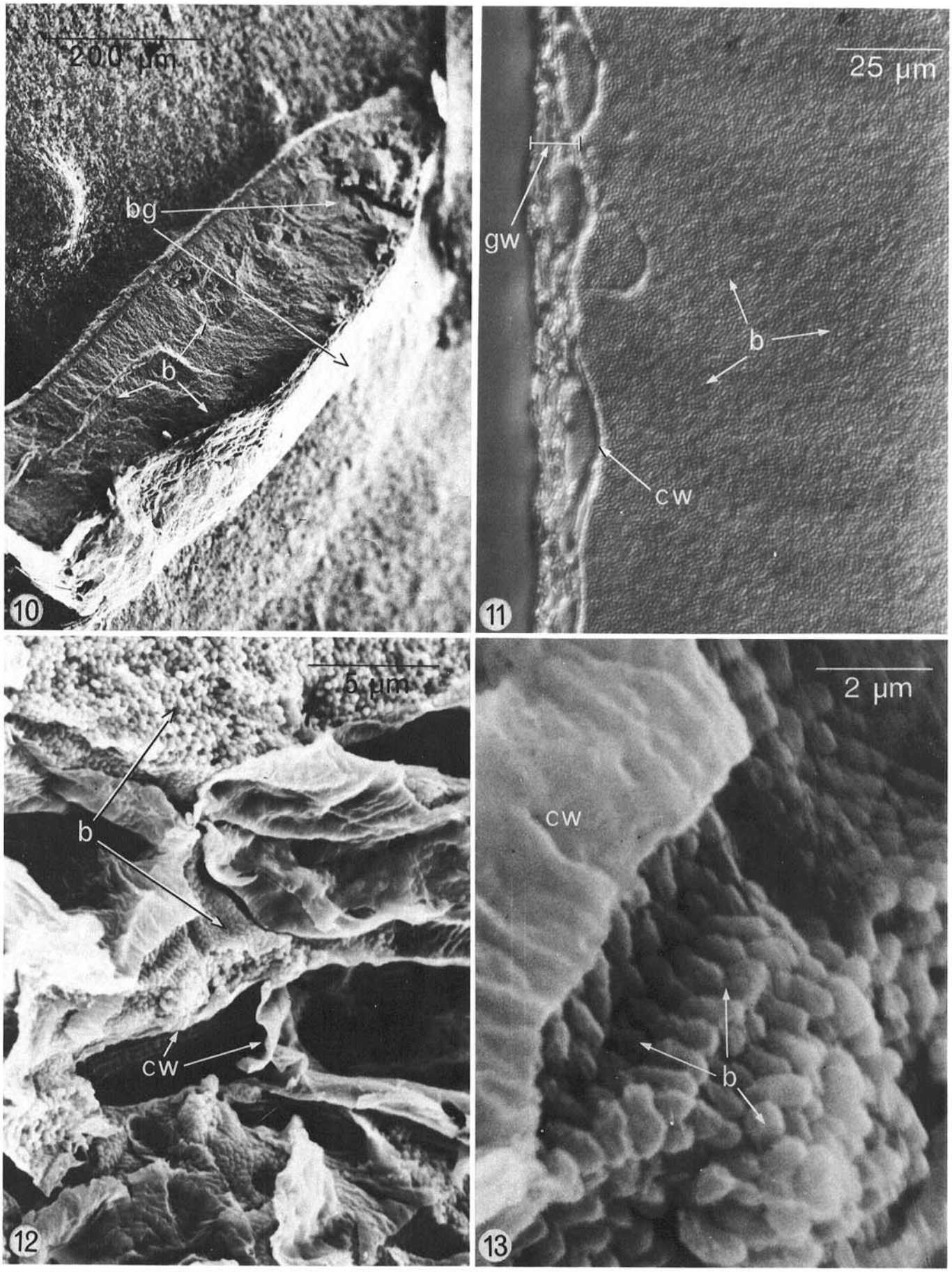

Fig. 10–13. 10, Scanning electron micrographs showing part of a bacteria-colonized *Lolium rigidum* seed gall. bg = gall colonized by bacteria, b = bacteria. **11,** Nomarski interference contrast photograph of a section through a gall colonized by bacteria. gw = gall wall, cw = cell wall, b = bacteria. 12–13 SEM photographs showing relationship between bacteria and the cell walls of the wall of a gall colonized by bacteria. **12,** cw = cell walls, b = bacteria. **13,** Higher magnification showing the close packing of the bacteria. cw = part of a cell wall, b = bacteria.

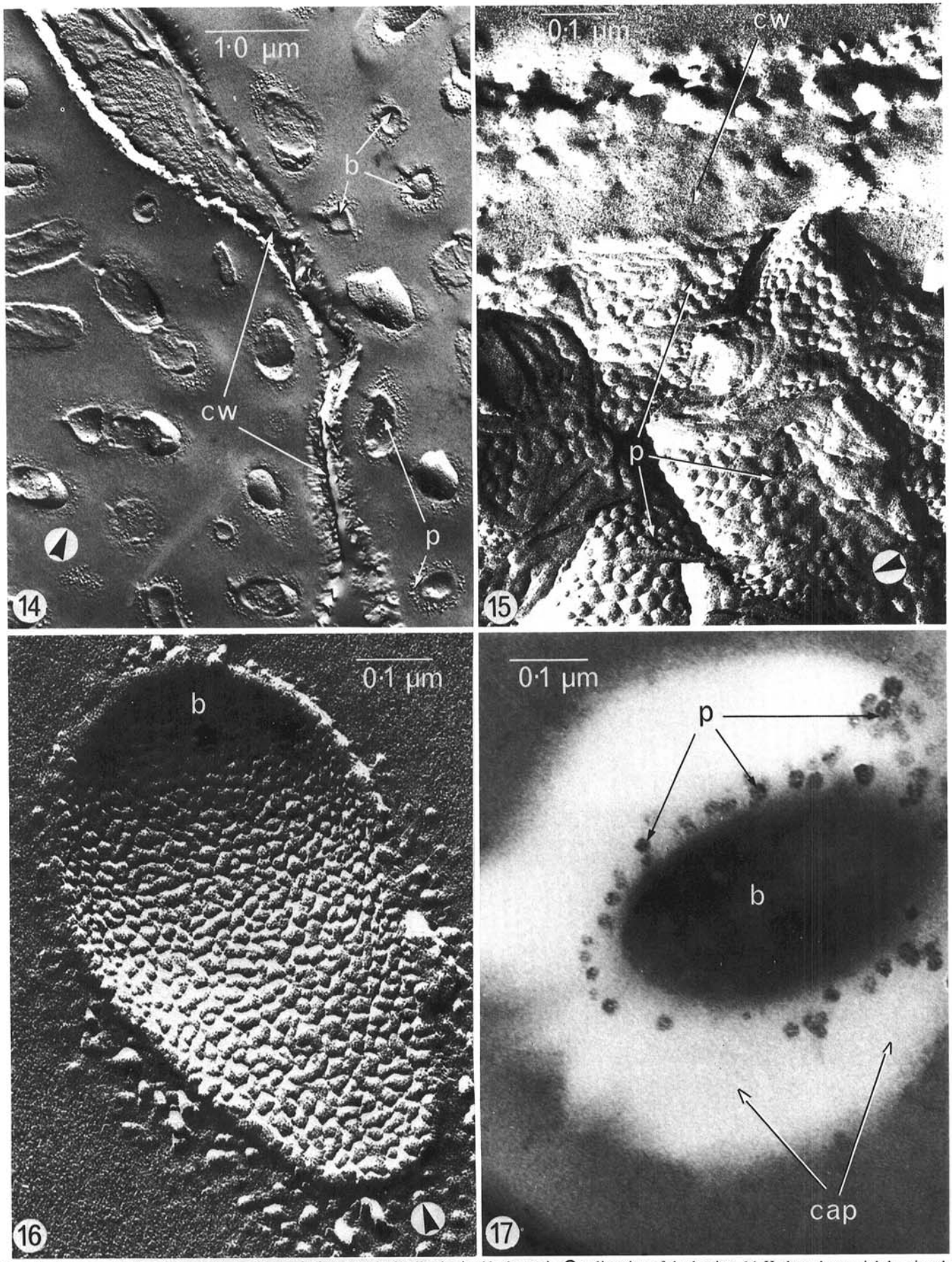

Fig. 14–17. Transmission electron micrographs of a freeze-etched gall colonized by bacteria. Ⓐ = direction of shadowing. **14,** Hydrated material showing the distribution of bacteria and particles adjacent to and within the cell walls respectively. cw = cell wall, b = bacteria, p = particles. **15,** Higher magnification showing arrangement of particles on the cell wall. cw = cell wall, p = particles. **16,** A bacterium at the same magnification as 15. b = bacterium. **17,** TEM photograph of a section cut through a hydrated gall colonized by bacteria. This material was fixed and stained in paraformaldehyde and osmium tetroxide. It was also stained in 2% phosphotungstic acid in absolute ethanol prior to being embedded in Araldite. b = bacterium, cap = bacterial capsule, p = particles.

SEM operating at 12 kV.

Transmission electron microscopy. Thin sections of material embedded in Araldite were cut with glass knives held in an LKB Ultratome and stained in a solution of saturated uranyl acetate in 50% methanol for 20 min at 24 C followed by lead citrate for 10 min at 24 C. These sections were observed in a Philips EM 400 transmission electron microscope (TEM) (Philips-Gloeilampenfabriken, Eindoven, The Netherlands) operated at 80 kV.

Freeze-etching. Fixed material for freeze-etching was mounted on gold specimen disks; if dry, it was mounted in 100% glycerol and if hydrated, it was mounted in 25% glycerol, quick-frozen in liquid Freon 22 at approximately −150 C, and plunged into liquid nitrogen.

Specimens were freeze-fractured and etched and replicas were made in a Balzer's apparatus (5) (Balzers-Aktiengesellschaft, Fiirstentum, Leichtenstein) operated with a sublimation time of 2 min at −100 C. These replicas were cleaned by treatment in 80% sulfuric acid at 60 C for 4–5 hr, washed several times in distilled water, treated overnight at 24 C in sodium hypochlorite containing 3.5% available chlorine, washed several times in distilled water, and picked up on collodion-coated grids.

RESULTS

The floret primordia of *L. rigidum* attacked by infective larvae of *A. agrostis* are modified and become seed galls that characteristically differ markedly in color depending on whether they become colonized by nematodes or bacteria (Figs. 1–6). Galls containing nematodes are dark brown whereas those colonized by bacteria are bright yellow. Coiled anhydrous nematodes in dry galls appear to the naked eye as a white woolly mass (Fig. 5). Examination at higher resolution under the SEM (Figs. 7,8) reveals that this white mass consists of hundreds of coiled, closely apposed infective larvae. The nematode-containing gall is similar to that of the closely related *Anguina tritici* that forms wheat seed galls in which the closely apposed and coiled infective anhydrobiotic larvae can remain viable for short periods of time at temperatures ranging from −190 C to 105 C (1). Similarly, in *A. agrostis* it is this larval stage in the dry gall that can withstand the dryness and heat of the Australian summer.

In the dry state, the walls of galls containing nematodes (Fig. 9) are about twice as thick as those of galls colonized by bacteria (Figs. 10,11). The necessary use of aqueous media, even though only for a short period, following dry fixation in glycerol, caused some movement of the packed nematode larvae away from each other and from the gall wall (Fig. 9) in galls cut into pieces to permit easier entry of other chemicals prior to embedding. Similar slight hydration of bacterially colonized gall material processed for sectioning did not produce such an obvious effect (Fig. 11). However, comparison of hydrated and dry bacterially colonized galls at higher resolution under the TEM, revealed a distinct difference in the spatial relationships of the packed bacteria; under anhydrous conditions the bacteria appeared to be much closer to each other.

Dry galls observed under the SEM (Figs. 10,12,13) were seen to be full of corynebacteria packed closely in a regular array, both along the walls of the cells making up the gall walls and within the gall itself. Observation of freeze-fractured, etched, bacteria-colonized galls at still greater resolution with the TEM showed numerous particles with diameters ranging 25–30 nm. These particles are associated with the bacteria and with the cell walls of the gall (Figs. 14–16). Also, thin sections of galls colonized by bacteria showed these particles within the capsules of the bacteria and in the gall walls (Fig. 17).

DISCUSSION

The reported differences in toxicity of the two types of galls to various animals and the localization of the toxin largely in the cell walls of galls containing bacteria (11) prompted this comparative study of their ultrastructure.

The thicker cell walls of the galls containing nematodes could reflect the plant's response to the nematodes' feeding habits.

The close packing of infective larvae (Fig. 8) and bacteria (Fig. 13) in anhydrous galls is well illustrated in the scanning electron micrographs. It seems that close contact between microorganisms plays an important role in survival under extreme environmental conditions (1).

Perhaps the most interesting of our observations is the discovery of the particles located in and around the bacteria and particularly in the gall wall, a site which is known to contain the greatest amount of toxin. We have detected these structures in freeze-etch preparations of cultured corynebacteria which introduces the possibility that they might be corynephages.

It is tempting to compare *C. rathayi* with *C. diphtheriae* in which a toxin is only produced by strains that are converted to toxin-producing forms by corynephages which carry the tox structural gene, the expression of this gene being regulated by the bacterium (6).

It seems unlikely, that if a bacteriophage was involved in some way with the production of toxin in galls of annual rye grass, that it would function like the phage of *C. diphtheriae*. *C. rathayi* is a plant parasite and host plant cells play an important role in the production of toxin. Toxicity has, so far, only been associated with galls containing bacteria and with callus tissue of *L. multiflorum* grown on artificial media and infected with this bacterium (10).

Furthermore, toxin production was not detected in cultures grown on various artificial media (11) although we have found particles associated with bacteria in cultures. It seems that the plant is involved in toxin production, but at the moment the mechanism is not clear.

One alternative hypothesis is that these particles are hexagonally-packed spherical sub-units of the bacterial wall (Fig. 16) similar to those described for some other species of bacteria (8). However, the sub-units associated with bacterial walls appear to be arranged in regular array and usually tend to be smaller, 8–12 nm in diameter, compared with the particles described above which are more randomly distributed and 25–30 nm in diameter (Fig. 17). Furthermore, they occur throughout the bacterial capsule as well as in the gall cell wall, so that these structures are not solely a component of the bacterial wall. This morphological study points to the need for further studies involving isolation and chemical analyses of these particles.

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