Colonization of the Sharpshooter Vectors, *Oncometopia nigricans* and *Homalodisca coagulata*, by Xylem-Limited Bacteria

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ABSTRACT

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Xylem-limited bacteria (often referred to as rickettsialike bacteria) were observed in the cibarium, the apodemal groove of the diaphragm, and the precibarium of the sharpshooters, *Oncometopia nigricans* and *Homalodisca coagulata*, by scanning electron microscopy. Bacteria colonized the cibarium, precibarium, and the apodemal groove of the diaphragm of *O. nigricans* after the insects had fed on Pierce's disease-affected grapes or periwinkle wilt-affected *Catharanthus roseus*. In *H. coagulata*, which were collected from orchards with phony peach disease or fed on plum leaf scald-affected plants, bacteria were attached to the floor of the cibarium, the apodemal groove of the diaphragm, and the walls of the precibarial area above and below the valve. Bacteria in both sharpshooter species were attached by one end to the walls of the cibarium and precibarium by means of extracellular material and possibly fimbraelike structures. Some dividing bacteria were observed.

Additional key words: Cicadellidae, Cicadellinae, leafhopper.

Sharpshooter leafhoppers (Tettigellidae, Cicadellidae) are known vectors of Gram-negative, xylem-limited bacteria (XLB), which either cause, or are associated with, a number of plant diseases (14). Pierce's disease (PD) of grape is caused by an XLB (7) that is transmitted by several leafhopper vectors (8,15). The bacteria associated with phony peach (PP) (10) and plum leaf scald (PLS) (11) have been cultured recently (6,17,19). Apparently, both diseases have the same causal agent (20). The causal agents of periwinkle wilt (PW) (12), PP, and PLS are transmitted by leafhopper vectors. Recently, Purcell et al (16) consistently isolated the PD bacterium from its leafhopper vector, *Graphocephala atropunctata* (Signoret). Light- and scanning electron microscopy of the cibarium and diaphragm of infective leafhoppers revealed that PD bacteria (PDB) attach to the floor of the cibarium and the apodemal groove of the diaphragm. Nymphs of *G. atropunctata* lose the ability to transmit PD after molting (15). Recently, Brlansky et al (3) reported the detection and transmission of an XLB in sharpshooters from a Florida citrus grove.

Florida has abundant populations of the sharpshooter leafhoppers, *Oncometopia nigricans* (Walker) and *Homalodisca coagulata* (Say), which are known vectors of the pathogens that cause PD (1), PP (18), and PW (12). All of these diseases (and also PLS) occur in Florida.

The purpose of this study was to investigate the location and means of attachment of these plant pathogenic, xylem-limited bacteria in their vectors.

MATERIALS AND METHODS

Adult *O. nigricans* were collected from a citrus grove in the southeast flatwoods in Martin County, FL, and were placed in a cage containing plants of grape (*Vitis vinifera* L. 'Chardonnay,' 'Mission,' 'Chenin Blanc,' or 'Cabernet Sauvignon'), periwinkle (*Catharanthus roseus* L. (G. Don)), ragweed (*Ambrosia artemisiifolia* L.), American elder (*Sambucus canadensis* L.), and rough lemon (*Citrus jambhiri* Lush) and allowed to feed. To obtain *O. nigricans* free of bacteria, young nymphs were removed from the above cages and placed on healthy plants. Sharpshooter nymphs lose the ability to transmit PD after molting (15). After the nymphs reached maturity, xylem squeezings from all caged plants were stained with 0.1% methylene blue and checked for bacteria by light microscopy (3). No symptoms of PD or PW developed on any of the grape or periwinkle plants, respectively, and they were free of XLB. Twelve nontransmitting adult *O. nigricans* were then placed on PD-affected grapes or PW-affected periwinkles, allowed to feed for 10–14 days, and then were prepared for SEM.

H. coagulata were collected in Madison County, FL, from peach orchards heavily infected with PP. One hundred thirty-three sharpshooters were prepared for SEM and examined for the presence of bacteria. Thirty adult sharpshooters were placed in a cage containing the aforementioned plants. These sharpshooters laid eggs, and the resulting hatched nymphs were reared to adulthood. After the nymphs hatched, the remaining 17 adults were removed from the cage, checked by SEM as described below, and found to be free of bacteria. The plants were checked as previously described. Twenty-five nontransmitting cage-reared adults were then allowed to feed on PLS-affected plums for 7–10 days and then were prepared for SEM.

Leafhoppers were prepared for SEM by initially fixing them in 3% glutaraldehyde in 0.06 M phosphate buffer (pH 6.8) for 1 hr. Heads were removed and fixation of them was continued at 4 C for 12–16 hr. The heads were washed three times in phosphate buffer to remove the glutaraldehyde and then postfixed in 1% osmium tetroxide in 0.06 M phosphate buffer (pH 6.8) for 2–4 hr. The specimens were washed three times in phosphate buffer and dehydrated in a 30–100% acetone series. Following a second change in 100% acetone, the specimens were transferred to graded series of acetone: freon mixtures and critical-point dried in a Bomar Critical-Point Dryer (The Bomar Co., Tacoma, WA 98401).

The cibarium and diaphragm were dissected from the head by using the following procedure. Heads were oriented on a Parafilm surface with the stylet pointing horizontally. Loose muscle tissue

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and internal organs were removed with INOX #5 forceps. The diaphragm was separated from the cibarium with forceps and the remaining exoskeleton of the head was carefully removed from the cibarium. The diaphragm and cibarium were mounted on SEM stubs, sputter-coated with 10 nm (100 Å) of gold-palladium, and viewed in a JEOL JSM 35 scanning electron microscope. Specimens found to contain bacteria were then further dissected by separating the epipharynx and hypopharynx halves, remounted, and the precibarium of both halves was sputter-coated and viewed.

For transmission electron microscopy (TEM), cibaria were dissected before fixation. Fixation and dehydration were performed as described for SEM through the 100% acetone step. The epipharynx and hypopharynx were separated and the hypopharynx was prepared for SEM as described. The epipharynx was transferred to two changes of 100% acetone and then infiltrated with Spurr's medium:acetone (1:1, v/v) overnight. They were then placed in a 2:1 Spurr's medium:acetone (2:1, v/v) mixture for 12 hr and transferred to 100% Spurr's overnight. The epipharynx halves were embedded in Spurr's medium and polymerized at 70 C.

Hypopharynxes were viewed with SEM to determine which of them contained bacteria. Only epipharynxes with hypopharynxes that contained bacteria were sectioned. Ultrathin sections were made on a Huxley LKB ultramicrotome, stained with uranyl acetate and lead citrate, and viewed with a Philips 201 electron microscope.

RESULTS

Bacteria were found in the cibaria of *O. nigricans* that had fed on PD-affected grapes and PW-affected periwinkle (Figs. 1 and 2). Bacteria presumed to be the Pierce's disease bacteria and PW

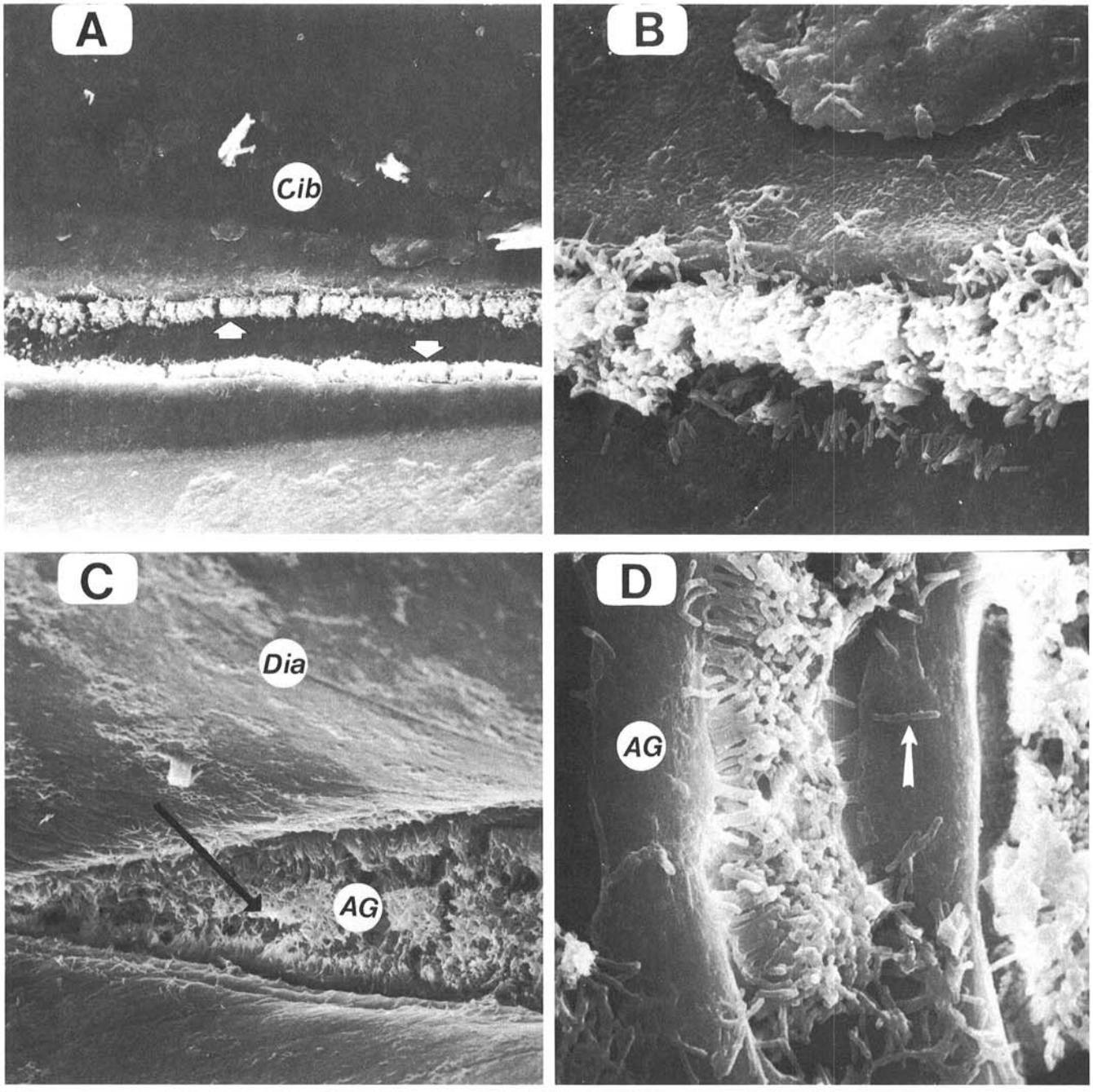

Fig. 1. Pierce's disease bacterium in the cibarium of *Oncometopia nigricans*. **A,** Bacteria (arrows) attached in groove of the floor of the cibarium (Cib) (×400). **B,** Enlarged view of the bacteria in A (×2,000). **C,** Bacteria colonizing the apodemal groove (AG) of the diaphragm (Dia) (×1,000). **D,** PD bacteria attached to groove in diaphragm (×3,000). Note two cells in D that appear to be dividing (arrow).

bacteria were found in the groove in the floor of the cibarium and at the entrance (food meatus) from the stylet into the cibarium (Figs. 1A and B, 2A and B). Both bacteria were found attached to the apodemal groove of the pump diaphragm (Figs. 1C and D, 2C and D) of leafhoppers that had fed on either PD- or PW-affected plants. Masses of bacteria were present as large mats and were usually polarly attached to the cibarium and apodemal groove (Figs. 1B and D, 2B). Both PD and PW bacteria appeared to undergo division (Figs. 1D and 2B). Bacteria were also found in the precibarium area of the epipharynx and hypopharynx. Bacteria usually were found anterior and posterior to the precibarial valve, but occasionally they were located only posterior to the valve. No bacteria were observed in the floor of the cibarium or in the apodemal groove of the diaphragm of *O. nigricans* fed on healthy grapes and healthy periwinkle. Two of 11 *O. nigricans* fed on the PD-affected grapes and three of 12 sharpshooters fed on PW-affected *C. roseus* contained bacteria.

H. coagulata collected from PP-affected orchards also contained bacteria in their cibaria. Of two collections of sharpshooters, bacteria were seen in only two of 50 from the first collection and one of 83 sharpshooters from the second. The presumptive PP bacteria were found in the groove of the floor of the cibarium (Fig. 3A), the entrance to the cibarium, and in the apodemal groove of the diaphragm. Again, bacteria appeared to be attached by one end to the wall in the floor of the pump and to the apodemal groove of the diaphragm. Division of the bacteria in the middle of the floor groove was noted. Extracellular material appeared to coat the PP bacteria and occasionally hindered the viewing of individual organisms (Fig. 3A).

Eight of 20 *H. coagulata* fed on PLS-affected plums contained bacteria in their cibarium. The bacteria were similarly attached in *H. coagulata* from a phony peach orchard (Fig. 3B); ie, attached in a polar orientation to the floor of the cibarium and diaphragm and surrounded by extracellular material (Fig. 3B and C). Bacteria also

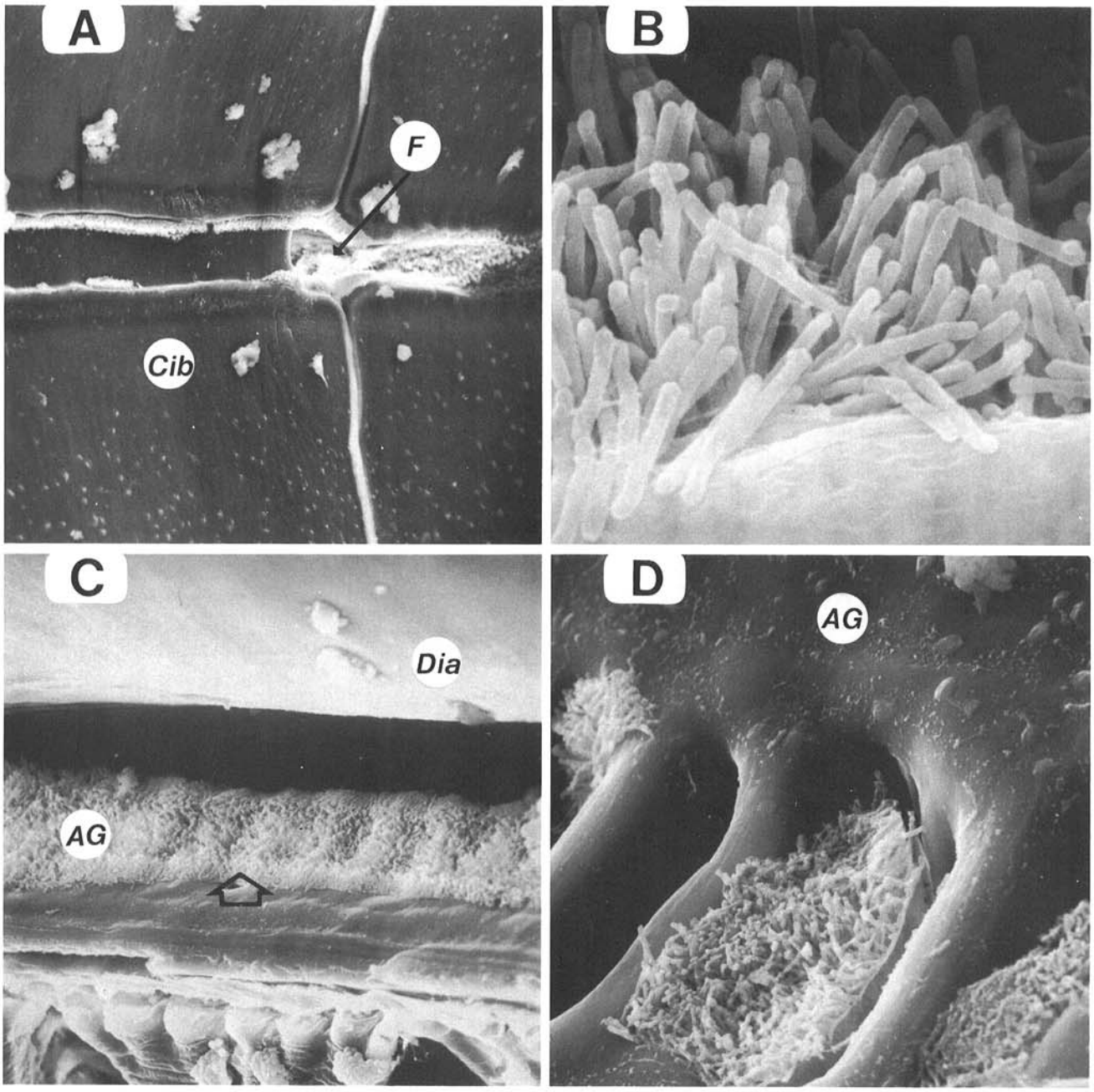

Fig. 2. Periwinkle wilt bacteria in the cibarium of *Oncometopia nigricans*. **A,** Bacteria attached to floor of cibarium (Cib), and growing in the area of the food meatus (entrance from the precibarium) (F) (×300). **B,** Enlarged view of bacteria colonizing groove in the floor of cibarium (×10,000). **C,** Lawn of bacteria (arrow) growing on the apodemal groove (AG) of the diaphragm (Dia) (×780). **D,** Bacteria growing in pocket of apodemal groove (×2,000).

were present in the epipharynx (Fig. 3C) and hypopharynx anterior and posterior to the precibarial valve (Fig. 3C–E).

No other morphologically distinct bacteria or other microorganisms were seen in the cibarium or precibarium of either leafhopper.

The internal structure of the PWB and its attachment to the lining of the precibarium was revealed by transmission electron microscopy (Fig. 4A–E). Bacterial structure was similar to that reported previously for PDB (7), PWB (12), PPB (10), and PLSB (11,19) in both plants and in culture media. The bacteria had a rigid rippled cell wall with a well-defined cytoplasmic membrane; the outer membrane and R-layer were visible (Fig. 4B–D). Bacteria measured 0.31–0.42 μm wide and 1.7–3.1 μm long. In most cases, bacteria were observed attached polarly to the lining of the precibarium (Fig. 4B and C). Dense-staining extracellular material was observed at the attachment end (Fig. 4B and C) and some fimbraelike structures were often observed mixed with this material at this end (Fig. 4C). It appeared that these materials caused the binding of bacteria to the sharpshooter (Fig. 4C), as well as to adjacent bacteria within the colony (Fig. 4B). Cell division was commonly observed (Fig. 4D), which resulted in bacterial cells being released into the matrix (Fig. 4E). Bacterial cells appeared to elongate prior to division. Evidence of this elongation also was seen with SEM.

DISCUSSION

In this study, *O. nigricans* and *H. coagulata* were colonized by various presumptive Gram-negative xylem-limited plant pathogenic bacteria. Bacteria were attached to the floor of the

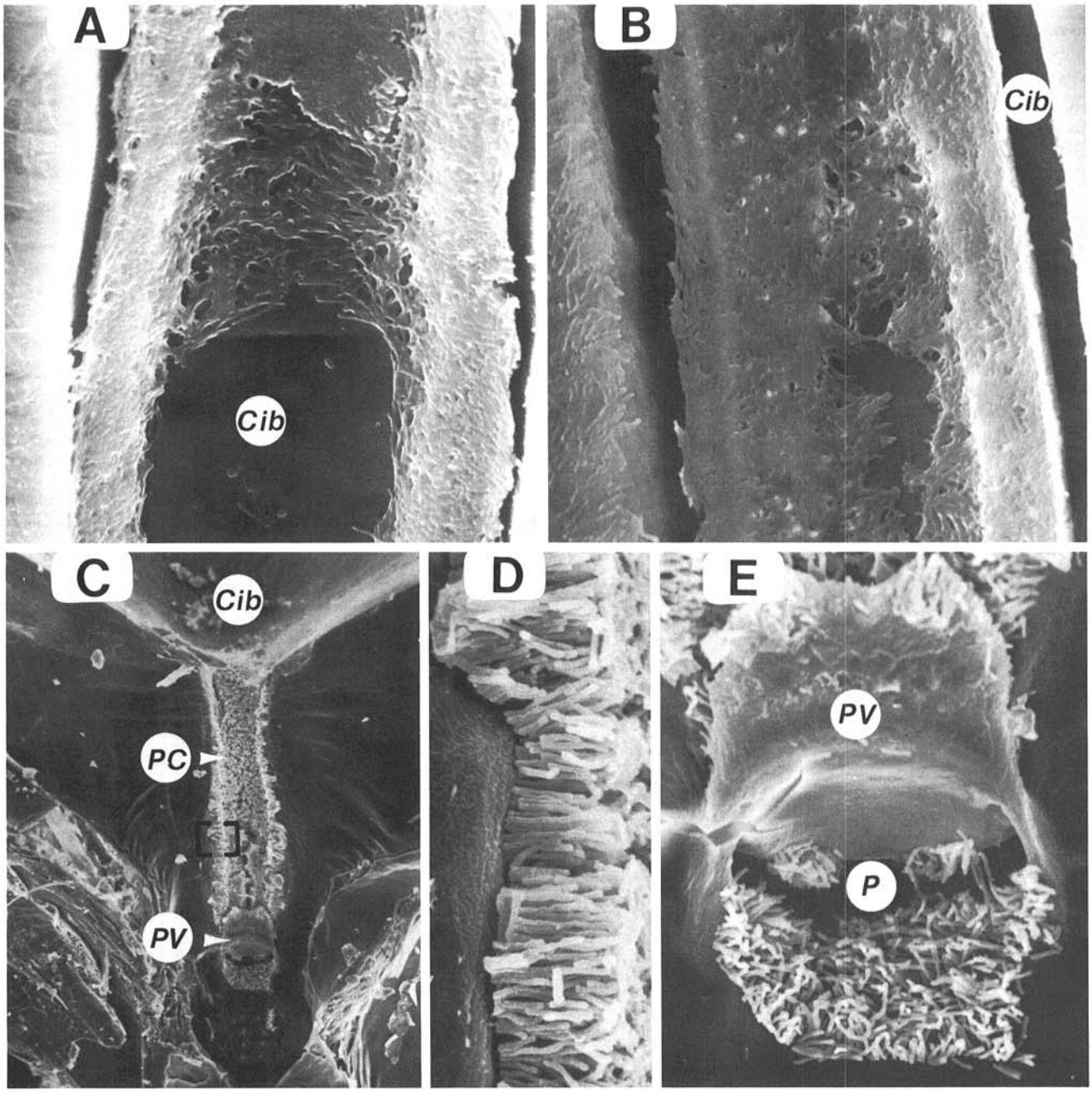

Fig. 3. Bacteria in cibarium (Cib) and precibarium (PC) of *Homalodisca coagulata*. **A,** Phony peach bacterium in floor of cibarium (×2,000). **B,** Plum leaf scald bacterium (PLSB) in floor of cibarium (×2,000). **C,** Plum leaf scald bacterium colonizing the precibarium (epipharynx side) anterior and posterior to the precibarial valve (PV) (×300). **D,** Enlarged view (boxed area in C) of the PLSB attached to the precibarium posterior to the precibarial valve (×3,000). **E,** Enlarged view of the plum leaf scald bacteria colonizing the area anterior to the precibarial valve and the precibarial pit (P) (×1,800).

cibarium, to the apodemal groove of the diaphragm, and to the walls of the precibarium in all four vector-bacterium combinations that were observed. Not all sharpshooters contained bacteria; the percentage was especially low in field-collected insects. The attachment of the bacteria to the cibarium and diaphragm surfaces was similar to that reported by Purcell et al (16) for PDB in *Graphocephala atropunctata*.

New evidence of the presence of bacteria in the precibarium of sharpshooter leafhoppers is presented. Bacteria in the precibarium were found both anterior and posterior to the precibarial valve. This valve was recently described by Backus et al (2) in their study on the sensory systems and feeding behavior of leafhoppers. The operation of the valve in sharpshooter feeding and the importance of the location of bacteria in relation to this valve are unknown, but may be important in vector transmission of XLB to plants. There is no information to indicate whether the XLB that are transmitted to plants originate anterior or posterior to the valve. The valve may preclude movement of bacteria from the cibarium into the plant xylem. Large colonies of bacteria near the precibarial valve (Fig. 3E) could possibly cause valve dysfunction.

Bacterial attachment in all combinations was polar. More bacterial growth was noted in the center of the floor of the cibarium of *H. coagulata* containing the PP or PLS bacteria than of *O. nigricans* and the PD or PW bacteria. With PP and PLS bacteria,

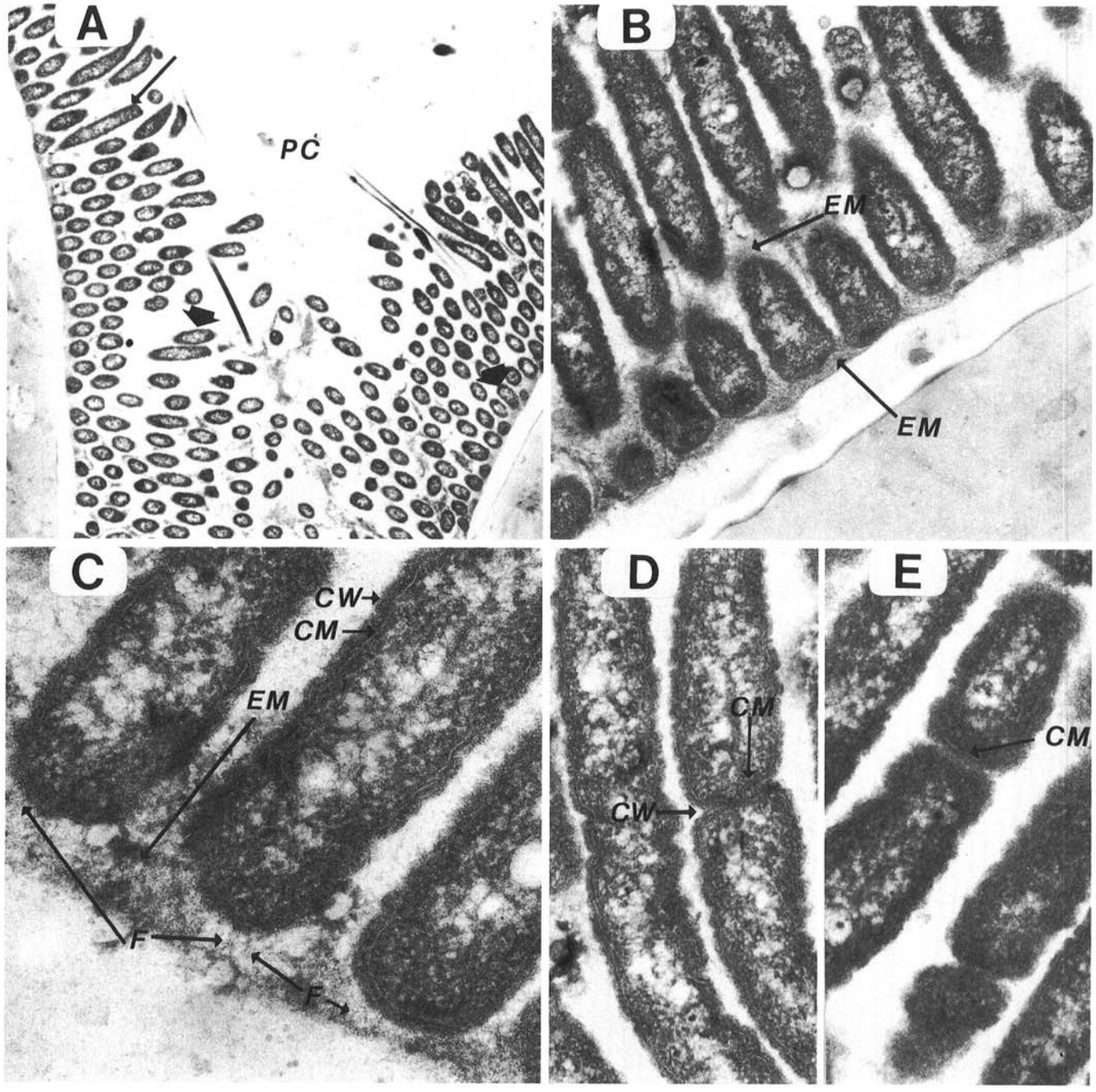

Fig. 4. Transmission electron microscopy of periwinkle wilt bacteria attached to the precibarium of the sharpshooter, *Oncometopia nigricans*. **A,** Bacteria (arrows) attached to the lining of the precibarium (PC) posterior to the precibarial valve (×8,580). **B,** Bacterial colony attached to the lining of the precibarium showing an extracellular material (EM) being produced which appears to attach bacteria to the sharpshooter and to each other (×30,400). **C,** Enlarged view of attached bacteria showing extracellular material and fimbraelike structures (F) extending from the bacteria cells (×74,385). **D,** Bacterium undergoing cell division in the precibarium (×46,500). Note the cytoplasmic membrane (CM), and cell wall (CW) bending. **E,** Two bacterial cells after cell division (×59,150). Note the appearance of the cytoplasmic membrane (CM).

extracellular material could be seen associated with the bacterial colonies. It is likely that these materials could be either the extracellular polysaccharides or glycocalyx commonly associated with some bacteria (4,5). Polar attachment of bacteria was probably due to the binding action of the extracellular material produced by the bacteria; however, fimbraelike structures (13) also were observed. Fimbrae are often seen on bacteria both in plants and from agar cultures. The bacterial colonies appeared to be in a protective matrix, which may provide a protected environment that could be analogous to bacteria found attached to rocks in a rushing stream (4). Bacteria not attached in a stream would be swept away and the stream itself would then be virtually sterile. Purcell et al (16) also observed a matrix material surrounding PDB aggregates in paraffin sections and suggested that attachment prevents the bacteria from being dislodged in the flow of ingested fluid, which he estimated passes through the precibarium at an average velocity of 8 cm/sec. Besides offering physical protection to the bacterial colony, this matrix may aid also in the extraction of nutrients from the fluid stream (4). Polysaccharide fibers of the glycocalyx are often negatively charged and thus can bind certain nutrients that pass by (4).

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