Physical Entrapment of Pseudomonads in Bean Leaves by Films Formed at Air-Water Interfaces

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ABSTRACT

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Water infiltrated into the intercellular spaces of bean leaves appears to dissolve materials from the cell wall surfaces. Subsequent transpiration causes intercellular fluid to recede until films, consisting of amorphous and/or fibrillar material condensing from the dissolved material, form at air-water interfaces. Bacteria introduced during water infiltration become trapped by films on cell wall surfaces or in corners between juxtaposed mesophyll cells. After infiltration, no difference in entrapment was observed at 3 hr between homologous or heterologous strains of *Pseudomonas syringae*, and the heterologous pathogens, *P. marginalis, P. pisi,* and *P. tomato*, or the saprophyte *P. fluorescens*. By 12 hr, bacterial multiplication was occurring with both the homologous *P. syringae* strain and heterologous *P. tomato* and colonies thereof, bounded by films, were expanding. Cells of pathogens or saprophytes were not attached to the cell wall and remained suspended in the intercellular fluid if the leaves were kept watersoaked for 12 hr. Under these conditions both pathogens and saprophytes appeared to multiply. Films observed in the water-soaked leaves were associated with gas pockets found in crevices between plant cells and not with bacterial cells. Leaf age greatly affected the amount of material that condensed in the films and the deposition of materials behind them. More films were formed and a greater amount of material was deposited in younger leaves. Vacuum infiltration of the electron microscopy fixative into tissue affected the integrity of the films. It is postulated that films may play a role in pathogenesis by assisting in the retention of moisture in the microenvironment surrounding the pathogen, thus enabling it to multiply.

Additional key words: host susceptibility.

Several plants are reported to immobilize and later encapsulate saprophytic (9,23,26), heterologous (4,9,12,17,19), or avirulent bacteria (23), but not virulent homologous pathogens. The postulated first step (23) in this process is the attachment or agglutination of incompatible bacteria to the plant cell wall. This is followed by a complex series of structural changes associated with the host plant actively immobilizing the bacteria with an engulfing membrane (3), wall cuticle (9,10), or pellicle (23).

Agglutination factors which apeared to affect host-parasite relationships were first reported around the turn of the century (20). Later, Berridge (2) established that potatoes contained a factor which agglutinated saprophytic bacteria but not potato pathogens. An agglutination factor isolated from potato has been characterized as a lectin (11,24). It is likely that a number of other agglutination factors are lectins (8,11).

The active immobilization process and the involvement of lectin or other specific agglutination compounds offers an attractive hypothesis to apply to the understanding of generalized plant defense mechanisms. However, a continuation of our studies with bean (26) has led us to question whether bacteria are actively immobilized by the plant. Also, some data are not supportive of the concept that rapid (9,23,26) immobilization and encapsulation occurs upon the ingress of bacteria into nonsuscept tissues. For example, multiplication of pathogens often occurs in nonhost plant leaves (1,5,7,31) although heterologous bacteria are reported to be rapidly encapsulated (4,9,12,17,19). Is this multiplication possible because some (12) or all (26) bacterial cells escape encapsulation, or because cells break out of the relatively fragile binding structures (4)? We have investigated the phenomenon of attraction and encapsulation further by studying the fine structure of the interaction of a variety of different bacteria with a single host species.

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MATERIALS AND METHODS

Pseudomonas syringae strain NCPPB 1004 isolated from common bean was used as a homologous pathogen. Heterologous pathogens were *P. syringae* strains NCPPB 1071 and NCPPB 1259 from lilac and pear, respectively, and *P. pisi* NCPPB 2353, *P. tomato* NCPPB 880, and *P. marginalis* 3 and 6. *Pseudomonas fluorescens* Biotype II strains 2 and 108 (28) were used as saprophytes because of their close relationship with *P. marginalis*. Bacteria were grown on nutrient agar for 18–24 hr at 22–24 C and inoculum was prepared by washing cells from agar slants with water. Primary leaves of 7- to 10-day-old bean plants (*Phaseolus vulgaris* L., 'Pinto') were infiltrated with approximately 10^9 bacteria per milliliter by submerging the leaves of potted plants in the inoculum and applying a vacuum (20 mm-Hg pressure) for 2 min. Leaves infiltrated with water were used for comparison. Intact, infiltrated plants either were incubated in the open, lighted laboratory for 3–12 hr or placed in plastic bags to maintain a water-soaked condition for these time periods. Visible evidence of watersoaking in the nonbagged plants visually disappeared after 1–2 hr although evidence of large water droplets in the intercellular spaces was observed up to 3 hr with the electron microscope (Fig. 15).

After the incubation period, areas which had been infiltrated were cut from the plant and placed in 4% glutaraldehyde fixation buffer with 0.08 M sodium cacodylate at pH 7.0. Sections of leaf tissue about 1-mm wide were cut from the immersed leaf pieces and placed in fresh fixative. During the early stages of the experimentation, samples were subjected to mild, intermittent vacuum infiltration for the first 30 min of a 2-hr fixation period. Although mild vacuum infiltration is the customary method of fixing leaf tissue, it contributed to the disruption of the delicate extracellular structures that are of primary importance to this study. Consequently, samples were fixed without vacuum, but with rotation for 2 hr. Cheesecloth plugs kept the tissue submerged in the fixative. All glutaraldehyde fixation was done at room temperature.

Fixed tissue was then rinsed in buffer with three changes during a

2-hr period. Samples were postfixed in sodium cacodylate-buffered 1.5% osmium tetroxide overnight at 4 C. Fixed tissue was washed with distilled water, dehydrated through an acetone series, and embedded in a low viscosity embedding media (27).

Silver sections were expanded with chloroform vapor, picked up on bare 300 mesh grids, and stained with aqueous uranyl acetate and Reynolds' (18) lead citrate. Specimens were examined and photographed with a Phillips 300 electron microscope operating at 60 to 80 Kv.

RESULTS

The noninfiltrated plant. The cell wall/intercellular space interface on noninfiltrated leaf tissue was typically smooth and unremarkable (Fig. 1). Films occasionally were found near cell junctures (Fig. 2), attached to walls and stretching across the intercellular spaces.

The water infiltrated plant. When distilled water alone was infiltrated into leaves about three to four times as many films were observed than in noninfiltrated leaves. The films appeared to form at a gas/liquid boundary of introduced intercellular water. In addition to observing films of the type found in noninfiltrated leaves (Fig. 3), there also were delicate films spanning long distances. At other times structures were seen that resembled those typically reported to engulf bacteria (Fig. 4, 5, and 7). Often a thickened structure was observed on the liquid side (behind) of a film in the area where the film and plant cell wall met. This thickened structure was termed the collar. The collar material may appear granular (Fig. 4) or fibrillar (Fig. 7).

The curvature of the film in Fig. 6 in contrast to those formed when water remained in the corners (Fig. 2 and 3) suggested that this film was laid down over a gas pocket in the corner. Also the cell wall surface behind the film was compact and smooth, a consistent feature of the wall/gas interface. Note the looseness of the cell wall on what we presume to have been the water side of the film. This loose condition has been associated with pathogenic bacteria; however, we interpret it as being due to the presence of liquid which solubilizes materials at the cell wall surface, freeing parts of the microfibrillar component.

The heterologous pathogen. At 3 hr after inoculation, most of the cells of the heterologous pathogens *P. tomato, P. pisi, P. marginalis,* and *P. syringae* (heterologous strains) were associated with films or other entrapment structures (Fig. 8–15). The entrapment structures varied in different sections. Some bacteria were enmeshed in a network of fibrillar material which presumably had aggregated upon evaporation of the water (Fig. 8). This structure appeared very similar to that seen in Fig. 7 where water only was infiltrated. Films were observed that were thin and well defined (Fig. 9) or thicker and looser (Fig. 10). Both fibrillar and amorphous material was present at the collar region of some films (Fig. 11). Bacteria were suspended at times between two films which formed bridges between plant cells (Fig. 14). This bridge appeared to have been formed when a bacterium was deposited against a film formed over a gas pocket and a second film was formed over the bacterium when the water evaporated (compare with Fig. 6). Occasionally bacteria were trapped in larger pockets of water which had not evaporated from the leaf (Fig. 15). Note how the bacterium was apparently floating freely in the fluid and was possibly dividing. Similar droplets or pockets of water containing freely floating *P. marginalis* cells also were observed.

Sometimes films were formed at a distance from the bacterial cell (Fig. 9) or were formed around the base of the bacterial cell to form a stalk (Fig. 12). There was no apparent filling in behind the films in some cases (Fig. 9), whereas in other leaf samples infiltrated with the same bacterium, the bacterial cells were completely encased in an electron-dense matrix (Fig. 13). Encasement occurred predominantly in physiologically younger leaves.

Examination of *P. tomato* and *P. marginalis* infiltrated tissues revealed that at 12 hr many cells of these heterologous pathogens were embedded in an electron-dense matrix (Fig. 16). With *P. tomato,* it appeared that multiplication occurred as approximately 3.5 times more bacterial cells per micrograph were observed in sister leaves at 12 hr than at 3 hr. The entrapment boundaries were pushed outwards, but not to the same degree as seen later with the homologous pathogen (Fig. 17). Cells in these colonies appeared to be tightly packed together. Occasionally, bacterial cells not associated with films were observed and these did not appear to be dividing (Fig. 18). As with other infiltrated samples, numerous films were observed in areas where bacteria were not present (Fig. 19). There was no evidence that multiplication occurred with *P. marginalis* as the average number of cells observed per micrograph was approximately the same (1.6 to 1.7).

The homologous pathogen. The cells of the compatible pathogen were entrapped by films (Fig. 20–23) at 3 hr, as with heterologous (compare Fig. 11 with 21) or saprophytic bacterial cells (compare Fig. 20 with 30). By 12 hr, however, the relationship of bacteria to entrapment differed from that with saprophytic or heterologous pathogens, since the pathogens were multiplying (4.3 times the number of cells per micrograph were seen) and forming colonies within the intercellular spaces. Nearly all colonies were bounded by discernible films (Fig. 24–27). Within the colonies, the bacteria were not packed tightly together as with *P. tomato* and were surrounded by varying amounts of loose fibrillar material and an electron-lucent matrix (Fig. 24–28) similar to that observed with *P. phaseolicola* in bean (13). Convex film boundaries were observed in some cases (Fig. 26) which appeared to indicate a reversal of pressure relationships between liquid and gas phase in the intercellular space. The amount of electron dense material aggregating in the films differed along the film (Fig. 26) and in different colonies in the same leaf (Fig. 27). Lack of electron dense material aggregating in the films prevented film detection in some cases although the colony was clearly contained to a portion of the intercellular space. Possible remnants of a collar and original entrapping material were seen in some sections which appeared to have been pushed out of the way after cell division began (Fig. 28).

The saprophyte. Both strains of *P. fluorescens* were encased in films at 3 hr (Fig. 29 and 30). Not much filling in behind the film was observed in either case. At 12 hr, most bacterial cells were embedded in an electron dense matrix (Fig. 31, 32b) although occasionally some were behind films only (Fig. 32a). No evidence of bacterial multiplication was observed in either the 3- or 12-hr specimens as determined by the number of bacterial cells per micrograph.

Bacteria in the water-soaked leaf. In water-soaked leaves both pathogens and saprophytes appeared to float freely in fluid in the intercellular spaces (Fig. 33, 34, 37) and were not bound by films or entrapment structures nor were they associated with the cell walls of the plant. Bacterial multiplication appeared to be occurring as often small groups of bacteria were observed and approximately 1.4 to 1.8 times as many cells per micrograph were observed. In one instance with *P. fluorescens,* a bacterial flagellum was observed (Fig. 38). The few films which were observed delimited what appeared to be gas pockets (Fig. 33, 34). The remnants of what were probably films present before watersoaking were seen occasionally disintegrating in the intercellular fluid (Fig. 35). Where a cell wall/water interface was present, the wall surface was loosely fibrillar (Fig. 36). It is of interest to note that all leaves infiltrated with either pathogens or saprophytes collapsed shortly after removal from the humidity chamber and drying out.

Plant age effect. When examining various tissue sections, there were differences in the amount of material accumulated behind films. This difference appeared to be associated with the physiological age of the plant (as judged by the proportion of intercellular space to mesophyll tissue) rather than to the presence of bacteria. This phenomenon was further investigated by comparing primary leaves from 7- and 10-day-old plants with a fully expanded primary leaf from a 24-day-old plant which was infiltrated with the homologous *P. syringae* strain.

The most films were observed in the 7-day-old leaves and greater deposits of middle lamella-like material were noted in these specimens (Fig. 39). An example of the more delicate films observed in the 10-day-old plant is shown in Fig. 40. Little evidence of film formation was observed in the 24-day-old leaf and where films were found associated with bacteria, the film was very thin and discontinuous (Fig. 41).

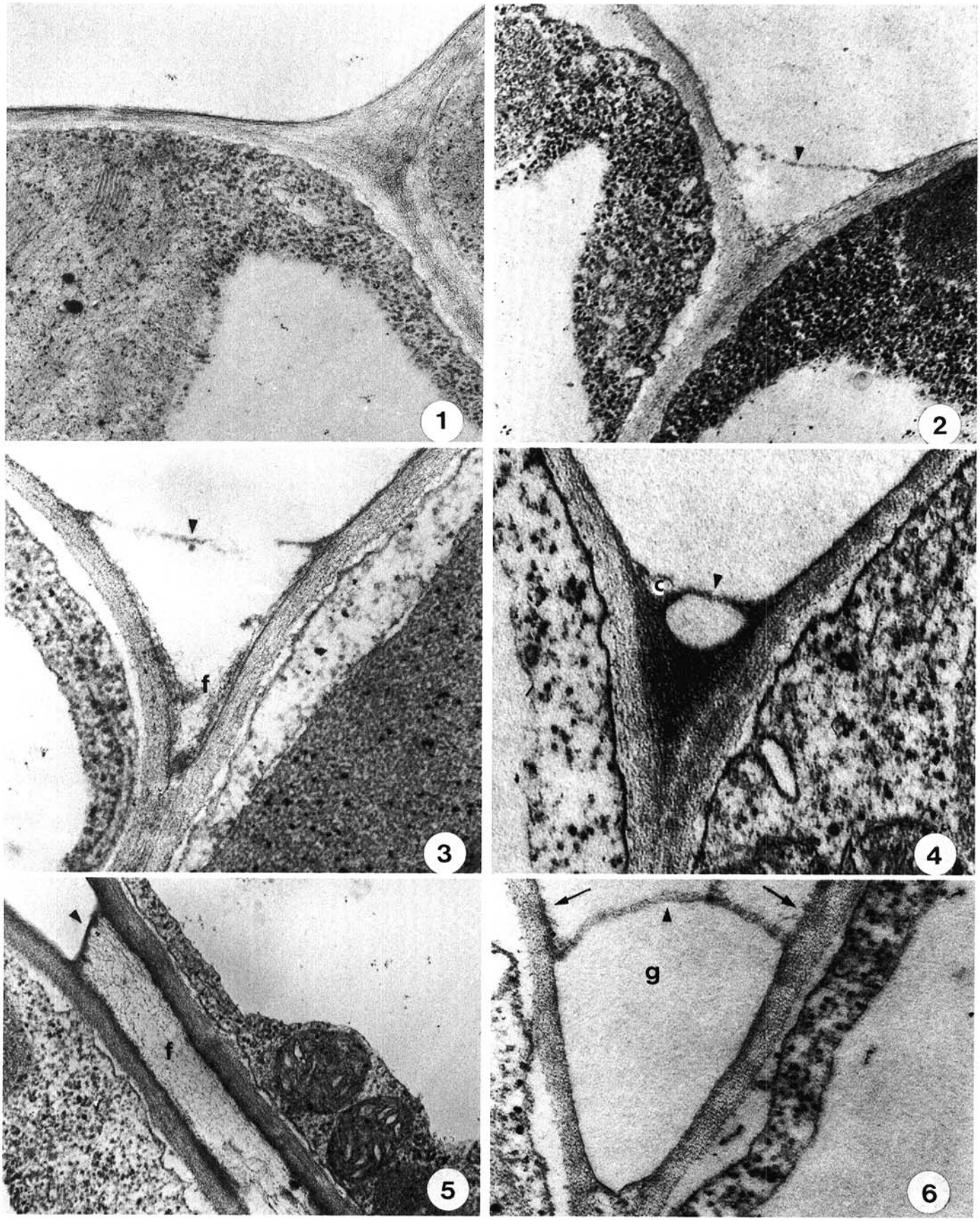


Fig. 1–6. Intercellular spaces of normal bean leaf mesophyll uninfiltrated (Fig. 1–2) or infiltrated with water and fixed 3 hr after infiltration (Fig. 3–6). Unlabeled arrowheads indicate films. **1,** A typical cell juncture; cell wall surfaces are smooth and no air-water interface films are present (×27,500). **2,** Film across cell juncture (×42,500). **3,** Film observed across cell juncture. Note abundance of fibrillar material (f) along the wall behind the film (×44,000). **4,** Film with dense deposit of material. Collarlike structure (film collar) (c) is associated with the film (×55,000). **5,** Film between two cells delimiting a chamber that contains a considerable amount of fibrillar material (×35,000). **6,** Film delimiting a presumed gas pocket (g). Note the smoothness of the cell wall where air was present behind the film and the roughness of the water-treated wall outside the film (arrows) (×71,250).

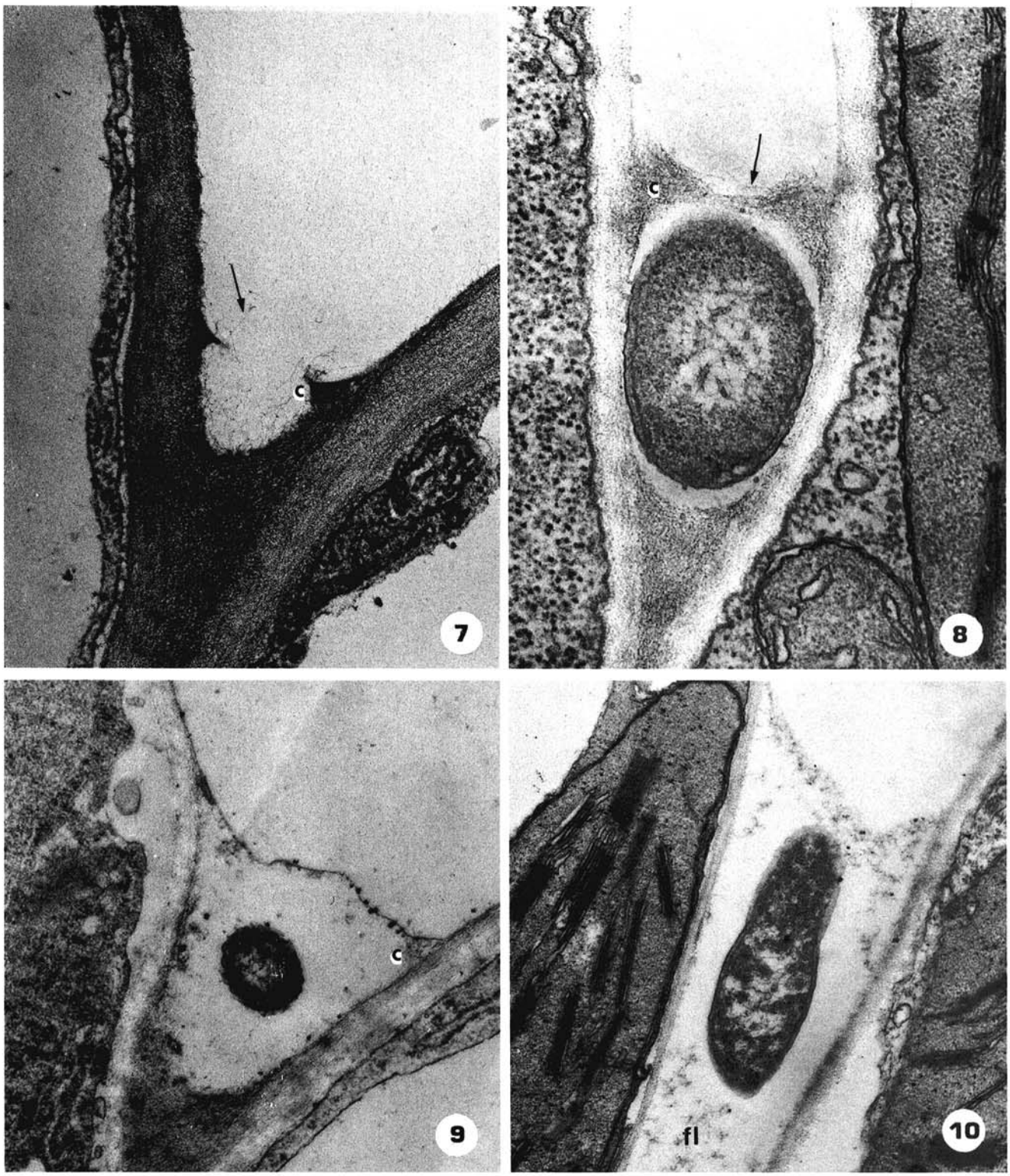


Fig. 7–10. Films formed in bean leaf mesophyll 3 hr after infiltration with water (Fig. 7) or with water plus heterologous pathogenic bacteria (Fig. 8–10). Collarlike structures (film collar) (c) composed primarily of fibrillar material (small arrows) frequently develop in regions where films stabilize in crevices between juxtaposed cell walls (Fig. 7–9). **7,** It appears that a delicate connection (film) across the film collars has been disrupted, leaving an artifact believed to be associated with vacuum infiltration of fixative (×39,330). **8,** Film collar with continuity of fibrillar material across the intercellular gap entrapping a *Pseudomonas pisi* cell. Note similarity of the chambers in Fig. 7 and 8 (×70,725). **9,** *P. marginalis* cell trapped by a film. A film collar has formed on one side of the film (×36,340). **10,** A heterologous *P. syringae* entrapped in flocculent (fl) intercellular material. In this example, no distinct film or film collar is present. Note similarity of this structure to that observed with the homologous *P. syringae* shown in Fig. 23 (×37,605).

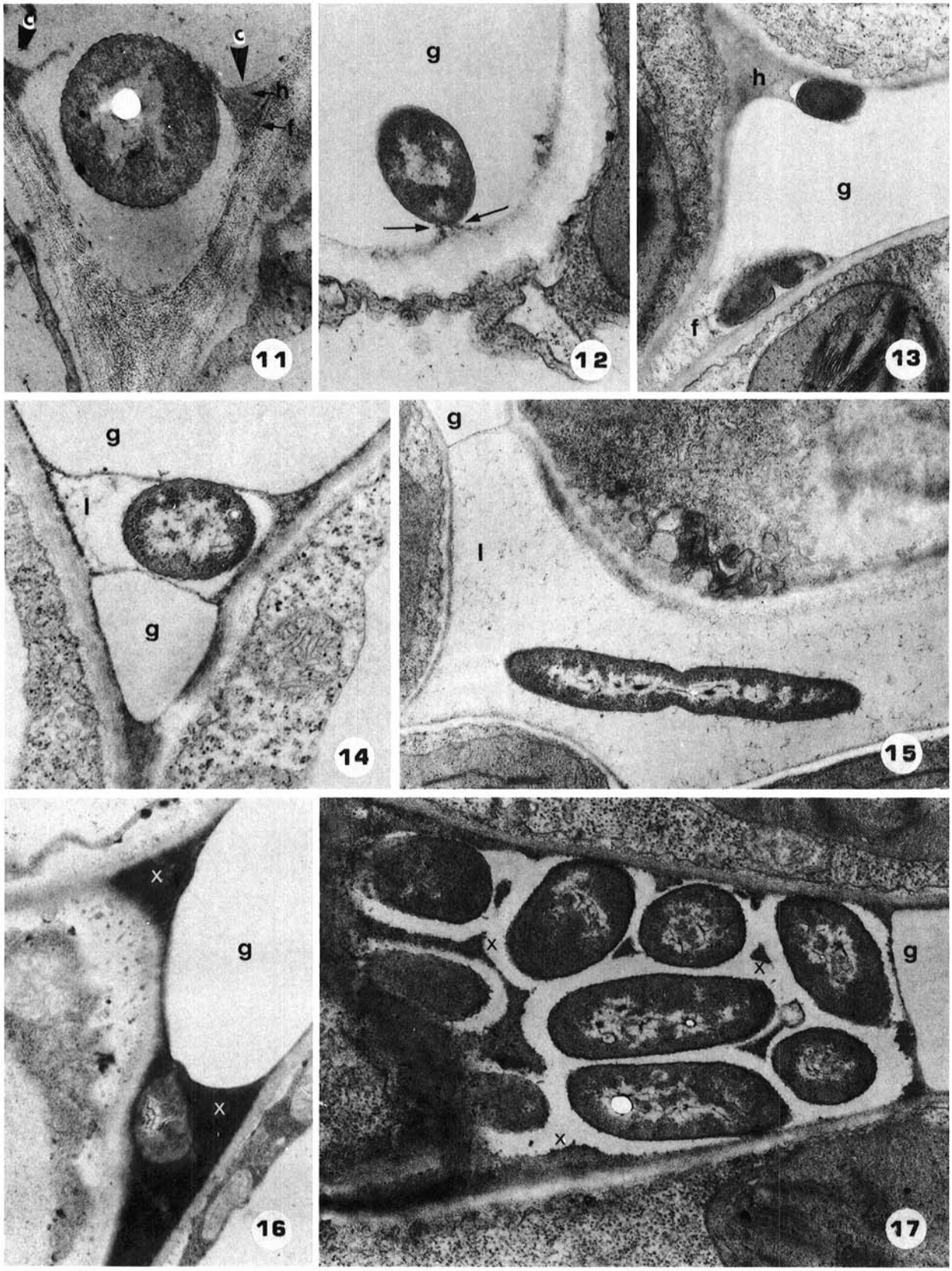


Fig. 11–17. Types of films associated in bean leaf mesophyll with heterologous pathogens 3 hr (Fig. 11–15) and 12 hr (Fig. 16–17) after infiltration. g = gas phase in intercellular space; l = liquid phase. **11,** Film covering a *Pseudomonas marginalis* cell. Note that the film collar (c) is composed of homogeneous-appearing component (h) and a granular or fibrillar component (f) (×70,800). **12,** A heterologous *P. syringae* cell attached by a stalk (arrows) to the host cell wall. The stalk may be formed from collapse of a film over the bacterium or the deposition of material around the base of the bacterial cell in a process similar to film collar formation (see Fig. 7–9, 11, 20) (×70,800). **13,** Cells of *P. tomato* which have been surrounded by a deposit of fibrillar (f) or homogeneous (h) material (×20,400). **14,** Bridge entrapping a *P. marginalis* cell formed by the recession of water to a trapped gas pocket (×34,800). **15,** *P. tomato* cell trapped in a large water pocket bounded by a film. Note that the bacterium appears to be dividing and is not associated with the plant cell walls (×18,000). **16,** A *P. marginalis* cell encased in an electron-dense matrix (x) (×21,600). **17,** A colony of dividing *P. tomato* cells. Note the closeness of the bacterial ceils to each other and the presence of considerable electron-dense material (x) between the cells (×24,000).

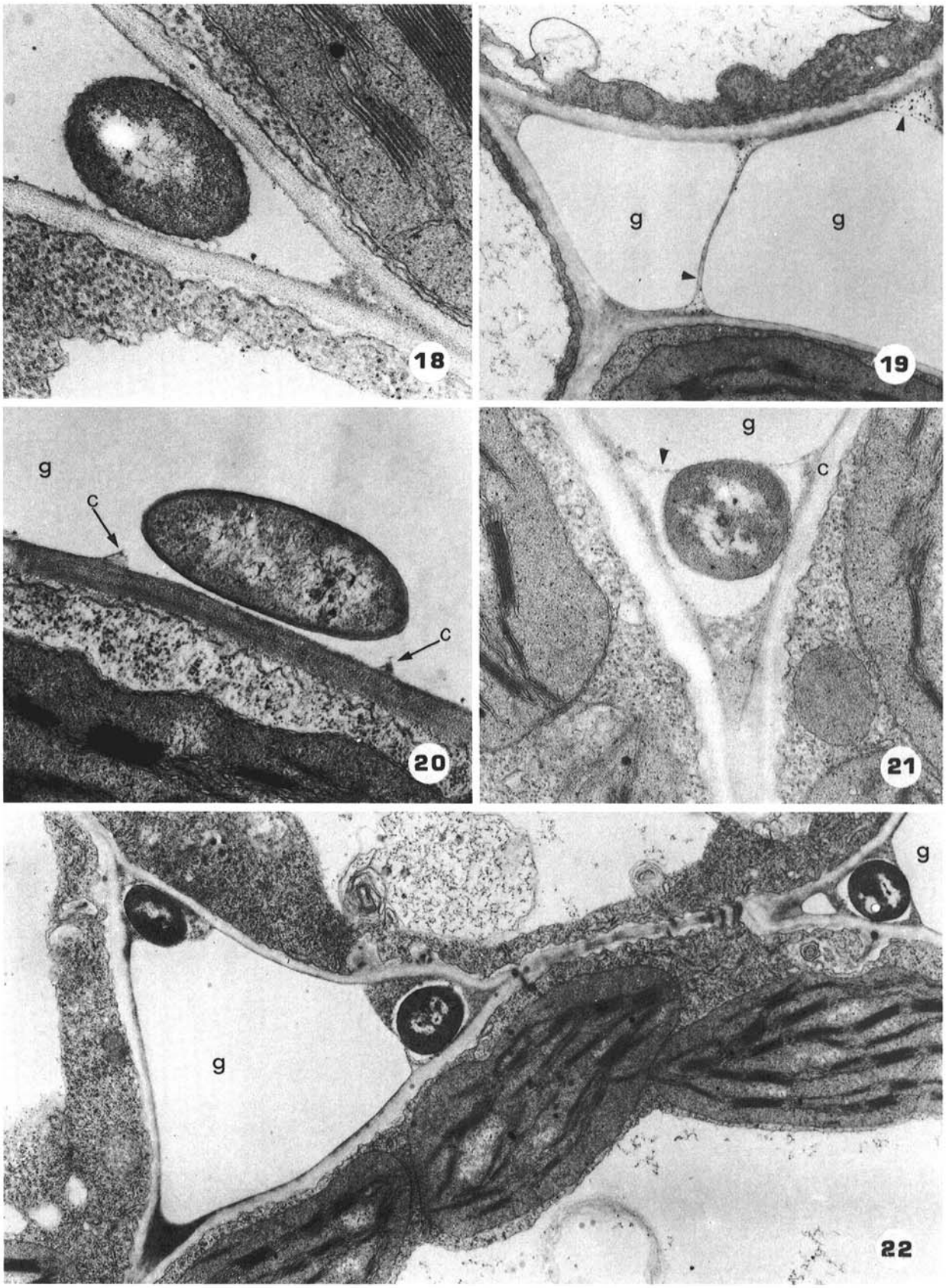


Fig. 18–22. Bean leaf mesophyll tissue 3 hr after infiltration with water suspensions of heterologous (Fig. 18–19) or homologous (Fig. 20–23) bacteria. g = gas phase in intercellular space; arrowheads indicate films. **18,** A *Pseudomonas tomato* cell in an intercullular space. No film is discernible, but there is some roughness of bean cell wall associated with the bacterium, which is suggestive of liquid being present (×54,840). **19,** Films (arrowheads) bounding an intercellular bridge and corner deposits in a leaf infiltrated with water and bacteria in an area where bacteria do not appear to be present (×18,000). **20,** Cell of the homologous *P. syringae* lying against the plant cell wall. A complete film is not discernible, but a collarlike base (film collar) (c) is indicative of film presence. Note the similarity of this bacterium-cell wall association with that of the saprophyte shown in Fig. 30 (×33,600). **21,** Cell of homologous *P. syringae* covered by a film. A film collar is indicated at c (×34,800). **22,** Cells of the homologous pathogen embedded in an electron-dense deposit on the walls of a young host leaf (×15,600).

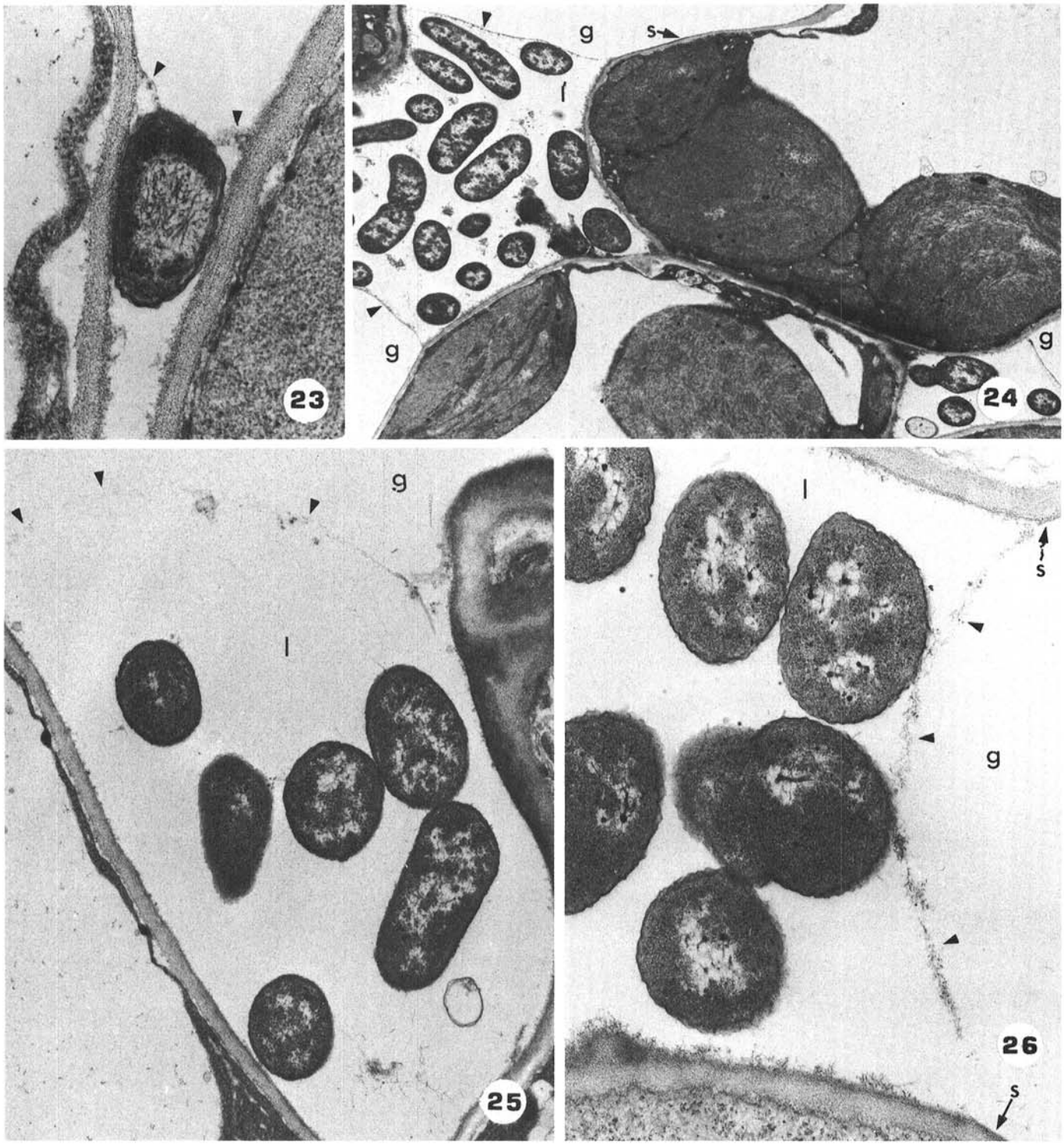


Fig. 23–26. Films in bean leaf mesophyll infiltrated with an aqueous suspension of the homologous strain of *Pseudomonas syringae* 3 hr (Fig. 23) or 12 hr (Fig. 24–26) after infiltration. Note a lack of crowding of the bacterial cells and a lack of intercellular matrix between the bacterial cells at 12 hr. Contrast Fig. 24–26 with Fig. 17 of a heterologous pathogen (*P. tomato*) which showed more closely packed cells and more intercellular matrix. g = gas phase in intercellular space; l, liquid phase; unlabeled arrowheads indicate films; s = smooth wall. **23,** bacterial cell trapped behind a film. Note the similarity of this film to that observed in Fig. 10 with the heterologous *P. syringae* (×51,600). **24,** Two colonies of *P. syringae* bounded by films (×13,200). **25,** The curvature of the film at the gas-liquid interface may indicate a pressure differential which provided a means for colony expansion (×29,040). **26,** Structure of the film covering a colony of *P. syringae* showing how the film is composed of aggregates of fibrillar and granular substances. Note that the host cell wall exposed to the gaseous intercellular space is smooth (s) whereas the wall exposed to the liquid phase around the colony has a fuzzy margin (×38,640).

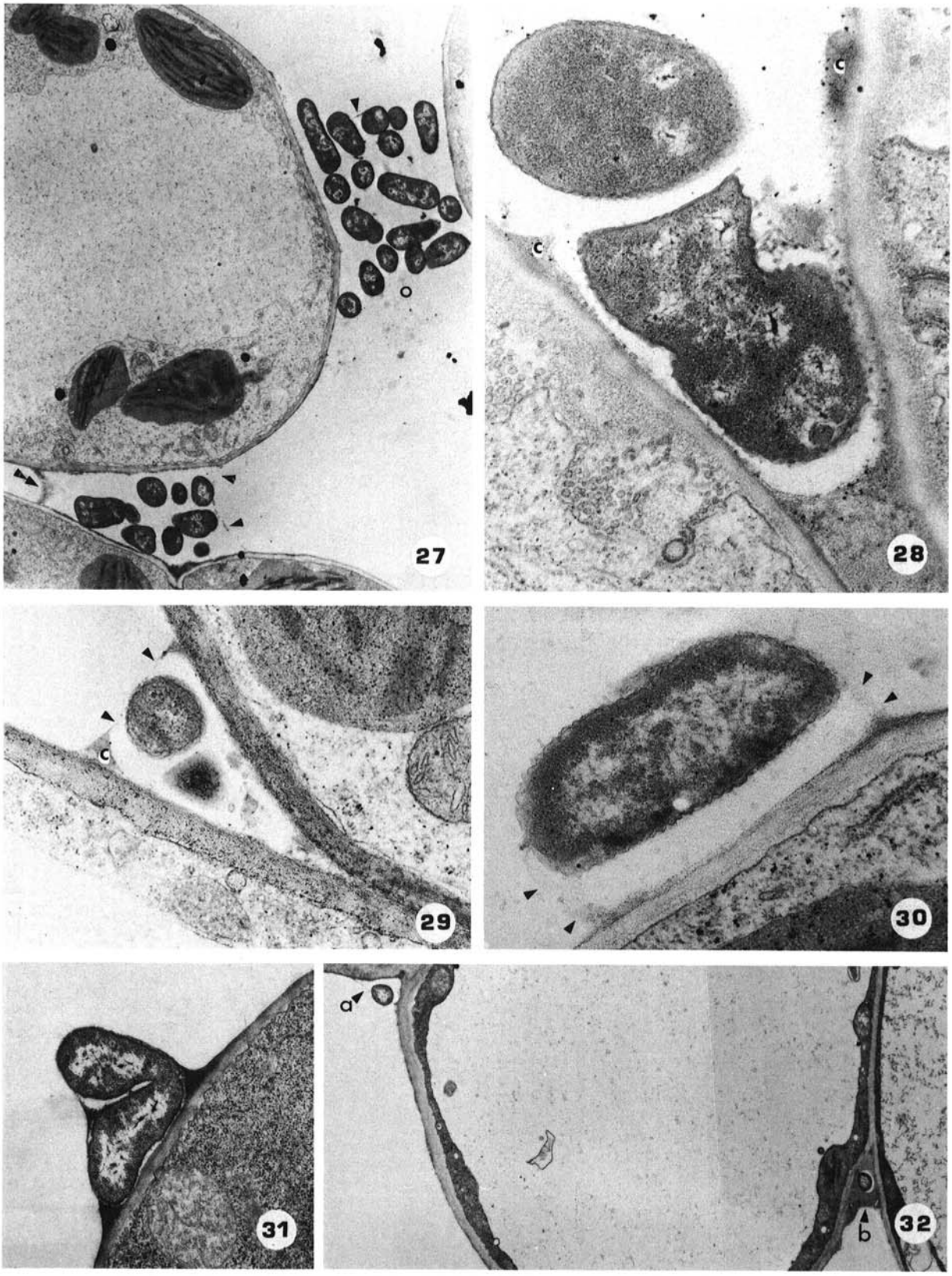


Fig. 27–32. Film structures associated with the homologous pathogen after 12 hr (Fig. 27–28) and a saprophyte after 3 hr (Fig. 29–30) or 12 hr (Fig. 31–32) unlabeled arrowheads indicate film; c = film collar. **27,** Colonies of homologous *Pseudomonas syringae.* Note the differences in the amount of stainable material in the individual films bounding the colonies. There are heavy films (double arrowhead), delicate films (arrowhead), and regions where we believe there are nondetectable films (0) (×6,600). **28,** Colony of *P. syringae* showing remnants of what is believed to be portions of a film collar of the original chamber formed before the colony began to expand (×38,400). **29,** *P. fluorescens* cells bounded by a film with a collar (×33,600). **30,** *P. fluorescens* cell after 3 hr showing a delicate film holding the bacterium to the bean cell wall. Note the similarity of entrapment of this bacterium to that shown in Fig. 20 with a homologous pathogen (×44,640). **31,** Cells of *P. fluorescens* at 12 hr showing deposit of electron-dense material around the cells (×19,800). **32,** Two cells of *P. fluorescens* adjacent to the same plant cell 12 hr after inoculation. Two different types of entrapment are shown: a simple, thin film (a) and a thick, encapsulating deposit (b) (×6,480).

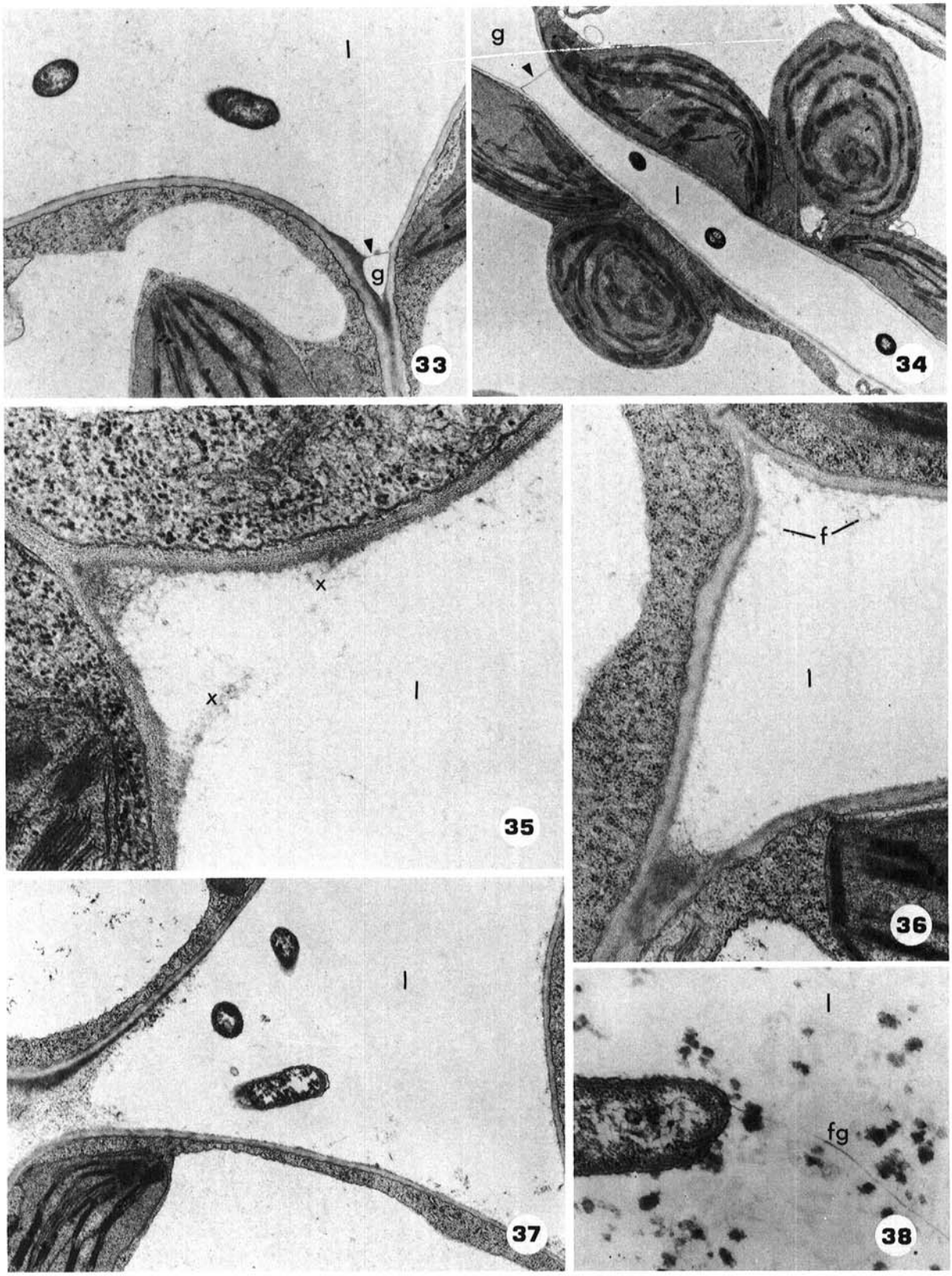
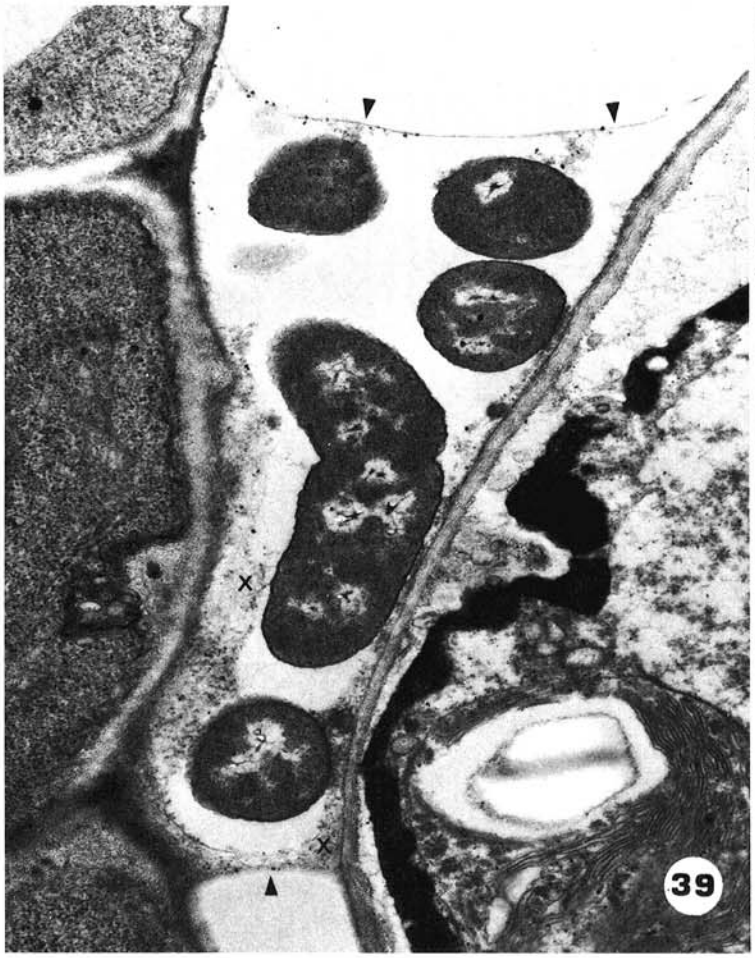
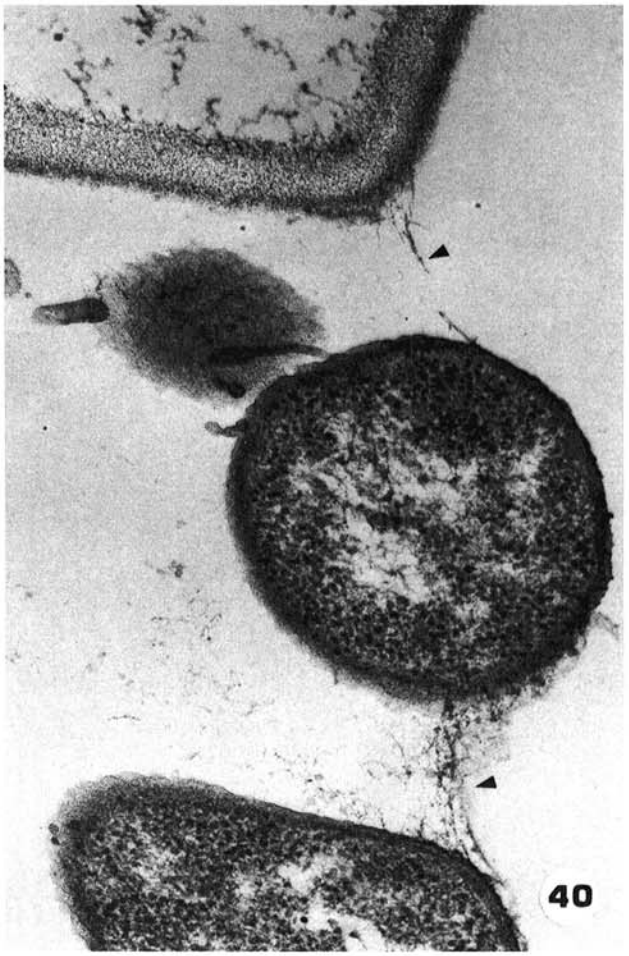


Fig. 33–38. Conditions in bean leaf mesophyll 12 hr after infiltration of heterologous pathogens or saprophytes into leaves that were kept continuously watersoaked. g = gas phase; l = liquid phase; arrowheads indicate films. **33,** *Pseudomonas fluorescens*. Note the lack of association of the bacterial cells with plant cell walls. A film covering a gas pocket is present (×13,200). **34,** *P. tomato* cells also are not associated with host cell walls. The film present delimits a large gas pocket within the watersoaked leaf (×7,200). **35,** The remnants of what was probably a film present before watersoaking, is indicated by the x's. (×56,880). **36,** Loose fibrils (f) are seen to be associated with cell walls of watersoaked leaves (×28,200). **37,** *P. marginalis* cells free in the intercellular liquid (×13,440). **38,** A *P. fluorescens* cell in a watersoaked leaf with an apparent flagellum (fg) (×36,000).





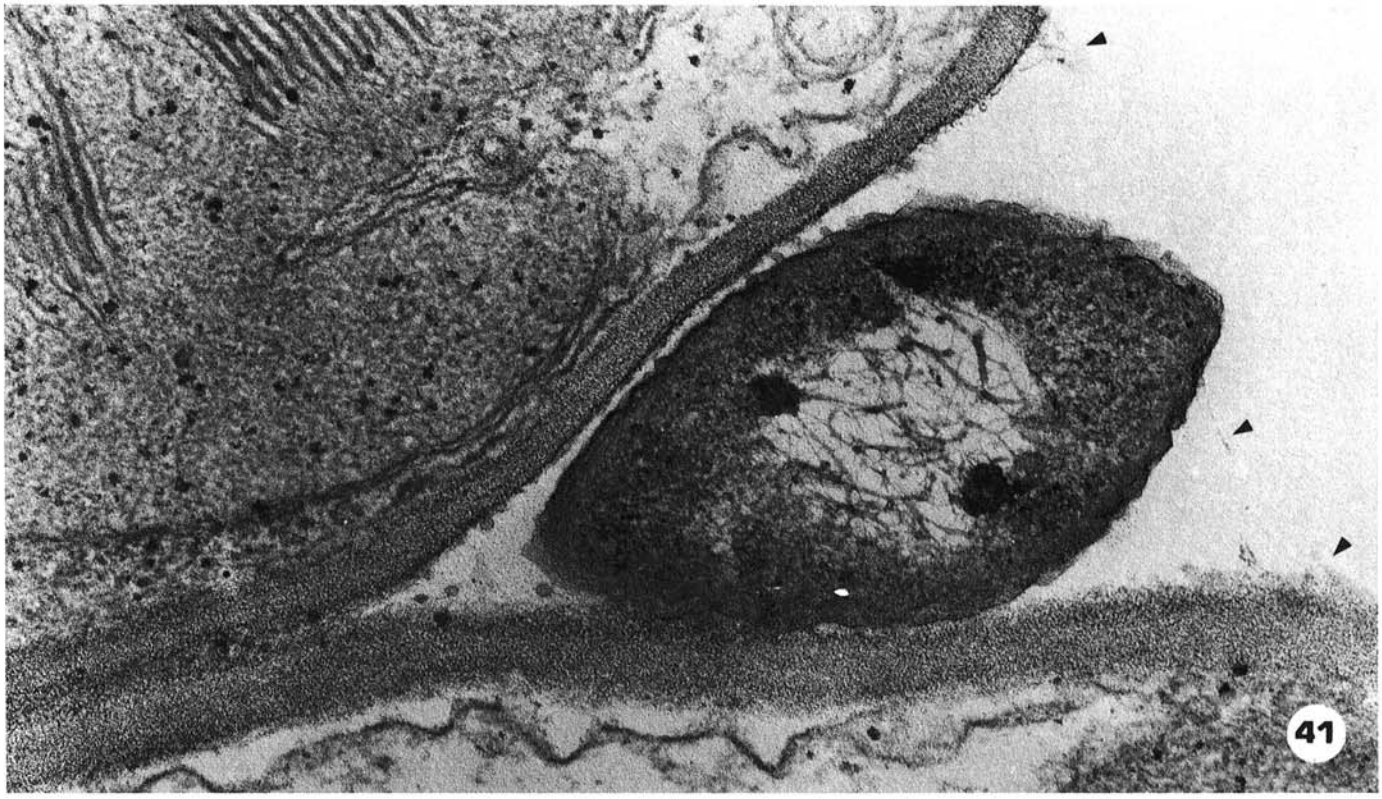


Fig. 39–41. The effect of leaf age on film characteristics in bean leaf mesophyll 12 hr after infiltration. Arrowheads indicate films. **39,** Colony of a homologous *Pseudomonas syringae* strain in a young (7-day) leaf. Considerable intercellular matrix (x) is aggregated around the bacterial cell. Films surrounding the colony are relatively heavy (×24,000). **40,** Colony of a homologous *P. syringae* strain in a moderately aged (10-day) leaf showing more delicate films (×61,800). **41,** Homologous *P. syringae* cell in an old (24-day) leaf showing only traces of a film (×83,520).

DISCUSSION

The interpretation of electron micrographs in studying attachment and encapsulation of bacteria in the intercellular spaces of leaves of plants is difficult and subject to opinion. We felt that some interpretations have been overly influenced by the hypothesis that saprophytic, heterologous, and avirulent pathogenic bacteria are attracted to plant cell walls, become engulfed or encapsulated by material from the host plant cell, and that this process is the result of a generalized defense mechanism against invading bacterial cells. Our studies with bean as well as examination of various published electron micrographs lead us to question whether this is a general phenomenon.

We postulate that many of the structures observed are not formed in response to bacteria, but are formed as a result of physical forces involved in water drying from the intercellular spaces. We believe that the water placed in the intercellular spaces during introduction of bacteria dissolves material on the plant cell wall surface. As the water evaporates from the intercellular spaces, the dissolved material becomes concentrated and some of it polymerizes or condenses forming a film or deposit. Because of the nature of the physical forces involved in transpiration of apoplastic water (30), these films and deposits usually form in corners between plant cells and around objects such as bacterial cells. Another observation consistent with the evaporation is the meniscus-like shape of the films associated with bacteria or formed elsewhere. These films and deposits in the corners of intracellular spaces were readily formed in tissues of uninoculated plants.

The interpretation that some bacteria are attached and encapsulated in contrast to others can be easily derived if close attention is not given to the age of the leaf and to the amount of water remaining in the intercellular spaces prior to fixation. No difference in type or degree of encapsulation was detected between homologous and heterologous pathogens, or saprophytes during the first 3 hr after inoculation in contrast with our earlier report (26). This is consistent with Sigee and Epton's findings (25) that *P. phaseolicola* in both susceptible and resistant beans were surrounded by what we term "films" in the early stages of pathogenesis. Others have reported that considerable fibrillar material was associated with homologous pathogens at certain stages of infection (12,14) or at times they were contained in "membrane"-bounded baglike structures (29). The differences we observed between pathogens and saprophytes relative to encapsulation appeared related to bacterial multiplication: the homologous pathogen multiplied and displaced films and other entrapment structures. One heterologous pathogen, *P. tomato,* also multiplied to a limited extent pushing films out a short distance or breaking them; another heterologous pathogen, *P. marginalis,* as well as the saprophytes did not appear to multiply, unless in water-soaked tissue, so films and other entrapment structures remained in place.

Further evidence that the so-called attachment of bacteria to cell walls in bean is not an active process, but is, instead, related to evaporation of intercellular water, lies with observations of the watersoaked condition. When leaves infiltrated with heterologous and saprophytic bacteria were not allowed to completely dry through transpiration no attachment occurred in the liquid containing areas within 12 hr. Bacterial attachment should not be prevented by the presence of water if this is an active process such as that evidenced by the attraction of *Agrobacterium* to plant cells (15) or root hairs (21). Also, under water-soaked conditions, both heterologous or saprophytic bacteria are reported to multiply (1,7,16,32).

The film, rather than related to a defense mechanism, more likely is important in pathogenesis. Bacteria enter plant leaves in a water phase and maintenance of a liquid phase around the bacteria cell is needed for multiplication. Films forming around bacteria may assist the pathogen in maintaining this liquid phase, especially in the early stages of incubation. The homologous pathogen in a susceptible host could maintain liquid around it through a combination of films, polysaccharides (6), and osmoticum. It is possible that homologous pathogens could not maintain the liquid phase around itself in normally susceptible hosts if adequate films were not produced during drying down. It seems possible that cell wall material may be less soluble in older tissues since the rate of cell wall synthesis has declined. Thus, less soluble material may be available for condensation and film formation, which could offer a partial explanation for the well-known resistance of older leaves to disease.

Some of the questions which we leave unresolved in bean concern the nature of the film: is it formed of material similar to those which form the intercellular cuticle described by Scott (22)? The nature of the processes which lead to the possible filling in behind films over the incubation period also remain unresolved although collar formation at the film/wall juncture could represent a preliminary step in the process. There is no question that there was variation in the types of films formed and the amount of filling in behind films in the different species examined. Our studies could not attribute this variation to differences between pathogens or saprophytes or even differences in host response to the presence of bacteria or water controls.

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