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Rhizobium sp. Degradation of Legume Root Hair Cell Wall at the Site of Infection Thread Origin

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Using a new microinoculation technique, we demonstrated that penetration of *Rhizobium* sp. into the host root hair cell occurs at 20 to 22 h after inoculation. It did this by dissolving the cell wall maxtrix, leaving a layer of depolymerized wall microfibrils. Colony growth pressure "stretched" the weakened wall, forming a bulge into an interfacial zone between the wall and plasmalemma. At the same time vesicular bodies, similar to plasmalemmasomes, accumulated at the penetration site in a manner which parallels host-pathogen systems.

The *Rhizobium* sp.-legume system has been studied for almost a century, and yet many of the early steps in the establishment of this symbiosis remain poorly understood. The earliest studies served primarily to strengthen the view that the thread and hair walls were one continuous structure (9, 14), a view substantiated by a later study (13) using serial sections. The thread tube, originating at the point of most acute curling, appeared to be an invagination of the hair wall. In contrast, recent studies (5) through 48-h-old specimens showed that the infection origin is not an invagination of the root tip, but is at a point where two portions of the wall of the curled hair form an enclosure. This work indicated a disintegration or degradation of the hair wall at the infection site. The thread wall appeared to be formed by the apposition of a new, fibrillar layer of wall material around the infection site containing the rhizobia.

The tropical legume *Macroptilium atropurpureum* (siratro) has a similar transient susceptibility to infection as soybean (3), where susceptible cells are located in a zone between the root tip and the smallest emerging root hairs, the zone moving acropetally with root growth. This knowledge, together with the use of a microinoculation technique (18), has enabled us to pinpoint the moment of infection, using the fast-growing *Rhizobium* sp. strain ANU240 (12), a derivative of NGR234 (17).

Late-log-phase cultures of ANU240 were microinoculated (ca. 20 nl) (18) onto the surface of the root between the root tip and the smallest emerging root hairs (3). Plants were grown in plastic growth pouches (3) at 85% relative humidity; 29°C day (13 h); 26°C night; photon flux density 200 to 220 μmol m^{-2} s^{-1} at midday from Sylvania Gro-lux VHO cool white fluorescent tubes. For transmission electron microscopy, infected tissue was sampled at 2-h intervals from 12 to 30 h after inoculation and at 4-h intervals from 30 to 50 h after inoculation, 20 samples per fixation time. Tissue was fixed in 2.5% glutaraldehyde–2.0% paraformaldehyde in 25 mM phosphate buffer at pH 6.8 for 6 h, postfixed (after three rinses in buffer) in 2% OsO_4 in distilled water for 2 h, rinsed, and dehydrated in a gradual acetone series. They were then infiltrated with Spurr's resin (16) gradually over 3 days and embedded in fresh resin overnight at 60°C. Sections were cut at approximately 70 nm on a Reichert-Jung Ultracut OMU4, mounted on 0.3% Formvar on slot grids, and stained with a saturated solution of uranyl acetate in 50% methanol for 15 min and lead citrate for 10 min with thorough washings after each stain. They were then viewed with an Hitachi H500 microscope at 75 kV.

The very early events of infection involve attachment of the *Rhizobium* sp. to the developing trichoblast and the start of root hair curling through active perturbation of the process of tip growth at this point (Fig. 1). By 5 to 6 h after inoculation the hair grows asymmetrically due to this effect of the bacteria, and within 9 to 11 h the hair contacts its own

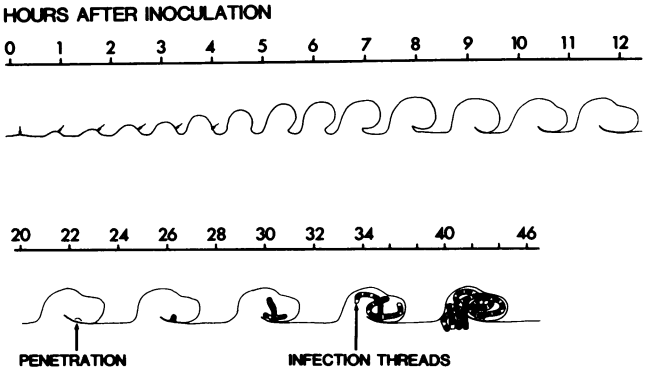

FIG. 1. Summary of infection events in siratro. (i) *Rhizobium* sp. attaches to the trichoblast at the point of root hair initiation within minutes of inoculation; (ii) *Rhizobium* sp. is moved outwards, away from the tip, and actively perturbs the process of tip growth at this point; (iii) by 5 to 6 h after inoculation the hair grows asymmetrically due to a one-sided "pinning down" effect of the bacteria; (iv) by 9 to 11 h the hair contacts its own basal part or neighboring cell and continues to grow along the epidermal surface, and the infected cell thickens its outer wall and appears brown under bright-field microscopy; (v) between 12 and 20 h *Rhizobium* sp. colonizes the small region between the epidermis and the overlying hair wall, and during this time wall-degrading enzymes are accumulated and the hair wall matrix has eroded; (vi) between 20 and 22 h *Rhizobium* sp. enters an interfacial zone between wall and plasmalemma; (vii) by 22 to 24 h tip growth ceases, infection thread synthesis commences, and subepidermal cell nucleoli enlarge in readiness for division; (viii) between 24 and 40 h infection threads ramify the hair cell, and subepidermal and cortical cell division commences to form a focus of cells immediately below the infection site; (ix) between 42 and 52 h threads orient towards the cortical tissue, penetrating and ramifying through the focus (or meristem) of cells.

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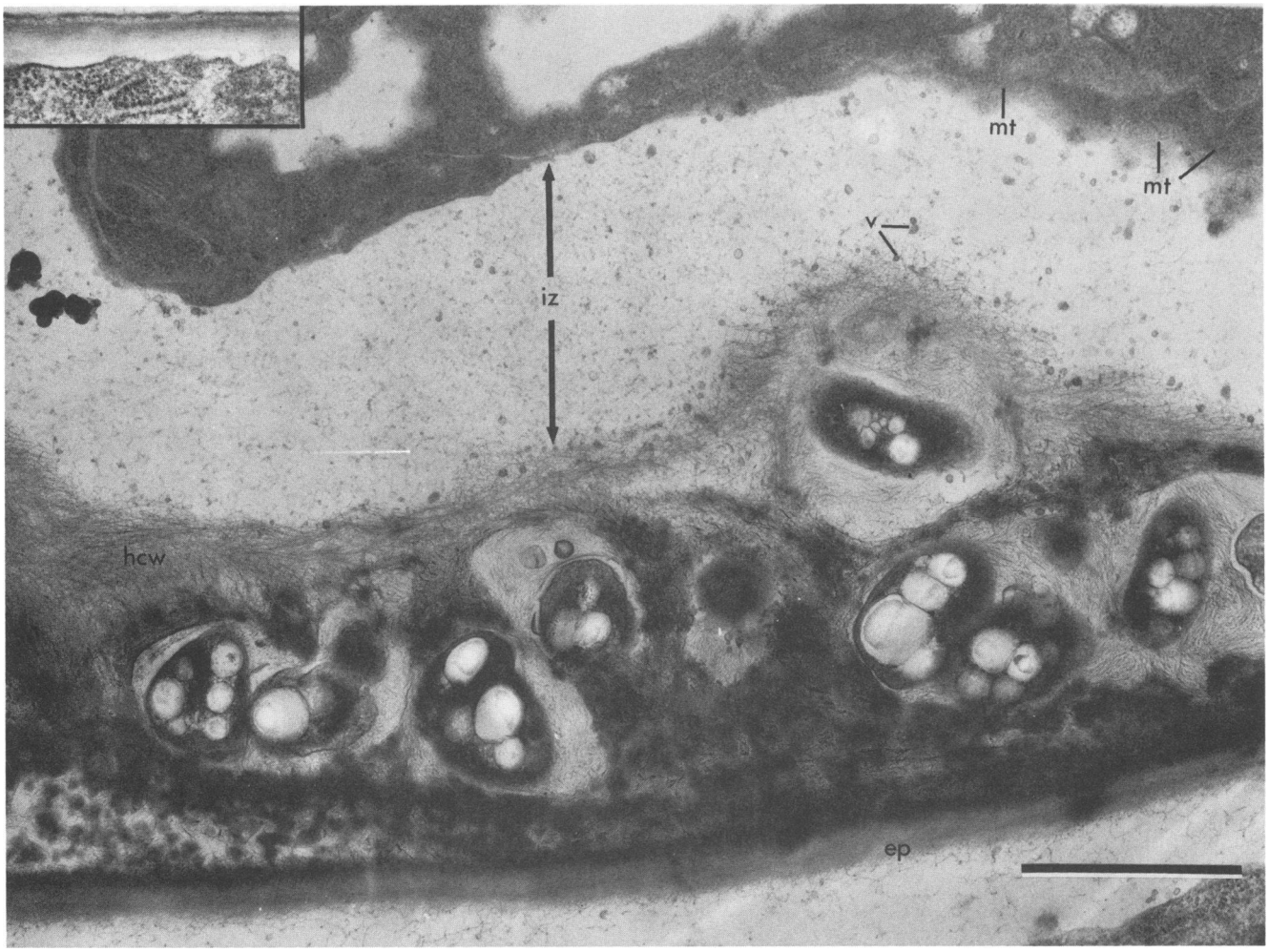

FIG. 2. Penetration by *Rhizobium* sp. into the hair cell 22 h after inoculation. A microcolony appears to have degraded the hair cell wall (hcw) matrix at the junction of the hair and epidermis (ep) and to have been pushing through the wall at the time of fixation. There are a large number of vesicular bodies (v) in an interfacial zone (iz), accumulating especially at the penetration site. Microtubules (mt) are distinguishable. Inset shows the hair wall structure from a minus-*Rhizobium* sp. control. Bar, 1 μm.

basal part or neighboring cell and continues to grow along the epidermal surface. Between 12 and 20 h *Rhizobium* sp. divides and colonizes the region under the curled hair. Accumulation of *Rhizobium* sp. enzymes in this deformed root hair enclosure may provide the necessary environment for degradative penetration of the hair cell wall. Micrographs representing the next stage of the infection, the actual penetration of the hair at 20 to 22 h, are presented in Fig. 2 and 3. These micrographs are representative of the numerous infection sites examined from the plant material prepared for microscopy. Figures 2 and 3 show a section through an infection site, the small zone between the curled hair and epidermis. The microcolony of rhizobia appears to have caused degradation of the cell wall matrix, leaving a layer of depolymerized wall microfibrils. A single *Rhizobium* sp. was in the process of penetrating this loosened wall material at the time of fixation (Fig. 3), possibly as a result of colony growth pressure. The host cytoplasm has moved away from its wall, leaving an interfacial zone. Accumulation of plasmalemma-like vesicular bodies may represent the first deposits by the host of infection thread-forming material. These vesicles may also contain molecules which are important in interactions with the invader. The hair cell cytoplasm is dense and granular, and microtubules are present next to the infection site, implying a role in the direction of infection thread wall material synthesis. In contrast to previous results (5, 13), the infection is not directly at the axis of the curl. The rhizobia enter into the hair cell in an area between the axis of the curl and the hair tip. These observations demonstrate that a region of the host cell is degraded by rhizobia, and it seems likely that the cell wall is altered by hydrolytic enzymes, either from the host or from the rhizobia. A polygalacturonase hypothesis (2, 11) is fully compatible with the evidence presented here; rhizobia multiply in an enclosed pocket and locally disrupt or degrade the hair wall, and a new layer of host cell wall-like material is deposited at the infection site. Whether the accumulation of this new wall-like material is a defense response is by no means proved, but is tenable. Certainly the localized deposition of wall-like materials such as papillae, collars, or sheaths at the point of penetration of microbial pathogens is well known (1, 4, 7, 15). In early stages of pathogenesis, marked structural modifications can occur in host cell walls (6, 9, 15, 19). For example, at the site of attachment of

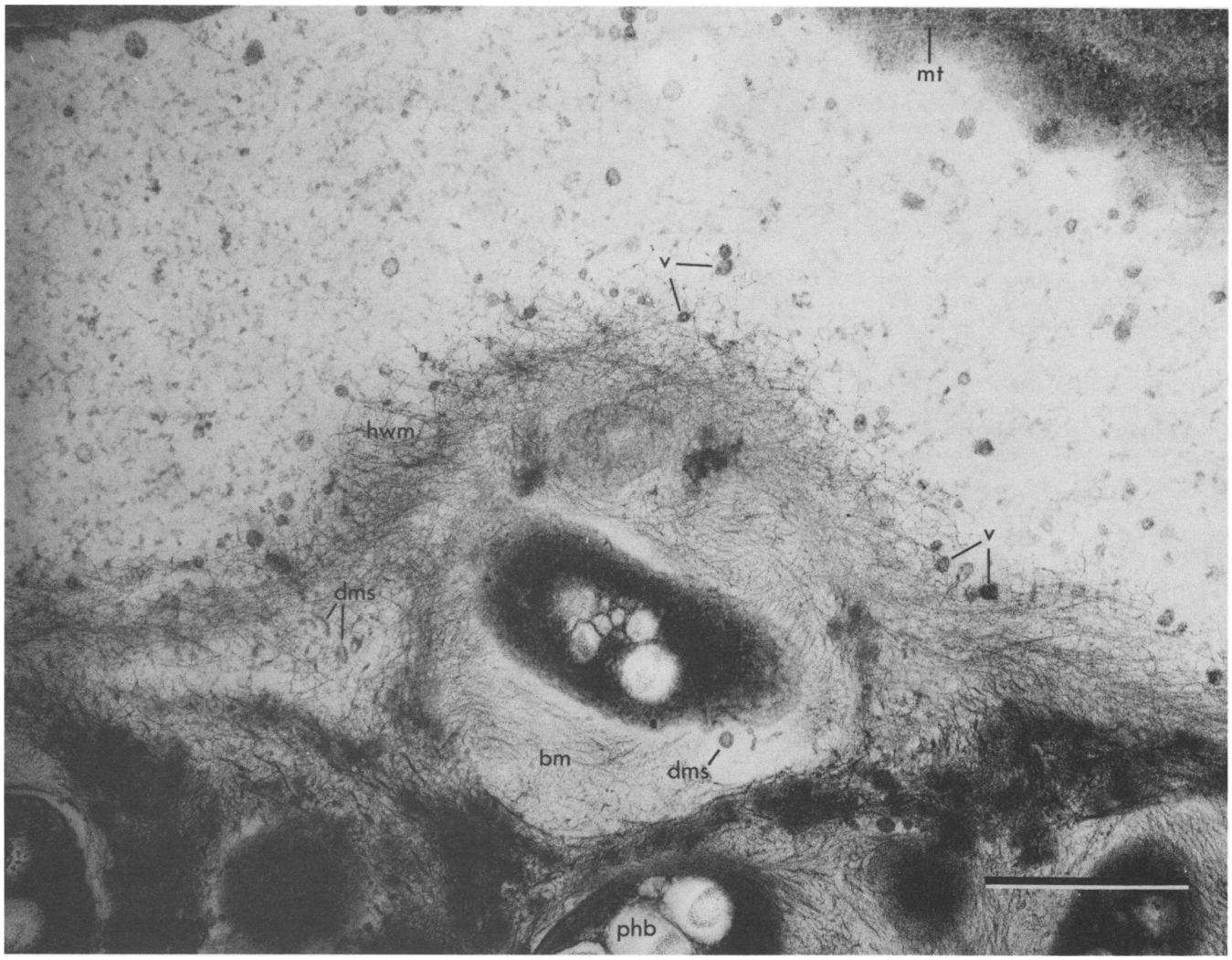

FIG. 3. Higher magnification of the central area of Fig. 2. Bacterially derived microfibril structures (bm) can be distinguished from hair wall microfibrils (hwm), which are more electron dense and have a more pronounced "beaded" structure. Note the double membrane structures (dms) near the bacteria. The rhizobia contain large white granules of polyhydroxybutyrate (phb). Bar, 500 nm.

Pseudomonas solanacearum to tobacco mesophyll cells, the host cell wall is frequently eroded, the plasmalemma separates from the cell wall and becomes convoluted, and numerous membrane-bound vesicles accumulate in the space between plasmalemma and the cell wall (15). Recent reports indicate that fragments of pectic polysaccharides from the host cell wall can act as potent elicitors of defense responses in plants (M. G. Hahn, A. G. Darvill, and P. Albersheim, Plant Physiol. **65**:136, abstr. 750, 1980; G. Lyon and P. Albersheim, Plant Physiol. **65**:137, abstr. 752, 1980). Thus, localized activation of host root polygalacturonase might result in both localized degradation of the hair cell wall and localized release of pectic elicitor substances that induce deposition of infection thread material (2).

Double membrane fragments, most likely of host origin, were observed within the microcolony, but not within the host wall microfibrils or interfacial zone (Fig. 3). It is tempting to hypothesize that these are host-secreted fragments, bound with proteins or glycoproteins, that function by signaling the initiation of the complex reactions leading to the symbiosis (10).

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