

Infection of Alfalfa Pollen by *Verticillium albo-atrum*

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ABSTRACT

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Light microscopy and scanning and electron transmission microscopy revealed that alfalfa pollen is susceptible to in vitro infection by *Verticillium albo-atrum*, the causal organism of Verticillium wilt of alfalfa. Infection occurred after pollen grains were mixed with spores of *V. albo-atrum* and incubated for 24 hr on potato-dextrose agar media. Hyphae of *V. albo-atrum* penetrated the exine wall layer with a thin penetration peg without forming appressoria. Penetration occurred more readily through the

germinative pores than through other parts of the host wall. During the early stage of hyphal invasion, the pollen showed plasmolysis, breakage of cytoplasmic membranes, and formation of large vacuoles in the cytoplasm. The invading hyphae filled the entire cell lumen by rapid hyphal proliferation. Eventually, the infected pollen ruptured, leaving a cluster of compact hyphal cells encircled by remnants of the pollen cell walls.

Additional key words: *Medicago sativa*.

Invasion of pollen by plant pathogens has been recorded for 37 viruses (8) but rarely for fungi despite numerous examples of fungal pathogens attacking blossoms of higher plants (1). Previous in vivo studies (4) showed that tripping flowers of alfalfa (*Medicago sativa* L.) with spores of *Verticillium albo-atrum* Reinke & Berthold resulted in infection of stigma and style and the presence of hyphae in pollen grains and pollen tubes. Furthermore, the infection of stigma and style could lead to the transmission of *V. albo-atrum* to the seed.

This paper describes the processes by which *V. albo-atrum* infects alfalfa pollen in vitro.

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MATERIALS AND METHODS

Suspensions of spores of *V. albo-atrum* in sterile distilled water (10^6 spores per milliliter) were flooded over the surface of potato-dextrose agar (PDA) or a selective medium (2) in petri dishes at 1 ml per dish. Pollen was collected from mature flowers of greenhouse-grown alfalfa cultivar Vernal by touching the keel petal with a toothpick. The pollen was dusted on the surface of plates containing a thin film of spores. Plates were incubated at room temperature, and samples of agar blocks containing a mixture of pollen and spores were collected after incubation for 7, 24, 32, and 48 hr on PDA or 1, 4, 7, 14, and 21 days on the selective medium. They were stained in acetocarmine and healthy and infected pollen were examined with a compound microscope.

For scanning electron microscopy (SEM) and transmission electron microscopy (TEM), specimens were collected from PDA plates after 7, 24, 32, and 48 hr of incubation and were fixed in 4% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.0, at 4 C

for 18 hr. They were postfixed in 2% osmium tetroxide, rinsed with buffer, and dehydrated in an ethanol series. For SEM, specimens then were critical-point dried, mounted with colloidal silver paste onto aluminum stubs, sputter-coated with gold, examined, and photographed in a Hitachi S500 scanning electron microscope. For TEM, specimens were infiltrated and embedded in Spurr's low-viscosity embedding medium (10), sectioned, stained with lead citrate and uranyl acetate, examined, and photographed in a Hitachi H500 transmission electron microscope.

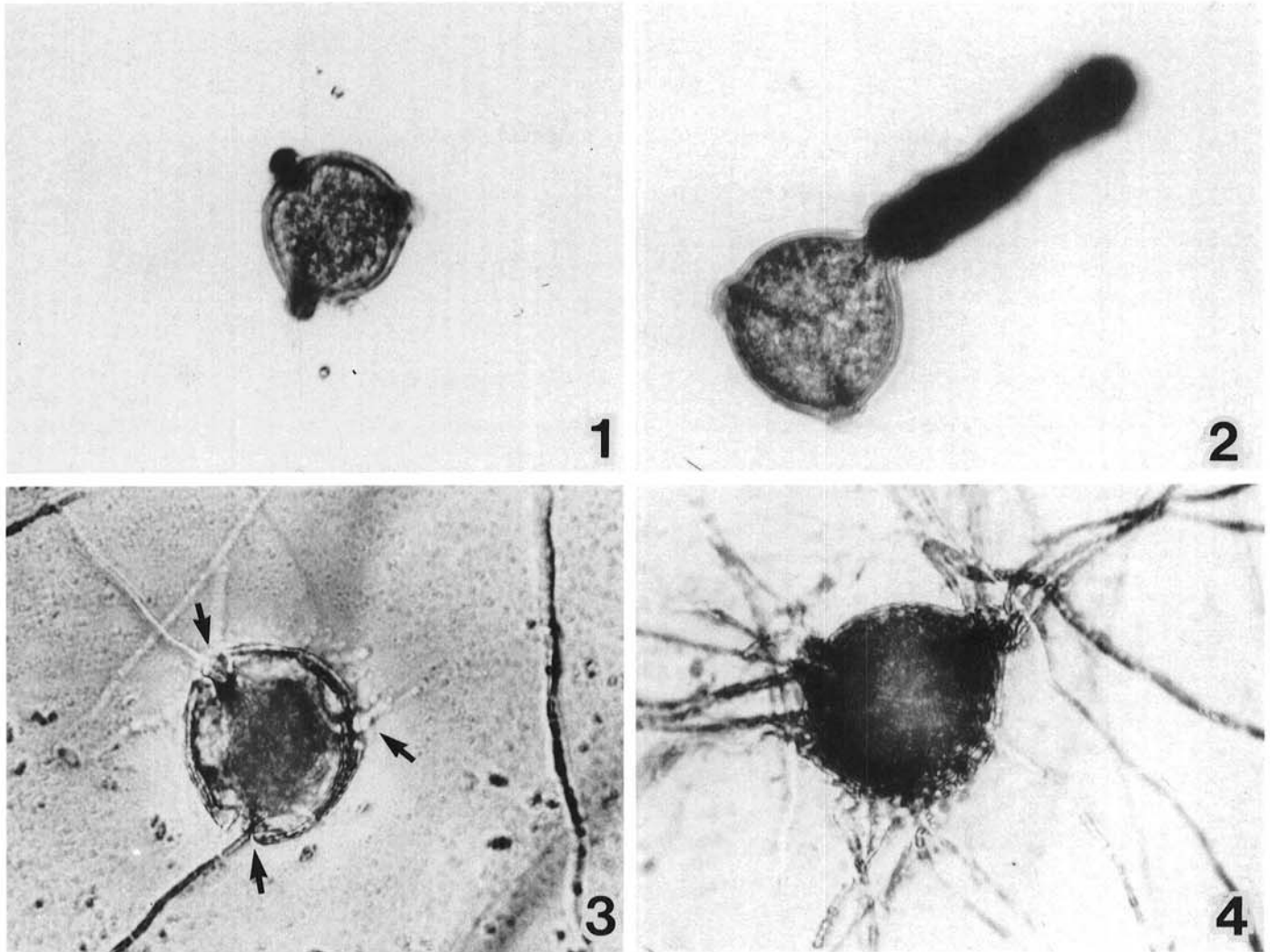
RESULTS

Light microscopy. The spherical alfalfa pollen cells contained three papillate germinative pores (Fig. 1). Some pollen germinated from one of the germinative pores and produced a pollen tube (Fig. 2) after incubation on PDA for 7 to 48 hr. Although many conidia of *V. albo-atrum* germinated after incubation on PDA and selective medium for 7 hr and 1 day, respectively, infection of pollen was observed in samples only after 24 hr and 4 or more days, respectively. The first signs of invasion of pollen by *V. albo-atrum* were the single hyphae that penetrated the germinative pores (Fig. 3), resulting in the appearance of three clusters of mycelia on each infected pollen grain during the advanced stages of infection (Fig.

4). Frequently, verticillate conidiophores were produced on the surface of the infected pollen incubated on the selective medium for 14 to 21 days.

Electron microscopy. SEM studies showed that each pollen grain had a rough surface (Figs. 5 to 8) and a spindle-shaped furrow surrounding each germinative pore (Figs. 6, 7, and 9). In samples incubated on PDA for 24 to 48 hr, single hyphae were attached to the pollen. The attachment was observed most frequently in the furrow (Figs. 5 and 6) and the germinative pore (Fig. 7) region and least frequently over the rough-surfaced wall (Fig. 8). Hyphal penetration occurred without the formation of appressoria (Figs. 5 to 8). In samples incubated on PDA for 48 hr, hyphae inside the pollen emerged and grew on the surface (Fig. 9).

TEM studies showed that an alfalfa pollen grain had two thick, well-defined wall layers, the exine and intine, a thin cytoplasmic membrane, and a dense cytoplasm (Fig. 10). The exine layer was thin and smooth in the region of the furrow and germinative pore (Figs. 10 and 13), but was thick and wavy in other regions (Figs. 10, 14, and 19). During infection, hyphae on the surface of the pollen grain produced a narrow hyphal peg which penetrated the double-layered exine (Fig. 11) or the single-layered exine wall at the furrow region (Figs. 12 and 13). In most cases, hyphal pegs were narrow and sharply constricted, but the constriction was less obvious at the germinative pores (Figs. 14 and 15). After penetration, the hypha in

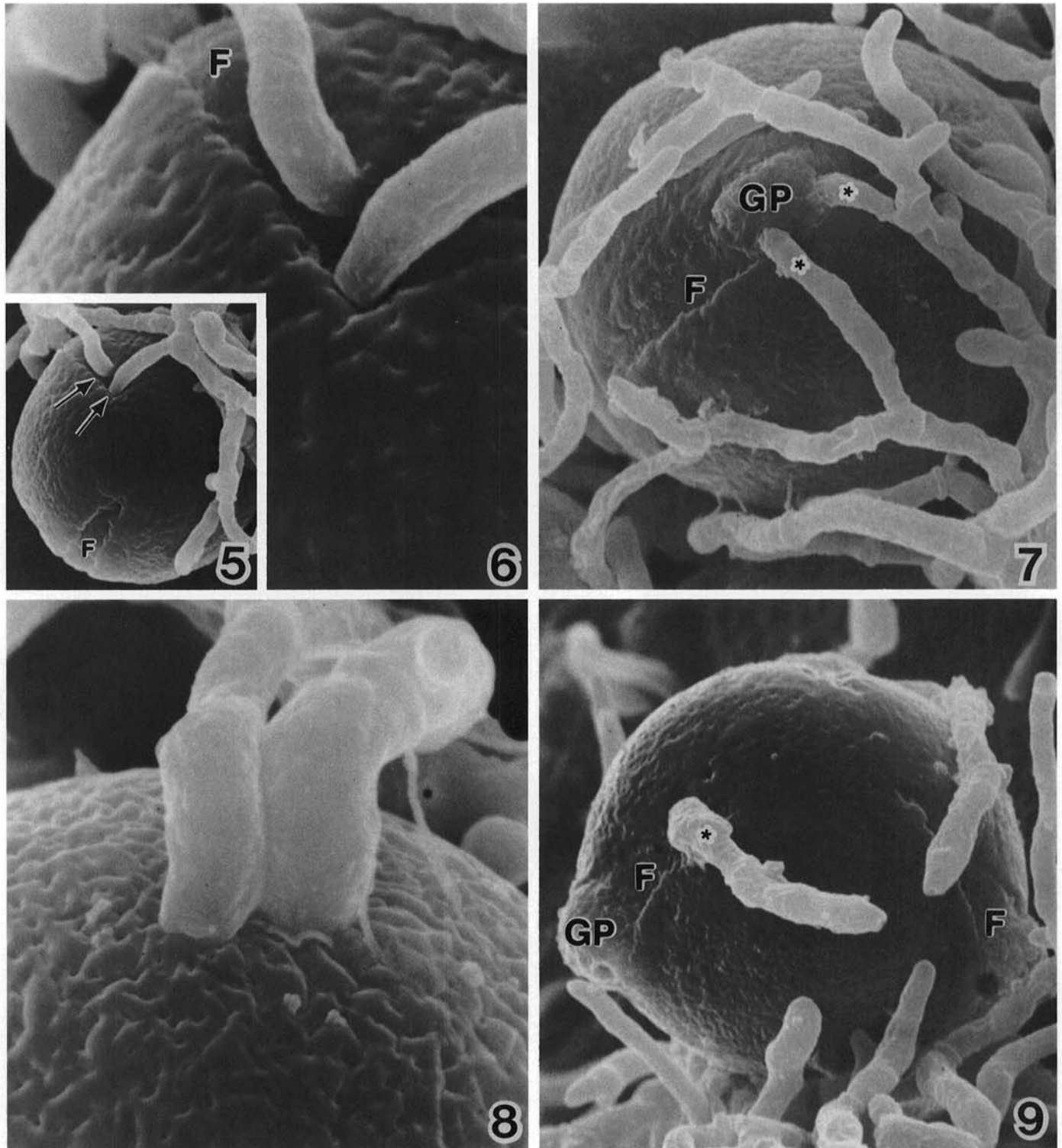


Figs. 1-4. Light microscopy of alfalfa pollen. **1,** An ungerminated pollen grain showing the three papillate germinative pores. ($\times 825$); **2,** A germinated pollen grain with an elongated pollen tube growing from one of the pores. On PDA for 24 hr. ($\times 1,025$); **3,** A pollen grain infected by hyphae of *Verticillium albo-atrum* entering through the germinative pores (arrows). On selective medium for 7 days. ($\times 1,025$); **4,** A pollen grain showing late stages of infection by *V. albo-atrum*. Note three clusters of hyphae protruding through the germinative pores. On selective medium for 21 days. ($\times 1,100$).

the pollen grain increased in diameter and developed into normal hyphal cells (Fig. 13). At each hyphal penetration site a small hole was produced in the exine layer (Figs. 11 and 13). Remnants from the degraded cell wall (Figs. 15, 17, and 18) were often observed near the penetration site.

After penetrating the exine layer, hyphae continued to invade the intine layer, the cytoplasmic membrane, and the cytoplasm of

pollen grains (Figs. 14 and 15). The host response in the early stages of infection included: the separation of cytoplasmic membrane from the intine layer due to plasmolysis, breakages of cytoplasmic membrane resulting in leakage of cytoplasm, and the presence of numerous large vacuoles in the cytoplasm (Figs. 14 and 15). Invagination of cytoplasmic membrane at the penetration region also was evident (Figs. 14 and 15).



Figs. 5-9. Scanning electron microscopy of alfalfa pollen infected by *Verticillium albo-atrum* (Figs. 5-8, on PDA for 24 hr.; Fig. 9, on PDA for 48 hr.). **5 and 6,** Invasion by two hyphae (arrows) through the furrow (F) of a germinative pore. ($\times 2,000$ and $\times 7,600$, respectively). **7,** Invasion by two hyphae (stars) through the germinative pore (GP) ($\times 3,300$). **8,** Invasion by two hyphae through the exine of the pollen grain without the formation of appressoria ($\times 8,600$). **9,** A hypha (star) emerging from the pollen grain through the furrow (F) region. ($\times 3,400$). Legend: F = furrow, GP = germinative pore.

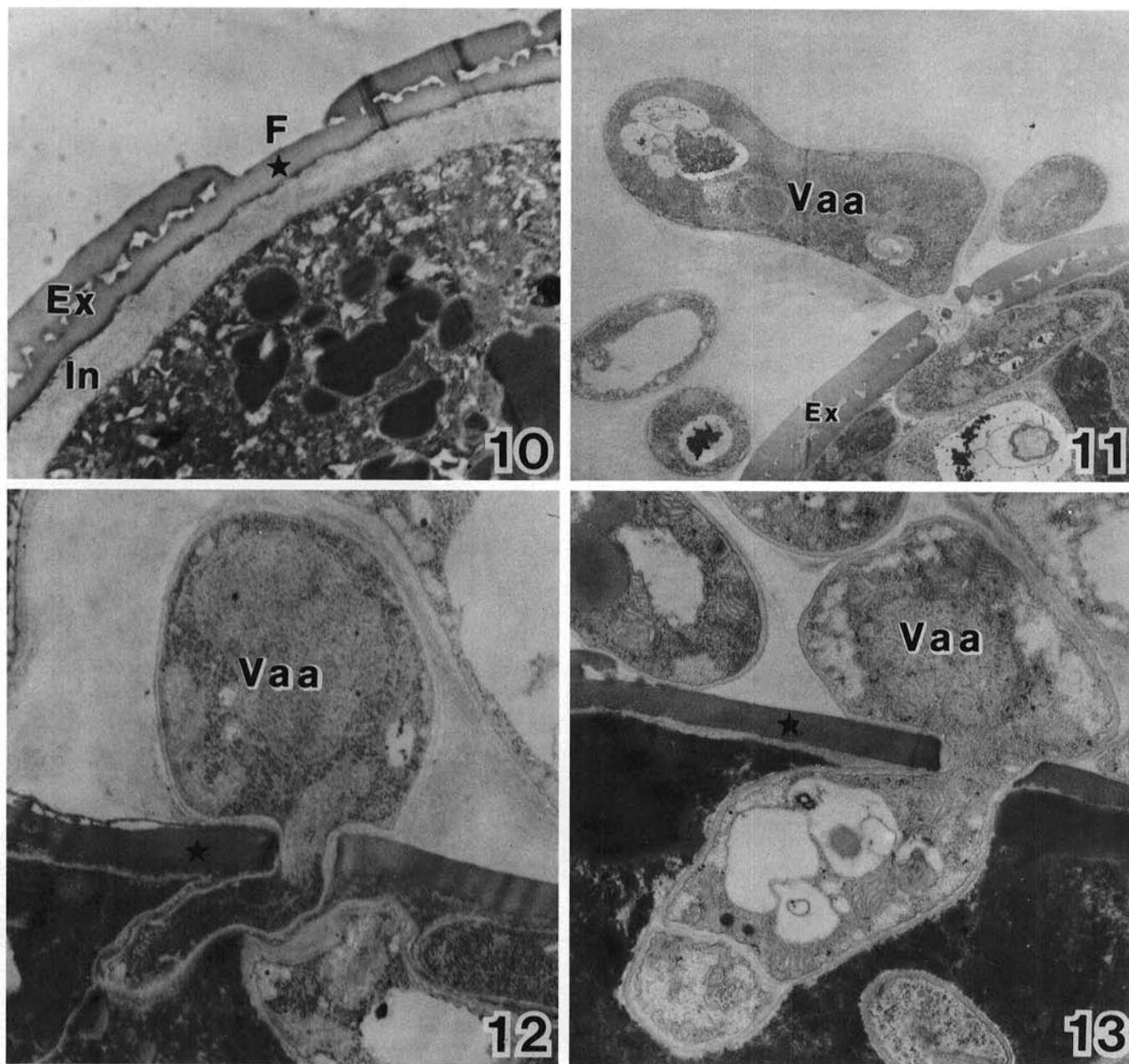
Hyphae of *V. albo-atrum* rapidly ramified within the infected pollen grain. They increased from a few after 24 hr (Fig. 14) to numerous after 32 hr (Fig. 16) of incubation on PDA. After 48 hr, hyphae in the pollen were dense and compact and, in most cases, part of the exine layer collapsed and disintegrated (Fig. 19). Signs of hyphae emerging from the infected pollen (Fig. 18), similar to those revealed by SEM (Fig. 9), were evident.

DISCUSSION

This study reveals that alfalfa pollen is susceptible to *in vitro* infection by *V. albo-atrum*. Rapid hyphal proliferation in the cells shows that the cytoplasm of alfalfa pollen is a suitable substrate for growth of this pathogen.

Hyphae of *V. albo-atrum* invade the exine layer of a pollen grain by a thin penetration peg without forming any specialized structure such as an appressorium. It appears that cell wall degradation by enzymes is involved in the penetration process since wall remnants are frequently observed at the point of penetration. However, the putative enzymatic activity seems to be restricted to the point of penetration because the holes created by penetration pegs are often small. This mode of penetration of alfalfa pollen by *V. albo-atrum* is similar to that observed in the infected style of alfalfa flowers (4). This study indicates that mechanical force also is involved in the infection of pollen grains by *V. albo-atrum* because invagination of the cytoplasmic membrane is evident at the hyphal penetration point.

The chief constituent of the exine layer of pollen is sporopollenin which is thought to be very resistant to oxidation

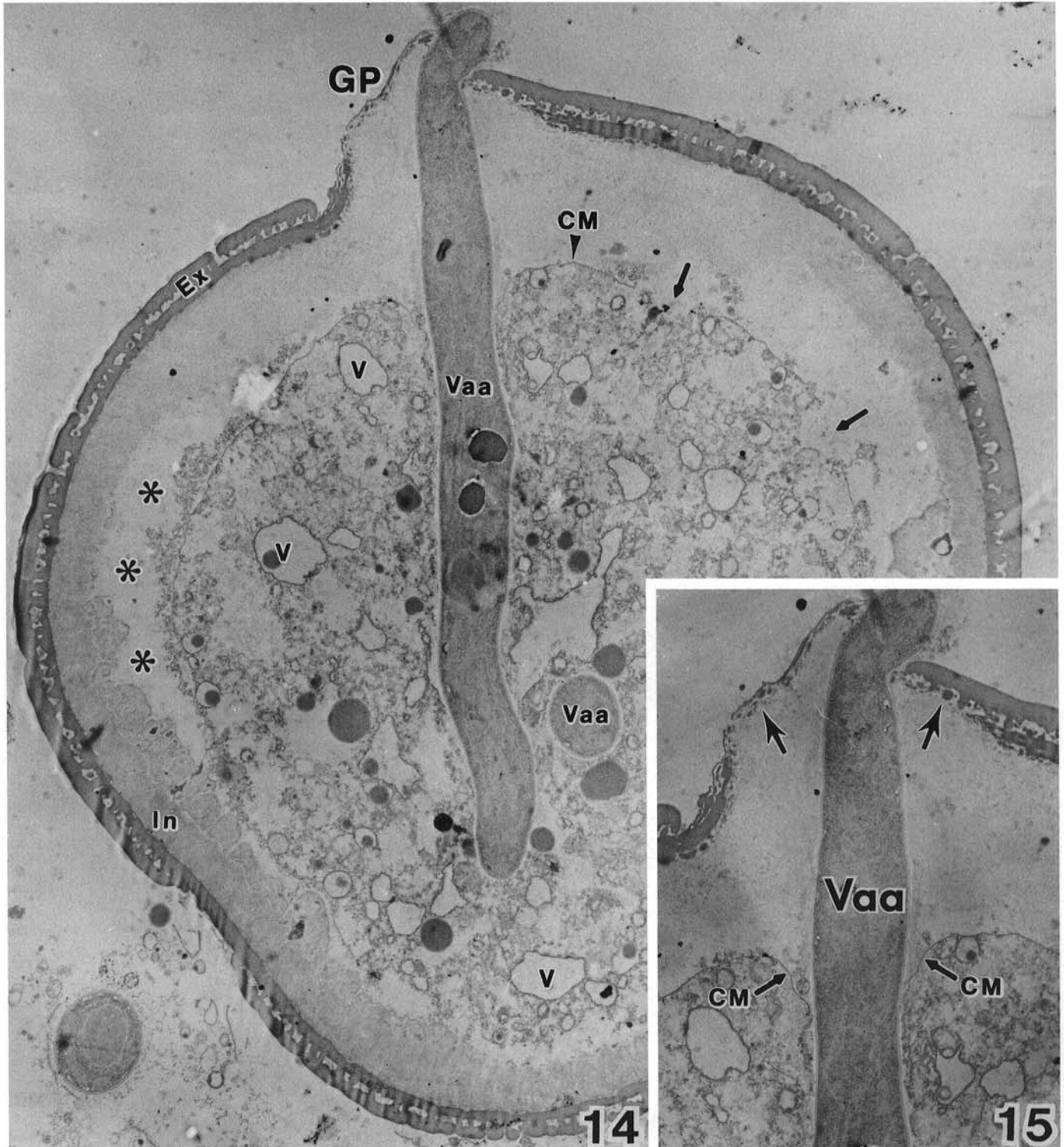


Figs. 10-13. Transmission electron microscopy of sectioned alfalfa pollen. (Fig. 10, healthy; and Figs. 11-13, infected by *Verticillium albo-atrum*). **10**, Section of a healthy pollen grain showing the exine (Ex) and intine (In) layers and the furrow (F and star) of a germinative pore region ($\times 10,200$). **11**, A hypha penetrating the thick exine layer (Ex) ($\times 7,600$). **12**, A hypha penetrating the thinner exine layer at the furrow region (star) via a narrow penetration peg ($\times 17,500$). **13**, Expansion of the penetration peg after penetration of the furrow region. ($\times 15,800$). Legend: Vaa = *V. albo-atrum*, Ex = exine layer, In = intine layer, F = furrow, and star = thinner exine layer of the furrow region.

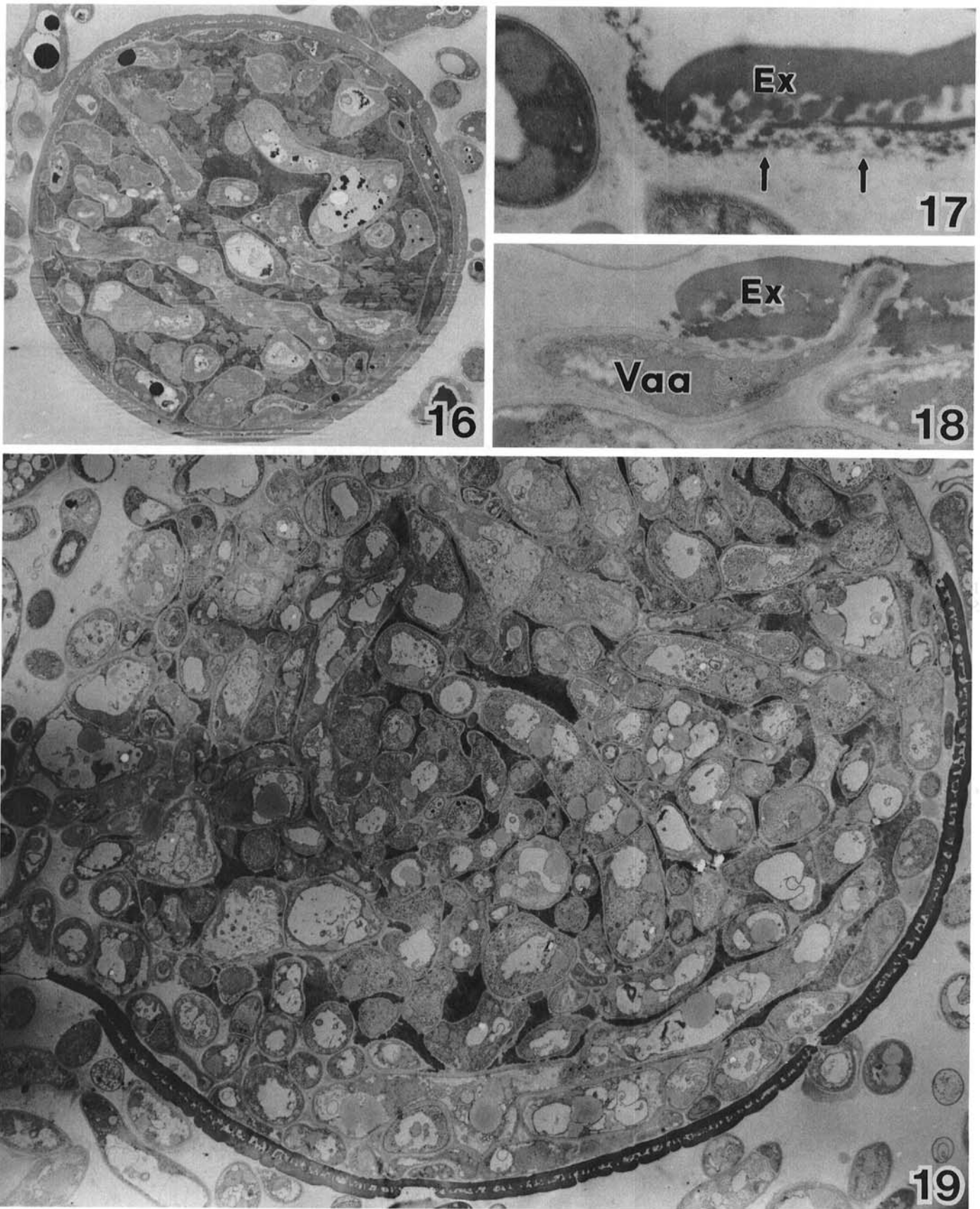
and hydrolysis (7,9). Nevertheless, this study indicates that hyphae of *V. albo-atrum* can cause breakdown of the exine layer, especially the thinner regions such as the three germinative pores and their surrounding furrows. The vulnerability of the germinative pores to fungal invasion may be coincidental with the reduced thickness of the exine layer in these regions.

Previous studies indicated that the pea aphid (5) and alfalfa weevil (3) are effective agents for dispersal of spores of *V. albo-*

atrum in alfalfa fields. Another study suggested that the alfalfa leafcutter bee could also spread *V. albo-atrum* in diseased alfalfa fields because the pathogen frequently occurred on leaf pieces forming bee cells (6) and on the insect (H. C. Huang and K. W. Richards, unpublished). Therefore, infection of alfalfa pollen by *V. albo-atrum* might occur under field conditions if the pathogen-contaminated insects visit and transport spores to the anther and stigma of an alfalfa flower. This means of spreading *V. albo-atrum*



Figs. 14-15. Transmission electron microscopy of an alfalfa pollen grain penetrated through the germinative pore (GP) by *Verticillium albo-atrum*. Host responses at early stages of hyphal invasion are: **14**, plasmolysis (stars), disruption of cytoplasmic membrane (arrows), and vacuolization of the cytoplasm (note remnants of the exine layer [arrows]) ($\times 6,600$) and **15**, invagination of cytoplasmic membrane (CM) at the point of hyphal penetration ($\times 9,300$). Legend: Vaa = *V. albo-atrum*, V = vacuole, Ex = exine layer, In = intine layer, CM = cytoplasmic membrane, and GP = germinative pore.



Figs. 16-19. Transmission electron microscopy of alfalfa pollen invaded by *Verticillium albo-atrum*. **16,** An infected pollen grain showing the extent of hyphal proliferation after 32 hr of incubation on PDA ($\times 2,800$). **17,** Remnants (arrows) of the exine wall ($\times 25,200$). **18,** A hypha exiting the exine layer from within the pollen ($\times 17,100$). **19,** An infected pollen grain showing the massive proliferation of the hyphae and rupture of the cell after 48 hr of incubation on PDA ($\times 4,100$). Legend: Vaa = *V. albo-atrum*, and Ex = exine layer.

to flowers is one of the mechanisms involved in the seedborne transmission of the pathogen in alfalfa (4). Since the alfalfa leafcutter bee is used as a pollinator for the production of alfalfa seed, it might be the most important insect in the transmission of *V. albo-atrum* via alfalfa pollen.

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