# Pathogenesis of *Venturia inaequalis* on Shoot-Tip Cultures and on Greenhouse-Grown Apple Cultivars

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## ABSTRACT

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Four apple (*Malus* × *domestica*) cultivars with different levels of resistance to *Venturia inaequalis,* the incitant of apple scab, were grown in the greenhouse and as shoot-tip cultures in vitro. Young, actively growing leaves were inoculated with a conidial suspension. Development of the fungus during the early events of pathogenesis and concurrent host responses were observed by light and electron microscopy. Prepenetration and penetration activities were indistinguishable in resistant and susceptible cultivars grown in vitro or in the greenhouse. After penetration, however, colonization and establishment of the fungus differed among cultivars and growing conditions. The resistant cultivar Freedom expressed a hypersensitive response 72 h after inoculation both in vitro and in the greenhouse, whereas the resistant cultivar Liberty showed reduced stroma formation with no sporulation. Typical stromata and sporulating lesions developed on leaves of greenhouse-grown plants of the susceptible cultivars Empire and McIntosh, but on shoot-tip cultures, the mycelia proliferated atypically above the cuticular leaf surface, and the number of appressoria increased significantly 5 days after inoculation. Although penetration and primary hypha formation occurred in vitro, the proliferation of secondary hyphae under the cuticular membrane was greatly restricted during the first 10 days after inoculation. Sporulation was observed in vitro 20–30 days after inoculation. The abnormal development of the fungus in shoot-tip cultures may be the result of a greater availability of nutrients on the leaf surface.

*Additional keywords:* apple tissue culture, screening for disease resistance.

Apple scab, caused by *Venturia inaequalis* (Cooke) G. Wint., is the most economically important disease of apples (*Malus* × *domestica* Borkh.) in the world (5). Most commercially grown apple cultivars are susceptible to scab. Despite numerous cytological and histological studies of the early stages of apple scab pathogenesis (for a recent review, see Becker [2]), our knowledge of the mechanism of infection of resistant cultivars by *V. inaequalis* and of the mechanism of resistance by the host is very limited. Little has been published on the early stages of pathogenesis of the apple scab fungus on promising resistant cultivars or hybrids that contain the Vf or Vm genes for scab resistance (6,7,11,32), and nothing has been published for the scab-resistant cultivar Freedom, which contains the Vf gene as well as genes for resistance from the cultivar Antonovka (20). Better information about the mode of action of scab resistance genes in new cultivars could help in the selection of more durable sources of resistance and in the evaluation of the risk associated with widespread introduction of these genes.

Tissue culture techniques are advantageous in the development of novel disease-resistant germplasm because they allow large-scale screening of germplasm in a small space and the study of host-parasite interactions in a controlled environment (9). Previous attempts to identify resistance to *V. inaequalis* in vitro with apple callus cultures were not successful (3,4,29). Because tissue organization may be critical for expression of resistance, we used shoot-tip cultures to test the response of four apple cultivars to *V. inaequalis.* Light microscopy (LM) and scanning (SEM) and transmission (TEM) electron microscopy were used to compare the early events of pathogenesis of *V. inaequalis* in the susceptible cultivars McIntosh and Empire and the resistant cultivars Liberty and Freedom, grown both in vitro and in the greenhouse.



## MATERIALS AND METHODS

Four apple cultivars were grown in vitro and in the greenhouse: Liberty, which carries the Vf dominant gene for resistance to *V. inaequalis* (21); Freedom, which carries an undefined gene or genes for resistance from the cultivar Antonovka in addition to Vf (20); and the susceptible cultivars McIntosh and Empire. Shoot-tip cultures were grown, as described previously (36), on Zimmerman's medium (37), containing (per liter) 1.0 mg of benzyladenine, 0.1 mg of indolebutyric acid, and 0.5 mg of gibberellic acid ($GA_3$). Initial explants were shoot tips 1.0–1.5 cm long. Shoot tips were placed vertically in baby food jars (five shoot tips per jar) containing 25 ml of medium. Potted apple trees 8–10 yr old were kept in cold storage and were moved to the greenhouse at 20 ± 5 C 5 wk before the start of the experiment.

New, actively growing leaves from shoot tips and the youngest expanded leaves from greenhouse plants were sprayed with a nonaxenic conidial suspension ($10^6$ conidia per milliliter). The conidial suspension included a mixture of *V. inaequalis* races 1–5 (isolates 1805-2, 1770-8, 1771-2, 1778-6, and 1810-1), originally obtained from E. B. Williams (Purdue University). After inoculation, greenhouse plants were incubated for 48 h in a mist chamber at 20 ± 1 C and 100% relative humidity and were then placed in the greenhouse at 20 ± 5 C. In vitro plants were incubated in a growth chamber at 19 ± 1 C with a 16-h photoperiod of white fluorescent light (40 $\mu Em^{-2}s^{-1}$ light intensity).

Nonaxenic inoculum of races 1–5 was used for most of the experiments described. Axenic inoculum was used only for a few experiments because of the slow growth of the fungal cultures and the loss of virulence over time. Axenic inoculum was used primarily to inoculate shoot-tip cultures on proliferation medium in order to reduce contamination problems during long incubation periods. This inoculum was prepared from two isolates representing races 1 and 5 of *V. inaequalis.* The fungus was grown in wick cultures (26) on 4% malt extract, and spores were collected, centrifuged (2,000 *g,* 5 min), and resuspended in distilled water. In vitro cultures were sprayed with a conidial suspension ($10^6$ conidia per milliliter) under a laminar flow hood.

For TEM and SEM studies, three leaves were collected per time interval and cultivar, cut into 3-mm$^2$ segments, and fixed in 3% glutaraldehyde in 0.05 M $KPO_4$ buffer, pH 7.0, at about 25 C for 2 h. Samples were rinsed in buffer for 1 h, postfixed in 1% osmium tetroxide for 2 h, rinsed in buffer for 1 h, and dehydrated in acetone series. For SEM, leaf samples were critical-point dried after fixation, mounted on stubs, sputter-coated with gold-palladium, and examined in a Hitachi S-530 SEM. For TEM, leaf sections were embedded by infiltration in a resin series (Epon Araldite) and polymerized in plastic in a vacuum oven at 70 C for 48 h. Thin sections of the leaf samples cut with a diamond knife on an MT-2 microtome were mounted on Formvar-coated slotted copper grids and stained for 15 min in 1% uranyl acetate and for 3–4 min in lead citrate. The stained sections were examined with a Hitachi H-500 H electron microscope operated at 80 kV.

For LM studies, leaves were decolorized overnight in 99% cold methanol and stained with periodic acid-basic fuchsin following Preece's (28) procedure. Actively growing inoculated leaves and comparable noninoculated leaves were sampled for each treatment every 3 h during the first 48 h after inoculation and 3, 5, 7, and 10 days after inoculation. Whole leaves from shoot-tip cultures and sections of greenhouse leaves were mounted on glass slides in glycerol and examined. The percentage of conidia that had germinated, the percentage that had formed appressoria, and the number of appressoria per conidium were determined on 100 spores selected at random per treatment. All experiments were replicated at least three times.

Angular transformation of the data and analysis of variance were performed with the SAS statistical package (SAS Institute Inc., Cary, NC) and were used to evaluate differences among means for each cultivar and growing condition at each time interval. A split-plot design was used to analyze the percentage of conidia that had germinated and formed appressoria over time. The main plots were cultivars, the subplots were the two growing conditions (in vitro and greenhouse), and each subplot was sampled at seven intervals (6, 12, 24, 36, 48, and 72 h and 5 days) after inoculation, with three replications.

## RESULTS

The percentages of conidia that germinated and formed appressoria were determined in LM preparations. No significant differences were detected among cultivars or growing conditions in the percentage of germination (Table 1), but significant differences were detected in the percentage of conidia with appressoria in resistant and susceptible cultivars grown in vitro and in the greenhouse (Table 2). The interaction between cultivar and growing condition was not significant. Therefore, most of the variation observed was due to these two main factors. Only 5% of the conidia on greenhouse cultivars formed multiple appressoria, compared to 40–50% for cultivars grown in vitro (Table 3). Multiple appressoria were detected on resistant cultivars 72 h after inoculation and on susceptible cultivars after 5 days.

SEM revealed differences between in vitro and greenhouse-grown plants in the anatomy of the leaf surface. Leaves from greenhouse plants had numerous trichomes, which were absent or sparse on leaves grown in vitro (Fig. 1A and B). TEM also revealed differences between leaves grown in the greenhouse and in vitro in the ultrastructure of the cuticular membrane. The adaxial *Malus* cuticular membrane or, in a broader sense, the plant cuticle consisted of a continuous layer of lipid material attached to the cell wall by an intermediate layer rich in pectin. TEM preparations of plant cuticles revealed two distinctive ultrastructural components, lamellae and fibrillae (14). In this study, the outer polylamellate layer was visible as an amorphous electron-translucent zone and was present in both greenhouse and in vitro leaves, whereas the inner reticulate zone, rich in osmiophilic deposits and fibrillae, developed only on greenhouse leaves (Fig. 1C and D). No significant differences were observed between resistant and susceptible cultivars in cuticle thickness, but the cuticular membrane of leaves grown in vitro was only half as thick as that of newly expanded leaves grown in the greenhouse (Table 4).

**Susceptible cultivars in the greenhouse.** Germination was observed 6 h after inoculation on susceptible cultivars in the greenhouse. Conidia developed germ tubes at their pointed apex (Fig. 2A). In most cases germination was monopolar, but some conidia also germinated in an intercalary fashion. A septum between the spore and the germ tube and a septal pore were observed under the TEM (Fig. 3B). Appressoria were observed 8 h after inoculation (Figs. 2B and 3A). Appressoria were usually formed at the juncture of epidermal cell walls. The majority were terminal, and very few were intercalary. During germ tube elongation and appressorium formation, cytoplasm migrated out of the spore into the germ tube.

Penetration occurred after differentiation of the appressorium by formation of a penetration peg and establishment of hyphae under the cuticle. The porelike area, observed with LM as a refractive spot, was interpreted as a penetration peg and was associated with the location of direct cuticular penetration. The characteristic penetration peg and the mucilaginous sheath that adheres the appressorium to the leaf surface were clearly observed

TABLE 1. Germination of conidia of *Venturia inaequalis* on apple leaves and analysis of variance (ANOVA)

| Cultivar | Growing condition | Germination[a] (%) after 6 h | 12 h | 24 h | 36 h | 48 h | 72 h | 5 days |
|---|---|---|---|---|---|---|---|---|
| Empire | In vitro | 76 ± 2.6 | 86 ± 1.0 | 92 ± 2.1 | 95 ± 2.0 | 96 ± 2.0 | 98 ± 1.2 | 98 ± 1.5 |
| | Greenhouse | 75 ± 2.0 | 83 ± 1.5 | 92 ± 2.5 | 93 ± 2.0 | 96 ± 1.5 | 96 ± 0.6 | 98 ± 1.0 |
| McIntosh | In vitro | 73 ± 2.0 | 85 ± 4.5 | 92 ± 1.0 | 93 ± 3.0 | 94 ± 2.1 | 96 ± 1.0 | 99 ± 1.0 |
| | Greenhouse | 77 ± 2.5 | 83 ± 2.6 | 87 ± 2.0 | 92 ± 3.0 | 94 ± 2.5 | 95 ± 3.1 | 98 ± 1.7 |
| Freedom | In vitro | 74 ± 1.5 | 83 ± 3.4 | 93 ± 2.5 | 96 ± 1.5 | 96 ± 2.0 | 96 ± 2.6 | 98 ± 1.0 |
| | Greenhouse | 73 ± 2.0 | 86 ± 1.7 | 92 ± 3.0 | 95 ± 1.2 | 97 ± 1.0 | 98 ± 2.0 | —[b] |

| ANOVA/source | df | Type III SS[c] | Mean square | *F* value | Pr > *F* |
|---|---|---|---|---|---|
| Cultivar (C) | 2 | 0.02078 | 0.01039 | 3.02 | 0.0863 |
| Growing condition[d] (G) | 1 | 0.00254 | 0.00255 | 0.74 | 0.4062 |
| C × G | 2 | 0.02095 | 0.01047 | 3.05 | 0.0849 |
| Error (a) | 12 | 0.04121 | 0.00343 | | |
| Time (T) | 6 | 2.27827 | 0.37971 | 156.43 | 0.0001 |
| C × T | 12 | 0.02606 | 0.00217 | 0.89 | 0.5561 |
| G × T | 6 | 0.00580 | 0.00097 | 0.40 | 0.8780 |
| C × G × T | 12 | 0.02637 | 0.00220 | 0.91 | 0.5458 |
| Error (b) | 72 | 0.17477 | 0.00243 | | |

[a]Values are means of three replicates ± standard error (100 spores were counted per treatment).
[b]On Freedom, spores collapsed following a hypersensitive response and could not be counted on greenhouse samples after 5 days.
[c]Sum of squares.
[d]In vitro or greenhouse.

TABLE 2. Formation of appressoria of *Venturia inaequalis* on apple leaves and analysis of variance (ANOVA)

| Cultivar | Growing condition | Conidia with appressorium[a] (%) after 6 h | 12 h | 24 h | 36 h | 48 h | 72 h | 5 days |
|---|---|---|---|---|---|---|---|---|
| Empire | In vitro | 30 ± 2.7 | 32 ± 3.5 | 34 ± 2.1 | 43 ± 3.0 | 46 ± 2.1 | 63 ± 3.8 | 92 ± 6.7 |
| | Greenhouse | 21 ± 1.5 | 22 ± 3.0 | 33 ± 2.5 | 42 ± 3.2 | 59 ± 4.6 | 62 ± 1.5 | 67 ± 2.7 |
| McIntosh | In vitro | 28 ± 1.5 | 43 ± 3.1 | 45 ± 2.0 | 48 ± 2.1 | 59 ± 3.1 | 62 ± 8.4 | 87 ± 7.6 |
| | Greenhouse | 20 ± 3.6 | 31 ± 6.1 | 45 ± 4.1 | 55 ± 3.8 | 56 ± 4.5 | 62 ± 3.8 | 65 ± 2.7 |
| Freedom | In vitro | 20 ± 3.8 | 23 ± 3.8 | 35 ± 4.2 | 42 ± 5.5 | 52 ± 6.7 | 55 ± 4.7 | 70 ± 8.9 |
| | Greenhouse | 17 ± 4.6 | 23 ± 2.7 | 30 ± 3.2 | 42 ± 4.0 | 53 ± 3.5 | 54 ± 4.5 | —[b] |

| ANOVA/source | df | Type III SS[c] | Mean square | *F* value | Pr > *F* |
|---|---|---|---|---|---|
| Cultivar (C) | 2 | 0.23187 | 0.11594 | 28.40 | 0.0001 |
| Growing condition[d] (G) | 1 | 0.09721 | 0.09722 | 23.81 | 0.0004 |
| C × G | 2 | 0.00083 | 0.00042 | 0.10 | 0.9039 |
| Error (a) | 12 | 0.04899 | 0.00408 | | |
| Time (T) | 6 | 3.55650 | 0.59275 | 259.09 | 0.0001 |
| C × T | 12 | 0.16473 | 0.01373 | 6.00 | 0.0001 |
| G × T | 6 | 0.26321 | 0.04387 | 19.17 | 0.0001 |
| C × G × T | 11 | 0.05259 | 0.00478 | 2.09 | 0.0323 |
| Error (b) | 70 | 0.16015 | 0.00229 | | |

[a]Values are means of three replicates ± standard error (100 spores were counted per treatment).
[b]On Freedom, spores collapsed following a hypersensitive response and could not be counted on greenhouse samples after 5 days.
[c]Sum of squares.
[d]In vitro or greenhouse.

TABLE 3. Percentage of conidia of *Venturia inaequalis* with one to five appressoria 5 days after inoculation of apple leaves[a]

| Cultivar | Growing condition | Number of appressoria per conidium 1 | 2 | 3 | 4 | 5 or more |
|---|---|---|---|---|---|---|
| Empire | In vitro | 47 ± 3 | 36 ± 4 | 11 ± 2 | 6 ± 3 | 0 |
| | Greenhouse | 95 ± 3 | 5 ± 1 | 0 | 0 | 0 |
| McIntosh | In vitro | 52 ± 4 | 33 ± 6 | 12 ± 4 | 3 ± 1 | 0 |
| | Greenhouse | 96 ± 3 | 4 ± 1 | 0 | 0 | 0 |
| Freedom[b] | In vitro | 60 ± 4 | 27 ± 2 | 6 ± 1 | 6 ± 1 | 1 |
| | Greenhouse | 96 ± 3 | 4 ± 1 | 0 | 0 | 0 |

[a]Values are means of three replicates ± standard error (100 spores were counted per treatment). Differences were significant ($P = 0.05$) between in vitro and greenhouse samples but were not significant among cultivars.
[b]Freedom conidia were counted 72 h after inoculation.

TABLE 4. Cuticle thickness of in vitro and greenhouse-grown apple leaves[a]

| Cultivar | Growing condition | Cuticle thickness (μm) |
|---|---|---|
| Empire | In vitro | 0.155 ± 0.033 |
| | Greenhouse | 0.333 ± 0.038 |
| Freedom | In vitro | 0.159 ± 0.018 |
| | Greenhouse | 0.345 ± 0.019 |

[a]Values are means of 20 leaf samples ± standard error. Differences between in vitro and greenhouse leaves were significant ($P = 0.05$).

by TEM (Fig. 3C). An electron-transparent sac with granular matrix, the infection sac, was observed by TEM during penetration (Fig. 3C). In some cases, penetration occurred without the formation of a germ tube or an appressorium.

Following formation of the appressorium, direct penetration of the fungus through the plant cuticle was observed 15 h after inoculation. Indirect evidence of enzymatic degradation of the cuticle during penetration and during subcuticular growth of the fungus was observed by TEM (Fig. 3C and D). After penetration, the infection hypha extended under the cuticle to the epidermal cell wall and flattened above it, forming the irregularly shaped primary hypha 24 h after inoculation (Fig. 2C). The primary hypha ramified into secondary hyphae (Fig. 2D), which branched radially to form a characteristic subcuticular stroma 24–48 h after inoculation (Figs. 2E and 3E). Separation of the cuticular membrane from the epidermal cell wall during proliferation of the subcuticular stroma was observed by TEM (Fig. 3E).

Sporulation occurred on susceptible cultivars grown in the greenhouse 7–10 days after inoculation. The subcuticular stroma produced conidiophores and conidia (Fig. 2F and G). The perforated cuticle formed a collar around the neck of the conidiophore (Figs. 2H and 3F). Sporulation of *V. inaequalis* (Fig. 2I) in susceptible cultivars is associated with the development of macroscopic symptoms.

**Susceptible cultivars in vitro.** Before penetration of the cuticle, the behavior of the fungus was similar on susceptible cultivars grown in vitro and in the greenhouse. After penetration, however, susceptible plants grown in vitro and in the greenhouse differed distinctly in the extent and nature of mycelial development.

In vitro, conidia not only germinated at the pointed apex, but also produced new germ tubes at the basal apex or in an intercalary fashion, and after ramification, a second and often a third appressorium was formed (Fig. 2J). Subcuticular stromata could not be detected by either LM or SEM in susceptible cultivars grown in vitro. The fungus appeared to grow only above the cuticle on the leaf surface, in a random (nondirectional) manner, and numerous "appressoriumlike" structures formed terminally as well as in an intercalary manner. Abundant mycelium was observed on the leaf surface above the cuticle 5 days after inoculation (Fig. 2K).

TEM studies of inoculated susceptible cultivars grown in vitro, however, showed that the cuticle was penetrated (Fig. 3G). However, after the primary hypha was formed under the cuticle, subcuticular proliferation of secondary hyphae was highly restricted (Fig. 3H) during the first 10 days after inoculation. Sporulation or macroscopic symptoms were observed on the shoot-tip cultures 20–30 days after inoculation, as well as random nonstromatic subcuticular hyphae in addition to the abundant mycelia above the cuticle.

**Resistant cultivars.** Prepenetration and penetration activities of *V. inaequalis* were indistinguishable on resistant and susceptible cultivars. After formation of the primary hypha, however, differences in colonization and establishment were detected between susceptible and resistant cultivars and between the two resistant cultivars.

On the resistant cultivar Freedom, a hypersensitive response was observed on plants grown in vitro and in the greenhouse. Typically only one cell, the primary hypha, developed after penetration of the cuticle (Fig. 3D). The hypersensitive response occurred 48–72 h after inoculation (Fig. 2L). The epidermal cells underlying the spreading hyphae exhibited brown discoloration and necrosis when viewed by LM. Epidermal cell necrosis was followed by the formation of the characteristic pit-type lesions.

In a few instances, subcuticular hyphae were observed in greenhouse-grown plants in areas where spore clumps accumulated during inoculation. No stroma formation or sporulation was detected, however.

On the Vf-resistant cultivar Liberty, formation of subcuticular stromata was observed, but limited development occurred 72 h after inoculation. Sporulation was not observed even 20 days after inoculation.

## DISCUSSION

Our results confirm earlier studies of apple scab pathogenesis demonstrating that conidia of *V. inaequalis* germinate, form appressoria, and penetrate the cuticle of both resistant and susceptible apple cultivars (6,7,10,11,23–26,32,34). Similar ultrastructure has been described for ascospores of *V. inaequalis* during germination and penetration of apple leaves (30). In addition, our study demonstrates that prepenetration and penetration activities of *V. inaequalis* are indistinguishable on apple plants grown in shoot-tip cultures and in the greenhouse. After penetration, however, distinctive differences became apparent between in vitro- and greenhouse-grown resistant and susceptible cultivars in colonization and establishment of the pathogen.

Expression of resistance occurred after penetration regardless of whether the plants were grown in vitro or in the greenhouse. This result confirms the observation that the cuticular membrane of mature and immature apple leaves is not an effective preformed physical barrier for the apple scab pathogen (32), nor is the undifferentiated cuticular membrane of in vitro plantlets. Recently, direct evidence has been obtained for the role of cutinase in the penetration and subcuticular colonization of apple leaves by *V. inaequalis* (18,19). Cutinase and cell wall-degrading enzymes (cellulose and pectin) have been implicated also in the degradation of the inner reticulate layer of the cuticular membrane (17,24,31), rich in cellulosic and pectin microfibrils (12,13,17), during establishment and proliferation of the subcuticular stroma.

The Vf and Antonovka-derived scab resistance in the cultivar Freedom was expressed as a hypersensitive reaction 48–72 h after inoculation under both greenhouse and in vitro conditions. Oxidation of polyphenols, release of phytoalexins, rapid cell collapse, and cytoplasmic aggregation of heterogeneous material, resulting in the brown discoloration of infected tissues, have been proposed as some of the mechanisms in plants that restrict ramification and development of fungi (1). The host cells and penetrating hyphae died rapidly enough to prevent establishment of a parasitic relationship. The fact that in a few cases the hypersensitive response was not observed and a subcuticular stroma was formed may be related to the high inoculum density and to the use of several races of the pathogen. The hypersensitive reaction is known to be race-specific (26).

Most of the scab-resistant cultivars selected in breeding programs contain the Vf gene from the wild species *M. floribunda* (27). Hybrids in which resistance is encoded by the Vf gene are known to express numerous intermediate symptoms between resistance and susceptibility (7). When the cultivar Liberty is inoculated with mixed populations of the pathogen, its Vf resistance is expressed as reduced stromatal growth both in vitro and in the greenhouse, as reported here and previously (32). In both Liberty and Freedom, fungal development was rapidly inhibited or reduced, giving rise to localized chlorotic lesions or small pinpoint lesions, respectively. That no conidiophore formation or sporulation was ever observed on either of these cultivars was expected, given that the selection criterion for the scab resistance breeding program in Geneva is lack of sporulation.

The typical subcuticular stroma was formed only on susceptible greenhouse plants. In vitro, abnormal elongation and branching of the germ tubes on the leaf surface and formation of multiple appressoria were observed on susceptible cultivars 5 days after inoculation and on the resistant cultivar Freedom after 72 h. Similar observations have been made with SEM for the susceptible cultivar Golden Delicious in vitro (8). Formation of multiple appressoria has been reported previously on older leaves of susceptible cultivars (ontogenic resistance) as well as on young and older leaves of the Vf-resistant cultivars Priscilla and Sir Prize (11). The percentage of multiple appressoria formed on our greenhouse material is similar to that previously reported. On ontogenically and genetically resistant cultivars, despite the formation of multiple appressoria, development of a subcuticular stroma

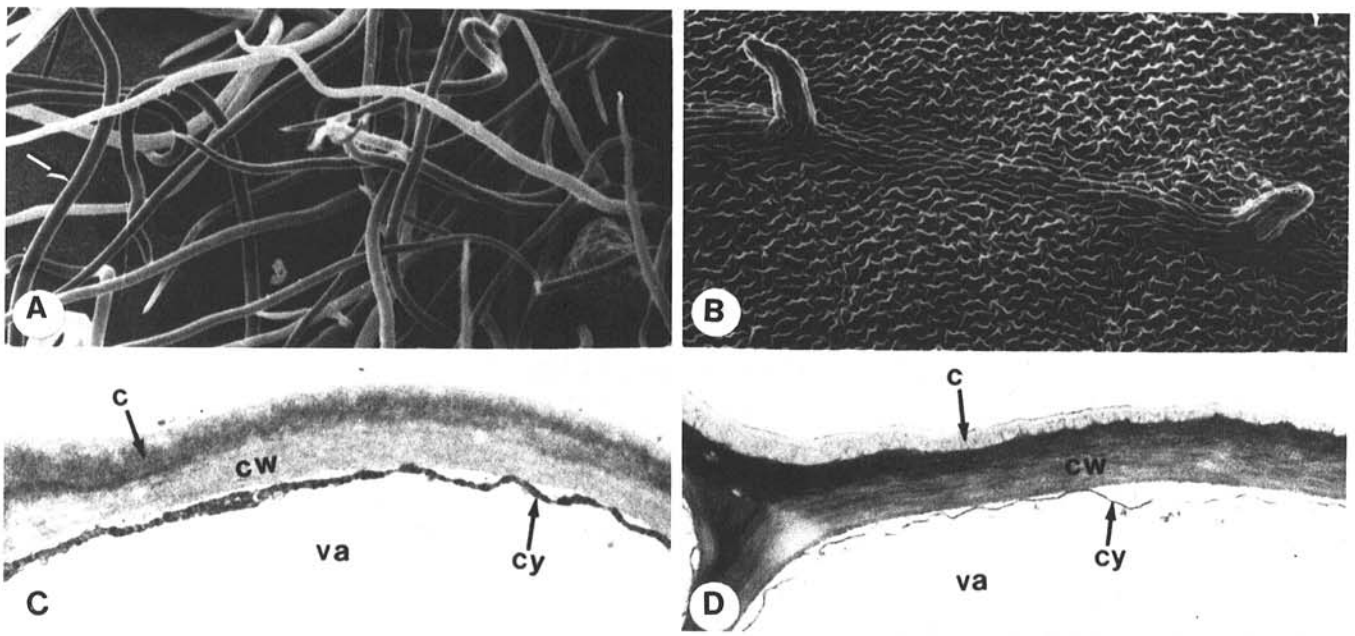



**Fig. 1.** Morphological differences between newly expanding apple leaves grown in vitro and in the greenhouse: **A,** Surface of a greenhouse-grown apple leaf as seen under a scanning electron microscope (SEM) (×50); note the abundant trichomes (arrow). **B,** Surface of a leaf of an apple plantlet grown in vitro, as seen under an SEM (×86); note the absence of trichomes. **C,** Transmission electron microscopy (TEM) cross section of a leaf from a greenhouse-grown plant (×10,000), showing the ultrastructure of the cuticular membrane (c) above the epidermal cells. The cuticular membrane (0.33 μm thick) appears under TEM to consist of two layers: a faint outer lamellate layer and an underlying electron-dense reticulate region. A narrow pectin layer is seen at the junction with the cell wall (cw). A thin layer of cytoplasm (cy) and the vacuole (va) are visible. **D,** TEM cross section of a leaf of a plantlet grown in vitro (×13,000); the cuticular membrane (0.15 μm thick) has a distinct lamellate layer but no reticulate region.

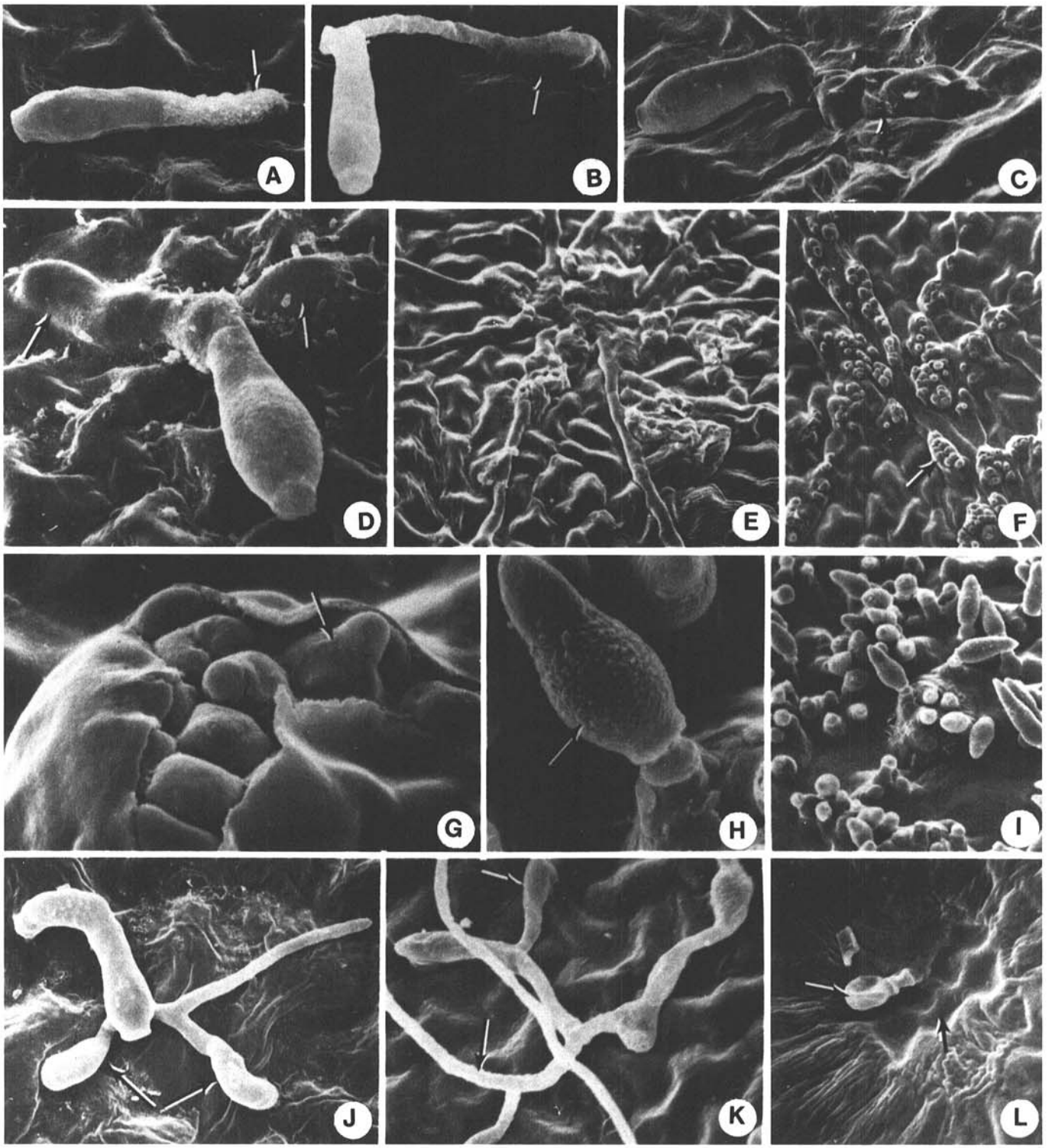
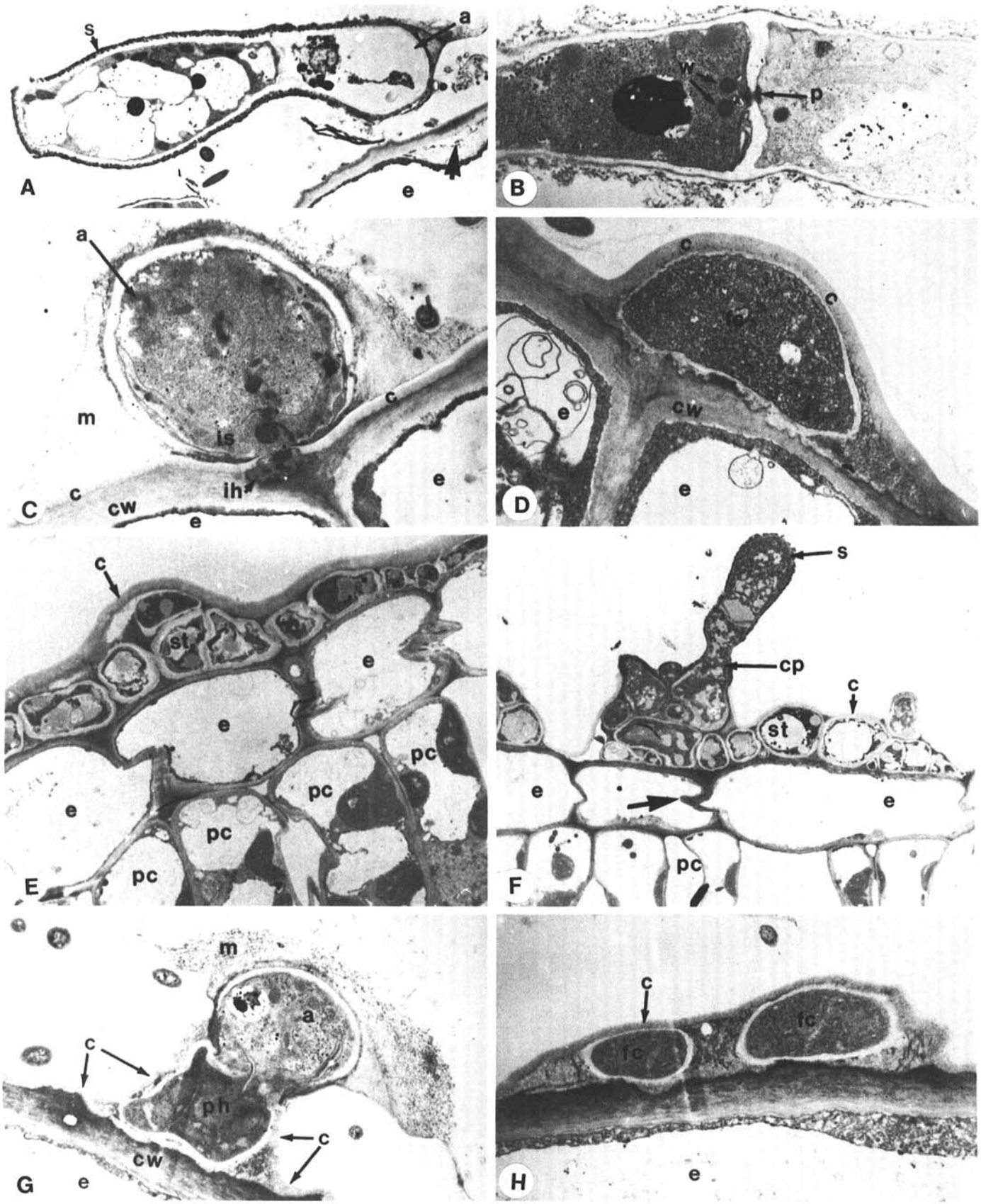



**Fig. 2.** Scanning electron micrographs of the pathogenesis of *Venturia inaequalis* on susceptible and resistant apple cultivars grown in the greenhouse **(A–I)** and in vitro **(J–L): A,** Conidium with a germ tube (arrow) on the surface of a McIntosh leaf 6 h after inoculation (×1,200); note the mucilaginous layer that adheres the germ tube to the leaf surface. **B,** Germinated conidium with appressorium (arrow) 9 h after inoculation (cultivar McIntosh) (×1,260). **C,** Primary hypha (arrow) is formed under the cuticle 24 h after inoculation (cultivar McIntosh) (×1,100). **D,** Primary hypha ramifies into secondary hyphae (arrows) (cultivar Empire) (×1,500). **E,** Secondary hyphae branch radially to form the characteristic subcuticular stroma 48 h after inoculation (cultivar Empire) (×400). **F,** Conidiophores (arrow) develop from the subcuticular stroma 7 days after inoculation (cultivar Empire) (×350). **G,** Close-up of the emerging conidiophores (arrow) breaking through the cuticle (cultivar Empire) (×1,600). **H,** Conidium (arrow) detaching from the conidiophore; notice how the cuticle forms a "collar" around the neck of the conidiophore (cultivar Empire) (×2,000). **I,** Mature conidia on cuticle surface (cultivar Empire) (×500). **J,** Germinated conidium with multiple appressoria (arrows) 72 h after inoculation (cultivar Empire) (×1,200). **K,** Ramification of the germ tubes leads to abundant mycelium above the cuticle (lower arrow) 5 days after inoculation; numerous appressoriumlike structures also were formed (upper arrow) (cultivar McIntosh) (×870). **L,** Collapse of the epidermal cells (lower arrow) underlying the primary hypha 72 h after inoculation (cultivar Freedom) (×600). The collapsed conidium above the epidermal cells is also visible (left arrow). The hypersensitive response was observed on plants grown in vitro and in the greenhouse.

is highly restricted. Interestingly, the fungus does not proliferate above the cuticle on resistant cultivars either in vitro or in the greenhouse.

Abnormal proliferation of *V. inaequalis* above the cuticle on susceptible cultivars grown in vitro may be the result of differences in the level of leaf differentiation. Alterations in the anatomy and physiology of apple leaves grown in vitro have been reported—for example, tissue culture plantlets do not develop trichomes as do greenhouse plants (16)—and high relative humidity inhibits the development of surface wax and cuticle (15,22,33). The morphological differences between tissue culture plantlets and normal plants may be related to the environmental conditions



of the tissue culture plants (e.g., very high and constant level of humidity, constant temperature and photoperiod, lack of wind, relatively constant $CO_2$ level, and the presence of growth regulators) (16).

The thin, undifferentiated cuticular membrane of in vitro plantlets may allow leakage of nutrients to the surface. The fungal proliferation above the cuticle on in vitro plants in the compatible interaction indicates that the pathogen is still able to derive nourishment from the underlying host tissue. Lack of direct contact between the fungus and the host cells, however, may slow the rate of proliferation of the fungus on in vitro leaves (e.g., stromata after 96 h were only one-fourth the size of those observed in greenhouse leaves). Sporulation was significantly delayed for the susceptible cultivars in vitro: from 7–10 days in the greenhouse to 20–30 days after inoculation in vitro. For Golden Delicious, sporulation has been reported to occur 40 days after inoculation (8).

Our results indicate that resistance to apple scab can be expressed at the shoot-tip level. Nonetheless, several questions remain unanswered: Is the in vitro test as reliable as the greenhouse test for selection of genotypes with apple scab resistance? Given the difficulties of producing contaminant-free inoculum because of the slow growth of *V. inaequalis* and the loss of virulence during subculture, can other methods be used to minimize contamination? Do culture conditions or composition of the medium interfere with the expression of resistance? We have conducted further studies to refine and simplify the conditions for successful selection of apple scab-resistant plantlets in vitro (35).

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**Fig. 3.** Transmission electron micrographs of the pathogenesis of *Venturia inaequalis* on susceptible and resistant apple cultivars grown in vitro and in the greenhouse: **A,** Penetration site on Empire apple leaf grown in the greenhouse 36 h after inoculation (×2,000), showing spore (s), germ tube, and appressorium (a). Note lipid bodies inside the spore. The host has responded to the fungal invasion by forming a papilla (arrow), a fibrillar deposit of cell wall material, on the inner surface of the epidermal cell (e). **B,** Enlargement of a longitudinal section through a germinated spore, showing the septal pore (p) with Woronin bodies (w) between the spore (right) and the germ tube (left) (×7,200). **C,** Cross section through the penetration site (cultivar Freedom grown in vitro) 36 h after inoculation (×5,000). Note that the appressorium formed at the junction of two epidermal cells (e). The host cell wall (cw) and the cuticle (c) are indicated. The two-layer walled appressorium (a) is covered by a mucilaginous layer (m) that adheres the fungus to the leaf surface. The infection sac (is) is in contact with the host cuticle at the penetration site. An infection hypha (ih) is seen below the place where the fungus entered the cuticle. **D,** Transverse section of a fungal cell (fc), the primary hypha, located below the cuticle (c) and along the junction of anticlinal epidermal cell walls, 36 h after inoculation (cultivar Freedom grown in the greenhouse) (×5,000). The hypersensitive response was initiated 48 h after inoculation. Note the osmiophilic deposits on the vacuole of one of the epidermal cells (e) and the cell wall (cw) lesions directly beneath the primary hypha. The cytoplasm of one of the epidermal cells appears coagulated. **E,** Subcuticular stroma (st) on cultivar Empire grown in the greenhouse, 10 days after inoculation (×1,200). The cuticle (c) appears stretched over the stroma. Epidermal (e) and palisade (pc) cells are still turgid with some osmiophilic drops. **F,** Sporulation on cultivar Empire grown in the greenhouse, 10 days after inoculation (×1,200). A conidiophore (cp) emerged through the cuticle (c). Note the spore (s) still attached to the conidiophore and the subcuticular stroma (st). **G,** Cuticle penetration on cultivar Empire grown in vitro, 36 h after inoculation (×5,000). Note the appressorium (a) surrounded by a mucilaginous sheath (m). The plane of section intercepts the penetration site. Cytoplasm migrated through a pore between the appressorium and the primary hypha (ph). The uplifted cuticle (c) covers the primary hypha on both sides (arrows). **H,** Subcuticular development on cultivar Empire grown in vitro, 72 h after inoculation (×5,000). Fungal cells (fc) are shown in transverse section below the cuticle (c). The proliferation of secondary hyphae is restricted.

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