Host-Pathogen Interactions Preceding the Hypersensitive Reaction of Malus sp. to Venturia inaequalis

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ABSTRACT

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Etiolated apple seedlings, either susceptible or hypersensitively resistant to each of four races of *Venturia inaequalis*, were inoculated simultaneously with spores from a particular race of the pathogen. Development of the fungus and concurrent host responses were followed in the living host-parasite system by light microscopy of epidermal strips from etiolated hypocotyls. Germination, appressorium formation, and penetration by *V. inaequalis* occurred at the same rate irrespective of susceptibility or hypersensitivity of the host. Following penetration of the cuticle of susceptible apple hypocotyls, growth of the fungus proceeded without interruption, and subcuticular stromata were formed. Penetration of hypersensitive hosts occurred at the same time as that on susceptible hosts, but the formation of subcuticular primary hyphae (which follows penetration) depended upon the pathogen. In addition, subcuticular stromata were not formed in hypersensitive host-pathogen combinations. Thus, inhibition of fungal growth in hypersensitive combinations occurred close to the time of penetration since primary hyphae either did not form or formed at a rate equal to or less than that in susceptible host-pathogen combinations. Granulation of cytoplasm in hypersensitively reacting cells and subsequent cell-browning occurred significantly later than the inhibition of subcuticular fungal growth. Similarly, changes in phloridzin and phloretin content of etiolated hypocotyls, which were measured by gas-liquid chromatography, did not occur until about 33 hours after inhibition of fungal growth. The data suggest that containment of *V. inaequalis* in the hypersensitive response is not mediated by changes in phloridzin or phloretin as previously assumed.

Additional key words: apple scab, physiological contact, *Phaseolus vulgaris*.

A significant problem in the study of disease resistance is the determination of the stage of pathogen development at which it is first affected by the host's resistance mechanism(s). This is important particularly in investigations built upon the hypothesis that stress compounds or phytoalexins constitute the primary resistance response of the host. If resistance is to be attributed solely to phytoalexins, their appearance in the host should coincide with an adverse effect on the pathogen.

The resistance of apple (*Malus* sp.) to *Venturia inaequalis* (Cke.) Wint. has been attributed to phytoalexin-like oxidation products of phloridzin (the major phenolic glycoside in apple) or its aglucone, phloretin (12, 19, 20, 23, 30). In several studies phloridzin, 3-hydroxyphloridzin, phloretin, and various derivatives of these compounds were shown to be inhibitory to certain bacteria and fungi, including *V. inaequalis* (2, 13, 19, 22, 29). Barnes and Williams (2) demonstrated that phloridzin and phloretic acid stimulated, and that phloretin inhibited, growth of *V. inaequalis* at concentrations between 5×10^{-3} and 10^{-2} M. Hunter (10) later demonstrated that phloretin (2×10^{-3} M) as well as phloridzin and phloretic acid stimulated the growth of *V. inaequalis*. However, these reports were based on the effect of these compounds on in vitro growth of the pathogen. The levels of phloridzin and phloretin in apple leaves are well documented (30). However, their location in the cell and the quantities available in vivo for conversion to inhibitory oxidation products (either by the host or pathogen) is unknown (30). Thus, the importance of phloridzin and phloretin to apple scab resistance is questionable.

The subcuticular growth habit of *V. inaequalis* provides a good system for comparing fungal growth in susceptible and hypersensitive interactions since microscopic observations are not hindered by host tissue. The use of epidermal tissue from etiolated apple hypocotyls (18) also allows observation of the living host-parasite combination in a single layer of host cells lacking pigments.

In the apple scab disease the mode of germination, appressorium formation, and penetration by *V. inaequalis* are identical irrespective of hypersensitive resistance, resistance, or susceptibility of the apple host (3, 15, 18, 21, 31). Nusbaum and Keitt (21) compared fungal development in resistant and susceptible host-pathogen interactions at 14, 24, 48, and 96 hours by

simultaneously inoculating differentially resistant apple hosts with a single isolate of *V. inaequalis*. Based on their data for formation of penetration pegs, no differences in the rates of penetration were observed. However, fungal development in a hypersensitive host-pathogen combination was not examined. In the hypersensitive host, the fungus occasionally produces subcuticular stromatic cells (15, 18). Typically, however, only one cell, the primary hypha, develops after penetration of the hypersensitive host cuticle. Development of a subcuticular stroma in resistant and susceptible hosts is more extensive (15, 18, 21). Therefore, if penetration of hypersensitive and susceptible apple hosts occurs at the same rate (as is the case with resistant and susceptible hosts), it should be possible to estimate a time for the initial inhibition of subcuticular fungal growth by comparing development of the fungus on both susceptible and hypersensitive hosts. Furthermore, if changes in levels of phloridzin, phloretin, or their oxidation products cause the initial inhibition of the fungus, such changes in infected tissue should be detected close to the time of fungal inhibition.

In this investigation we examined: (i) the rate of appressorium formation and penetration on hypersensitive and susceptible hosts, (ii) the time of the first visible host cell response after inoculation of the hypersensitive host, (iii) the time that subcuticular growth of the pathogen ceases or is inhibited with respect to detection of a visible host response, and (iv) the correlation of changes in levels of phloridzin and phloretin in infected host tissues and inhibition of fungal development.

MATERIALS AND METHODS

Culture of the fungus.—Isolates representing races 1 to 4 (30) of *V. inaequalis* were grown in wick culture (2) on 4% malt extract at 19 C for 14 days. Spores were centrifuged (2,000 *g*, 5 minutes) and resuspended three times in distilled water. Spore concentrations were determined with a hemacytometer.

Plant material.—Etiolated apple hypocotyls were selected for microscopic observation because the tissue response to scab infection is similar to that of green leaves and the in vivo host-parasite interaction can be observed at the microscopic level (15, 18). Progeny of the following apple cultivars and crosses were used: Cortland (open-pollinated), McIntosh (open-pollinated), both susceptible to *V. inaequalis;* Cortland × 1197-1 (527-1 = *Malus micromalus* pit type × 317-22 = *Malus atrosanguinea* 804 pit type), McIntosh × 1197-1, all hypersensitive to races 1 to 4 of *V. inaequalis*. Seedlings, grown and prepared for inoculation as previously described (18), were sprayed with spore suspensions (1.5 to 3.0×10^6 spores per milliliter) and incubated in the dark at 19 C.

The penetration peg and the primary hypha.—The penetration peg is defined as that area of the appressorium from which penetration of the host cuticle occurs (15, 21). It is recognized as a circular area about 2 μm in diameter in the appressorium (15, 21).

A primary hypha is the first subcuticular cellular growth of the pathogen to emerge through the site of penetration (the penetration peg of the appressorium). Primary hyphae give rise to subcuticular stromata of the fungus (15, 18, 21, 31).

Preparation of tissues for microscopic observation.—Penetration pegs are formed on etiolated apple hypocotyls (15); however, their detection by light microscopy often is difficult, apparently due to cuticle thinness (18). Etiolated bean hypocotyls (*Phaseolus vulgaris* L. 'Perry Marrow') were more suitable for observation of penetration pegs. Bean seedlings were grown in moist chambers by the method of Elliston et al. (9) and their hypocotyls were inoculated and incubated as described for apple.

Apple and bean hypocotyls were inoculated at the same time with spores of individual isolates of *V. inaequalis*. Epidermal strips were removed from etiolated hypocotyls and mounted in water for observation of living host cells (18). Epidermal strips mounted in lactophenol - 0.1% (w/v) cotton blue were used for counts of spore germination and formation of appressoria, penetration pegs, and primary hyphae. Microscopic observation was initiated 12 hours after inoculation and continued at 3-hour intervals for up to 24 hours. Formation of appressoria, penetration pegs, and primary hyphae were determined and expressed as percentage of germinated spores. Spore counts ranged from 200 to 500 per time interval for each tissue mounted in water as well as mounted in lactophenol-cotton blue.

Phloridzin and phloretin content in etiolated apple hypocotyls.—Etiolated seedlings of Cortland and Cortland × 1197-1 (susceptible and hypersensitive to races 1 through 4 of *V. inaequalis,* respectively) were grown and inoculated as described. Hypocotyls were excised at the desired intervals after inoculation and immediately were placed in powdered dry ice. After freezing, the tissues were ground with dry ice in a mortar, lyophilized, ground to a fine powder, and 100-mg portions were extracted in a micro-Soxhlet apparatus with 30 ml of pentane-hexane mixture (2:1, v/v) for 24 hours (20 cycles per hour). The pentane- and hexane-soluble fraction was discarded and the residue similarly was extracted with 30 ml of ethyl acetate for 24 hours. The ethyl acetate extracts then were evaporated to dryness at 70 C and maintained at −20 C until analyzed by gas-liquid chromatography.

Ten μliters of a 0.04 g/ml solution of phenanthrene (Matheson, Coleman and Bell, Norwood, OH 45212 in pyridine was added to the ethyl acetate extracts (phenanthrene served as the internal standard). The solvent was removed under nitrogen and 1.0 ml of a freshly prepared mixture of hexamethyldisilazane (Applied Science Laboratories, Inc., State College, PA 16801), trimethylchlorosilane and pyridine (3:1:9, v/v/v) was added. Prior to use, the pyridine was distilled over a 0.4-nm (4 Å) molecular sieve. Samples were agitated for 1 minute, held at room temperature for 15 minutes, and 4.0 μliters were chromatographed in a Hewlett-Packard F&M 810 Dr-12 gas chromatograph equipped with dual glass columns and a flame ionization detector. Glass columns (183 cm in length) were made from 3-mm internal diameter tubing fused to 17.8 cm and 3.2 cm tubing (4-mm internal diameter) at the injection port and detector ends, respectively. Columns were packed with Chromosorb Q (39.4/47.2-mesh per linear cm) coated with 3% OV-1 (Applied Science Laboratories, Inc., State

College, PA 16801). The carrier gas (He), H_2, and air flow rates were 50, 30, and 590 ml/minute, respectively. Injection port and flame ionization detectors were set at 330 C. The temperature was programmed from 158 C (held for 4 minutes) to 300 C (held for 5 minutes) at a rate of 10 C/minute increase. Concentrations of phloridzin

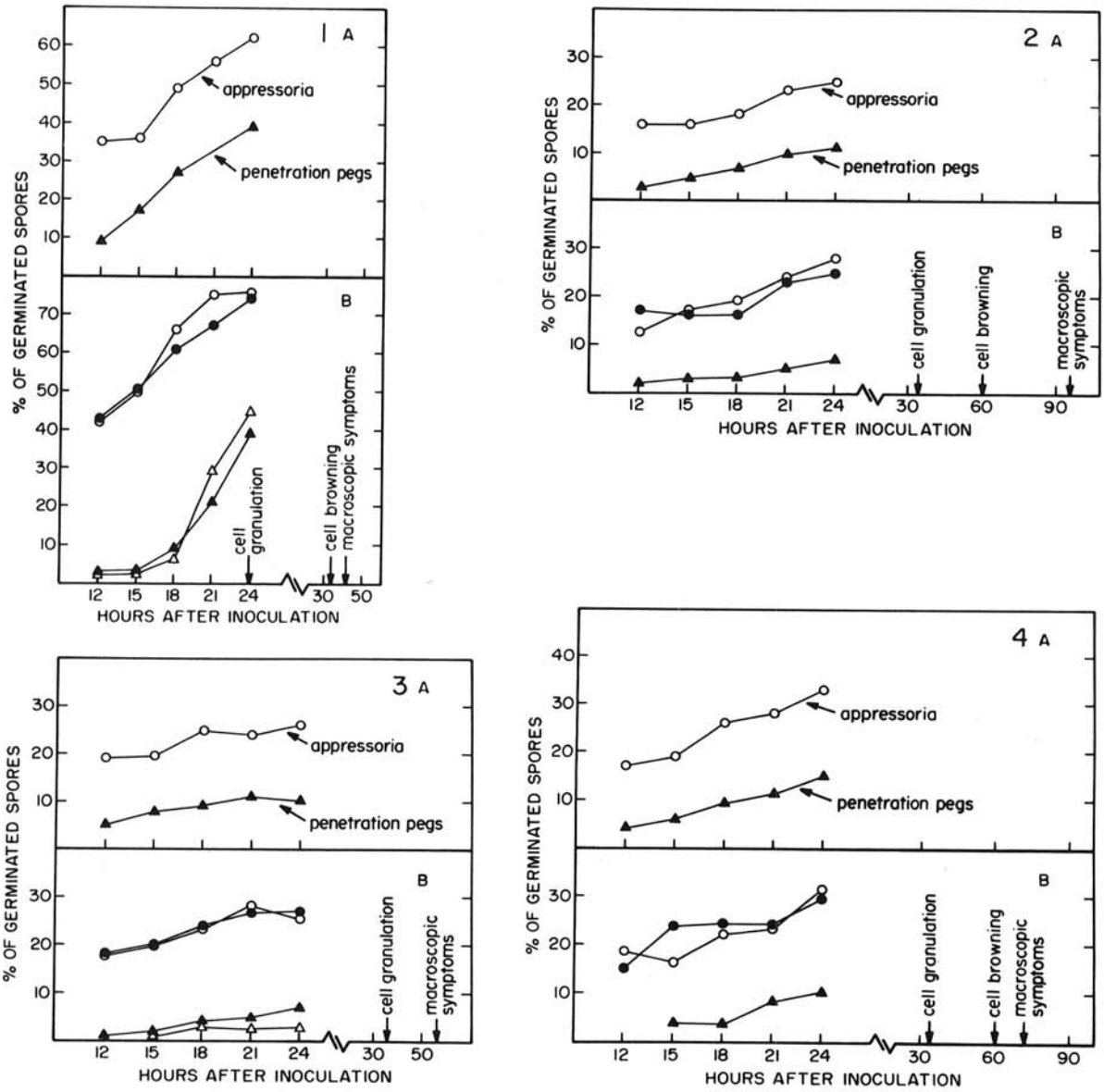

Fig. 1-4. The infection process in apple scab leading to the expression of the hypersensitive response to *Venturia inaequalis*. Inoculum: 1.5 to 3.0 × 10^6 spores/ml; 400 to 500 spores counted per time interval per host except for counts of 200 to 400 spores made at 12 hours after inoculation. **Part A** of each figure shows formation of appressoria and penetration pegs by *V. inaequalis* on etiolated bean hypocotyls (*Phaseolus vulgaris* L. 'Perry Marrow'). **Part B** of each figure shows the formation of appressoria and primary hyphae on etiolated apple hypocotyls in susceptible and hypersensitive host-pathogen combinations. Note that penetration peg formation could not be determined accurately on apple due to thinness of the apple cuticle. o = appressoria in hypersensitive combinations, ● = appressoria in susceptible combinations, △ = primary hyphae formed in hypersensitive combinations, ▲ = primary hyphae formed in susceptible combinations. The time after inoculation when host cell granulation, cell browning, and macroscopic symptoms were first observed in the hypersensitive response is shown. **1)** Race 2, isolate 1770-8, 79% and 81% spore germination at 12 and 24 hours after inoculation, respectively. Apple hosts were Cortland (susceptible) and Cortland × 1197-1 (hypersensitive). **2)** Race 3, isolate 1771-1, 55% and 66% spore germination at 12 and 24 hours after inoculation, respectively. Apple hosts were Cortland (susceptible) and Cortland × 1197-1 (hypersensitive). Note that primary hyphae did not develop in the hypersensitive host-pathogen combination. **3)** Race 4, isolate 1773-2, 61% and 64% spore germination at 12 and 24 hours after inoculation, respectively. Apple hosts were McIntosh (susceptible) and McIntosh × 1197-1 (hypersensitive). **4)** Race 1, isolate 1769-5, 47% and 60% spore germination at 12 and 24 hours after inoculation, respectively. Apple hosts were Cortland (susceptible) and Cortland × 1197-1 (hypersensitive). Note that primary hyphae did not develop in the hypersensitive host-pathogen combination.

and phloretin were determined by the internal standardization method against standard durves. Detection of phloridzin and phloretin was accurately reproducible between 0.5 and 22 and 0.5 and 14 μg per injection volume, respectively. Duplicate extracts (ethyl acetate extracts of 0.1 g apple hypocotyl tissue) for each of three samples were chromatographed twice for each sample time at instrument attenuation levels of 320 and 640. Peak areas were measured with a polar planimeter. Noninoculated tissue treated in the same manner served as controls.

RESULTS

Development of Venturia inaequalis on apple and bean hypocotyls.—At each observation period the percentage and rate of appressorium formation on susceptible and hypersensitive apple and on bean was nearly identical (Fig. 1, 2, 3, and 4). Therefore, spores of an isolate of a given race germinated and formed appressoria to the same extent and at the same rate on each host.

After formation of the appressorium the fungus penetrates the cuticle by means of the penetration peg. That penetration has occurred is apparent when the site of penetration, the penetration peg (15, 21), is observed within the appressorium (Fig. 7). Since the rates of spore germination and appressorium formation were equivalent on bean and apple, we assumed that the rate of penetration on apple was identical to that observed on bean.

Following penetration of the cuticle, the fungus began subcuticular growth. The appearance of the first cell of the subcuticular stroma (Fig. 7), called the primary hypha by Nusbaum and Keitt (21), was evidence that subcuticular growth and development had begun. Penetration pegs on bean hypocotyls were observed prior to or were formed at a greater rate than primary hyphae formation on apple (Fig. 1, 2, 3, and 4). This confirmed the assumption that development of the fungus from germination through penetration was equivalent on susceptible and hypersensitive apple hypocotyls as well as on the bean hypocotyl.

Within 24 hours after inoculation, primary hyphae (Fig. 7) consistently were observed on susceptible apple (Fig. 1-B, 2-B, 3-B, and 4-B). Depending upon the race isolate of the fungus, primary hyphae on hypersensitive apple either did not develop (Fig. 2-B and 4-B) or appeared at a rate equal to (Fig. 1-B) or slightly less than that on susceptible apple (Fig. 3-B).

Subcuticular development of each race of *V. inaequalis* on hypersensitive apple did not progress beyond the primary hypha stage. However, on susceptible apple, spore germlings of each race isolate had developed subcuticular stromata by 24 hours after inoculation (Fig. 8). Development of the subcuticular stroma on susceptible apple occurred without interruption from the time of primary hypha formation.

Host response to infection.—As previously reported (18), the first response of hypersensitive apple epidermis observed consistently was cytoplasmic granulation of one or two cells closest to the appressorium. Cytoplasmic browning of hypersensitive apple cells was observed from 11 to 26 hours after granulation (Fig. 1-B, 2-B, and 4-B). Macroscopic hypersensitive symptoms in the form of restricted brown lesions (18), were observed from 21 to 62 hours after granulation (Fig. 1-B, 2-B, 3-B, and 4-B). No changes in susceptible apple epidermis were detected throughout the observation period.

Change in phloridzin and phloretin content of etiolated apple hypocotyls.—In the hypersensitive response to the race 1 isolate of *V. inaequalis,* no substantial change (in

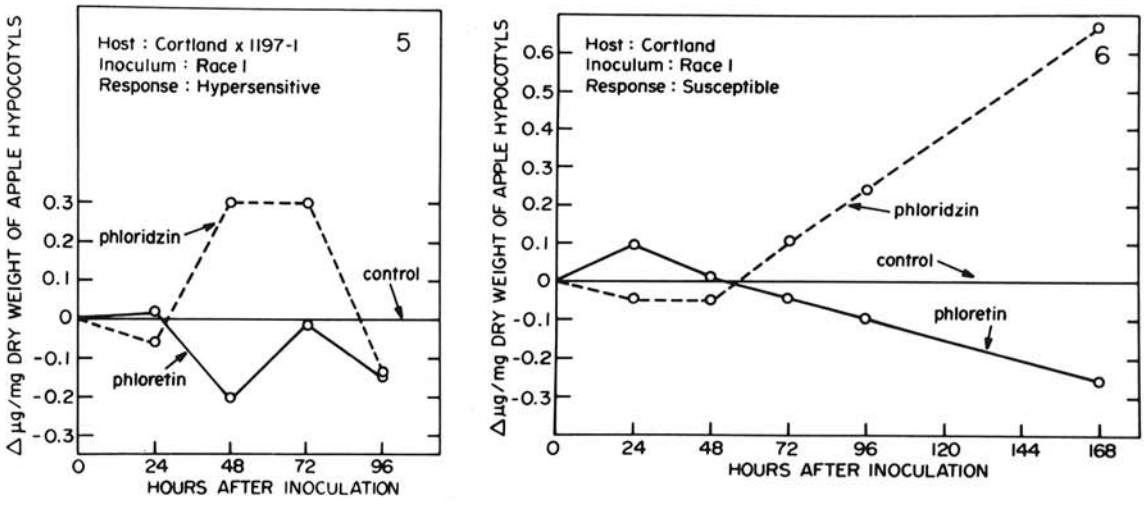

Fig. 5-6. Change in phloridzin and phloretin content of etiolated apple hypocotyls after inoculation with *Venturia inaequalis* race 1 (isolate 1769-5). Extracts were from tissue samples used in the experiment shown in Figure 4-B. Thus, phloridzin and phloretin content correspond, with respect to time after inoculation, to pathogen development and host response data shown in Figure 4-B. Phloridzin and phloretin were measured by gas liquid chromatography. **5)** Hypersensitive host-pathogen combination. The levels of phloridzin and phloretin in control tissue were approximately 12.73 and 1.80 μg/mg dry weight, respectively. **6)** Susceptible host-pathogen combination. The levels of phloridzin and phloretin in control tissue were approximately 14.54 and 1.70 μg/mg dry weight, respectively.

comparison with the noninoculated controls) in phloridzin or phloretin levels were observed prior to 24 hours after inoculation (Fig. 5). Within 48 hours after inoculation, phloridzin had increased by approximately 0.3 μg/mg dry weight whereas phloretin had decreased by approximately 0.2 μg/mg dry weight. Hypersensitive hypocotyls inoculated at the same time with the race 1 isolate exhibited cell granulation at 34 hours after inoculation and cell browning at 60 hours after inoculation (Fig. 4-B). In addition, no primary hyphae were evident, indicating that the fungus had not developed beyond the stage of penetration. Thus, a maximum of 33 hours would have elapsed between inhibition of fungal growth (assuming 15 hours after inoculation for initial penetration and inhibition, Fig. 4-B) and the change in phloridzin and phloretin content observed first at 48 hours after inoculation (Fig. 5). This suggests that the fungus was inhibited close to the time of penetration and prior to a significant change in phloridzin and phloretin content.

Primary hyphae on etiolated susceptible hypocotyls first were observed at 15 hours after inoculation (Fig. 4-B). The levels of phloridzin and phloretin in susceptible hypocotyls inoculated at the same time did not change significantly prior to 48 hours after inoculation (Fig. 6).

DISCUSSION

The validity of sequentially estimating the events of a host-parasite interaction depends in part on the pathogen establishing physiological contact with resistant and susceptible hosts at the same time. Physiological contact in the apple scab interaction is not easily defined because of the subcuticular growth habit of *V. inaequalis*. However, for spore populations of single isolates the rates of germination and appressorium formation in susceptible combinations were nearly the same as those in hypersensitive combinations (Fig. 1-B, 2-B, 3-B, and 4-B). This indicates that prior to penetration, development of the pathogen was independent of the apple host.

Primary hyphae consistently were produced on susceptible apple. However, on hypersensitive apple, primary hyphae sometimes did not develop (Fig. 2-B, 4-B), or in other fungus race-hypersensitive host combinations, were formed at a rate equal to or less than that on susceptible apple (Fig. 1-B, 3-B). It was necessary, therefore, to establish whether the inconsistent appearance of primary hyphae was the result of the inability of the fungus to penetrate the hypersensitive host at the same rate as the susceptible host or whether penetration occurred at the same rate, but subcuticular development was inhibited immediately following penetration.

Although it was impossible to obtain consistent data for penetration peg formation on apple hypocotyls, the following suggests that the rate of peg formation on both apple hosts was the same as that observed on bean. (i) The extent of germination and appressorium formation on bean was nearly the same as that on apple (Fig. 1, 2, 3, and 4). (ii) Penetration occurred before subcuticular growth, and peg formation on bean preceded, or occurred to a greater extent than, primary hypha formation on apple. (iii) Dependent upon the fungus race, primary hyphae were formed at the same rate on both apple hosts (Fig. 1). Thus, penetration would have occurred at the same rate on both apple hosts. (iv) Nusbaum and Keitt (21) found

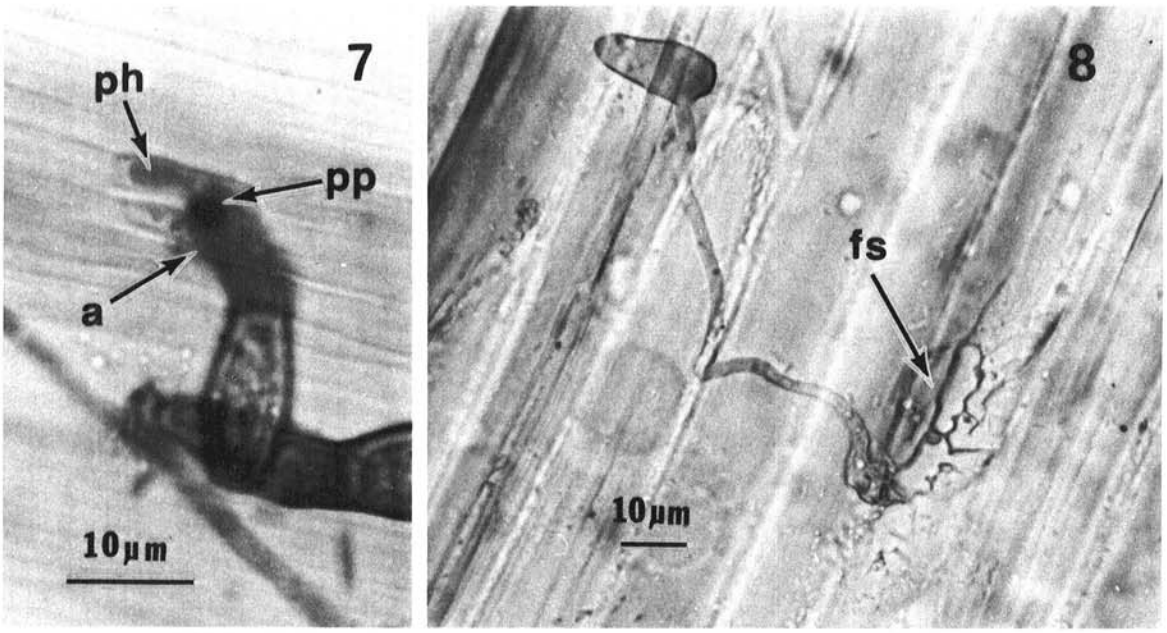

Fig. 7-8. *Venturia inaequalis* (race 1, isolate 1769-5) on epidermis of etiolated Cortland (susceptible) apple hypocotyl. **7)** Photographed at 15 hours after inoculation. The tissue was stained with lactophenol-cotton blue to demonstrate more clearly the circular penetration peg (pp) in the appressorium (a) and the fungal primary hypha (ph) beneath the host cuticle. **8)** Unstained preparation showing the subcuticular fungal stroma (fs) at 24 hours after inoculation.

equal amounts and rates of penetration of differentially resistant and susceptible hosts. Therefore, one can assume that no significant time delay existed between penetration and primary hypha formation on susceptible apple.

Primary hypha formation on hypersensitive apple depended on the race isolate of the fungus (Fig. 1-B, 2-B, 3-B, and 4-B). In addition, the hypersensitive response occurred even when primary hyphae were not formed. This suggests that inhibition of subcuticular growth occurred either at, or close to, the time of penetration of the host cuticle.

Inhibition of fungal growth at or close to the time of penetration also is suggested by the observation that subcuticular development of the fungus on hypersensitive apple did not progress beyond the primary hypha, whereas the fungus formed a stroma on susceptible apple within 24 hours after inoculation (Fig. 8).

The in vivo development of a variety of pathogens in both compatible and incompatible interactions has been investigated (1, 4, 5, 6, 7, 8, 11, 14, 16, 17, 24, 25, 26, 27, 28). Those studies which have dealt with a comparison of host necrobiosis, phytoalexin accumulation, and inhibition of the pathogen are of particular importance with respect to the present investigation.

Rahe et al. (24) demonstrated that the accumulation of phaseollin in incompatible combinations of bean and *Colletotrichum lindemuthianum* was associated with browning of host cells. Similarly, an increase in phaseollin coincided with host cellular browning and phaseollin increased in both compatible and incompatible (hypersensitive) bean anthracnose interactions (1). Skipp and Deverall (25) demonstrated that incompatibility in bean anthracnose was not associated with the inability of *C. lindemuthianum* races to form appressoria and penetrate the host. In fact, intracellular hyphal growth occurred in incompatible interactions but was more restricted in host cells undergoing visible browning. These authors could not determine the time at which hyphal growth first was inhibited, but their results suggested that it was concurrent with the host browning response and therefore coincided with the accumulation of phaseollin. Kitazawa and Tomiyama (11) and Tomiyama (28) demonstrated that hyphal growth of *Phytophthora infestans* was not inhibited until 2.5 hours after "hypersensitive death" and "browning" of cells of an incompatible potato cultivar. Similarly, host cell death has been suggested to precede the cessation of growth of *Bremia lactucae* in hypersensitive interactions of this fungus with lettuce (14). These studies suggest that host necrobiosis and resistance are associated closely with the inhibition of growth of the pathogen.

In contrast, Brown et al. (4) demonstrated that the collapse of wheat cells during a hypersensitive response to *Puccinia graminis tritici,* does not in itself inhibit the development of the rust colony and suggested that host necrosis may be the consequence rather than the cause of resistance to the pathogen.

Phenolic oxidation in apple scab results in the characteristic browning of the hypersensitive reaction and has been suggested to be responsible for inhibition of *V. inaequalis* (2, 12, 19, 20, 22, 23, 30). However, for each fungus race studied, cellular browning of the host was not observed until 8, 12, and 36 hours before symptoms were observed macroscopically (Fig. 1-B, 4-B, and 2-B, respectively). The histological data of our study indicate that inhibition of *V. inaequalis* occurs close to the time of penetration and prior to 24 hours after inoculation. This would require a change in phloridzin and/or phloretin at an extremely early time in disease development if changes in the levels of these compounds or their oxidation products primarily are responsible for inhibition of the pathogen. However, no substantial changes in either phloridzin or phloretin could be detected in the hypersensitive response prior to 24 hours after inoculation (Fig. 5). In addition, host cell granulation which precedes cell browning in the hypersensitive response (18) did not occur until after cessation of fungal development (Fig. 1, 2, 3, and 4). The data presented in Fig. 4 were taken from hypocotyls inoculated at the same time and with the same spore suspension used for data in Fig. 5 and 6. Thus, we contend that inhibition of the fungus occurs just after penetration of the cuticle and that neither phloridzin and phloretin nor their oxidation products represent the primary means of host resistance in the hypersensitive response. These compounds may, however, represent a "secondary" source of resistance since they and/or their oxidation products have been shown to inhibit in vitro growth of *V. inaequalis* (2, 19, 20, 23).

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