The Hypersensitive Reaction in Malus species: Changes in the Leakage of Electrolytes from Apple Leaves After Inoculation with Venturia inaequalis

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ABSTRACT

Conductance measurements indicated higher leakage of electrolytes from apple leaves of hypersensitive than either resistant or susceptible host-parasite combinations. The pattern of electrolyte leakage was verified using leaves from trees in an orchard and trees and seedlings grown in a greenhouse. A twofold increase in leakage was detected 22 hr before symptom expression in a clonal selection inoculated with races of the fungus to which it is hypersensitive, whereas leakage remained constant after inoculation with a race to which it is susceptible. These results suggest that increased cell permeability is associated with host response to the pathogen, and the magnitude of increase is greatest in hypersensitive host-pathogen combination. Some questions are raised concerning the induction of increased respiration and metabolism of phloridzin in relation to decompartmentalization and loss of host cell permeability. Phytopathology 60:373-376.

Williams & Kuć (12) reviewed literature pertaining to resistance in *Malus* spp. to *Venturia inaequalis* (Cke.) Wint. Spore germination, appressorial formation, and cuticular penetration are similar on both susceptible and resistant hosts regardless of host-isolate combination. After penetration, the fungus establishes itself between the cuticle and epidermal cells, and in susceptible host-parasite combinations it ramifies in this stratum. Within 10-14 days, conidiophores erupt through the cuticle and develop conidia. Resistant reactions range from a pinpoint necrosis (hypersensitive) to larger necrotic lesions with no sporulation (type 2). In the hypersensitive reaction, rapid cellular collapse occurs 40-72 hr after inoculation (8), while the type 2 reaction is visible 7-12 days after inoculation (12).

Several investigators (1, 3, 4, 5, 9, 10, 11) have reported changes in cell permeability during host-parasite interaction. Thatcher (9) was one of the first to recognize that such changes could be detected in infected tissue prior to visible symptoms. He suggested that, during the disease process, changes in cell permeability affected the development of the pathogen, and that impairment of membrane semipermeability would alter compartmentalization of normal biochemical functions in host cells. Changes in host cell permeability have also been reported by Goodman (4) in a hypersensitive reaction. Leaves of *Nicotiana tabacum* L. 'White Burley' were inoculated with two bacteria, *Erwinia amylovora* and *Pseudomonas syringae*. Changes in selective permeability of host cells were observed, but infection with a yellow saprophyte, 35A (not capable of inducing hypersensitive reactions), did not alter permeability of tobacco leaf tissue.

These studies suggest that modification or disruption of host cell membranes may be important in pathogenesis. The purpose of this investigation was to study the effect of infection by *V. inaequalis* on cell permeability of apple leaves. The following points were considered: (i) changes in the permeability of host tissue after infection, as measured by leakage of electrolytes; (ii) the duration of the host-parasite interaction required to initiate permeability changes; (iii) the contribution of the pathogen to the conductance of the bathing solution; and (iv) the time relationship between permeability changes and initiation of the visible hypersensitive response.

MATERIALS AND METHODS.—*Venturia inaequalis* was grown on 4% Difco malt medium for 10-14 days in 8 oz prescription bottles, and spore suspensions were prepared as described by Barnes & Williams (2). Spores were centrifuged once at 2,000 *g*, followed by resuspension in deionized water. The concentration of spores was determined on a microscopic grid (Petroff-Hausser bacteria counter, C. A. Hausser & Son, Philadelphia, Pa.).

Seeds of open-pollinated McIntosh, McIntosh × *Malus atrosanguinea* (12) '1197-1', and McIntosh × *M. floribunda* (12) '955-1' were planted in plastic multipots (77/flat) containing soil. Controlled growing conditions were used (14 hr day at 19 C). In a second study, rapidly growing shoots were acquired in early April from *M. atrosanguinea* (12), Geneva, *M. floribunda*, and Golden Delicious trees in an orchard. To maintain turgor, shoots were kept in Hoagland-Snyder nutrient solution during inoculation. Clonal selection OR45T132 (*M. atrosanguinea* 333-9) was used to compare responses to races 2, 4, and 5 of *V. inaequalis*.

Inoculation and Conductance Measurements.—Leaves from 4- to 6-week-old seedlings or shoots were washed with deionized water, and their surfaces sprayed with a spore suspension. An equal number of noninoculated leaves served as controls. Inoculated and control leaves were placed in a chamber at 19 C and 99% humidity. After an initial incubation period, as indicated in each figure, the leaves were excised and rinsed twice in deionized water, then floated with their upper surface down in trays containing 75 ml of distilled water at

19 C. To assess the degree of changes in permeability, conductance measurements were taken periodically of the bathing solutions until symptoms appeared on leaves of hypersensitive host-parasite combinations. The conductance of the bathing solutions was determined with a conductivity bridge (Model R.C. 16-B2, Industrial Instruments, Inc., Cedar Grove, N.J.), using a conductivity cell (cell constant 0.1, Beckman Instruments, Palo Alto, Cal.). When hypersensitive symptoms appeared (Fig. 1-a, 2-a, 3-a, 4, 5), leaves were homogenized in their bathing solutions and their conductance determined. The conductance values (mho) obtained in each experiment were corrected for initial conductivity of the bathing solution and expressed as a percent of the homogenate ($\Delta\%$ H). The $\Delta\%$ H values were calculated from the equation:

$$\Delta\%\ \mathrm{H} = \frac{\text{mhos at } t_{hr} - \text{mhos at } t_o}{\text{mhos H}} \times 100\%$$

where H = conductivity of homogenate
t_o = conductivity at time zero
t_{hr} = conductivity periodically measured until symptom expression.

RESULTS.—*Influence of infection by V. inaequalis on electrolyte leakage from apple leaves.*—In the first experiment (Fig. 1-a, b), seedling plants were inoculated with a mixture of races 1, 2, and 4 and compared to healthy tissue. McIntosh × *M. micromalus* is hypersensitive when infected with one or a combination of these races. McIntosh × *M. floribunda* and McIntosh are resistant (type 2) and susceptible, respectively.

In the second experiment (Fig. 2-a, b, 3-a, b), young leaves from orchard trees were inoculated. Infection of *M. atrosanguinea* or *M. micromalus* with races 1, 2, or 4 results in a hypersensitive reaction. Geneva and *M. floribunda* are resistant host-parasite combinations, and Golden Delicious is susceptible.

The rate of electrolyte leakage from inoculated hypersensitive tissue was greater than from resistant or susceptible combinations. In all cases, and if symptoms appeared on diseased intact leaves, the conductance of the bathing solution was higher for inoculated tissue than for controls. Comparison of $\Delta\%$ H in control leaves (Fig. 1-b, 2-b, 3-b) indicates that electrolyte leakage was also highest in apple leaves hypersensitive to races 1-4 of the fungus, and lowest in susceptible leaves. Differences between $\Delta\%$ H (inoculated) and $\Delta\%$ H (controls) (Table 1) were greater in inoculated leaves that are hypersensitive to one or a combination of races 1-4 of *V. inaequalis* than in either resistant or susceptible combinations. These differences in $\Delta\%$ H suggest that the rate of change in conductance is due to increased permeability of host cells.

To minimize variation from host tissue, a clonal selection, OR45T132, was tested. This selection is hypersensitive to races 1-4, but susceptible to race 5 of *V. inaequalis*. Leaves were inoculated with a single isolate of race 2, 4, or 5. Healthy leaves served as controls. Greater conductance occurred in hypersensitive than in susceptible host-parasite combinations (Fig. 4, 5). This loss of selective permeability in host cell membranes begins approximately 22 hr prior to symptom expression. The rate of leakage doubles (Table 2). These data also indicate the level of extra-

TABLE 1. Change in conductivity of apple (*Malus* spp.) leaf-bathing solution 5 hr prior to visible hypersensitive symptoms

Plant Material	Δ % H inoculated			Δ % H control			Δ % H-Δ % H[c] inoc.-cont.		
	M[a]	r4[b]	r1[b]	M[a]	r4[b]	r1[b]	M[a]	r4[b]	r1[b]
Seedlings									
McIntosh × *M. micromalus*	8.50	7.10		5.00	4.15		3.50	2.95	
McIntosh × *M. floribunda*	6.80	7.00		3.95	4.85		2.85	2.15	
McIntosh	5.40	5.50		2.85	3.50		2.75	2.00	
Orchard trees									
M. atrosanguinea	8.00	10.20	5.25	5.00	5.90	2.25	3.00	4.30	3.00
M. micromalus	6.60	8.00		3.80	5.10		2.80	2.90	
Geneva	2.70			2.80			0.00		
M. floribunda	2.00		1.90	1.50		1.90	0.05		0.05
Golden Delicious	1.00	2.40	1.75	1.20	2.00	1.00	0.00	0.40	0.75

[a] M = Leaves inoculated with a mixture of races 1, 2, and 4.
[b] r4 = Race 4 (1×10^6 spores/ml), r1 = race 1 (5×10^5 spores/ml).
[c] Differences in Δ % H between inoculated and control tissue.

TABLE 2. Change in conductivity of apple leaf bathing solution 22 hr prior to visible hypersensitive symptoms

Clonal selection: OR45T132	Δ % H inoculated			Δ % H control	Δ % H-Δ % H[c] hyper.-cont.	Δ % H-Δ % H[d] suscept.-cont.
	r2[a]	r4[a]	r5[b]			
Exp. 1	15.00		10.00	8.00	7.00	2.00
Exp. 2		14.00	8.00	4.00	10.00	4.00

[a] r2 = Race 2, r4 = race 4; both initiate hypersensitive reactions.
[b] r5 = Race 5; initiates susceptible reaction.
[c] Differences in Δ % H between hypersensitive host-parasite combination and control.
[d] Differences in Δ % H between susceptible host-parasite combination and control.

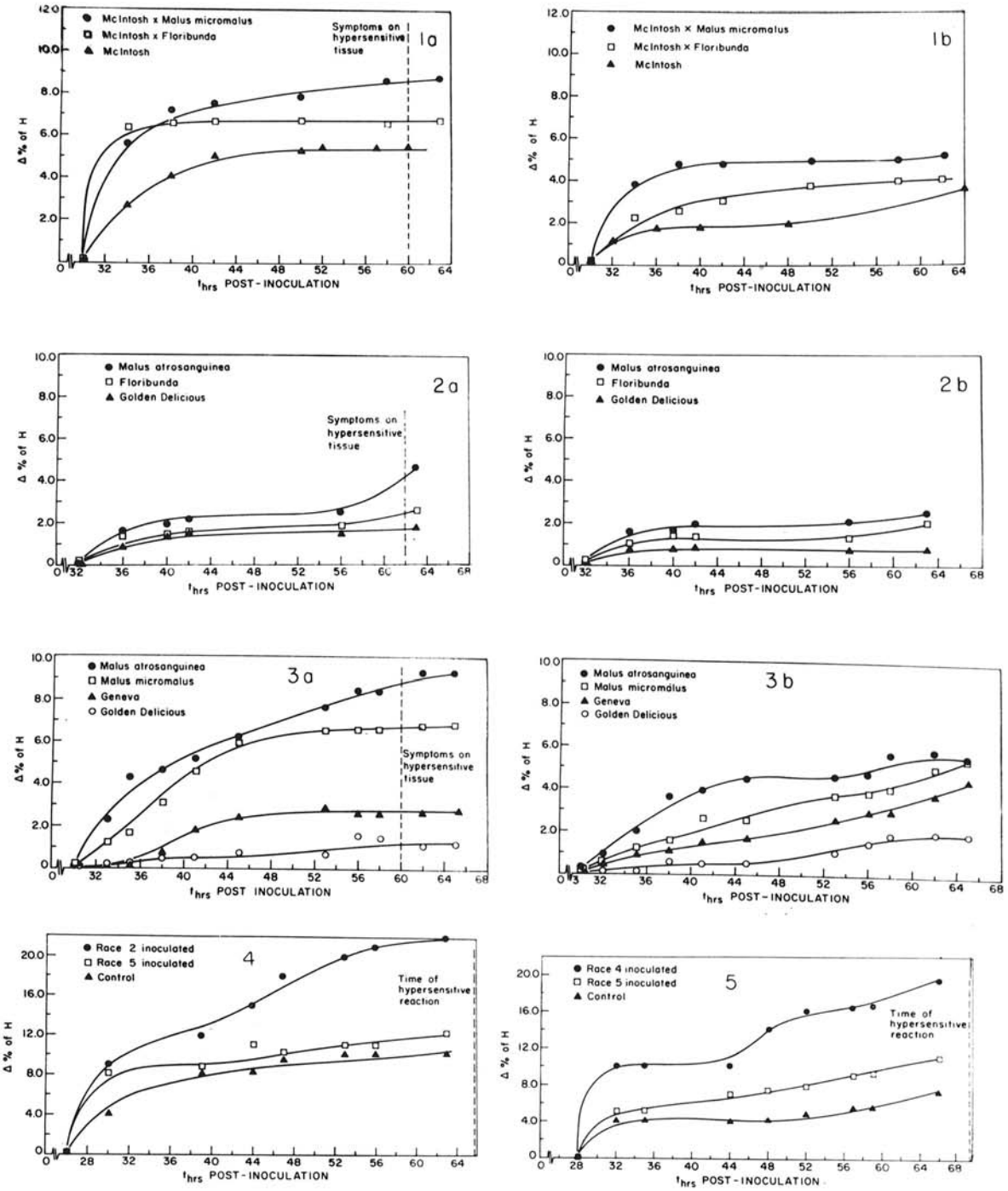

Fig. 1-5. 1-3. Change in conductivity of apple (*Malus* spp.) leaf-bathing solutions. **1a)** After inoculation with a mixture of races 1, 2, and 4 (7.5×10^5 spores/ml) of *Venturia inaequalis*. McIntosh × *M. micromalus* = hypersensitive; McIntosh × *M. floribunda* = type 2 reaction; McIntosh = susceptible. **1b)** Noninoculated control. **2a)** After inoculation with same mixture as above (1×10^6 spores/ml). *M. atrosanguinea* = hypersensitive; *M. floribunda* = resistant; Golden Delicious = susceptible. **2b)** Control. **3a)** After inoculation with mixture of races (5×10^5 spores/ml). Geneva = resistant. **3b)** Control. Each curve represents results of at least two replications. **4-5.** Change in conductivity of apple leaf-bathing solution. **4)** After inoculation with race 2 or 5 and water control. Selection, OR45T132, hypersensitive to race 2, susceptible to race 5 (Race 2 and 5 were adjusted to equal concentrations of 1×10^6 spores/ml). **5)** Inoculated with race 4 or 5 and water control. Selection is hypersensitive to race 4 (Race 4 and 5 adjusted to 1.5×10^6 spores/ml). Each figure is an average of four replications.

cellular electrolytes contributed by the fungus and control leaves.

DISCUSSION.—An increase in electrolyte leakage from inoculated apple leaves was detected prior to symptom expression. This suggests impairment of the semipermeable nature in host cell membranes. Physical or chemical changes induced by a pathogen could alter the normal osmotic conditions necessary for the host cells. For a compatible host-parasite relationship, the pathogen obtains nutrition from host cells. An increase in permeability to water and solutes by the plasma membrane of contiguous host cells would be desirable, but a complete loss of semipermeability would result in cellular collapse typical of an incompatible response.

Since changes in permeability could be detected 22 hr before symptom expression, the mechanism initiating the hypersensitive response must begin before this alteration, but after penetration of the cuticle by the pathogen. At the time of hypersensitive symptoms, the resistant and susceptible combinations appear to be compatible, and little increase in conductance was detected.

Whether respiration increases before or after the observed changes in permeability is not known.

Does substrate oxidation result in disruption of cell membranes or does the disruption of cell membranes by another mechanism cause substrate oxidation? Loss of membrane integrity which leads to mixing of enzymes and substrates separately compartmentalized in a healthy cell could initiate the oxidation reactions suggested to be associated with necrosis.

Phloridzin, a major phenolic glycoside in apples, is enzymatically hydrolyzed to phloretin in vitro by an apple leaf extract and oxidized to quinones which immediately polymerize (6, 7). It was also shown that substrate oxidation occurred before enzymic hydrolysis (7).

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