Leakage of Electrolytes and Phenols from Apple Leaves Caused by Virulent and Avirulent Strains of Erwinia amylovora

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ABSTRACT

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Apple leaves infiltrated with virulent and avirulent strains of *Erwinia amylovora* showed a linear relationship of leakage of electrolytes and total phenols during the first 5 hours. Thereafter, the rate of increase of electrolyte and phenol concentrations was nonlinear. With the avirulent strain leakage of phenol was 1 hour earlier than the leakage of electrolytes. With the virulent strain leakage of phenols and electrolytes occurred simultaneously. Both phenomena occurred sooner in tissues infiltrated with the avirulent strain. Both occurred well in advance of visible browning symptoms which were evident about 12 hours after infiltration.

Additional key words: hypersensitive reaction, *Erwinia amylovora,* electrolyte leakage, phenols, and resistance.

Klement and Goodman (7) have described loss of turgor, followed by complete desiccation of the affected tobacco leaf tissues to a papery-thin condition within 24 hours during the hypersensitive reaction induced by a number of plant pathogenic bacteria. From these symptoms, they suggested that a change in permeability of host cell membrane may have occurred. In studies relative to the hypersensitive reaction in tobacco, Goodman (5) demonstrated significant changes in the loss of electrolytes of host tissue cells inoculated with inoculum containing 5×10^7 cells/ml of *Erwinia amylovora* and *Pseudomonas syringae*. Loss of electrolytes as indicated by changes of conductance of the ambient solution was taken as evidence for the change in permeability of cell membranes. Working with Jonathan apple (*Malus pumila* L.), Burkowicz and Goodman (1) reported that leaves infiltrated with inoculum containing 10^9 cells/ml of either a virulent or an avirulent strain of *E. amylovora* showed symptoms of browning which closely coincided with the onset of significant electrolyte loss.

There are a number of reports of accumulation of phenolic compounds during pathogenesis or hypersensitive reaction (2, 4, 8) which suggest that these compounds may play a role in the hypersensitive reaction. It is a possibility that phenolic substances in the middle lamella and other components of the wall may be released first by the degradative effect of the bacteria on the host cell wall and that these phenolic substances subsequently may be oxidized to quinones which in turn could bring about cell membrane permeability alterations which would allow leakage of electrolytes as well as additional aromatic substances. I have examined the possibility that phenols accumulate prior to and may be causally related to the conductivity changes observed in bacteria-infiltrated leaves.

MATERIALS AND METHODS

Following the infiltration procedures previously reported (1), eight leaf disks infiltrated with 10^8 cells/ml of virulent or avirulent strains of *E. amylovora* were shaken in 25 ml of distilled water in 250-ml Erlenmeyer flasks for 20 minutes and the conductance of the ambient solutions was measured with a conductivity bridge (Model 31) and a conductivity cell of k = 0.1 (Yellow Springs Instrument Co., Yellow Springs, Ohio) at 0.5-hour intervals for a period of 15 hours. The conductance values, when multiplied by the cell constant (k), provide conductivity values in micromhos. The same samples of leaf leachates were partially purified using the following method before the determination of total phenols. The leachates were passed through a Millipore filter (0.22 μm pore size) to remove the bacteria and then evaporated to dryness in a rotary evaporator at a temperature not exceeding 40 C. Each of the residue samples was suspended in 5-ml aliquots of 80% aqueous ethanol (v/v) and centrifuged at 12,100 *g* for 15 minutes in a Sorvall centrifuge to remove the protein precipitate. The supernatant solution was decanted and filtered again through a Millipore filter (0.22 μm pore size) and stored at 2-4 C for subsequent determination of total phenols.

Total phenols were determined according to Spies (9), using the Folin-Ciocalteu Reagent (2N in acid) (Fisher Scientific Co.). In this procedure, 10 ml of 14% aqueous sodium carbonate solution was added to 5 ml of the methanolic solution of partially purified leachate, and to this was added 3 ml of diluted phenol reagent (phenol reagent-water, 1:2, v/v) with agitation. The precipitate

formed was removed by centrifugation at 3,020 *g* for 15 minutes. One hour after mixing, the absorbance of the samples was determined in 1-cm cells at 650 nm in a Beckman DB-G spectrophotometer. A standard curve was prepared with 7.5 to 120 μg/ml chlorogenic acid which yielded absorbance values of 0.66 to 0.96. All the experiments were repeated once and the average values were used for the analysis.

RESULTS AND DISCUSSION

Electrolyte leakage and phenol content of the samples of leachates from leaves inoculated with avirulent and virulent strains showed linear relationships during the first few hours and a nonlinear trend thereafter (Fig. 1). In the control samples, the linear relationship was maintained throughout the period of the experiment.

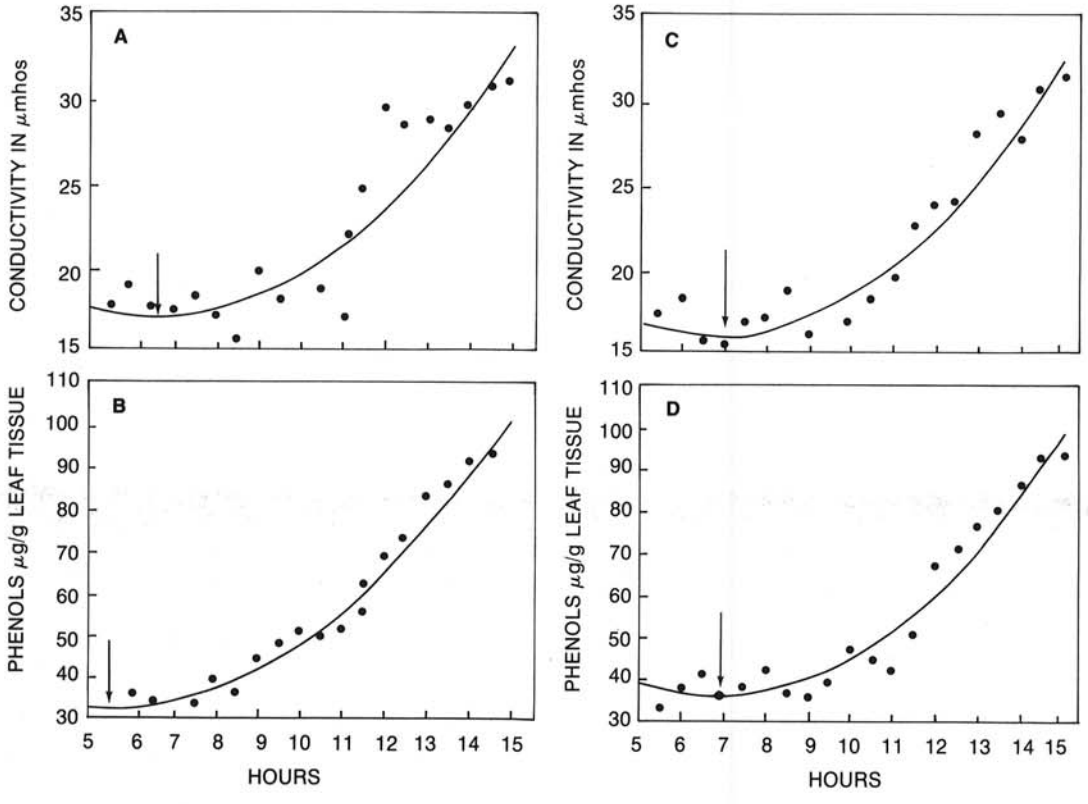

Fig. 1-(A to D). Time course of conductivity values and phenols in water leachates of apple leaves infiltrated with **A, B)** avirulent, and **C, D)** virulent strains of *Erwinia amylovora*. The arrow indicates the time in hours when response begins to increase at a quadratic rate.

TABLE 1. Regression equations used to estimate the point of nonlinearity

Treatment	Equation	R^2 [a]
	Term 1[c] Term 2[c] Term 3[c]	
C_{AV}[b]	$Y = 27.33 - 3.06^{*}_{(1.40)} \times Time + 0.23^{**}_{(0.07)} \times Time^2$	0.8296
C_V	$Y = 29.13 - 3.68^{**}_{(0.88)} \times Time + 0.26^{**}_{(0.04)} \times Time^2$	0.9338
P_{AV}	$Y = 57.17 - 8.68^{**}_{(2.49)} \times Time + 0.78^{**}_{(0.12)} \times Time^2$	0.9769
P_V	$Y = 83.56 - 13.68^{**}_{(2.79)} \times Time + 0.98^{**}_{(0.14)} \times Time^2$	0.9552

[a] R^2 = Multiple correlation squared = [Corr (Y. Y)]^2.

[b] C_{AV}, C_V, P_{AV}, and P_V represent conductivity values and total phenols for leachates of leaves infiltrated with avirulent and virulent strains of *Erwinia amylovora*.

[c] Term 1 = intercept;
Term 2 = time linear regression coefficient; and
Term 3 = time squared regression coefficient.
Figures in parentheses below the regression coefficients indicate the corresponding Standard errors.
Asterisks indicate probability of significant difference: * indicates $P = 0.05$; and ** indicates $P = 0.01$.

Statistical analysis (3) revealed that the phenol content and conductivity values were highly correlated. The correlation coefficients were 0.93 for the samples inoculated with the avirulent strain and 0.96 for the virulent strain. Also the two strains both induced similar permeability changes in the leaf tissues as revealed by the correlation coefficient of conductivity values of 0.92. Similarly, the phenol content of leachates from leaves infiltrated with either strain had a coefficient of correlation of 0.98.

Since the time-course of permeability changes and phenol leakage might have a bearing on pathogenesis, it was of interest to determine more precisely the rates at which conductivity values and total phenols increased. Specifically, differences in host response to virulent and avirulent strains would be revealed if conductivity values and total phenol concentrations became nonlinear at different times. The equations utilized to detect nonlinearity are given in Table 1.

The point of nonlinearity was calculated from the formula: − time linear regression coefficient divided by twice the time squared regression coefficient. The analysis of data in Fig. 1 shows that the leakages of phenol and electrolytes began at 5.5 and 6.6 hours, respectively, in the case of the avirulent strain whereas in the case of the virulent strain the leakages of phenols and electrolytes began almost simultaneously at 6.9 and 7.0 hours, respectively. Both phenomena occurred sooner in tissues infiltrated with the avirulent strain, and well in advance of visible browning symptoms which were evident about 12 hours after infiltration.

Thus, it appears that changes in permeability and phenol leakage are almost simultaneous in the compatible combination (virulent strain). In the case of the avirulent strain, however, these changes appeared 0.4 hour and 1.4 hours earlier, respectively. This supports the contention of Burkowicz and Goodman (1) and Goodman (6) that symptoms (flaccidity and electrolyte leakage) appear sooner in the incompatible combination (hypersensitive reaction). This is significant because even though phenolic compounds and the permeability changes were produced in both host-parasite combinations, the rates of response of the host were more rapid in the incompatible combination. These data suggest that the rapidity with which the host plant responds to the invading pathogen is an important factor in disease resistance. It also is possible that rates of response might be even more divergent if the inoculum levels used were less than 5×10^6 cells/ml (10). Finally, the fact that phenol leakage precedes loss of electrolyte in both incompatible and compatible combinations, and by a greater interval in the case of the former, supports the possibility that the two phenomena are causally related.

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