The Quantitative Relation Between Plant and Bacterial Cells Involved in the Hypersensitive Reaction

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ABSTRACT

Dead plant cells, selectively stained with Evans blue, were detected in symptomless tobacco leaves inoculated with the incompatible pathogen, *Pseudomonas pisi*, at concns lower than those required to give a confluent hypersensitive reaction (HR). With each concn of *P. pisi*, plant cell death was first detected within 1-2 h, and rose to a max 6 h, after inoculation. No further plant cell death occurred after this interval. A 1:1 ratio was observed between the number of cells of *P. pisi* introduced and the number of dead plant cells present 6 h or more after inoculation. In all cases, dead plant cells occurred singly and were randomly distributed throughout the tissue. Confluent HR occurred when the ratio of bacteria to total plant cells was 1:4 or lower. In tissues inoculated with *Pseudomonas tabaci*, the compatible pathogen, cell death was detected only 18 h after inoculation, and it increased as the typical disease syndrome developed. No cell death was detected in tissues inoculated with the saprophyte, *Pseudomonas fluorescens*. We concluded that HR will occur at the cell level, invisible to the unaided eye, in tissues naturally inoculated with small numbers of incompatible bacteria.

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Additional key words: cell death.

The hypersensitive reaction (HR) induced by the introduction of high concns of phytopathogenic bacteria into nonhost plants, is recognized by the rapid collapse and desiccation of tissues in the inoculated zone (11). Bacterially-induced HR was first described by Klement et al. (10) in incompatible host/pathogen combinations between tobacco leaves and phytopathogenic pseudomonads. By contrast, slow but progressive disease development took place in leaves inoculated with equivalent concns of the compatible pathogen, *Pseudomonas tabaci*, and no symptoms were detected in leaves inoculated with the saprophyte, *Pseudomonas fluorescens*. Similar observations by other workers using a variety of host/pathogen combinations have revealed that the ability to induce HR in nonhost leaves is an attribute of the vast majority of phytopathogenic bacteria

(6, 10, 15). The requirement of critically high concns of bacteria ($\geq 5.0 \times 10^6$ cells/ml) for the induction of HR has not been adequately explained (11). With few exceptions (10), the introduction of concns of phytopathogenic bacteria lower than those required to give confluent HR, causes only the appearance of slight chlorosis (11) or faster maturation (1) of the inoculated tissues. Since only small numbers of phytopathogenic bacteria are introduced into plant tissues under natural conditions (17), the validity of the concept of HR as a possible disease resistance mechanism rests on the hypothesis that plant cell death is occurring, but is undetected, in tissues inoculated with low concns of incompatible bacteria (9, 10, 11).

To provide insight into the significance of the bacterially-induced HR, we report here on the effect of low concns of *Pseudomonas pisi* on the viability of tobacco leaf cells in intact leaves.

MATERIALS AND METHODS.—Tobacco plants, *Nicotiana tabacum* 'Samsun', were grown from seed in vermiculite and watered twice daily with half-strength Hoagland's solution. These plants were maintained under 14 h, 16,660 lux, 24 C day and 10 h, 21 C night conditions at a minimum relative humidity (RH) of 55%.

Washed cells of *Pseudomonas pisi* (Sackett) [American Type Culture Collection (ATCC) No. 11055], *Pseudomonas tabaci* (Wolf and Foster) Stevens (ATCC No. 11528) and *Pseudomonas fluorescens* Migula (ATCC No. 13525) from 20-h nutrient broth shake cultures were adjusted in a spectrophotometer at 500 μm to give an absorbance corresponding to a known concn. The cell number was verified by standard plate-count procedure. A hypodermic syringe was used to infiltrate dilutions of the standard concn into panels of each of the seventh, eighth and ninth leaves of 9-wk-old tobacco plants. To determine changes in plant cell viability in the inoculated leaf panels, we employed Evans blue (Fisher Scientific Company, St. Louis), a nonpermeating pigment of low phytotoxicity, to detect dead cells in intact tissues (2). A 1.0% aqueous solution of this pigment was infiltrated into the inoculated panels with a hypodermic syringe, and segments of these leaf panels were examined by light microscopy after a 15-min equilibration period. The selective staining of dead cells with Evans blue depends upon exclusion of this pigment from living cells by the intact plasmalemma, whereas it passes through the damaged plasmalemma of dead cells and accumulates as a blue protoplasmic stain. We confirmed the selective properties of this dye by observing that it was taken up only by the damaged, but still turgid, cells at the periphery of a needle puncture in tobacco leaves. Cell damage was detected by the swelling or clumping of plastids and the cessation of cyclosis. When the dye was applied 24 h after the needle puncture injury, the blue stain was detected only in similar numbers of collapsed cells located around the wound. Our studies and those of others (2) suggest that the ability of plant cells to take up this dye signifies an irreversible change which leads to cell death. For clarity, we refer to such stained cells as dead. Dead mesophyll cells in leaf panels inoculated with *P. pisi* were counted in segments viewed from the upper and lower surfaces, respectively. Total dead cells/cm^2 of leaf surface in one replicate was calculated by pooling the numbers of dead palisade and dead mesophyll cells observed in each of 60 grids (0.004 cm^2) placed randomly on each surface of the nine inoculated panels per plant. We found 15 ± 8 dead cells per cm^2 in control tobacco leaves infiltrated with water. This value was subtracted from the number of dead cells counted in leaves inoculated with different concns of *P. pisi*. The number of plant cells/cm^2 in tobacco leaves was calculated by a microscopic examination of 1-μm-thick sections cut from tissues prepared by a previously described procedure (5). The intercellular volume of these leaves was estimated by weighing the amount of water which could be introduced into a known area of the leaf.

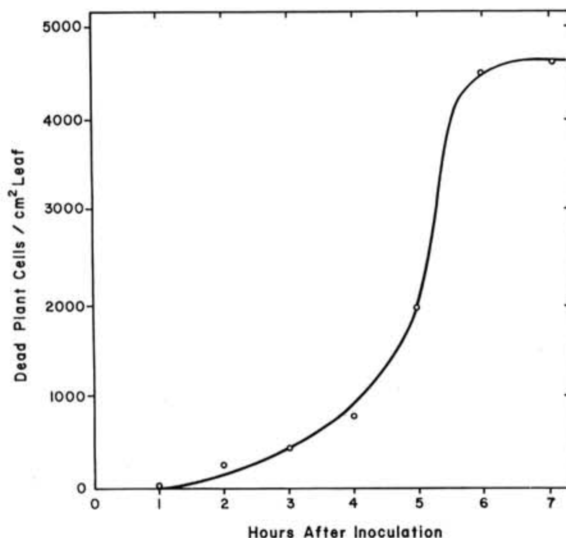

Fig. 1. Dead mesophyll cells, detected by their selective staining with a 1.0% aqueous solution of Evans blue, in tobacco leaves at intervals after the introduction of 5.0×10^3 cells/cm^2 leaf of the incompatible pathogen *Pseudomonas pisi*. Each point represents the mean of three replicates.

RESULTS.—Assuming the density of water to be 1.0 g/ml, and an average leaf thickness of 0.04 cm, the intercellular volume of tobacco leaves used in these experiments, was calculated to be 0.01 ml/cm^2 of leaf. An inoculum concn expressed as bacteria per ml would therefore introduce two orders of magnitude less bacteria per cm^2 of leaf.

When 5.0×10^3 cells of *P. pisi* were introduced per cm^2 leaf, dead mesophyll cells were first detected 1 to 2 h after inoculation, and their number increased to a maximum of 4.5×10^3 dead cells/cm^2 at 6 to 7 h after inoculation (Fig. 1). Figure 2 (upper left) shows the typical symptoms observed 48 h after the introduction of 5.0×10^1, 5.0×10^2, 5.0×10^3, and 5.0×10^4 cells of *P. pisi*/cm^2 into panels A, B, C, and D, respectively, of a tobacco leaf. Confluent HR developed only in panel D. No macroscopic symptoms other than slight chlorosis are seen in panels inoculated with the lower concns of *P. pisi*. Patterns of dead cells (arrows) selectively stained with Evans blue and viewed from the upper (u) and lower (l) surfaces of panels A, B, C, and D at 6 h after inoculation are shown in Fig. 2-a, b, c, and d, respectively. At this interval, panel D was completely flaccid, but not necrotic, and approximately

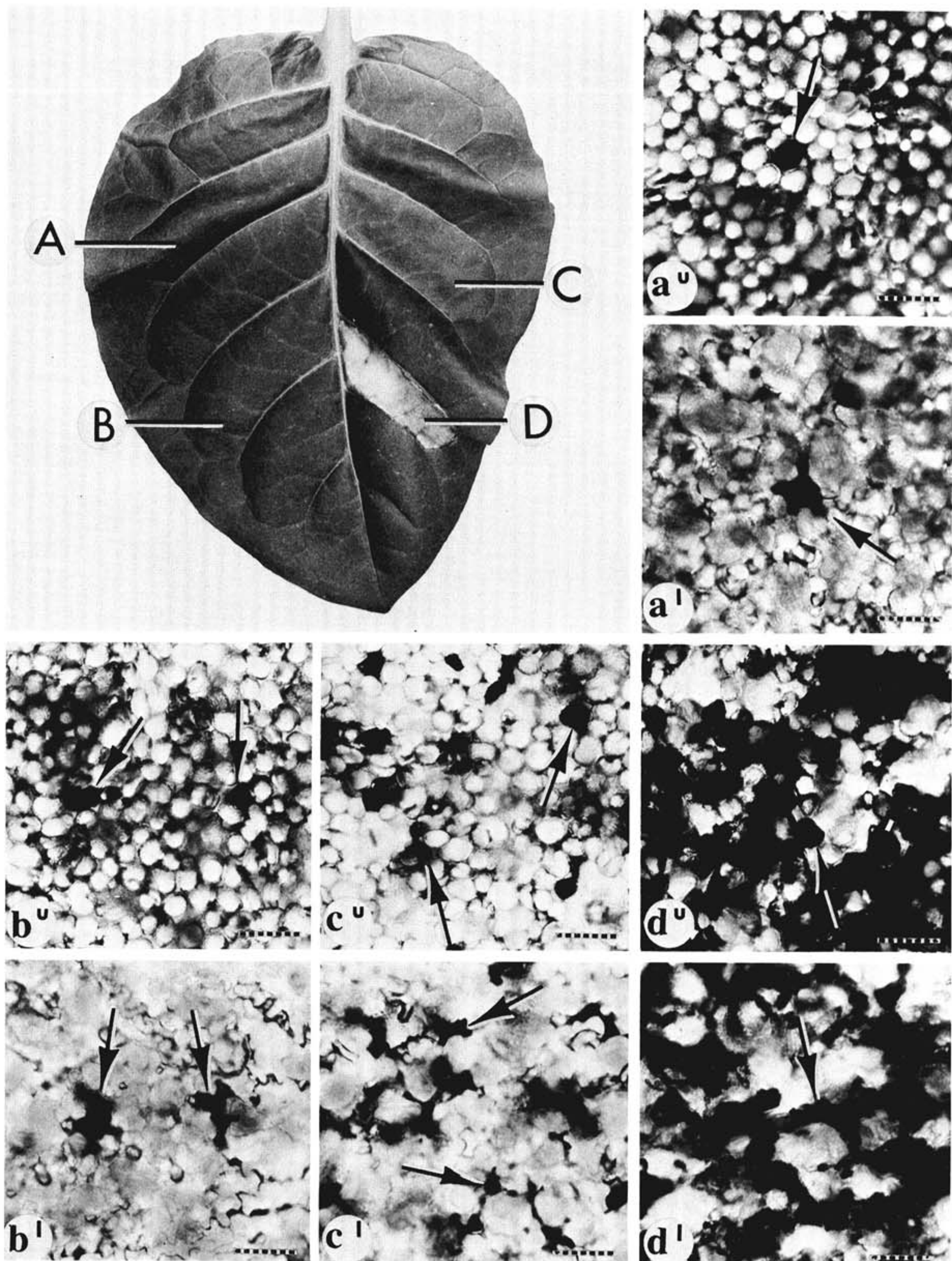

Fig. 2-(a to d). Macroscopic and microscopic symptoms observed in tobacco leaves inoculated with different concns of the incompatible pathogen, *Pseudomonas pisi*. (Upper left) Macroscopic symptoms produced 48 h after the introduction of 5.0×10^1, 5.0×10^2, 5.0×10^3 and 5.0×10^4 cells *P. pisi*/cm^2 leaf into panels A, B, C, and D, respectively. a, b, c, and d are light micrographs of both the upper (u) and lower (l) surfaces of tobacco leaves and show patterns of dead mesophyll cells (arrows), selectively stained with a 1.0% aqueous solution of Evans blue, in panels A, B, C, and D, respectively, 6 h after inoculation.

one-half of the mesophyll cells were dead, as evidenced by their selective staining with Evans blue (Fig. 2-d). In panels A, B, and C, dead mesophyll cells occurred singly and were randomly distributed throughout the tissue (Fig. 2-a, b, and c). The number of dead mesophyll cells is approximately proportional to the numbers of bacteria introduced. Dead mesophyll cells were counted at intervals up to 96 h after the introduction of 5.0×10^1 (A), 5.0×10^2 (B) and 5.0×10^3 (C) cells of *P. pisi*/cm^2 leaf (Fig. 3). In each case it was observed that plant cell death reached a maximum 6 h after inoculation. When the number of cells of *P. pisi* introduced/cm^2 of leaf was plotted against the number of dead mesophyll cells/cm^2 leaf, observed at 6 h after inoculation, a linear relation was obtained with a slope of 1.0 (Fig. 4).

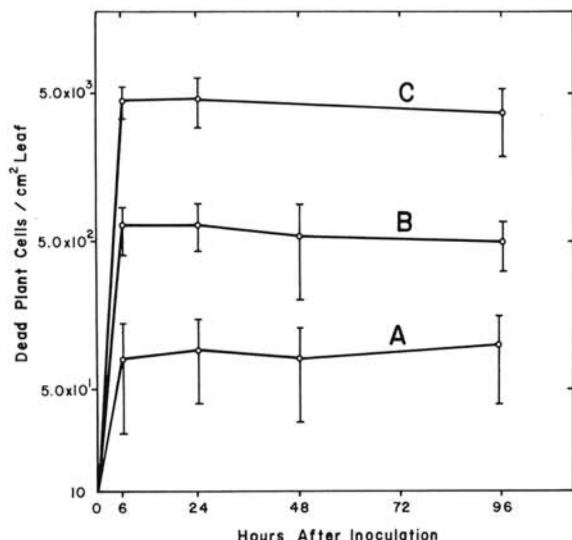

Fig. 3. Dead mesophyll cells, detected by selective staining with a 1.0% aqueous solution of Evans blue, in tobacco leaves at intervals after the introduction of 5.0×10^1 (A), 5.0×10^2 (B) and 5.0×10^3 (C) cells/cm^2 of leaf of the incompatible pathogen, *Pseudomonas pisi*. Each point represents the mean of eight replicates, and the vertical bars the range.

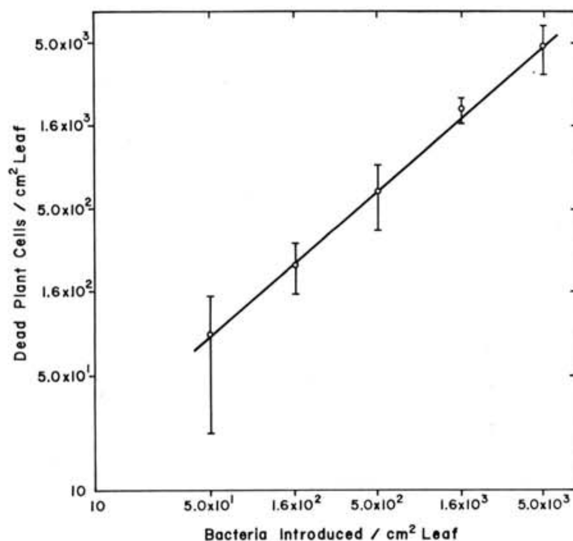

Fig. 4. Relation between the number of cells of the incompatible pathogen *Pseudomonas pisi* introduced into tobacco leaves and the number of dead mesophyll cells detected in those leaves 12 h after inoculation by selective staining with a 1.0% aqueous solution of Evans blue. Each point represents the mean of six replicates, and the vertical bars the range.

Randomly dispersed, collapsed plant cells were seen in sections of tobacco leaves sampled 24 h after the introduction of 5.0×10^3 cells of *P. pisi*/cm^2 leaf (Fig. 5-a, b). No vacuole was detected in these collapsed cells, and an electron microscope examination revealed that they contained only starch grains embedded in a homologous matrix. Since these collapsed cells occurred only very infrequently in the water-infiltrated control leaves, we suggest that they would become selectively stained with Evans blue in intact leaves and be recorded as 'dead'. Plant cell death was not detected until 18 h after inoculation of the compatible pathogen, *P. tabaci*. In this host-pathogen combination, plant cell death increased progressively, and groups of 10 or more dead cells occurred throughout the inoculated region 24 h after inoculation (Fig. 5-c). Typical disease symptoms appeared as these groups of dead cells enlarged and coalesced. The introduction of 5.0×10^3 cells of *P. fluorescens*/cm^2 into tobacco leaves caused no detectable plant cell death (Fig. 5-d).

DISCUSSION.—The results presented here indicate that plant cell death does occur in symptomless tobacco leaves inoculated with low concns of *P. pisi*, and that it has many characteristics of the confluent HR induced by high concns of this incompatible pathogen (Table 1). Further correlations between appearance of cell death and the macroscopic symptoms produced in tobacco leaves inoculated with compatible (*P. tabaci*) and saprophytic (*P. fluorescens*) bacteria (Table 1), demonstrate that we can distinguish between these three types of bacteria on the basis of plant cell death.

We found it difficult to make accurate counts of the very large number of dead mesophyll cells observed in tobacco leaves following the introduction of greater than 5.0×10^3 cells of *P. pisi*/cm^2. From an examination of thin-sections and intact leaves, we have calculated that tobacco leaves used in these experiments contain 2.0×10^5 cells/cm^2. The ability of a dose of 5.0×10^4 cells of *P. pisi*/cm^2 to cause HR suggests that confluent tissue necrosis will develop if the ratio of bacteria to total plant cells is 1:4, when, by extrapolation of Fig. 4, 5.0×10^4 plant cells/cm^2 (25%) are killed. In agreement with this prediction, living mesophyll cells were detected in these tissues 6 h after inoculation (Fig. 2-d). However, the panel becomes completely desiccated after 48 h (Fig. 2, upper left, D). We suggest that tissue collapse in this case occurs because of the death of a greater proportion plant cells than can be tolerated by the tissue. This is supported by our observations that all the plant cells in the inoculated zone appear dead at 6 h after the introduction of more than 5.0×10^4 cells of *P. pisi*/cm^2 leaf. Confluent tissue necrosis, induced by $>5.0 \times 10^4$ cells *P. pisi*/cm^2, may therefore be rationalized as the macroscopic symptom of

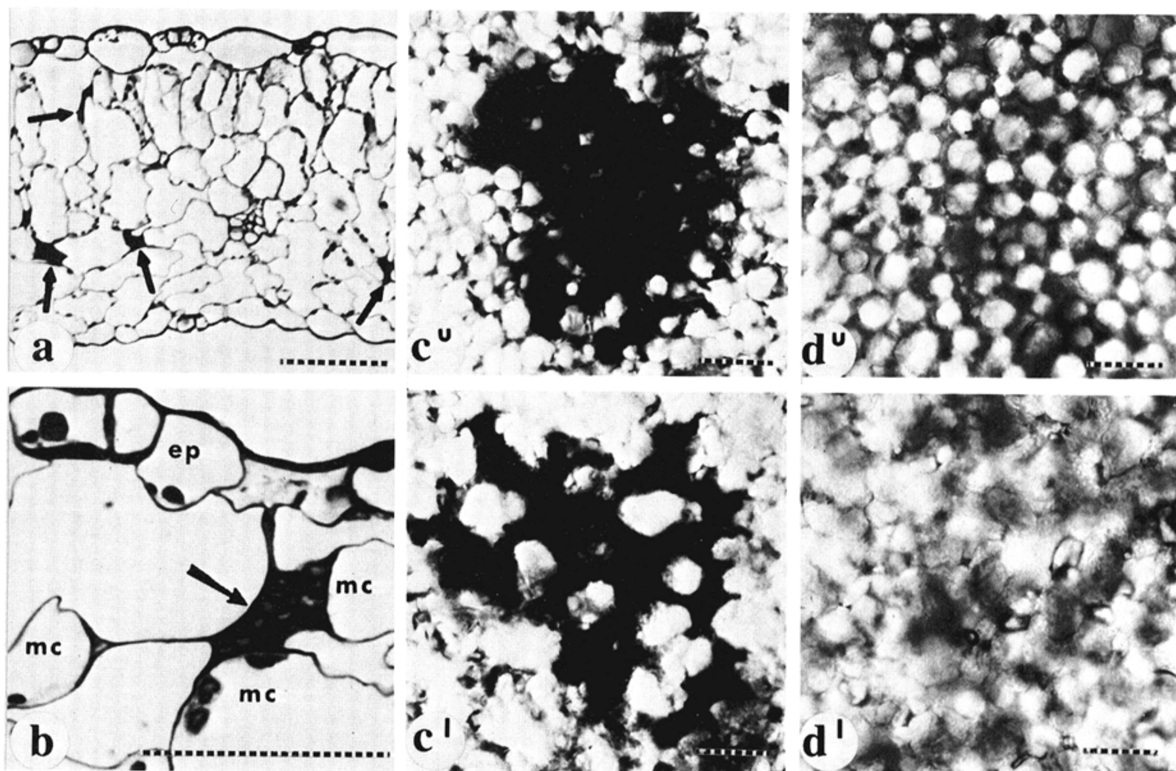

Fig. 5-(a to d). Dead mesophyll cells in tobacco leaves 24 h after the introduction of 5.0×10^3 cells/cm^2 of incompatible (*Pseudomonas pisi*), compatible (*Pseudomonas tabaci*) and saprophytic (*Pseudomonas fluorescens*) bacteria. **a)** Four collapsed, dead, mesophyll cells (arrows), located in leaf tissue inoculated with *P. pisi*. Section, 1-μm thick, stained with methylene blue. **b)** Higher magnification of a) showing a collapsed dead cell (arrow) between the epidermis (ep) and three intact mesophyll cells (mc). **c)** Groups of dead mesophyll cells, detected by their selective staining with a 1.0% aqueous solution of Evans blue, in the upper (u) and lower (l) surfaces of leaves inoculated with *P. tabaci*. **d)** Upper (u) and lower (l) surface of a leaf inoculated with *P. fluorescens* and treated with a 1.0% aqueous solution of Evans blue. No dead cells were detected. Bar represents 0.1 mm.

TABLE 1. Response of tobacco leaf cells to inoculation with incompatible, compatible and saprophytic bacteria

Time after inoculation	*Pseudomonas pisi* and *Erwinia amylovora*		*Pseudomonas tabaci*		*Pseudomonas fluorescens*	
(h)	Low[a]	High	Low	High	Low	High
3	Rapid increase in plant cell death[b] (Fig. 1)	Earliest detected change in plant membrane structural protein (7).	-[c]	-	-	-
6	Maximum plant cell death (Fig. 3)	Wilting, electrolyte leakage and cell disorganization in inoculated region (3, 4)	-	-	-	-
24	Randomly dispersed dead cells appear collapsed (Fig. 5-a,b)	Confluent tissue collapse and desiccation (11).	Groups of dead cells (Fig. 5-c)	Disease symptoms	-	-

[a]Low:
$<10^4$ cells/cm^2;
$<10^6$ cells/ml

High:
$>10^4$ cells/cm^2
$>10^6$ cells/ml

[b]Cell death detected by selective staining with 1.0% aqueous solution of Evans blue.
[c]No detected plant cell death or macroscopic symptoms.

massive plant cell death caused by bacteria acting independently of each other. The observation of small necrotic lesions in tobacco leaves inoculated with concns of *P. syringae* lower than those required to give confluent HR, led Klement et al (10) to similar conclusions on the quantitative aspects of HR. However, necrotic lesions are not produced when low concns of most other species of phytopathogenic bacteria are introduced into nonhost tissues (6, 18). Indeed, the absence of symptoms in such cases has led other workers to suggest that the response of leaves to low concns of bacteria is different from the response of tissues undergoing HR (1). The HR has also been interpreted as an artifact produced by the unnaturally high concns of phytopathogenic bacteria used (16). Our data, however, indicates that HR occurs at the cell level in leaves containing only a few incompatible bacteria. Studies on hypersensitive tissues (3, 4, 7) have, therefore, recorded events which will occur in individual plant cells undergoing hypersensitive death induced by small numbers of bacteria introduced naturally.

The hypersensitive response of plants to fungal and viral infection is generally held to be at least symptomatic of a major disease resistance mechanism whereby the pathogen is localized in a small necrotic lesion in nonhost tissues (8, 13). It remains uncertain whether bacterially-induced HR operates in a similar fashion. When 5.0×10^5 cells *P. pisi*/ml are inoculated into a tobacco leaf, plant cell death reaches a maximum after 6 h (Fig. 1). Cells of *P. pisi* surviving in tobacco leaves at this interval after inoculation then enter a logarithmic growth phase, which continues for a further 18 h, and then enter a stationary phase (14). Leakage from dead cells (3) possibly provides nutrients sufficient for the log growth phase of these bacteria. Collapse and desiccation of the dead cells 18 h later (Fig. 5-a, b) may result in physical localization of the bacteria. That tobacco leaves inoculated with low concns of *P. pisi* show reduced sensitivity to the subsequent introduction of an HR-inducing inoculum, suggests that other factors may operate in the expression of the incompatible interaction (12, 14).

The lowest concn of *P. pisi* which is capable of consistently causing confluent HR in tobacco leaves is 5.0×10^6 cells/ml. Higher concns of *Erwinia amylovora* and a number of other species of phytopathogenic bacteria, are required to induce a similar response (6). Our preliminary investigations with tobacco leaves inoculated with concns of *E. amylovora* lower than those required to give confluent HR indicated that in that incompatible interaction, the ratio of bacteria introduced to plant cells killed is 10:1.

The quantitative responses of phytopathogenic bacteria to host and nonhost tissues are well documented in population studies. However, little data is available on altered plant cell viability in these interactions. Techniques employed here may be applied to other host/pathogen studies to provide precise quantitative information on this important plant cell response.

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