Hypersensitivity of Suspension-Cultured Tobacco Cells to Pathogenic Bacteria

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Portion of a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree, North Carolina State University, Raleigh.

Journal Series Paper 9389 of the North Carolina Agricultural Research Service, Raleigh 27695-7601.

The authors thank G. A. Payne, M. E. Daub, J. S. Kahn, and C. J. Baker for reviewing the manuscript.

The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named, or criticism of similar ones not mentioned.

Accepted for publication 23 July 1985 (submitted for electronic processing).

ABSTRACT

Atkinson, M. M., Huang, J.-S., and Knopp, J. A. 1985. Hypersensitivity of suspension-cultured tobacco cells to pathogenic bacteria. Phytopathology 75:1270-1274.

The hypersensitive reaction of suspension-cultured tobacco cells to pathogenic bacteria was investigated. Net electrolyte efflux, an initial stimulation and subsequent decline in respiration rate, the development of brown pigmentation, and finally cell death were observed in tobacco cells after inoculation with *Pseudomonas syringae* pv. *pisi* (incompatible with tobacco) or avirulent isolate B1 of *P. solanacearum* strain K60. The specificity of the reaction was tested by measuring electrolyte efflux and cell death induced by *P. solanacearum* K60 and *Agrobacterium tumefaciens* (both compatible with tobacco), *P. fluorescens* (a saprophyte), and cells of heat-killed *P. s.* pv. *pisi*. With the exception of *P. solanacearum* K60, these bacteria induced little electrolyte loss and did not kill tobacco cells. These results demonstrate that the hypersensitive reaction of suspension-cultured tobacco cells is similar to that of tobacco leaf tissue with respect to symptom development and specificity of induction. Suspension-cultured tobacco cells thus provide a model system for further studies of hypersensitivity.

Additional key words: *Nicotiana tabacum*.

The hypersensitive reaction (HR) of higher plants is associated with resistance to a wide range of plant pathogens (22). It is characterized by the rapid death of plant cells at the site of pathogen invasion and the localization of the pathogen at that site. Bacterial pathogens induce a rapid HR which has been studied in leaf tissue of various plant species (24,26,34). Symptoms associated with the HR include a net efflux of electrolytes (5,6,15), an increase and subsequent decline in respiration rates (30), decreased RNA synthesis (1), and browning (25). These events, which end in cell death, are generally complete within 12–24 hr after infiltration of leaves with an HR-inducing bacterium. During this time, bacterial numbers are reduced and bacteria are localized within the hypersensitive lesion (25,36). Induction of HR requires live and metabolically active bacteria (27,32) and physical contact between plant and bacterial cell walls (23,38).

A given host plant is generally considered hypersensitive only to bacteria that induce rapid cell death. This includes avirulent and incompatible (nonhost) bacterial pathogens. Although compatible (host) pathogens may induce electrolyte loss (2,5,7) and respiratory stimulation (30), these symptoms are relatively mild and do not lead to rapid cell death. The basis for this specificity of induction is not known.

The biochemical mechanism for the HR is not understood. Investigations into this problem would be facilitated by the availability of suitable plant cell suspension cultures. These cultures provide populations of plant cells that can be easily manipulated as a whole or as individual units. Experimental conditions can be rigorously controlled and the removal or addition of cell or solution samples can be performed in a nondestructive manner. However, since the response of cultured plant cells to pathogenic invasion is not always similar to that of the intact plant (4), it must first be shown that such cells express the HR to a particular pathogen. Numerous studies of callus cultures (9,17–19) have described the expression of resistance to fungal pathogens but most have not dealt specifically with the question of hypersensitivity. An exception is the study by Haberlach and co-workers (17) who concluded that resistance of tobacco callus to *Phytophthora parasitica* was accompanied by a "hypersensitive" reaction. Relatively little work has been done on bacterially induced hypersensitivity of cultured cells. Huang and Van Dyke's report (20) that population trends of compatible, incompatible, and saprophytic pseudomonads on tobacco callus were similar to those observed in leaf tissue is consistent with the expression of hypersensitivity. However, the inoculation of suspension-cultured soybean cells with pathovars of *Pseudomonas syringae* resulted in phytoalexin elicitation but not in an HR since cell death did not occur (12,13). Kennedy and co-workers (21) have also reported the absence of an HR in soybean callus inoculated with pathogenic bacteria. Furthermore, studies with leaf tissue (7,38) suggest that bacterial induction of the HR may not occur efficiently in an aqueous environment. Because valid questions are raised by these reports, it is essential that the use of a suspension-cultured cell system for studying hypersensitivity to bacterial pathogens be supported by strong evidence for expression of the HR.

The purpose of the studies reported here was to test the degree of similarity of the HR to *Pseudomonas syringae* pv. *pisi* of suspension-cultured tobacco cells and tobacco cells in intact leaves. In these systems we wanted to compare the chronologies of symptom developments and their relationship to plant cell death caused by selected bacteria ranging from tobacco-compatible pathogens to saprophytes.

MATERIALS AND METHODS

Tobacco cell cultures. Tobacco callus was derived from *Nicotiana tabacum* 'Hicks' as previously described by Huang and Van Dyke (20). Suspension cultures were initially established by transfer of callus tissue from Schenk and Hildebrandt (SH) agar medium (33) to SH broth. Callus tissue was pressed through 1-mm-

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diameter nylon netting to exclude large cell aggregates. Cells were grown in 125- or 250-ml Erlenmeyer flasks at room temperature (25 ±3 C) on a rotary shaker at 130 rpm. Transfer to an equal volume of fresh medium was performed weekly, and 3- to 4-day-old cultures were used for experimentation. The fresh weight of these logarithmic-phase cultures was 0.2–0.3 g/ml with approximately 5 × 10^6 cells per gram. Most cells were in aggregates which ranged in size from a few cells to as many as 200. Some single cells were also present. All cells were nonchlorophyllous.

Bacterial cultures. The sources of bacterial isolates are given in Table 1. Prior to each experiment, bacteria were streaked onto nutrient agar plates and incubated at 25 C for 16–20 hr. Bacterial cells were suspended in sterile deionized water, washed once by centrifugation, and resuspended to an inoculum density of approximately 2 × 10^8 viable bacteria per milliliter of SH broth. Heat-killed cells of *P. s.* pv. *pisi* were prepared by autoclaving a bacterial suspension at 121 C and 15 psi for 15 min.

Inoculation and preparation of tobacco cells for experimentation. Tobacco suspension cultures were inoculated with bacteria (1 ml of bacterial inoculum in 10 ml of tobacco cell culture) at the beginning of each experiment to give a final concentration of 3–5 × 10^7 bacteria per milliliter of tobacco cell culture. This protocol resulted in an initial ratio of approximately 30 bacteria per tobacco cell and produced a near-maximum hypersensitive reaction with *P. s.* pv. *pisi*. Inoculated tobacco cultures were incubated at 25 ±1 C on a rotary shaker at 160 rpm and tobacco cells were collected by filtration through Miracloth (Calbiochem, La Jolla, CA) at intervals after bacterial inoculation. Bacteria were not retained by Miracloth. Tobacco cells were washed with assay medium [0.175 M mannitol, 1 mM MES (2-*N*-morpholinoethane sulfonic acid) buffer adjusted to pH 6.0 with tris] at a volume of 50 ml/g (fresh weight) of tobacco cells. Unattached bacteria and SH broth were removed from the tobacco cells by washing, and the cells were used immediately for the experiments described below. All experiments were performed at least twice and most were performed three times.

Net electrolyte loss. Inoculated and control (uninoculated) washed tobacco cells (1.0 g) were added to 10 ml of assay medium in 50-ml polystyrene beakers and incubated at 25 ±1 C with shaking for 30 min. The low ionic strength of this medium, as opposed to SH broth, was required for rapid electrolyte loss from hypersensitive cells. The contents of each beaker were filtered and the conductivity of the filtrate was determined with a conductivity bridge and electrode (K = 1.0/cm). These data were used to calculate the rate of electrolyte loss at various times after inoculation (Fig. 1). Total conductivity was determined on 1.0-g samples of cells frozen and thawed in 10 ml of 1% Triton X-100 and then stirred for at least 2 hr. Net electrolyte loss from control tobacco cells was slow and did not vary with time. This background electrolyte loss was subtracted from the loss exhibited by inoculated tobacco cells.

The 12-hr cumulative electrolyte loss (Table 1) was determined by a modified procedure. Tobacco cells (1.0 g, removed from culture medium 3 hr after inoculation) were washed and transferred to 15 ml of assay medium supplemented with 0.5 mM $CaCl_2$. Preliminary experiments showed that this concentration of $CaCl_2$ decreased nonspecific electrolyte loss and cell death in the control but did not prevent hypersensitive loss of electrolytes or cell death. Cell filtrates for conductivity measurements were prepared as described above. Under these conditions, control tobacco cells exhibited a net uptake of electrolytes while hypersensitive cells exhibited a net loss.

Respiration rates. Tobacco cells (0.5 g) were washed to remove bacteria and added to 9.5 ml of air-saturated SH broth in a glass chamber equipped with a magnetic stirring bar. Oxygen consumption was monitored for 3 min with a YSI model 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH). Temperature within the glass chamber was maintained at 25 C.

Browning and death of tobacco cells. Tobacco cell cultures or color photographs of tobacco cultures were visually assessed for the presence of orange-brown pigmentation. Cell death was determined by Evans blue exclusion (14).

Bacterial populations in tobacco cultures. One-milliliter aliquots were removed from tobacco cell cultures and filtered through Miracloth. Serial dilutions of each filtrate were made in sterile PBS (10 mM Na^+, K^+, PO_4^{2-}, and 180 mM NaCl, pH 7.0) and concentrations of viable bacteria were determined by dilution plate count on nutrient agar.

Attachment of bacteria to tobacco cells. Samples of inoculated tobacco cells (0.5 g) were washed with sterile assay medium and then ground in 5 ml of sterile PBS in a mortar and pestle containing 0.2-mm-diameter glass beads. The number of viable bacteria in each homogenate was determined by dilution plate count.

Infiltration of tobacco leaves with bacteria. Tobacco plants (cultivar Hicks) were grown in a greenhouse for 8–12 wk after being transplanted, then they were moved into the laboratory for experimentation. Bacterial suspensions were prepared as described above except that cell density was adjusted to 5 × 10^7 bacteria per milliliter. A 26-1/2 gauge hypodermic needle fitted to a syringe was

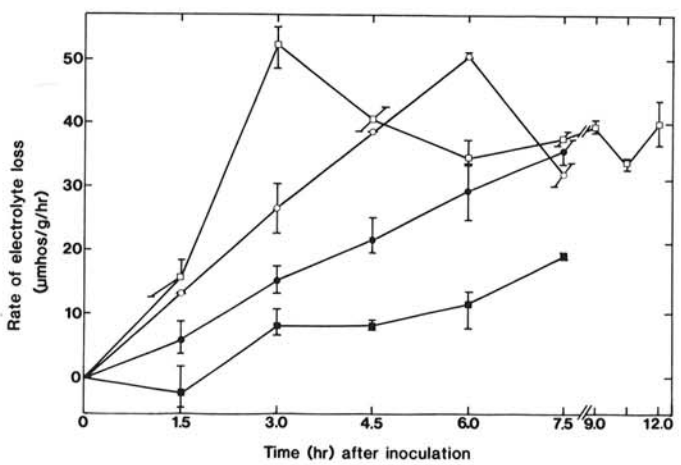

Fig. 1. Rate of net electrolyte loss from suspension-cultured tobacco cells inoculated with bacteria. Tobacco cells (1 g fresh weight) were harvested at 1.5-hr intervals after inoculation and then incubated for 30 min in 10 ml of assay medium. Data represent the difference between rate of electrolyte loss from inoculated and uninoculated (control) tobacco cells during this 30-min period. Lysis of the tobacco cell membranes by detergent treatment gave a total conductivity of approximately 450 μmhos. Data are means and standard deviations based on three replications. *Pseudomonas syringae* pv. *pisi*, —□—; *P. solanacearum* B1, —○—; *P. solanacearum* K60, —●—; and *Agrobacterium tumefaciens*, —■—.

TABLE 1. Electrolyte loss, viability, and brown pigmentation of suspension-cultured tobacco cells 15 hr after inoculation with bacteria

Inoculum[x]	Source	Electrolyte loss[y] (μmhos)	Viability[z] (%)	Browning
Pseudomonas syringae pv. *pisi*	R. N. Goodman	169 ± 9.6 a	27.5 ± 4.4 a	+
P. solanacearum B1	S. F. Jenkins	104 ± 8.7 b	37.3 ± 11.7 a	+
P. solanacearum K60	S. F. Jenkins	76 ± 7.4 c	69.8 ± 8.1 b	±
Agrobacterium tumefaciens	M. Sasser	7 ± 4.2 d	76.2 ± 9.0 bc	+
P. fluorescens	M. Sasser	10 ± 4.3 d	76.0 ± 6.4 bc	−
Heat-killed *P. s.* pv. *pisi*		−1 ± 3.4 e	88.3 ± 5.5 c	−
Control		0 ± 4.2 e	86.9 ± 9.0 c	−

[x] The initial inoculum density was 5 × 10^7 viable bacteria per milliliter.

[y] Represents the loss of electrolytes from tobacco cells to the assay medium. Data are the differences between inoculated and control (uninoculated) cells. Means and standard deviations of four replicates. Means with the same letter are not significantly different (P = 0.05).

[z] Means and standard deviations of four replicates. Means with the same letter are not significantly different (P = 0.05).

used to infiltrate bacterial suspensions into the intercellular spaces of tobacco leaves (25). Fully expanded upper leaves were chosen for infiltration. Symptom development was recorded for up to 4 days after infiltration.

RESULTS

Net electrolyte loss. Relative to the control, *A. tumefaciens, P. fluorescens*, and nonviable *P. s.* pv. *pisi* induced little or no net electrolyte loss in tobacco cells between 3 and 15 hr after inoculation (Table 1). In contrast, *P. solanacearum* K60, *P. solanacearum* B1, and *P. s.* pv. *pisi* induced significant electrolyte losses. Of these bacteria, K60 was the weakest and *P. s.* pv. *pisi* was the strongest inducer. The difference between conductivities of control cell suspensions and those inoculated with *P. s.* pv. *pisi* indicated a net loss of 35–40% of total electrolytes during the 12-hr incubation period. Release of all electrolytes by detergent treatment of cells indicated a total conductivity of approximately 450 μmhos.

Investigation of the rate of electrolyte loss showed that *P. s.* pv. *pisi* induced this symptom within 1.5–2 hr after inoculation (Fig. 1). The rate of loss increased to a maximum at 3 hr after inoculation and then decreased to approximately two-thirds the maximum rate. This lower rate was maintained for up to 12 hr after inoculation. *P. solanacearum* B1 induced similar rates of electrolyte loss except that the maximum was not reached until 4.5–6 hr after inoculation. Electrolyte loss induced by *P. solanacearum* K60 developed more slowly but reached comparable rates within 7.5 hr. *A. tumefaciens* induced very low rates of electrolyte loss from tobacco cells. *P. fluorescens* and heat-killed *P. s.* pv. *pisi* were not investigated.

Respiration rates. The respiration rate of uninoculated tobacco cells was approximately 15 nmoles O_2/g/min. All bacteria tested stimulated respiration in tobacco cells (Fig. 2). This stimulation was observed in cells that had been washed free of unattached bacteria. In each case the stimulation of respiration began at approximately the same time as electrolyte efflux. *P. solanacearum* B1, and *P. s.* pv. *pisi* induced the most rapid stimulation. Cells inoculated with these bacteria, however, exhibited an abrupt decline in respiration which began about 3 hr after inoculation. This decline continued until 6 hr, after which respiration rates leveled off at 50–85% of the control rate. Vital staining of tobacco cells indicated that most remained viable during this time period. *P. solanacearum* K60 and *A. tumefaciens* induced little or no significant decline in respiration during the 7.5-hr experimental period. *P. fluorescens* and heat-killed *P. s.* pv. *pisi* were not investigated.

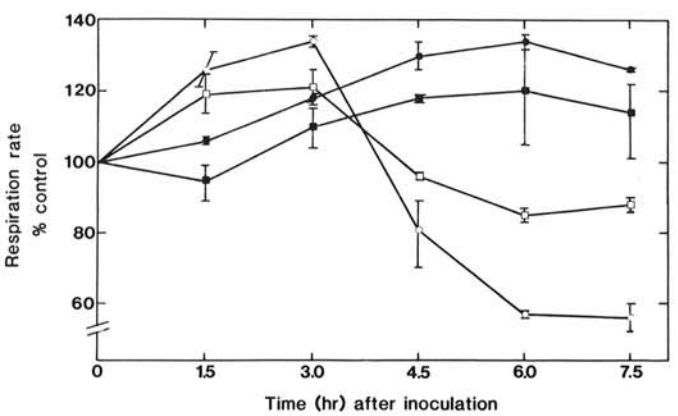

Fig. 2. Respiration rates of suspension-cultured tobacco cells inoculated with bacteria. Measurements were made on tobacco cells washed free of unattached bacteria. The control rate was that of uninoculated tobacco cells. Data are means and standard deviations based on at least two replications. *Pseudomonas syringae* pv. *pisi*, —□—; *P. solanacearum* B1, —o—; *P. solanacearum* K60, —●—; and *Agrobacterium tumefaciens*, —■—.

Browning and tobacco cell death. Uninoculated suspension-cultured cells were off-white in color. All bacteria, including nonviable *P. s.* pv. *pisi* and *P. fluorescens* induced a light brown coloration within 2 hr after inoculation. *P. s.* pv. *pisi, P. solanacearum* B1, and *A. tumefaciens* induced a more intense orange or yellow-brown coloration in tobacco cells (Table 1). *P. solanacearum* K60 induced intense browning in some experiments, but not in most of them.

Tobacco cell death appeared to be correlated with the severity of electrolyte loss induced by bacteria. However, cell death did not occur unless inoculated cells were transferred to the low-ionic-strength assay medium. Tobacco cells lost electrolytes more rapidly in this medium than in the high-ionic-strength culture medium (*unpublished*). Within 15 hr under these conditions, more than 70% of the tobacco cells were killed after inoculation with *P. s.* pv. *pisi*, 60% with *P. solanacearum* B1, 30% with *P. solanacearum* K60, and 20% with *A. tumefaciens* or *P. fluorescens*. Approximately 10–15% of control cells and those inoculated with heat-killed *P. s.* pv. *pisi* were dead.

Bacterial populations in tobacco cultures. Bacterial strains differed in their abilities to multiply and remain viable in tobacco cell cultures (Fig. 3). *P. solanacearum* K60 more than doubled its population density within 9 hr after inoculation. In contrast, population density of the avirulent isolate, B1, dropped rapidly. Population density of *P. s.* pv. *pisi* declined during the first 3 hr after inoculation but then began to increase slowly. The population density of *A. tumefaciens* also declined during the first few hours after inoculation and then remained relatively constant. *P. fluorescens* was not investigated. The data reported in this section represent the density of viable bacteria in the culture medium and do not take into account bacteria which were attached to tobacco cells.

Attachment of bacteria to tobacco cells. Rates of bacterial attachment were constant for 30–60 min after inoculation (*unpublished*). All bacterial strains tested attached to tobacco cells with similar efficiency during a 30-min attachment period (Table 2).

Infiltration of tobacco leaves with bacteria. *P. s.* pv. *pisi* induced a rapid HR in tobacco leaf tissue; brown necrotic lesions developed within 12–15 hr after infiltration (*unpublished*). *P. solanacearum* B1 induced similar lesions within 15–18 hr. Chlorosis developed within 1–2 days after infiltration of leaf tissue with *P. solanacearum* K60 and was followed by a hypersensitive-type brown necrotic lesion within 3–4 days. Nonviable *P. s.* pv. *pisi* and *P. fluorescens* induced mild chlorosis.

DISCUSSION

Our results demonstrate that suspension-cultured tobacco cells express the HR when inoculated with certain phytopathogenic

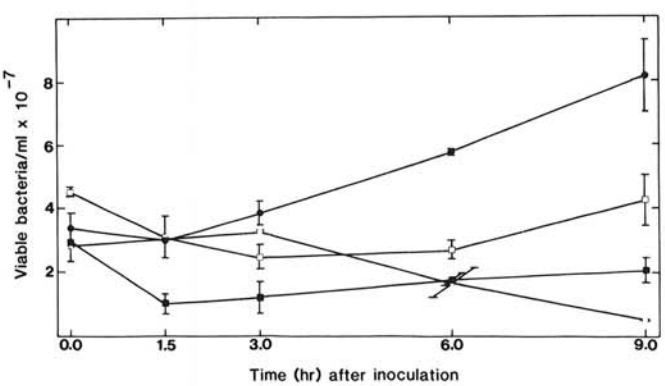

Fig 3. Bacterial populations in tobacco cell suspension cultures. Data represent the density of viable, unattached bacterial cells in tobacco cell culture. Data are means and standard deviations based on at least two replications. *Pseudomonas syringae* pv. *pisi*, —□—; *P. solanacearum* B1, —o—; *P. solanacearum* K60, —●—; and *Agrobacterium tumefaciens*, —■—.

bacteria. This conclusion is based on the appearance of characteristic HR symptoms of electrolyte efflux, respiratory stimulation, subsequent decline, and the development of brown pigments. These symptoms were followed by cell death. It should be noted however, that cultured cells differ from mature leaf cells in some important aspects. Suspension-cultured tobacco cells are rapidly growing and dividing, are nonchlorophyllous, and exist in an aqueous enviornment. The extracellular environment of cultured tobacco cells may provide more readily available water, nutrients, and minerals for bacterial growth than does the interior of a leaf. These and other differences should be kept in mind when interpreting results obtained with suspension-cultured cells. For example, hypersensitive cultured tobacco cells lost viability only when transferred to a low-ionic-strength assay medium. We believe that the high concentrations of K^+ and other electrolytes in the culture medium (33) inhibited electrolyte loss and thereby prevented cell death. This would be expected if the HR involves a passive efflux of electrolytes.

The specificity of HR induction in cultured tobacco cells was similar in most respects to that observed in leaf tissue. Three of four controls (*A. tumefaciens, P. fluorescens*, and heat-killed *P. s.* pv. *pisi*) induced few if any of the physiological changes associated with the HR. These bacteria were chosen because they are not known to induce the HR in any host. However, *P. solanacearum* K60 induced most hypersensitive symptoms and a small, but significant, loss of viability in tobacco cells. In this case, specificity was expressed only as relatively small reduction in the severity or rapidity of symptom expression. A review of the literature shows that this quantitative type of specificity also occurs in leaf tissue. For example, virulent strains of *Xanthomonas vesicatoria* (37) and *Erwinia amylovora* (5) induce significant electrolyte losses from their respective hosts and *P. syringae* pv. *tabaci*, a tobacco pathogen, induces respiratory stimulation in tobacco (30). In some cases, inoculation with virulent bacteria may be followed by hypersensitive-type necrosis. *P. s.* pv. *tabaci* can induce rapid necrosis in tobacco leaves if an inoculum containing more than 2×10^8 bacteria per milliliter is used (28, and *unpublished*). Klement and coworkers (28) concluded that the mechanism of cell death induced by this bacterium is the same as for the HR. Even *P. solanacearum* K60, a highly virulent tobacco pathogen, induces delayed necrosis in leaf tissue; this was reported by Lozano and Sequeira (29) and agrees with our results in the present study. These observations support the conclusion that at least some compatible bacterial pathogens have the capacity to induce symptoms associated with the HR (5,34). Weak expression of these symptoms may actually aid pathogenesis because movement of limited quantities of water, electrolytes, and perhaps nutrients into the intercellular spaces of plant leaves would favor bacterial growth without rapidly killing host cells (5). An incompatible interaction may differ from this only in that symptoms of the HR are severe enough to cause rapid host cell death.

Despite these quantitative aspects of HR specificity, the rapid formation of necrotic lesions in leaf tissue and the death of cultured tobacco cells seem to reflect a qualitative or "all or none" specificity. In most cases, a bacterium-plant interaction can be classified as either HR(+) or HR(−), incompatible or compatible, with little difficulty. This is consistent with a threshold effect (5) for hypersensitive cell death which is supported by our data. We believe that late symptoms of hypersensitivity (respiratory decline, decompartmentalization, and browning) followed by cell death occur only when the severity of electrolyte loss, or some other early symptom, rises above a threshold tolerance level. Necrotic lesion formation, the most widely used criterion for the HR in the past, is therefore simply an indication that this threshold has been reached. For many purposes, this is a valid and useful criterion, but it is not suitable for investigations into the physiology and biochemistry of HR induction. Measurements of electrolyte loss or respiratory stimulation should provide more sensitive assays for this purpose.

An additional question concerning the specificity of HR induction is the attachment of bacteria to plant cells. While the necessity of bacterial attachment to plant cells for HR induction has been demonstrated (23,38), its contribution to the specificity of HR induction is unclear. The efficiency of attachment of bacteria to tobacco cell walls is not always correlated with HR induction (3, and our results). However, others have shown that *P. solanacearum* K60 attaches less efficiently to tobacco cell walls than its avirulent isolate B1 and that factor appears to be important to the specificity of HR induction (10,35). This difference in attachment was not observed when the pH of tobacco cell cultures was below 6. Our tobacco cell cultures maintained an external pH below 5.5, which may explain why we did not observe a difference in attachment or a greater difference in HR induction by these two strains. Several workers have associated the phenomenon of bacterial envelopment by host cell walls with HR induction (8,11,16,35). However, this phenomenon apparently occurs after the initial attachment of bacteria (24) and is not dealt with in our present study.

TABLE 2. Attachment of bacteria to suspension-cultured tobacco cells

Bacteria	Attachment[a,b] (%)
Pseudomonas syringae pv. *pisi*	6.52 ± 0.69
P. solanacearum (B1)	3.73 ± 1.44
P. solanacearum (K60)	4.30 ± 1.50
Agrobacterium tumefaciens	4.79 ± 0.63

[a] Percent of bacterial inoculum attached to tobacco callus cells during a 30-min incubation.
[b] Means and standard errors of three experiments.

Bacterial growth patterns in tobacco cell cultures were not always similar to those observed in tobacco leaf tissue. HR-inducing organisms typically lose viability or enter a static phase during the HR (25,35). The abrupt decline in population density of *P. solanacearum* B1 between 3 and 6 hr after inoculation may be attributable to an HR effect. *P. s.* pv. *pisi*, however, did not exhibit this abrupt population decline. Desiccation of leaf tissue is considered an important factor in the reduction of bacterial populations during the HR (33). Clearly, this factor is not present in our system. Finally, *A. tumefaciens* did not multiply well in tobacco cultures even though an HR was not induced. However, it should be noted that host infection by this organism is not associated with rapid proliferation of bacteria.

The molecular mechanism of HR induction is not known. Electrolyte efflux has been regarded by many either as a primary, or very early, symptom of the HR (5,6,15,31). Indeed, we were able to detect this symptom within 1.5 hr after inoculation. Interestingly, respiratory stimulation began at approximately the same time, indicating that these two symptoms are closely associated. Understanding the mechanism of induction of the HR will, therefore, require a thorough investigation of the molecular basis for these two symptoms. Recent results suggest that hypersensitive electrolyte loss begins as a specific plasmalemma K^+ efflux/H^+ influx exchange (2). These results warrant further investigation.

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