Prevention of the Hypersensitive Reaction in Tobacco Leaves by Heat-Killed Bacterial Cells

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ABSTRACT

The hypersensitive reaction (HR) induced by infiltration of tobacco leaves with cell suspensions (5×10^9 cells/ml) of race 2 isolates of *Pseudomonas solanacearum* was prevented by prior infiltration (18 hr) with a similar suspension of heat-killed cells of either race 2 or race 1. A similar protective effect was induced by heat-killed cells of *P. lachrymans* and *Xanthomonas axonopodis*, but not by those of *Escherichia coli*.

In leaf areas infiltrated with heat-killed cells, protection was first evident when leaves were challenged with live cells 18 hr later; thereafter, the protective effect spread to neighboring intercostal areas not previously infiltrated with heat-killed cells. By 48 hr after infiltration, inhibition of the HR was also evident in leaves immediately above the infiltrated one.

In leaves infiltrated with heat-killed cells and subsequently challenged with live cells of compatible (K60) or incompatible (S210) isolates, the populations of both bacteria decreased. Populations of K60 decreased very gradually (100-fold in 48 hr), but did not induce the typical compatible, necrotic reaction. Populations of S210 decreased sharply (100-fold in 12 hr) in the protected tissues at a rate that was more rapid than during the course of the HR, but no symptoms other than slight yellowing were present.

Prevention of the HR was light-dependent. In leaves infiltrated with heat-killed cells and incubated in the dark for 24 hr before infiltrating with live S210 cells, the HR was not prevented. Increasing the exposure to light resulted in progressively better protection against the HR. In leaves infiltrated with heat-killed cells in alternate intercostal areas, a 24-hr exposure to light before challenging with live cells completely prevented the HR over the entire laminae. Phytopathology 60:875-879.

The hypersensitive reaction (HR) induced by phytopathogenic bacteria in incompatible hosts (5, 6, 7, 12, 13), or by avirulent mutants of these bacteria in compatible hosts (6), is characterized by rapid death of host cells in contact with the pathogen. The pathogen becomes localized and may die. Infiltration of tobacco leaves with incompatible strains of *Pseudomonas solanacearum* E. F. Sm. results in a rapid HR, and the bacterium dies rapidly in infiltrated tissues (12, 13).

Induced resistance or protection against viral or bacterial infections occurs following injection of plants with living or heat-killed bacteria (1, 2, 3, 9, 10, 11). Protection against local lesion development by several viruses may also result from the effect of previous systemic or localized viral infections (8, 14, 15). This protection results in smaller or fewer lesions from challenge inoculations, as compared to symptoms on leaves of plants not previously infected. Viral local lesions can also be reduced in size and number by pretreatment with heat-killed bacterial cells (9). In preliminary studies it was determined that the HR induced by incompatible strains of *P. solanacearum* in tobacco leaves could be prevented by heat-killed preparations of different bacteria. The objectives of this study were to determine (i) the populations of heat-killed bacteria necessary to prevent the HR; (ii) the changes in bacterial populations following challenge inoculations with compatible and incompatible strains of *P. solanacearum* in protected leaves; and (iii) the effect of light on the protective effect of heat-killed bacteria.

Materials and Methods.—*Nicotiana tabacum* L. 'Bottom Special' seeds were sown on vermiculite, and, after 30 days, seedlings were selected for uniform height and planted in coarse silica sand in 6-inch pots. Hoagland's nutrient solution was applied on alternate days. All plants were grown for 30 days in growth chambers at 28 C, 50% relative humidity (RH), and a 12-hr photoperiod (1,800-2,000 ft-c) provided by Sylvania Grolux and General Electric cool-white fluorescent lamps and Mazda incandescent bulbs.

Two isolates of *P. solanacearum*, K60, isolated from tomato (*Lycopersicon esculentum* Mill.), and S210, isolated from plantain (*Musa* group AAB) (12, 13), were used in most experiments. Isolates of *P. lachrymans* (Smith & Bryan) Carsner (Wis. B-73), *Xanthomonas axonopodis* Starr & Garces (ICPB No. XA 127) (17), and *Escherichia coli* (Migula) Castellani & Chalmers (NDA W3110) were used to compare the effects of heat-killed cells of these species with those of *P. solanacearum*. Each isolate was streaked on Kelman's tetrazolium agar medium (TZC) (4) and grown for 48 hr at 30 C. Wild-type colonies of each isolate of *P. solanacearum* were selected on the basis of their fluidity, color, and other morphological features. *P. solanacearum* isolates were stored in sterile distilled water at 20 C to reduce mutant formation, and the other species of bacteria were transferred periodically on nutrient-dextrose agar slants.

For protection experiments, heavy suspensions in distilled water were prepared from the 48-hr growth of each of the above isolates on TZC medium. Suspensions were autoclaved at 121 C for 30 min or heated in a water bath at 90-95 C for 20 min. The cells were washed by centrifuging (13,000 *g*), resuspending the

pellet in distilled water, and centrifuging the cells again. The suspensions were adjusted to an OD (600 mμ) equivalent to 5×10^9 cells/ml, and they were infiltrated on alternate intercostal areas of fully expanded tobacco leaves by means of a hypodermic syringe fitted with a fine needle (5). A small area ($2\ cm^2$) in the center of each panel was challenged 24 hr later by infiltrating a similar suspension of live cells of an isolate of *P. solanacearum* (S210) that normally induces the HR in tobacco leaves (13) at a minimum population of 3.5×10^7 cells/ml.

To determine the period required to induce the protective response, 54 tobacco leaves from 28 plants were infiltrated as above with heat-killed S210 cells and six of these leaves, as well as six infiltrated with distilled water, were challenged at 6-hr intervals with live S210 cells during a 48-hr period.

The numbers of bacteria required to prevent the HR were determined by infiltrating tobacco leaves with heat-killed S210 suspensions at 450, 150, 67, 30, and 9.2×10^7 cells/ml and challenging 24 hr later with similar populations of live S210 cells. Three leaves were infiltrated with each population. The intensity of the HR was recorded on 18-20 challenged areas for each population in either pretreated or control intercostal areas according to the following index: 1, no symptoms; 2, yellowing; 3, small dark spots within the yellow infiltrated area; 4, small, necrotic, dark areas within a light brown infiltrated area; 5, entire infiltrated area light brown and surrounded by a dark halo; and 6, typical hypersensitive reaction.

Bacterial populations in leaf tissues infiltrated as above were determined during a 48-hr incubation period by removing at 6-hr intervals a $1\text{-}cm^2$ disc from the center of each of five infiltrated areas with a corkborer. Each disc was comminuted with a TenBroeck tissue grinder in 1 ml sterile distilled water. The supernatant fluid was diluted with distilled water in a standard logarithmic series. A 0.1-ml sample from each dilution up to 10^{-10} was pipetted onto the surface of TZC medium in a petri dish and spread evenly with an L-shaped glass rod. Individual colonies typical of each isolate were counted after 48-hr incubation at 32 C.

The effect of light on prevention of the HR was determined by infiltrating 20 tobacco leaves with heat-killed S210 bacteria (5×10^9 cells/ml) and immediately covering the laminae with aluminum foil. The plants were then incubated at 28 C, 50% relative humidity (RH), and continuous light (1,800 ft-c). At each 6-hr interval during a 24-hr incubation period, three of the pretreated leaves were uncovered. Twenty-four hr after infiltrating with heat-killed cells, all treated leaves were challenged by infiltrating with live S210 bacteria (5×10^8 cells/ml).

RESULTS.—When leaves infiltrated with heat-killed S210 cells were challenged 24 hr later with live cells of the same isolate, the HR was prevented in the pretreated leaf areas. The live cells caused only a slight chlorotic area in the protected intercostal sections, but a normal HR was obtained in the unprotected ones. Bacterial cells that were killed by treatment at 90-95 C gave more complete protection than those killed by autoclaving.

The protective response was not specific, either in terms of the bacterial cells used to induce protection, or of the organisms used in the challenge inoculation. For instance, heat-killed cells of the following isolates of *P. solanacearum*, B-1 (a nonvirulent mutant of K60), K60 (a compatible strain), and S210 (an incompatible strain), and isolates of *P. lachrymans* and *X. axonopodis*, all induced similar protection against the HR induced by S210 or *P. lachrymans*. The protection by heat-killed S210 cells was more complete than that induced by heat-killed K60 cells; with K60, small necrotic spots were visible within the challenged area. Moreover, heat-killed *E. coli* cells did not prevent the HR induced by S210. Our preparations from *E. coli* evidently did not contain the factor responsible for the protective response.

Leaves challenged immediately after infiltrating with heat-killed S210 cells showed a normal HR, but, as the period allowed for induction of protection increased from 6 to 12 hr, the HR became less marked, with separate necrotic spots rather than total collapse of the cells. Leaves challenged 18 hr after infiltrating with heat-killed S210 cells were fully protected and did not show an HR. After 18 hr, the protective effect spread to neighboring intercostal areas not previously infiltrated with heat-killed cells. By 48 hr, two leaves immediately above the protected leaf also showed incomplete development of the HR, indicating partial protection. Comparable leaves of plants that were not treated with heat-killed cells showed a normal HR when infiltrated with S210. Generally the HR was completely prevented on those intercostal areas where the concen-

TABLE 1. Number of heat-killed *Pseudomonas solanacearum* (S210) cells required to prevent the hypersensitive reaction in tobacco intercostal areas infiltrated with live S210 cells

No. S210 heat-killed cells[a] ($\times 10^{-7}$)/ml	Hypersensitive reaction index[b] 24 hr after challenging with: S210 live cells[c] ($\times 10^{-7}$)/ml				
	450	150	67	30	9
0[d]	4.0	4.0	4.5	2.1	1.7
450	1.5	1.8	1.5	1.5	1.3
0[d]	5.4	5.1	5.0	3.8	2.5
150	4.1	2.5	2.1	2.0	2.0
0[d]	5.7	5.8	5.6	4.1	2.8
67	5.4	4.1	4.0	2.4	2.1
0[d]	5.2	5.1	5.0	4.3	2.0
30	5.7	5.3	5.0	4.1	3.0
0[d]	5.4	5.7	5.2	4.1	4.0
9	5.7	5.2	5.1	5.0	3.0

[a] Infiltrated in alternate intercostal areas only.

[b] HR index: 1 = no symptoms; 2 = yellowing; 3 = small dark spots within the yellow infiltrated area; 4 = small, necrotic, dark areas within a light brown infiltrated area; 5 = entire infiltrated area light brown and surrounded by a dark halo; and 6 = typical hypersensitive reaction.

[c] Infiltrated in all intercostal areas 24 hr after infiltration with heat-killed cells.

[d] Alternate intercostal areas not infiltrated with heat-killed bacteria.

tration of heat-killed cells was equal to or higher than that of the live cells in the challenge inoculum (Table 1). When the population of heat-killed cells was at least twice that of the live cells, the protective effect spread to adjoining untreated intercostal areas as well. At populations of heat-killed cells lower than those of live cells, prevention of the HR was either partial or absent in the treated areas.

Populations of both K60 and S210 bacteria decreased in leaf tissues previously (24 hr) infiltrated with heat-killed cells (Fig. 1). The gradual decrease in the population of K60 (100-fold in 48 hr) in protected leaves was in contrast to the sharp increase (140-fold in 48 hr) detected in the nonprotected inoculated leaves. Moreover, protected tissues did not show the typical dark brown necrotic reaction induced by this isolate (13). In the protected panels there was only a slightly chlorotic discoloration of the challenged area even after 96 hr. The population of S210 decreased rapidly (from 1×10^8 to 1×10^2 cells/leaf disc in 36 hr) in the pretreated leaf tissue. This was a more rapid decrease than that found during the course of the HR in unprotected leaf tissue.

Prevention of the HR was light-dependent. In leaves that were infiltrated with S210 heat-killed cells and covered with aluminum foil for 24 hr, the typical HR was obtained when challenged with S210 live cells. Increasing the length of exposure of leaves to light immediately after infiltration with heat-killed cells, however, reduced the hypersensitive response. A 24-hr exposure to light completely prevented the HR in both treated and untreated leaf panels (Fig. 2, 3).

When the two bottom leaves of each of six 1-month-old tobacco plants were pretreated with heat-killed S210 (5×10^9 cells/ml) and the plants were exposed to light for 24 hr, up to three leaves immediately above the treated ones in each plant also showed partial protection (HR index 4.0) when challenged with live S210 (1×10^8 cells/ml).

Discussion.—The induction of protection against infection by bacterial, fungal, or viral pathogens as a consequence of previous infections has been reported frequently (3, 8, 9, 10, 11, 15). However, prevention of the bacterial HR, which is generally considered a defense mechanism, has not been reported previously. The protection against TMV local lesion formation, a hypersensitive phenomenon, by injecting heat-killed cells of *P. syringae* (9) or by the systemic effect of localized viral infections (14, 15) appears to be similar to the prevention of the HR induced by incompatible isolates of *P. solanacearum* or by *P. lachrymans* in tobacco.

The prevention of the HR in tobacco leaf tissues by prior treatment with heat-killed bacteria, and consideration of the factors affecting this phenomenon, suggest the following conclusions:

1) Since the HR was prevented by heat-killed cells of *P. solanacearum, P. lachrymans,* or *X. axonopodis,* but not of *E. coli,* at populations that were as high or higher than those in the challenge (S210) inoculum, the factor responsible for the protective response is probably nonspecific and common to several, but not all, bacteria. In viruses, according to Ross (15), almost any

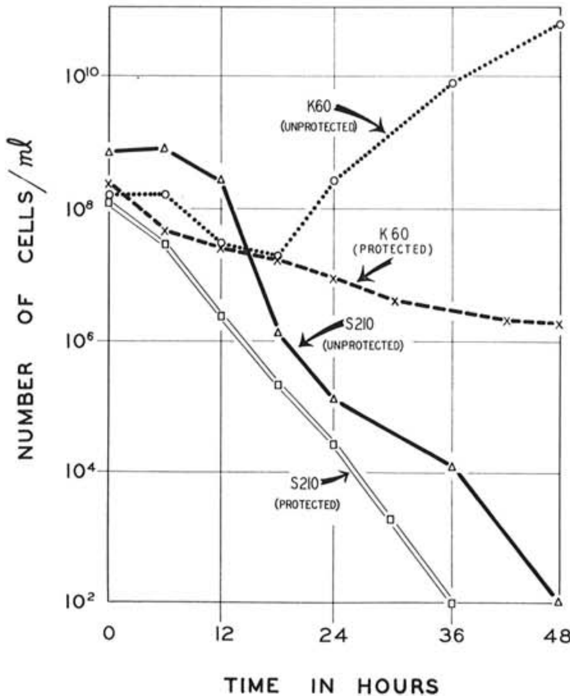

Fig. 1. Effect of infiltration with heat-killed *Pseudomonas solanacearum* S210 (5×10^9 cells/ml) on survival of compatible (K60) and incompatible (S210) isolates of *P. solanacearum* infiltrated 24 hr later in tobacco leaves. Number of cells per ml = number of cells per cm^2 of infiltrated leaf tissue.

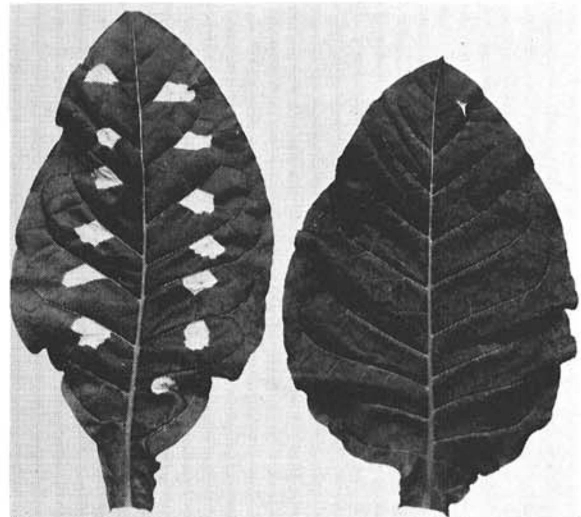

Fig. 2. Effect of continuous light (24 hr) on prevention of the HR with heat-killed bacteria. (Left) Control leaf infiltrated with live *Pseudomonas solanacearum* isolate S210 (1×10^8 cells/ml); (right) leaf infiltrated with heat-killed S210 (5×10^9 cells/ml) and incubated for 24 hr under continuous light (1,800 ft-c) before challenging with live S210 (1×10^8 cells/ml). Photograph taken 60 hr after infiltration with live bacteria.

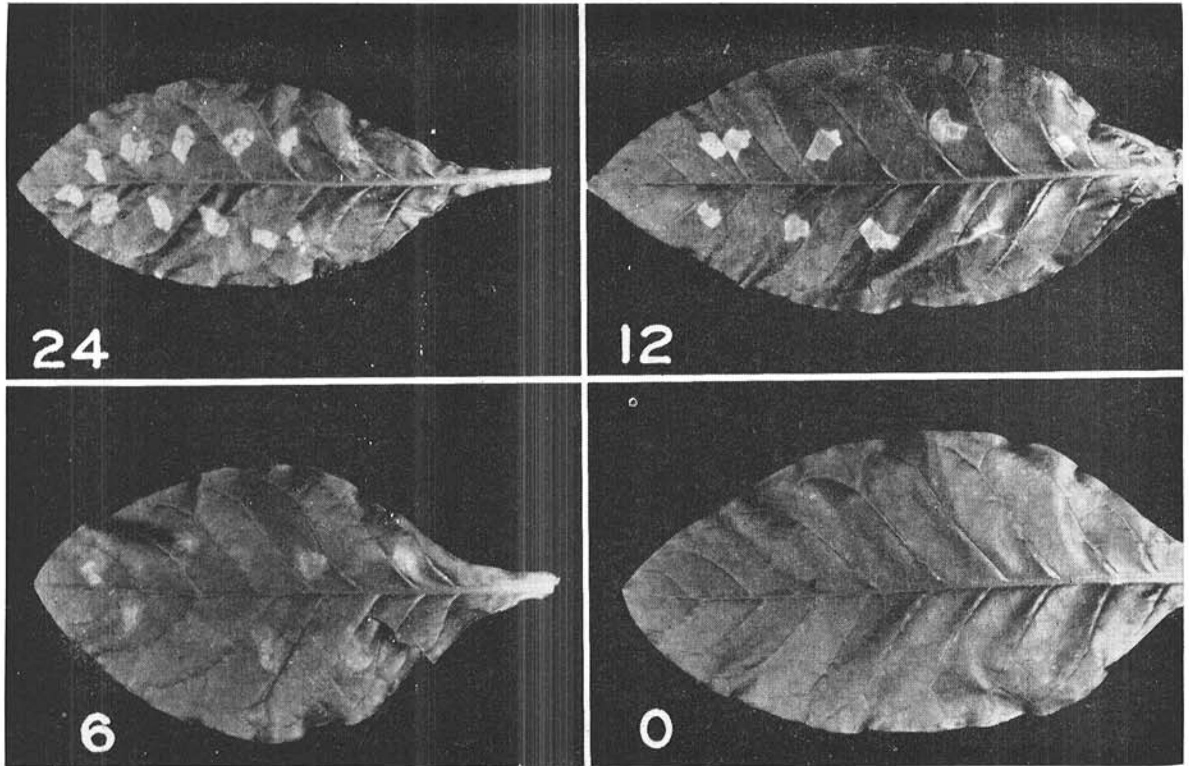

Fig. 3. Effect of light on prevention of the HR. Leaves infiltrated with heat-killed *Pseudomonas solanacearum* isolate S210 (5×10^9 cells/ml) and incubated for 24 hr before challenging with live S210 (5×10^8 cells/ml): **24)** 24 hr of darkness; **12)** 12 hr of darkness and 12 hr of light; **6)** 6 hr of darkness and 18 hr of light; **0)** 24 hr of light. Photograph taken 60 hr after infiltration with live bacteria.

virus that induces necrotic local lesions will induce a systemic protective response, which indicates involvement of general induction mechanisms common to several viruses.

2) Since the HR was not prevented until 18 hr after treatment with heat-killed cells, protection apparently requires a sequence of metabolic changes that occur over a period of several hr. Comparable results were obtained by Lovrekovich & Farkas (10) in preventing growth of *P. tabaci,* a compatible pathogen, in tobacco leaf tissues treated with heat-killed bacterial cells, and by Ross (15) in the prevention of virus local lesions in plants infected with other viruses. Presumably, the immediate release of some preformed antibacterial or antiviral factor is not involved.

3) The mechanism that results in the HR and that which prevents it are both light-dependent (12, 13). A light period of at least 12 hr was necessary for the preventive effect to be manifested in leaves infiltrated with heat-killed bacteria, and further increases in photoperiod also increased the preventive effect.

4) Since infiltration with heat-killed bacteria resulted in a rapid decrease in the populations of both S210 (incompatible race 2) and K60 (compatible race 1), the treatment apparently induces the production of nonspecific inhibitory substances. Such substances prevent the initial establishment of the pathogen and, thus, interfere both with the HR caused by incompatible strains and the dark necrotic response characteristic of compatible ones.

5) The substance(s) that prevents the HR can spread from the treated areas and protect untreated areas as well. Similar evidence for transport of a resistance-inducing substance or substances from one area to another of the same leaf or from one leaf to another has been reported in the prevention of virus local lesions after systemic virus infection (15) and in the prevention of tobacco wildfire by heat-killed bacteria (11). The movement of substances induced by heat-killed bacteria appears to be influenced by (i) the length of the incubation period following initial infiltration; (ii) the length of the photoperiod during incubation; and (iii) the relative concentrations both of the heat-killed cells used to induce protection and of the live cells used in the challenge inoculation.

Since the substances produced during the HR induced by *P. solanacearum* appear to be toxic to the leaf tissues (13), they are probably different from those produced as the result of infiltration with heat-killed cells. In the latter case, only a slight yellow discoloration is produced within 48 hr. The nature of the protective compounds remains unknown. Live S210 cells that normally induce the HR in tobacco leaves (13) must contain both the HR-inducing factor and the substance that can induce the host to prevent the HR. When live cells are used, the first factor apparently predominates. The

protection factor is released or activated only after the cells are killed by heat. Some bacterial species (*X. axonopodis*) only have the protective factor, since live cells are not able to induce the HR (13), whereas others (*E. coli*) have neither factor. Initial studies (16) indicate that both factors can be separated from cell-free preparations by appropriate chromatographic procedures.

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