Different Induction Periods for Hypersensitivity in Pepper to *Xanthomonas vesicatoria* Determined with Antimicrobial Agents

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ABSTRACT

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The minimum inhibitory concentration (MIC) of 12 antimicrobial agents to *Xanthomonas vesicatoria* was determined for growth in nutrient broth and for induction of the hypersensitive reaction (HR) in pepper leaves. The latter was determined by additions of an agent to inoculum or to inoculated leaves. The MICs for growth and induction of HR were similar for chloramphenicol (chl), rifampicin (rif), and tetracycline, but much lower for growth than for induction of HR for the other antimicrobial agents. The longest period of time between inoculation of pepper leaves with an incompatible strain of *X. vesicatoria* and infiltration with an agent that prevented cell collapse (induction period) was variable with the 12 antimicrobial agents. The longest estimate of an induction period, 3 hr when cell collapse began near 5 hr after inoculation, was obtained with chl and rif. Mutants resistant to chl and rif induced HR when mixed with the chemicals to which they were resistant.

Additional key words: bacterial spot of pepper, disease resistance.

Klement and Goodman (7) first proposed an induction period for bacteria to initiate a hypersensitive reaction (HR) in tobacco leaves. The period was determined by injections of streptomycin into inoculated leaves at specific times after inoculation. They concluded that only 15–20 min were needed for cells of *Erwinia amylovora* or *Pseudomonas syringae* to initiate HR in tobacco leaves. Collapse of inoculated cells occurred 8–9 hr after inoculation of control leaves in their experiments.

Klement (5) proposed that three distinct phases occurred during development of HR. These were induction time, latent period, and cell collapse. The induction time was the period of time needed for bacteria to initiate HR, after which living bacteria were thought not to be necessary. The latent period was the time between the end of induction and the beginning of cell collapse, and presumably was the time necessary for completion of processes in the plant leading to cell collapse. Cell collapse was characterized by increases of electrolyte leakage from plant cells.

Klement (5) obtained an induction time of 3–4 hr and a latent period of 5–6 hr with cells of *P. phaseolicola* in tobacco. The times were determined by injections of streptomycin at intervals of time after inoculation to "kill" the bacterium. The length of the induction times was influenced by the age of cultures used as inoculum. Sequeira (10) reported an induction period of 3 hr for avirulent cells of *P. solanacearum* in tobacco. Durbin and Klement (3) obtained an induction period of 90–105 min with *P. syringae* in tobacco with injections of streptomycin into inoculated leaves or by transferring inoculated plants to 37 C.

The concept of an induction period for HR has received relatively little attention since its proposal, but it may be important to the eventual understanding of HR. Any proposed mechanism of induction of HR must be consistent with the time needed for induction. The purpose of this research was to compare induction periods as determined with antimicrobial agents that have different mechanisms of bacterial inhibition.

MATERIALS AND METHODS

Leaves of pepper plants (*Capsicum annuum* L.) of the experimental line 10R were inoculated in these experiments. The pepper line is nearly isogenic to the cultivar Early Calwonder and is homozygous for a gene for hypersensitivity to certain strains of *Xanthomonas vesicatoria* (Doidge) Dows. Plants were cultivated

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in 10-cm-diameter pots in a greenhouse and then transferred to a growth chamber after leaves were inoculated. The chamber was kept at 30 C with a light intensity of 6,500 lux 30 cm from the source, which was the distance from the light to the inoculated leaves.

Cells of a strain of *X. vesicatoria* obtained from pepper plants in Florida and which caused HR in 10R were used. Inocula were prepared from nutrient broth cultures that were in the log phase of growth. Cultures were centrifuged at 2,500 *g* for 10 min and resuspended in either sterile, double-distilled water or sterile tap water. Suspensions were adjusted to an OD_{600nm} of 0.3 or 0.6A and assumed to contain 10^8 or 2×10^8 cells/ml, respectively (12). Inocula of lower concentrations were obtained by dilution. Inoculations were always accomplished by the injection-infiltration technique (6).

Electrical conductivity measurements were made with a Model 31 Conductivity Bridge (Yellow Springs Instrument Co., Yellow Springs, OH 45387). Fifteen leaf disks, 15 mm in diameter, were placed in 20 ml of double-distilled water, infiltrated under vacuum, and shaken for 1 hr. The procedure for determination was as previously described (11).

The sources of the 12 antimicrobial agents were: colistin methane sulfonate, sodium salt (col), ethidium bromide (eb), erythromycin (ery), mitomycin C (mit), novobiocin, sodium salt (nov), puromycin dihydrochloride (pur), rifampicin (rif), (Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178); chloramphenicol (chl) and tetracycline hydrochloride (tet) (U.S. Biochemical Corp., P.O. Box 22400, Cleveland, OH 44128); streptomycin sulfate (str) (ICN Nutritional Biochemicals, 26201 Miles Rd., Cleveland, OH 44128); nalidixic acid (nal) (Swartz-Mann, Mountain View Ave., Orangeburg, NY 10962); and hexadecyltrimethyl-ammonium bromide (htab) (Matheson, Coleman and Bell, Norwood, OH 45212). Solutions of the materials were made in sterile double-distilled water. Sterile solutions were obtained by passage through a 0.45 μm membrane filter.

The minimum inhibitory concentration (MIC) of each antimicrobial agent to *X. vesicatoria* was determined in three ways. The MIC for growth in nutrient broth was determined in tube cultures containing twofold serial dilutions of the agents from a maximum amount of 10 μg/ml. The cultures were started with 10^3 cells per milliliter and growth was rated positive if turbidity occurred within 7 days. The MIC for development of HR was determined in inoculum and in inoculated leaves. Equal volumes of twofold serial dilutions of the chemicals and inoculum containing 2×10^8 cells per milliliter were mixed. Intercostal areas of pepper leaves were infiltrated 30 min after the mixing. Ten concentrations of a chemical, beginning at 500 μg/ml, were tested. Confluent collapse within 9 hr after inoculation was indicative of HR development. The MIC for HR in inoculated leaves was determined with twofold serial dilutions of the chemicals in water and infiltrated into pepper leaves 1 hr after inoculation with *X. vesicatoria* at 10^8 cells per milliliter. Ten concentrations, beginning at 500 μg/ml, were infiltrated into intercostal areas. Cell collapse at 9 hr after inoculation was indicative of HR. Three replicates of each concentration of antimicrobial agent were included in each experiment and three experiments were completed.

Induction periods for HR in pepper leaves infiltrated with *X. vesicatoria* at 10^8 cells per milliliter were determined with chl and rif. Eb, nal and str were included as references. The concentrations of the antimicrobial agents were not constant, but each was above the MIC for inhibition of HR in inoculated leaves. Three leaves on each of 12 plants were completely infiltrated with inoculum and the time of inoculation was recorded for each plant. Hourly, thereafter, one leaf from a pair of plants was harvested for determination of electrolyte leakage and the other five leaves on the pair of plants were totally infiltrated with the antimicrobial agents, one per leaf. This procedure was continued until infiltration was impossible due to collapse of cells. Bacteria only and bacteria suspended in the agents were also done at time zero. Electrolyte leakage from each leaf that had been inoculated and infiltrated with an antimicrobial agent was determined 9 hr after inoculation. Four replications were used and the experiment was repeated three times.

Colonies of *X. vesicatoria* that were resistant to chl and rif were selected from plates of nutrient agar containing 100 μg/ml of an antibiotic. One-half milliliter of a nutrient broth culture of *X. vesicatoria* in a stationary phase of growth was placed in each of 10 plates. Colonies of resistant bacteria appeared within 7 days. Only pathogenic mutants were used in experiments.

RESULTS

The MICs of the 12 antimicrobial agents for growth of *X. vesicatoria* in nutrient broth and for HR by additions to inocula or to inoculated leaves are in Table 1. All chemicals prevented growth in nutrient broth at 10 μg/ml or less, but wide variation of MICs occurred with HR. With most materials, a higher concentration of a chemical was needed to inhibit HR in plants than to prevent growth in culture. Also, a higher amount of chemical was usually needed to inhibit HR in inoculated leaves than with additions to inoculum.

All materials except ery, nal, and pur inhibited HR at 500 μg/ml in the inoculum. Col, htab, nov, and str in inoculum inhibited HR, but did not in inoculated leaves. Five other chemicals, chl, tet, rif, eb, and mit inhibited HR by addition to inoculum and to inoculated leaves, but chl, rif, and tet seemed to be unusual in that a low MIC occurred with tests of growth in nutrient broth and tests with HR. Of the three materials rif had the lowest MIC in HR tests. In subsequent tests, only chl and rif inhibited HR in leaves inoculated 2 hr previously. No chemical inhibited HR in leaves inoculated 3 hr previously. Electrolyte leakage from control leaves (the beginning of cell collapse) began 3 and 4 hr after inoculation in these tests.

A more critical comparison of the effects of chl and rif on inhibition of HR in inoculated leaves was made in experiments in which electrolyte leakage was used to determine cell collapse. An increase in electrolyte leakage was presumed to be associated with HR development (2). Electrolyte leakage was minimal in inoculated-nontreated leaves for 0–5 hr after inoculation but increased significantly at determinations made at 6 hr (Table 2). Both chl and rif inhibited HR when placed in leaves 0–3 hr after inoculation and partially inhibited HR at 4 hr. The two chemicals provided the same measure of the induction period.

Ethidium bromide and str inhibited HR when added to inocula, but did not totally inhibit cell collapse in inoculated leaves. Reduced electrolyte leakage occurred after these materials were placed in leaves at 1–4 hr after inoculation. With both materials, cell collapse appeared to be less strong around infiltration points. Possibly, these materials were rapidly absorbed to mesophyll cells

TABLE 1. Minimum inhibitory concentrations of 12 antimicrobial chemicals for growth of *Xanthomonas vesicatoria* in nutrient broth and for inhibition of hypersensitivity in pepper when mixed with inoculum or infiltrated into inoculated leaves

Antimicrobial chemical	Minimum inhibitory concentration (μg/ml)[a]		
	Growth in culture	Hypersensitive reaction	
		Inoculum	Leaf
Colistin	1	125	(500)[b]
Hexadecyl trimethyl ammonium bromide	1	31	(500)
Chloramphenicol	3	8	8
Erythromycin	5	(500)	(500)
Novobiocin	1	125	(500)
Puromycin	10	(500)	(500)
Streptomycin	1	125	(500)
Tetracycline	1	4	8
Rifampicin	1	1	1
Ethidium bromide	1	8	125
Mitomycin C	1	4	62
Nalidixic acid	5	(500)	(500)

[a] Figures were rounded to nearest whole number. Mean of three replicates.
[b] Parentheses means no inhibition, and the number is highest concentration tested.

near infiltration points in pepper leaves and were at higher concentrations in those areas of the leaves.

Nalidixic acid did not inhibit HR, even when added to inoculum. Partial inhibition of HR seemed to occur with treatments at 2–4 hr after inoculation, but this was probably an artifact. Leaves normally remained water-soaked for about 30 min after infiltration, but with nal at 2–4 hr after inoculation leaves remained water-soaked for unusually long periods. HR development stops during water-soaking (12), but continues after leaves become dry. The delay in drying of leaves could account for the partial inhibition of HR.

Mutants that were resistant to chl and rif caused HR when suspended in solutions of 100 μg/ml of the materials to which they were resistant. The parent cultures suspended in the solutions did not cause HR. The effects of chl and rif apparently were on the bacterium rather than the host.

DISCUSSION

The induction period for development of HR most often has been estimated after infiltration of inoculated leaves with str (3,5,7). With str and the pepper-*X. vesicatoria* system the induction period was determined to be less than 1 hr, which was in agreement with Klement and Goodman (7) for the induction period for HR in tobacco inoculated with *E. amylovora* and *P. syringae*. However, str did not provide the longest induction period in the pepper-*X. vesicatoria* system. Apparently str does not affect *X. vesicatoria* in pepper leaves as rapidly as in cultures of the bacterium. Colony-forming units of *X. vesicatoria* were reduced in culture from 10^8 cells per milliliter to zero within 0.5 hr after addition of 500 μg/ml of str, but when the same concentration of str was infiltrated into inoculated pepper leaves 8 hr were required to reduce the population 100-fold (8). Pepper leaves did not inactivate str, because equal zones of inhibition of *X. vesicatoria* in agar overlays of the bacterium occurred over an 8-hr period with leaf disks taken from leaves infiltrated with str (8). Some mechanism other than inactivation must account for the reduced effect of str on the bacterium in the pepper leaves.

Chloramphenicol and rif provided the longest induction period in the pepper-*X. vesicatoria* system. Inhibition of the causal bacterium in culture and in the leaf also occurred at relatively low concentrations of the chemicals. The latter is important because the chemicals could interfere with host processes involved in HR development.

The time of induction of HR by *X. vesicatoria* in pepper as determined with chl and rif was between 3 and 4 hr. That time may not be constant because the time between inoculation and cell collapse varies with conditions (1,5) and the induction time would vary accordingly. Under conditions of our experiments at least three-fifths of the time from inoculation to cell collapse must be attributed to induction period. That time probably underestimates the time for the true induction period with the pepper-*X. vesicatoria* system. Some time is needed after treatment for uptake and the inhibition of processes in the bacterium that lead to HR induction. That time may be relatively long in leaf tissue, but cannot readily be determined because of the lack of knowledge of HR induction. The time must be added to the determined induction period and concomitantly substracted from the latent period. In fact, it may be that the latent period is really the time needed for the "shutting down" of processes in the bacterium that are responsible for induction of HR rather than for completion of processes in the plant leading to cell collapse. If the latter is true, then the actual induction period is the time from inoculation until the beginning of cell collapse.

Antimicrobial agents of diverse mechanisms of action inhibited HR when placed in inoculum. Materials were used that are known (4) to inhibit membrane function (col and htab), protein synthesis (chl, nov, str, and tet), RNA replicase (rif), and DNA function (mit and eb). Many of these did not inhibit HR in inoculated leaves, probably because of interaction with host tissues.

No relationship existed between reversibility of inhibitory action on the bacterium and inhibition of HR in inoculated leaves. For example, chl inhibition could be reversed by transferring the treated bacteria to distilled water, but str inhibition could not be reversed by this procedure. Rif inhibition could not be reversed (8).

Three materials did not inhibit HR even though they inhibited growth of *X. vesicatoria*. Two of these materials are reported to inhibit protein synthesis (ery and pur) and the other to inhibit DNA replication (nal) (4). Possibly, ery and pur did not inhibit HR when placed in inocula because 30 min was not sufficient for action of these materials with *X. vesicatoria* before inoculation. On the other hand, nal did not inhibit HR even when mixed with inoculum for 24 hr (R. E. Stall, *unpublished*). Treatment of *X. vesicatoria* with nal stopped cell division (8). Therefore, cell division is apparently not essential for HR development. The latter point had not been clarified in HR research (3).

Other authors have concluded (7,9) that protein synthesis is essential during induction of HR by bacterial plant pathogens. Functional DNA also appears to be essential which is implied by inhibition of HR with rif, mit, and eb (10). Thus, gene(s) for HR induction seem to be transcribed after inoculation. It would be interesting to learn if such genes are derepressed after contact with pepper cells.

TABLE 2. Electrical conductance of water containing pepper leaf tissue inoculated with *Xanthomonas vesicatoria*

Hours after inoculation	Conductance (μS)[a]					
	Control	Chl[b]	Rif	Str	Eb	Nal
0	44 a	33 a	33 a	28 a	34 a	456 bc
1	43 a	47 a	40 a	398 b	192 b	475 bc
2	35 a	44 a	37 a	343 b	172 b	338 ab
3	41 a	49 a	44 a	337 b	141 b	363 ab
4	38 a	294 b	353 b	313 b	299 c	335 ab
5	82 a	430 c	417 c	440 bc	420 d	479 c
6	318 b					
9	437 c					

[a] At hourly intervals conductance from control leaves was measured and antimicrobial agents were infiltrated. Values under antimicrobial agents were obtained 9 hr after inoculation of leaves with *X. vesicatoria* (10^8 cells per milliliter).

[b] Abbreviations are: chl (chloramphenicol); rif (rifampicin); str (streptomycin); eb (ethidium bromide), and nal (nalidixic acid). Concentrations used were 25, 25, 250, 125, and 500 μg/ml, respectively. Values associated with same letter are not significantly different, $P = 0.05$. Figures for same agent can be compared, but not for different agents.

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