Effect of Low Concentrations of Xanthomonas vesicatoria Infiltrated into Pepper Leaves

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ABSTRACT

Infiltration of *Capsicum annuum* leaves with pathotypes of *Xanthomonas vesicatoria* below the concentration required to induce visible hypersensitive response predisposed such leaves to increased electrolyte loss following reinfiltration with the same or greater bacterial concentration. The increase in electrolyte loss, over the control, was inversely related to concentration of bacteria in the second inoculation. Enhanced electrolyte loss was neither dependent on reinfiltration of inoculated leaves with the same bacterial pathotype nor consistently affected by temperature or light during incubation. The influence of reinoculation was effective between 6 and 42 hours after the first inoculation. Reinfiltration with water alone was nearly as effective in accentuating electrolyte loss as inocula containing live bacteria, but the effect of reinfiltration was reduced by calcium nitrate.

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The biological phenomenon referred to as "hypersensitive response" (11) has been demonstrated repeatedly with phytopathogenic bacteria (6, 7). A number of factors have been found to influence development of this basic response (3, 4, 5), but complete understanding of the cause remains to be established. Considerable attention has been given to preventing full development of the hypersensitive response by pre-inoculation treatment with bacteria of different types subjected to various treatments (8, 9, 12), including disintegration by sonication (10, 13, 14). A consistent conclusion from these studies has been that inocula must contain bacterial cells equal to, or in excess of, the minimal concentration required to induce a demonstrable plant response. Death of isolated host cells following infiltration with low inoculum concentrations of bacteria has been reported (16). The studies reported herein are offered as evidence of pathologic effects not previously noted from inoculation with bacterial concentrations below that required to cause confluent hypersensitivity.

MATERIALS AND METHODS.—Isolates 71-14 and E-3 of *Xanthomonas vesicatoria* (Doidge) Dows. (tomato strain and pepper strain, race 2, respectively) (1, 3) were used exclusively and inocula prepared with cells from 24-hour nutrient broth shake cultures. Pelleted cells obtained from cultures centrifuged 10 minutes at 1,500 *g* were resuspended in sterile distilled water to give 50% photometric transmittance (625 nm), which was equivalent to 10^8 cells/ml. Appropriate dilutions were made to obtain inoculum concentrations indicated.

Plants of the pepper (*Capsicum annuum* L.) cultivars 23-1-7 (23-1) [a breeding line homozygous for hypersensitive response to the pepper strain, race 2, of the bacterium] and Yolo Y (YY) were used throughout these studies. All inoculations were accomplished by hypodermic infiltration of leaves, and test plants were maintained in temperature-controlled (± 2 C) growth rooms. Light (approximately 6,460 lux) was supplied from "Cool-white" and "Gro-lux" fluorescent tubes approximately 45 cm above the plants.

TABLE 1. In vivo concentrations of *Xanthomonas vesicatoria* (tomato strain) in Yolo Y pepper leaves after indicated incubation intervals at 30 C

Inoculum concns (cells/ml)	Bacterial cells/mm² leaf tissue × 10³				
	Hours of incubation				
	0	8	16	24	48
10^5	...	...	...	0.5[a]	1.6
10^7	1.3	1.4	6.7	10.7	...
(10^5 and 10^7)[b]	1.9	0.9	2.7	1.4	...

[a]Average of six replicates; all other figures represent nine replications.
[b]Second inoculation (10^7) administered 24 hours after first inoculation.

TABLE 2. Effects of temperature and light on electrolyte loss elicited by double inoculation of Yolo Y pepper leaves with *Xanthomonas vesicatoria* (tomato strain)

Temperature/light	Electrolyte loss (ratio test × control)		
	Ratios of bacterial concns of first and second inocula		
	1:1[a]	1:10	1:100
30 C—Intermittent light	9.5(17)[b]	4.4(15)	2.4(90)
30 C—Complete darkness	13.8(7)	2.4(20)	1.2(12)
25 C—Complete darkness	12.5(5)	2.4(6)	...

[a]First inoculation always accomplished with 10^5 cells per ml.
[b]Figures in parentheses indicate total number of replicates.

Bacterial multiplication in vivo and electrolyte loss were assessed as described previously (1, 2, 4, 15), except that eight disks 16 mm in diameter were suspended in 10 ml of sterile distilled water. Individual experiments consisted of three replications. The effect of reinfiltration of leaves was assessed as μmhos conductivity of leachates from test leaves compared to μmhos conductivity of leachates from control leaves always inoculated with the same inoculum and incubated 24 hours under the same environmental conditions as test leaves. Variability inherent in numerical measure of conductivity of leachates in successive experiments prompted presentation of results as the ratio of the numerical conductivities of leachates from test leaves and companion control leaves.

RESULTS.—Infiltration of YY pepper leaves with 10^5 cells/ml of isolate 71-14 (tomato strain) did not induce a detectable increase in electrolyte loss after 24 hours of incubation at 30 C. Reinoculation of the leaves, again with 10^5 cells per ml of the same bacterial isolate (1:1 ratio of inocula concentrations) caused a decided increase in loss of electrolytes (average of 9.7 × control for 14 replications) after incubation for an additional 24 hours at the same temperature. A ten-fold increase in concentration of the inoculum used for reinoculation (1:10 ratio of bacteria in the two inocula) produced greater total electrolyte loss, but a lower relative effect compared to control inoculations (average of 6.1 × control for nine replications). This trend continued, and was more exaggerated (average of 2.8 × control for 66 replications) when inoculum for reinoculation contained 100 × more bacterial cells than the original inoculum (1:100 inoculum concentration ratio). Similar results were obtained with 23-1 pepper inoculated with bacterial isolates 71-14 and E-3 (pepper strain, race 2).

The influence of the second inoculation on electrolyte loss was not related to in vivo multiplication of bacteria as determined by recovery isolations (Table 1). The effect of reinoculation was not dependent on infiltration with the same bacterial pathotype used in the original inoculation. Reinfiltration with bacteria of another pathotype (100× original inoculum) caused an average increase of 2.3 × control (15 replications) electrolyte loss. The discovery that reinoculation with heat-killed bacteria caused an increase (average of 9.0× control for three replications) in electrolyte loss prompted reinfiltration of inoculated leaves with sterile, distilled water that also caused increase in electrolyte loss (average of 8.2 × control for 11 replications) comparable to the 1:1 inoculum ratio. Further, the enhancement of electrolyte loss from inoculated leaves demonstrated by reinfiltrating with water only was reduced by addition of calcium nitrate to the reinfiltration solution [average of 2.9× control for six replications with 0.15 N $Ca(NO_3)_2$], and the degree of reduction was correlated with the concentration of calcium nitrate infiltrated [average of 7.2 × control for three replications with 0.07 N $Ca(NO_3)_2$]. No consistent effect of light or continual darkness could be established, and temperature (25 vs. 30 C) did not noticeably affect the response (Table 2). Increase in electrolyte loss induced by reinoculation was not demonstrable until more than 6 hours after the original inoculation, reached a maximum effect at 24 hours after original inoculation (2.5× control) and was only slight 42 hours after original inoculation.

Infiltration with soluble materials from sonicated bacteria was ineffective in altering electrolyte loss from leaves subsequently inoculated with intact bacterial cells and induced no effects on inoculated leaves distinguishable from reinfiltration with water only. Neither inoculation with *Pseudomonas fluorescens* or *Erwinia herbicola* nor reinfiltration with either of these saprophytic bacterial species following inoculation with pathogenic *X. vesicatoria* elicited enhanced electrolyte loss distinguishable from reinfiltration with water only.

DISCUSSION.—Leaching of electrolytes from inoculated leaves has been employed as a means of quantitatively assessing severity of some disease manifestations (17). The technique used in these experiments was simple and apparently effective, but the results permit only speculation concerning pathological processes involved. Variability was a constant consideration and prompted evaluation of individual experiments on the basis of simultaneous control treatments.

The studies that involved reinoculation with phytopathogenic bacteria of the same pathotype could be considered evidence of sensitization of plant leaf tissue. This supposition is strengthened by the absence of significant bacterial multiplication in vivo when electrolyte loss was noticeably affected. The observation that calcium influenced electrolyte loss in direct proportion to concentration in the second infiltration was consistent with results obtained in earlier studies, but does not contribute significantly to elucidation of the biological processes involved.

In previous experiments with isolate 71-14 and YY pepper plants, both light and temperature were found to consistently influence development of the hypersensitive response characteristic of this host-pathogen combination. Failure to demonstrate any consistent effect of either environmental condition in these studies does not detract greatly from the possible concept of sensitization, but neither does it contribute measurably to an understanding of the biological nature of hypersensitivity to pathogenic bacteria. The fact that these results are in direct opposition to those published by other investigators (12) supports the suggestion that different pathogens may induce hypersensitive responses by dissimilar biological processes (5).

Probably the most intriguing aspect of these studies is the effect on electrolyte loss of infiltrating water into previously inoculated leaves. The possibility of physical damage to the plant by infiltration of water alone could not be demonstrated in control inoculations. Furthermore, no influence from previous infiltration with water or the contents of sonicated bacteria was noted following reinfiltration with bacterial inoculum. Heat-killed bacteria in the first inoculation did not elicit demonstrable response after reinfiltration with either water alone or bacterial inoculum. Thus, for reinfiltration to cause notable effect, it was essential that the first inoculation be accomplished with live, whole pathogenic bacteria. Preliminary attempts to extract from inoculated leaves material(s) capable of inducing electrolyte loss from infiltrated leaves were unsuccessful. These results do demonstrate, however, that in vivo concentration of pathogenic bacteria insufficient to induce macroscopic symptoms can cause significant effects on host tissue in addition to killing isolated cells.

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