Evidence Against the Involvement of Hydrogen Peroxide in Bacterial Leaf Spot of Pepper

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ABSTRACT

Hydrogen peroxide (H_2O_2) was found to be highly toxic to *Xanthomonas vesicatoria* and to *Xanthomonas phaseoli* in vitro, but did not cause visible damage when injected into pepper leaves. Isolation of bacteria from inoculated pepper leaves was not affected by subsequent injections of H_2O_2, an enzymatic generator of H_2O_2, catalase, manganese chloride, or malate. Extracts of intercellular space fluid from healthy pepper leaves had H_2O_2-decomposing ability which was lost within 48 h following inoculation with bacteria. The lack of sensitivity in vivo by the plant or the bacteria, suggest that H_2O_2 is not a major causal factor in either pathogenesis or disease resistance in the system studied.

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Hydrogen peroxide is a strong oxidant which may be involved in many reactions in bacterial pathogenesis of plants or in resistance to bacterial pathogens. Hydrogen peroxide oxidizes sugars and fatty acids (10), solubilizes pectins (5), inactivates dehydrogenases (6) and degrades DNA (18). *Mycoplasma pneumoniae* produces H_2O_2 extracellularly and this is considered to be a factor in virulence (2). Production of H_2O_2 has been related to

wood decay by fungi (9) and to ineffectiveness of some *Rhizobium* spp. in legume root nodules (3). Mussell (15) reported that treatment of cotton leaf tissue with H_2O_2 or with enzymatic systems capable of generating H_2O_2 resulted in tissue damage similar to that caused by infection by *Verticillium albo-atrum*. Bacterial formation of H_2O_2 is probably extracellular while bacterial catalase is intracellular and offers protection from H_2O_2 which may enter the cell (12). Low levels of H_2O_2 are inhibitory or toxic to bacteria (13, 14, 16) and release of this common product of plant metabolism (17) as a disease resistance mechanism is possible.

This paper reports studies on the role of H_2O_2 in pathogenesis and in disease resistance to bacterial infections of pepper leaves. The results relate to H_2O_2 production by bacteria, tolerance of H_2O_2 by bacteria and by pepper leaves, bacterial catalase levels, H_2O_2 levels levels in extracts of pepper leaf intercellular fluid, and the influence of H_2O_2, catalase, malate or $MnCl_2$ on the recovery of bacteria from inoculated leaves.

MATERIALS AND METHODS.—Pepper *(Capsicum annuum* L. 'Early Calwonder') plants were grown on a greenhouse bench at 27 ± 2.5 C and all in vivo experiments were conducted there. Fully expanded nonsenescent leaves were used for experimental purposes.

Xanthomonas phaseoli (E. F. Sm.) Dows. (Xp-517) and *Xanthomonas vesicatoria* (Doidge) Dows. (Xv-728) were grown in nutrient broth, harvested by centrifugation at 1,700 *g*, resuspended in sterile distilled water and the suspension used as inoculum in the various experiments.

Bacterial production of H_2O_2 was assayed by streaking cells of *X. phaseoli* and *X. vesicatoria* on manganese dioxide (MnO_2) agar and on heated blood *o*-dianisidine agar (18).

Pepper plant sensitivity to reagent H_2O_2 (not stabilized) was tested by hypodermic injection of leaves with H_2O_2 in serial tenfold dilutions from 1.0 M to 10 μM. Evaluation was by visual examination for tissue discoloration or collapse, 24 h following treatment. The effect of enzyme-generated H_2O_2 on pepper leaves was determined by hypodermic injection of a solution containing either 10 or 200 units/ml of glucose oxidase [(E C 1.1.3.4), Type V:, (Sigma Chemical Co.)] into leaves, followed 2 h later by injection of a solution of glucose at serial tenfold dilutions from 0.1 M to 10 μM. Evaluation was again by visual examination of the leaves.

The toxicity of H_2O_2 to *X. phaseoli* and to *X. vesicatoria* was tested in vitro using 1 ml of bacteria suspended in sterile distilled water and 1 ml of reagent H_2O_2 so that the culture tube contained 1.6×10^4 cells/ml of *X. phaseoli* or 1.7×10^4 cells/ml of *X. vesicatoria* with H_2O_2 at levels of 1, 2, 4, 8, 10, 20, 40, 80, and 100 μM. The treatment period was 4 h at 25 C on a rotary shaker and toxicity was determined by serial tenfold dilutions and plating on nutrient agar of five replicates of each treatment.

The toxicity to *X. phaseoli* and to *X. vesicatoria* of H_2O_2 generated by the action of glucose oxidase on glucose was also evaluated by an in vitro experiment. The reaction mixture contained either 4.3×10^4 cells/ml of *X. phaseoli* or 4.5×10^4 cells/ml of *X. vesicatoria*, either 10 or 200 units of glucose oxidase and 10 μM, 100 μM, 1 mM or 10 mM glucose. The incubation period was 4 h at 25 C on a rotary shaker, and assay was by serial dilution of the reaction mixture and plating of five replicates.

The in vivo bactericidal effect of reagent H_2O_2 was assayed by injection of inoculum of 1×10^7 cells/ml of *X. phaseoli* or of *X. vesicatoria* into pepper leaves and followed 4 h later with an injection of water or of H_2O_2 at concns of 1.0 M to 10 μM in logarithmic dilutions. Enzymatically generated H_2O_2 was similarly assayed, substituting for reagent H_2O_2 an injection of either 10 or 200 units of glucose oxidase 2 h after inoculation, followed 2 h later by glucose at 0.1 M to 10 μM in logarithmic dilutions. A 4-h incubation period was allowed following the treatments, and then bacterial populations were assayed.

The effect of supplemental catalase [(E.C.1.1.11.1.6) (Sigma, C-40)] on bacterial populations in plant leaves was determined by suspending 1×10^7 cells/ml of *X. vesicatoria* or of *X. phaseoli* in water or in water containing 100 units/ml of catalase and injecting the suspension into pepper leaves. Incubation was for 48 h on a greenhouse bench and assay was by cutting four disks (4-mm diam) of leaf tissue which were triturated in 2 ml of sterile water using a sterile mortar and pestle. Following trituration, serial tenfold dilutions were made in sterile water and the dilutions plated on nutrient agar. Similar treatments were made using 50 μg/ml of malate and 100 μM $MnCl_2$.

Extracts of the intercellular space fluid of healthy pepper leaves were made by the technique of Klement (8). The leaves were from pepper plants that had been exposed to 6 h of sunlight on a greenhouse bench prior to extraction. The extracts were assayed for H_2O_2 using the fluorescence technique of Keston and Brandt (7).

Assays of the catalase activity of disrupted bacterial cells were performed on *X. phaseoli* and on *X. vesicatoria*. Suspensions containing approximately 1×10^8 cells/ml were prepared by growth of the bacteria for 18 h in nutrient broth, harvesting by centrifugation at 1,700 *g*, and resuspension in sterile distilled-water. The cells were disrupted by subjecting the bacterial suspensions to 0.98 kg-force/mm^2 (1,400 psi) of N_2 gas, allowing them to equilibrate for 20 min followed by sudden release through an exit port (4). Three passages resulted in disruption of more than 98% of the bacteria. The preparation was passed through a 0.45-μm filter to remove any remaining intact cells; as was verified by plating of an aliquot of the resulting suspension. Bacterial catalase activity was determined by use of a polarographic oxygen probe which measured the rate of O_2 evolution from catalase decomposition of H_2O_2. The sample chamber contained 3.0 ml of 8 mM H_2O_2 which was vacuum-desaturated of dissolved O_2 to approximately 20% and was held at 24 C in a water bath. A 0.1-ml aliquot of the disrupted bacterial suspension was introduced into the chamber through a capillary tubing inserted in the vent slot and the rate of decomposition of H_2O_2 was plotted by a recorder.

The intercellular space fluid was extracted from pepper leaves subsequent to inoculation with water, or 1×10^8 cells/ml of *X. phaseoli,* or of *X. vesicatoria*. The extracts were prepared by the technique used for extracts from healthy leaves. Bacteria were removed from the extracts by filtration using 0.2-μm filters. Extracts from water-

injected leaves and from *X. phaseoli*- and *X. vesicatoria*-inoculated leaves were tested for activity in decomposition of H_2O_2, and for their effect on bacterial catalase decomposition of H_2O_2. The tests were performed using the same procedure as for the bacterial catalase assay. The extracts were assayed at 1, 24, and 48 h following injection of the leaves.

RESULTS.—Colonies of *X. phaseoli* and of *X. vesicatoria* on heated blood *o*-dianisidine agar developed the green halo characteristic of H_2O_2-generating microorganisms (18). A confirming result was obtained by a slight clearing of the MnO_2 agar (18).

Hypodermic syringe injection of reagent H_2O_2 or of the glucose - glucose oxidase H_2O_2-generating system did not result in visible damage to the plant leaves at any concn evaluated. When 1.0 M reagent H_2O_2 was injected, small bubbles rapidly began to exude from stomates, probably indicating very rapid decomposition of the H_2O_2.

Reagent H_2O_2 at 40 μM in in vitro assays killed all treated *X. phaseoli* cells whereas 20 μM H_2O_2 only reduced numbers of viable cells, and lesser concns of H_2O_2 had no significant effect (Table 1). Cells of *Xanthomonas vesicatoria* were killed by 20 μM H_2O_2, but not by lesser concns (Table 1).

Hydrogen peroxide generated by the action of glucose oxidase on glucose in vitro was lethal to *X. phaseoli* and *X. vesicatoria* at the 10 mM level of glucose (Table 2). Assuming total conversion of the added glucose, and no decomposition of H_2O_2, the generating system would have produced an H_2O_2 concn of 10 mM at the glucose level that corresponded to total kill of the bacteria. The lower enzyme level (10 units/ml) was found to significantly reduce bacterial populations at the 1 mM glucose level, whereas the 200-unit level of glucose oxidase did not lead to a similar reduction. The explanation of this seemingly anomalous result is not known.

Recovery of *X. phaseoli* or of *X. vesicatoria,* from inoculated leaves that were subsequently injected with reagent H_2O_2 or H_2O_2-generation system, did not differ ($P = 0.01$) from the water-injected control. Addition of 100 units/ml of catalase, 50 μg/ml of malate, or 100 μM $MnCl_2$ to aqueous suspensions of *X. phaseoli* or *X. vesicatoria* did not cause differences ($P = 0.01$) in recovery from the numbers found in the nonamended controls.

The fluorescence assay indicated that detectable amounts of H_2O_2 were not present in the extract from the intercellular spaces of pepper leaves.

Bacterial catalase activity caused evolution of 0.48 $\pm$ 0.03 μl O_2/min/ml of 8 mM H_2O_2 in the cell-free preparation from 1×10^8 cells of *X. phaseoli* and 0.45 $\pm$ 0.03 μl of O_2/min/ml of 8 mM H_2O_2 in the *X. vesicatoria* preparation. Extracts of intercellular space fluid from pepper leaves 1 h after inoculation had higher H_2O_2-decomposing activity than did the bacterial preparation, but within 24 h and 48 h following inoculation the leaf extract had apparently lost all H_2O_2-decomposing activity (Table 3). Addition of an equal quantity of a 48-h extract to the disrupted bacterial cell preparation caused loss of all catalase activity in the latter.

DISCUSSION.—The ability of pepper leaves to withstand injections of 1 M H_2O_2 without visible damage, indicates that bacterial production of H_2O_2 is probably not a major factor in pathogenesis. These results are in contrast to those reported by Mussell (15) in which he found that cotton leaf tissue was injured by H_2O_2 treatment and by infiltration of 1,000 units of glucose oxidase and 25 mM glucose. The vigorous decomposition of H_2O_2 injected into pepper leaves is not readily accounted for by the limited decomposing ability of either the intercellular fluid or of the bacteria, and suggests a bound material which is a strong H_2O_2 decomposer. Cohen and Somerson (2) suggested that H_2O_2 production over extended time periods may be a factor in virulence of *Mycoplasma pneumoniae*. Cumulative damage due to a continuous low level of H_2O_2 production may also be true in plant leaves but seems unlikely due to the great tolerance of the leaves to H_2O_2.

TABLE 1. Toxicity of reagent H_2O_2 to *Xanthomonas phaseoli* and to *Xanthomonas vesicatoria*

H_2O_2 added[a] (μM)	Bacterial cells/ml	
	X. phaseoli	*X. vesicatoria*
0	15,560x[b]	17,420x
1	15,480x	16,800x
2	15,520x	17,680x
4	15,600x	17,680x
8	15,840x	17,000x
10	15,320x	17,560x
20	9,240y	0y
40	0z	0y
80	0z	0y
100	0z	0y

[a]Treatment was for 4 h at 25 C in vitro.
[b]Numbers are means of four replicates. Numbers in a column not having the same letter differ, $P = 0.05$, by Duncan's Multiple Range Test.

TABLE 2. Toxicity of H_2O_2 generated by action of glucose oxidase on glucose to *Xanthomonas phaseoli* and to *Xanthomonas vesicatoria*. Treatment was for 4 h at 25 C in vitro

Additions		Bacterial cells/ml	
Glucose oxidase (Units)	Glucose (mM)	*X. phaseoli*	*X. vesicatoria*
0	0	21,800x[a]	18,700x
10	0	22,000x	18,500x
10	0.01	19,800x	18,900x
10	0.1	13,900y	11,400y
10	1	72z	91z
10	10	0z	0z
200	0	20,700x	18,800x
200	0.01	17,800x	16,900x
200	0.1	18,200x	17,800x
200	1	16,000x	15,700x
200	10	0z	0z

[a]Numbers are means of four replicates. Numbers in a column not followed by the same letter differ, $P = 0.05$, by Duncan's Multiple Range Test.

TABLE 3. Decomposition of H_2O_2 in vitro by disrupted cells of *Xanthomonas vesicatoria* or of *Xanthomonas phaseoli*, by intercellular space fluid from healthy or diseased pepper leaves, and by disrupted bacteria plus extract from diseased leaves

Additions[a]	Treatment		
	Water	*X. phaseoli*	*X. vesicatoria*
Disrupted bacteria (1×10^8 cells/ml)	-	0.48 ± 0.03[b]	0.45 ± 0.03
Extract from 1-h water-injected leaves	0.81 ± 0.02	-	-
Extract from 1-h inoculated leaves	-	1.02 ± 0.02	0.93 ± 0.03
Extract from 24-h inoculated leaves	-	0.35 ± 0.02	0.00 ± 0.01
Extract from 48-h inoculated leaves	-	0.00 ± 0.01	0.00 ± 0.01
Disrupted bacteria + 48-h extract (1:1)	-	0.01 ± 0.03	0.00 ± 0.02

[a]A polarographic oxygen probe was used to record O_2 evolution when a 0.1-ml aliquot of one of the additions was added to 3.0 ml of 8 mM H_2O_2.

[b]Units are in μl/ml/min of O_2 evolved at 24 C. Values are the means of four replicates and the standard error of the means.

When it was determined that plant leaves would withstand 1 M H_2O_2, whereas bacteria were killed in vitro by concns less than 40 μM, it seemed as if this considerable difference in sensitivity might constitute a disease resistance mechanism through the release of H_2O_2 into the intercellular spaces of the leaf. The failure of H_2O_2 at levels up to 1.0 M to reduce bacterial populations in pepper leaves preinoculated with bacteria, greatly diminishes the prospect that H_2O_2 is itself a major resistance factor. Substantiation of this was obtained when supplemental catalase did not affect bacterial populations in the leaf. Manganese ions caused decomposition of H_2O_2 in vitro (19) but were found to have no effect on numbers of bacteria recovered from pepper leaves. Malate inhibits catalase activity (11) but also was found to not affect the number of bacteria recovered from leaves in either a compatible or incompatible host-pathogen situation.

The reason for the loss of H_2O_2-decomposing action in the extracts from inoculated leaves is not known, but could be due to plant cell leakage of materials such as dicarboxylic acids which may inhibit catalase activity (11). That such an inhibitor could be released, is supported by the loss of activity in the bacterial preparation to which was added the 48-h extract from inoculated leaves. No explanation is offered for the differences between the two extracts made 24 h postinoculation. Although Abo-El-Dahab and El-Goorani (1) found *Pseudomonas solanacearum* catalase levels did not correlate with virulence of the isolate, the total inactivation of bacterial catalase by a plant substance might be a factor in disease resistance.

Hydrogen peroxide may be involved in many reactions within the plant and especially as a substrate for peroxidase, but a specific role in pathogenesis or in disease resistance was not established in the current research.

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