Enhancement of the Bactericidal Activity of a Peroxidase System by Phenolic Compounds

N. V. Rama Raje Urs and J. M. Dunleavy

Post-doctoral Research Associate, Department of Botany and Plant Pathology, Iowa State University, Ames 50010; and Plant Pathologist, Agricultural Research Service, U. S. Department of Agriculture, and Professor, Department of Botany and Plant Pathology, Iowa State University, respectively.

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ABSTRACT

A reaction mixture bactericidal to *Xanthomonas phaseoli* var. *sojensis*, a pathogen of soybeans, consisted of horseradish peroxidase, potassium iodide, and hydrogen peroxide. Incubating the bacteria (10^6/ml) with the reaction mixture for 30 minutes at 37 C decreased the viable cell count from 10^6 to 10^4 cells/ml. Catechol, scopoletin, guaiacol, hydroquinone, 8 - hydroxyquinoline, and ferulic, caffeic, cinnamic, and protocatechuic acids enhanced the bactericidal activity, but coumarin, resorcinol, and gallic acid had no effect at concentrations tested.

Preincubation of the reaction mixture for 30 minutes and incubation with the bacterial cells for 30 minutes revealed that the oxidative products of 8 - hydroxyquinoline, hydroquinone, catechol, and guaiacol reduced the viable cell number to fewer than 30 cells/ml.

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Additional key words: enzyme, phenols.

In the presence of hydrogen peroxide (H_2O_2), peroxidase can oxidize mono- and di-phenols, mono- and di-amines, and inorganic iodide and nitrate (13, 14). Kojima (6) reported that peroxidase can increase the bactericidal effects of phenols in the presence of H_2O_2. Viral RNA and a number of plant viruses have been inactivated in vitro by enzymatically oxidized polyphenols (8, 12, 18). We have shown that horseradish peroxidase (HRP) and peroxidase isolated from soybean plants [*Glycine max* (L.) Merr.] were bactericidal against *Xanthomonas phaseoli* (Smith) Dows. var. *sojensis* (Hedges) Starr Burkh. in the presence of H_2O_2 and potassium iodide (KI) (16, 17). Because peroxidase and phenols commonly occur in plants (11, 13, 14), and because peroxidase can oxidize phenols to quinones, it seemed logical that the antibacterial mechanism may be caused by the oxidation of phenols to toxic quinones. The purpose of this study was to determine whether phenols enhance the bactericidal activity of a peroxidase system.

MATERIALS AND METHODS.—*Bactericidal assay.*—*Xanthomonas phaseoli* var. *sojensis*, the inciting agent of bacterial pustule disease of soybeans, was used as the test organism. The preparation of buffers, bacterial cells, and the assay for the bactericidal activity was described earlier (16). The assay components in a final volume of 2 ml were placed in sterile tubes (12×95 mm) in a temperature-controlled shaker incubator and incubated for 30 minutes at 37 C. Unless otherwise noted, the components of the assay mixture were added as follows: 0.33 ml of citrate phosphate buffer (pH 4.1), 0.42 ml of KI (10 μM), 0.14 ml of H_2O_2 (3×10^{-3}M), 0.5 ml of bacterial cells (10^6/ml), 0.4 ml of the appropriate concentration of a specific phenol, and 0.6 μg/ml of HRP (Nutritional Biochemicals, RZ = 3.2) and enough distilled water to yield a final volume of 2 ml. The concentrations of the phenols used in all the experiments ranged from 10^{-2} to 10^{-6}M. When any reactants were omitted, they were replaced by an appropriate volume of sterile distilled water. After incubation, triplicate samples were diluted with trypticase soy broth, spread on trypticase soy agar plates, and further incubated for 24-48 hours before the bacterial colonies were counted. The viable cell count was determined, and the percentage of survival was calculated (16).

The effects of the components of the reaction mixture on bacteria were determined by preincubating the assay mixture, containing an appropriate volume of buffer, HRP, H_2O_2, KI, and a specific phenol (10^{-3}M), for 30 minutes at 37 C and further incubating for an additional 30 minutes at 37 C after bacterial cells were added. The requirement of KI in the reaction mixture in the presence of a specific phenol (10^{-3}M) also was determined by incubating bacterial cells with the reaction mixture, with and without KI.

RESULTS.—Addition of catechol, scopoletin, guaiacol, hydroquinone, and 8 - hydroxyquinoline at concentrations of either 10^{-5} or 10^{-6}M increased the bactericidal activity of the peroxidase system, resulting in a decrease in viable cells from 10^6 to 10^2 cells/ml. In contrast, the peroxidase system alone decreased the viable cell count from 10^6 to 10^4 cells/ml (Fig. 1-5). The same compounds at concentrations of 10^{-3} or 10^{-4}M further enhanced the bactericidal activity of the system, resulting in fewer than 10 viable cells/ml (Fig. 1-5). Caffeic, cinnamic, ferulic, and protocatechuic acids enhanced the bactericidal activity of the assay system only at a concentration of 10^{-3}M, but coumarin, gallic acid, and resorcinol had no effect (Table 1). Only hydroquinone and cinnamic acid were bactericidal at 10^{-3}M when tested alone (Table 1, Fig. 4).

Adding a specific phenol to the system resulted in a decrease in the number of viable bacterial cells. This does not indicate, however, whether bacteria are inhibited during the reduction of H_2O_2 or whether phenols are oxidized to more potent bacterial inhibitors. An experiment was designed to answer this question. The

assay mixture was incubated for 30 minutes at 37 C for the oxidation reaction to proceed and then assayed for bactericidal activity by incubating with bacterial cells for an additional 30 minutes. Oxidized product(s) of 8 - hydroxyquinoline, hydroquinone, catechol, and guaiacol were highly bactericidal, resulting in fewer than 30 viable cells/ml from an initial population of 10^6 cells/ml (Fig. 6).

Peroxidase can liberate iodine from an iodine donor

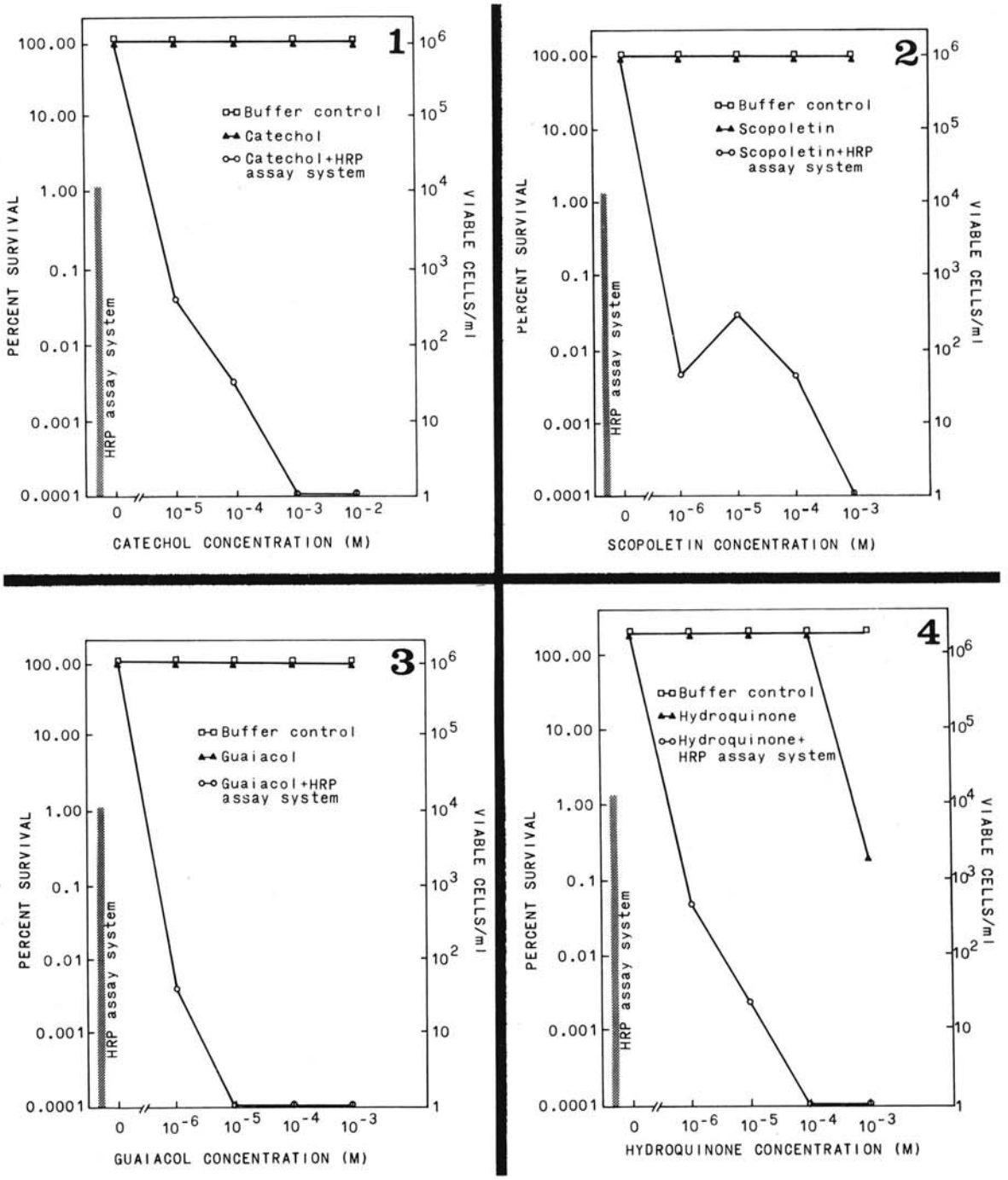

Fig. 1-4. Enhancement of the bactericidal effect of the peroxidase system on *Xanthomonas phaseoli* var. *sojensis* by phenolic compounds: **1)** catechol, **2)** scopoletin, **3)** guaiacol, **4)** hydroquinone. HRP assay system consisted of, 0.33 ml of citrate phosphate buffer (pH 4.1), 0.42 ml of KI (10 μM), 0.14 ml H_2O_2 (3×10^{-3}M), 0.5 ml of bacterial cells (10^{-3}M), 0.4 ml of appropriate concentration of a specific phenol, 0.6 μg/ml of HRP, and enough distilled water to yield a final volume of 2 ml. Incubation period 30 minutes at 37 C.

TABLE 1. Bactericidal effect of various concentrations of seven phenols, alone and in the peroxidase system[a] on *Xanthomonas phaseoli* var. *sojensis*

Phenol	Concentration(M)	Viable cells/ml[b]	
		Peroxidase system plus phenol	Buffer plus phenol (control)
Caffeic acid	10^{-3}	2×10^{2}	1×10^{6}
	10^{-4}	3×10^{5}	1×10^{6}
	10^{-5}	5×10^{4}	1×10^{6}
	10^{-6}	4×10^{4}	1×10^{6}
Cinnamic acid	10^{-3}	8×10^{3}	7×10^{4}
	10^{-4}	3×10^{4}	1×10^{6}
	10^{-5}	6×10^{4}	1×10^{6}
	10^{-6}	4×10^{4}	1×10^{6}
Ferulic acid	10^{-3}	7×10^{1}	1×10^{6}
	10^{-4}	2×10^{4}	1×10^{6}
	10^{-5}	6×10^{4}	1×10^{6}
	10^{-6}	1×10^{5}	1×10^{6}
Coumarin	10^{-3}	7×10^{4}	1×10^{6}
	10^{-4}	8×10^{4}	1×10^{6}
	10^{-5}	2×10^{5}	1×10^{6}
	10^{-6}	4×10^{4}	1×10^{6}
Gallic acid	10^{-3}	5×10^{5}	1×10^{6}
	10^{-4}	2×10^{5}	1×10^{6}
	10^{-5}	3×10^{5}	1×10^{6}
Protocatechuic acid	10^{-3}	3×10^{1}	1×10^{6}
	10^{-4}	3×10^{5}	1×10^{6}
	10^{-5}	2×10^{5}	1×10^{6}
	10^{-6}	6×10^{5}	1×10^{6}
Resorcinol	10^{-2}	6×10^{4}	1×10^{6}
	10^{-3}	5×10^{4}	1×10^{6}
Horseradish peroxidase system	...	1×10^{4}	...

[a]Peroxidase system contained 0.33 ml of citrate phosphate buffer (pH 4.1), 0.42 ml of KI (10 μM), 0.14 ml of H_2O_2 (3×10^{-3}M), 0.5 ml of bacterial cells (10^6/ml), 0.4 ml of appropriate concentration of a specific phenol, 0.6 μg/ml of HRP and enough distilled water to yield a final volume of 2 ml. Incubation period 30 minutes at 37 C.

[b]Data represent mean of three replications.

such as KI in the presence of H_2O_2. Other researchers (3, 4, 15) reported that the presence of either iodide or chloride was essential for bactericidal activity of the peroxidase system. An experiment was designed to determine whether KI is required. When potassium iodide was omitted from the reaction mixture the bactericidal activity increased in the presence of caffeic acid, and protocatechuic acids whereas it decreased in the presence of cinnamic acid, coumarin, ferulic acid and scopoletin. However, bactericidal activity of the system was unaffected by catechol, guaiacol, hydroquinone and 8 - hydroxyquinoline (Table 2).

TABLE 2. The effect of KI on the survival of *Xanthomonas phaseoli* var. *sojensis* in a peroxidase system[a] with a specific phenol added

Phenol (10^{-3}M)	Viable cells/ml[b]	
	With KI	Without KI
Caffeic acid	2×10^{2}	<10
Catechol	<10	<10
Cinnamic acid	8×10^{3}	1×10^{5}
Coumarin	7×10^{4}	1×10^{5}
Ferulic acid	7×10^{1}	2×10^{2}
Guaiacol	<10	<10
Hydroquinone	<10	<10
8-Hydroxyquinoline	<10	<10
Protocatechuic acid	3×10^{1}	<10
Scopoletin	<10	8×10^{4}

[a]Peroxidase system contained 0.33 ml of citrate phosphate buffer (pH 4.1), 0.42 ml of KI (10 μM), 0.14 ml of H_2O_2 (3×10^{-3}M), 0.5 ml of bacterial cells (10^6/ml), 0.4 ml of a specific phenol (10^{-3}M), 0.6 μg/ml of HRP and enough distilled water to yield a final volume of 2 ml. Incubation period 30 minutes at 37 C.

[b]Data represent mean of three replications.

DISCUSSION.—This study showed that certain phenols enhance the bactericidal activity of a peroxidase-mediated antibacterial system. The degree of antibacterial activity depends on the compound oxidized. Viral RNA and several plant viruses have been inactivated by a polyphenol-polyphenol oxidase system (8, 12, 18). Our data suggest that a similar mechanism may be active against bacteria in the presence of a peroxidase-phenol-H_2O_2 system.

Earlier researchers (3, 4, 15) reported that a halide is required for bactericidal activity in the peroxidase system. Our results indicate that KI is not required if it is replaced by a suitable oxidizable substrate such as catechol.

Miller (7) and Qualliotine et al. (9) reported that the bactericidal activity of the system was based on the formation of unstable free radicals, whereas Strauss et al. (15) believed that aldehyde formation was involved. Our

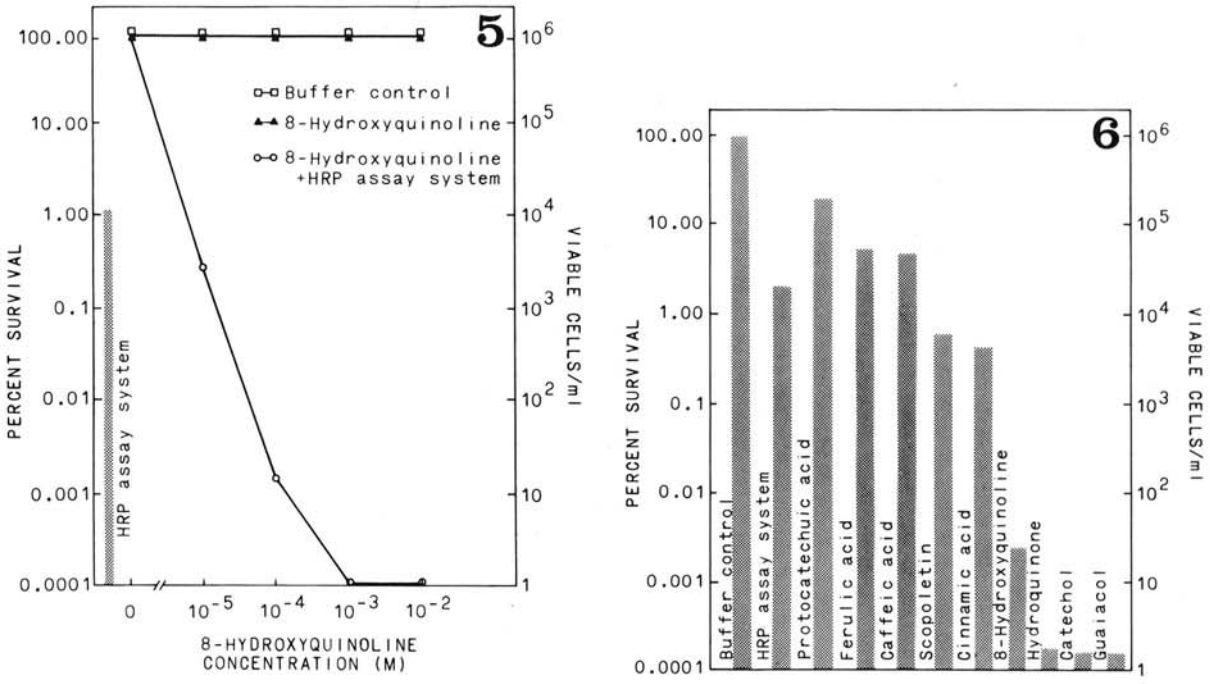

Fig. 5-6. 5) Enhancement of the bactericidal effect of the peroxidase system on *Xanthomonas phaseoli* var. *sojensis* by 8-hydroxyquinoline and **6)** by oxidation products of phenolic compounds. HRP assay system consisted of, 0.33 ml of citrate phosphate buffer (pH 4.1), 0.42 ml of KI (10 μM), 0.14 ml H_2O_2 (3×10^{-3}M), 0.5 ml of bacterial cells (10^{-3}M), 0.4 ml of appropriate concentration of a specific phenol, 0.6 μg/ml of HRP, and enough distilled water to yield a final volume of 2 ml. Incubation period 30 minutes at 37 C.

data on the effect of preincubation of the peroxidase system with phenolic compounds suggests that some compounds are oxidized to stable toxic products. In the presence of H_2O_2, peroxidase mediates the oxidation of catechol to *O*-benzoquinone (12). Polyphenol oxidase also mediates the oxidation of catechol to *O*-benzoquinone, which inactivates several plant viruses (8, 12, 18). If such a mechanism exists in the peroxidase system that we tested, we should be able to obtain bactericidal products. When we allowed the oxidation of catechol to proceed before the addition of bacteria, we observed that bactericidal activity of the system was enhanced. Similar results were obtained when hydroquinone and guaiacol were used instead of catechol. These results agree with those of Kojima (6), who suggested that peroxidase can oxidize phenols to toxic quinones, but not with Geiger's (2) findings, which indicate that the mechanism of the antibacterial action of quinones and hydroquinones was the formation of free radicals. Peroxidase also can mediate the oxidation of scopoletin in the reaction mixture, but the product formed is not known (10). Clarke (1) suggested that scopoletin can give rise to scopolin, a fluorescent compound that accumulates in response to infection. In our study, we observed that a scopoletin concentration of 10^{-5} to 10^{-6}M in the reaction mixture was enough to enhance the bactericidal activity of the system.

Xanthomonas phaseoli var. *sojensis* secretes H_2O_2 extracellularly (17). Koenigs (5) has shown also that many wood-rotting fungi secrete H_2O_2 extracellularly. He postulated that H_2O_2 produced by the fungi might be involved in plant pathogenesis. Because peroxidase and phenols occur commonly in plants, and because bacteria can secrete H_2O_2 (a primary substrate for peroxidase), we believe that the peroxidase - H_2O_2 - phenol system may be involved in plant resistance to *X. phaseoli* var. *sojensis*.

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