Peroxidase-Generated Hydrogen Peroxide as a Source of Antifungal Activity In Vitro and on Tobacco Leaf Disks

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ABSTRACT

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Hydrogen peroxide was generated in vitro by horseradish peroxidase in the presence of NADH or NADPH. When peroxidase was inactivated by heating (30 min at 100 C) or catalase was added to the reaction mixture, little or no hydrogen peroxide was generated as measured by a guaiacol oxidation assay. In the absence of either H_2O_2 or peroxidase, guaiacol was also not oxidized. Guaiacol oxidation was also observed when either NADH was replaced by NAD and malate in the presence of malate dehydrogenase or NADPH was replaced by NADP and glucose-6-phosphate in the presence of glucose-6-phosphate dehydrogenase. Oxidation was not observed when the enzymes were heat-inactivated. Hydrogen peroxide at 2.61×10^{-5} M totally inhibited the spore germination of Peronospora tabacina, Cladosporium cucumerinum, and Colletotrichum lagenarium in vitro and markedly inhibited germination at 6.5×10^{-6} M. When a sporangiospore suspension in pH 7.0 phosphate buffer was incubated with NADH or NADPH and peroxidase at 18–20 C for 18 h, the germination of sporangiospores of P. tabacina was inhibited by 72–78%. When the same mixture of sporangiospores with NADH or NADPH and peroxidase was applied to the upper surface of tobacco leaf disks, blue mold development was markedly inhibited. If peroxidase was inactivated or catalase was added, the inhibitory effects on spore germination and disease development were abolished. When NADH was replaced by NAD and malate in the presence of malate dehydrogenase, spore germination was inhibited in vitro and disease development on leaf disks was reduced.

Edreva and co-workers (8) demonstrated that increased peroxidase activity in tobacco plants infected with P. tabacina was of host origin. The rise in activity occurred earlier and was greater in tobacco resistant to blue mold than in susceptible tobacco (6). Numerous isozymes of peroxidase increased in activity after infection. No new isozymes specific for plants infected with P. tabacina or resistance were observed (4). Strong localization and high intensity of the peroxidase response around the sites of infection were characteristic of systemic resistance induced by Mg^{2+} in tobacco seedlings (7).

The role of oxidoreduction processes in plant pathogenesis has frequently been studied (7). The accumulation of oxidized metabolites and the formation of active oxygen species that are deleterious to the host cells could contribute to damage caused by pathogens as well as serve as antimicrobial agents (7). Data are available on the enhancement of respiration and the activity of peroxidase, polyphenoloxidase, and lipoxygenase in plants infected by obligate parasites (7). These enzymes create an oxidative state in the host cells and are associated with necrosis formation (7,9). Lignin biosynthesis includes the polymerization of three cinnamyl alcohols and is mediated by the peroxidase-H_2O_2 system. Cell wall-bound peroxidases are probably involved not only in the oxidative polymerization of hydroxylated cinnamyl alcohols but also in the generation of hydrogen peroxide necessary for lignification (7,10,11,17). Lignification has been suggested as a mechanism for disease resistance, particularly in disease induced by fungal pathogens (1–3,15,16,22,23).

It has been reported that hydrogen peroxide was generated at the expense of NADH and that this process was mediated by cell wall-bound peroxidase (10,12–14,18,20). Plant cell wall-bound malate dehydrogenase can use malate to reduce NAD if an oxaloacetate-withdrawing auxiliary system is present (10,14,19). Malate dehydrogenase increases in tobacco infected with P. tabacina (5,7).

The present studies were carried out to investigate: 1) the generation of hydrogen peroxide in vitro by horseradish peroxidase in the presence of NADH or NADPH; 2) the generation of hydrogen peroxide in vitro by horseradish peroxidase and either malate, malate dehydrogenase, and NAD or glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP; 3) the antifungal activity of H_2O_2; and 4) the inhibitory effects of H_2O_2 and peroxidase on the germination of sporangiospores of P. tabacina in vitro and disease development of blue mold on tobacco leaf disks.

MATERIALS AND METHODS

Enzymes and chemicals. *Enzymes.* Peroxidase (EC 1.11.1.7) from horseradish (one unit will form 1.0 mg of purpurogallin from pyrogallol in 20 s [pH 6.0 at 20 C]); malate dehydrogenase (EC 1.11.1.37) from bovine heart (one unit will convert 1.0 μmole of oxalacetate and β-NADH to L-malate and β-NAD per minute [pH 7.5 at 25 C]); catalase (EC 1.11.1.6) from bovine liver (one unit will decompose 1.0 μmole of H_2O_2 per minute [pH 7.0 at 25 C]); and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from baker's yeast (one unit will oxidize 1.0 μmole of glucose-6-phosphate to 6-phosphogluconate per minute [pH 7.4 at 25 C] in the presence of NADP) were obtained from Sigma Chemical Company, St. Louis, MO.

Chemicals. β-Nicotinamide adenine dinucleotide (reduced and oxidized), β-nicotinamide adenine dinucleotide phosphate (reduced and oxidized), hydrogen peroxide (30% solution), L(−)malate, guaiacol, p-coumaric acid, manganese chloride (solution, 10% $MnCl_2$ in H_2O), and glucose-6-phosphate were obtained from Sigma.

Plants and pathogens. Burley tobacco (*Nicotiana tabacum* L. 'Ky-14') plants were grown in 2-L pots containing Pro-Mix (Premier Peat Crop Marketing, New York) in the greenhouse (diurnal temperature 20–26 C during fall and winter and 20–33 C during spring and summer, photoperiod of 16 h with daylight supplemented by fluorescent and incandescent light). The pots were saturated with a 0.1% 15:16:17 (N:P:K) fertilizer solution (Peters Fertilizer, W.R. Grace and Co., Fogelsville, PA) five times a week. Plants were used for experiments 8 wk after seeds were planted.

Isolate 79 of *P. tabacina* was collected from tobacco plants in a field near Georgetown, KY, in 1979. Inoculum was obtained from freshly sporulating infected leaves on 7- to 8-wk-old plants 6 or 7 days after inoculation. Sporangiospores were gently brushed into a small quantity of distilled water, washed three times with distilled water on a 0.8-μm filter and then resuspended in a pH 7.0 phosphate buffer to make a sporangiospore suspension (5 × 10⁵ sporangiospores per milliliter). Race 1 of *C. lagenarium* (Pass.) Ell. & Halst. and *Cladosporium cucumerinum* Ell. & Arth. were maintained on green bean pod agar and V8 juice agar, respectively, at 24 C in the dark. Conidia of *C. lagenarium* from 8-day-old cultures were washed with distilled water three times on a 0.22-μm filter and resuspended in a pH 7.0 phosphate buffer to make a conidial suspension (4 × 10⁵ conidia per milliliter). Conidia of *C. cucumerinum* were resuspended in a 0.1% sucrose solution to make a conidial suspension (4 × 10⁵ conidia per milliliter).

Generation of hydrogen peroxide by horseradish peroxidase in the presence of NADH, NADPH. The generation of hydrogen peroxide was determined in pH 7.0 phosphate buffer containing 4 mM guaiacol, 14 mM NADH (16 mM NADPH), 0.6 mM *p*-coumaric acid, 20 μl of 10% $MnCl_2$, and horseradish peroxidase to give a final volume of 5.0 ml. After a 3-min shaking, the visible color reaction was observed. Controls were peroxidase and catalase inactivated by heating for 30 min at 100 C. Cofactors were *p*-coumaric acid and $MnCl_2$.

Generation of hydrogen peroxide by horseradish peroxidase using either malate, malate dehydrogenase, and NAD or glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP. The generation of hydrogen peroxide was determined in a reaction mixture containing 4 mM guaiacol, 14 mM NAD, 33 mM malate, 0.6 mM *p*-coumaric acid, 20 μl of 10% $MnCl_2$, horseradish peroxidase (50 activity units), malate dehydrogenase in pH 7.0 phosphate buffer (50 activity units) with a final volume of 5.0 ml.

In other experiments, 16 mM NADP, 50 mM glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were substituted for NAD, malate, and malate dehydrogenase. After a 3-min shaking, the color reaction was observed. Controls contained heat-inactivated enzymes.

Inhibitory effect of hydrogen peroxide on spore germination. Sporangiospore suspensions of *Peronospora tabacina* in pH 7.0 phosphate buffer were mixed with different volumes of 30% H_2O_2 to give a final volume of 3.0 ml (5 × 10⁵ sporangiospores per milliliter). Spores were germinated in petri dishes at 18–20 C for 18 h in the dark. Conidial suspensions of *C. cucumerinum* in 0.1% sucrose solution and *C. lagenarium* in pH 7.0 phosphate buffer were treated in the same manner except that germination was at 20–23 C. After 18 h, spores were killed with formalin and spore germination was determined using a hemacytometer under a light microscope.

Inhibitory effects of NADH, NADPH, and horseradish peroxidase on the germination of sporangiospores of *P. tabacina* in vitro and disease severity of blue mold on leaf disks. A sporangiospore suspension of *P. tabacina* in pH 7.0 phosphate buffer was mixed with 7 mM NADH or 8 mM NADPH, 0.3 mM *p*-coumaric acid, 2 μl of 10% $MnCl_2$, horseradish peroxidase (50 activity units) to give a final volume of 5.0 ml (5 × 10⁵ sporangiospores per milliliter). Controls contained catalase or peroxidase that was inactivated by heating (30 min at 100 C). Cofactors were *p*-coumaric acid and $MnCl_2$. Immediately after mixing, the spore suspension was either added to leaf disks or used to determine spore germination.

For the spore germination assay, the mixture of sporangiospores with chemicals and enzymes was incubated in petri dishes at 18–20 C for 18 h in the dark, and the spore germination was determined using a hemacytometer. For the disease severity assay, leaf disks (10 mm in diameter) were cut from the third fully expanded leaf from the top of tobacco plants. Leaf disks were placed with their upper surface up on wet, sponge-rubber pads in moistened transparent plastic boxes (35 × 27 × 9 cm high). A 50-μl droplet of the mixture of sporangiospores with chemicals and enzymes was placed on the upper surface of each leaf disk

and leaf disks were incubated in moistened transparent plastic boxes. The boxes were kept at 18–20 C for 18 h in the dark and then held in a growth chamber (20–23 C, 86.5 μE s⁻¹ m⁻², 12 h of light per day) for 7 days. After 7 days, disease development of blue mold on leaf disks was recorded. Symptoms on each leaf disk were individually rated using a visual scale of 0–4 (0 = no disease, 1 = 1–25% disease, 2 = 26–50% disease, 3 = 51–75% disease, 4 = 76–100% disease). Twelve leaf disks were used for each treatment.

Inhibitory effects of NAD, malate, and horseradish peroxidase in the presence of malate dehydrogenase on the germination of sporangiospores of *P. tabacina* in vitro and disease severity of blue mold on leaf disks. A sporangiospore suspension of *P. tabacina* (5 × 10⁵ sporangiospores per milliliter) in pH 7.0 phosphate buffer was mixed with 7 mM NAD, 16 mM malate, 0.3 mM *p*-coumaric acid, 2 μl of 10% $MnCl_2$, horseradish peroxidase (50 activity units), and malate dehydrogenase (50 activity units) with a final volume of 5.0 ml. Controls were peroxidase and malate dehydrogenase inactivated by heating for 30 min at 100 C. Cofactors were *p*-coumaric acid and $MnCl_2$. Spore germination and disease severity assays were the same as previously described.

RESULTS

Guaiacol is oxidized to colored oxidation products in the presence of hydrogen peroxide and peroxidase. A color reaction was not evident in the absence of either hydrogen peroxide or peroxidase. Hydrogen peroxide was generated, as apparent from the color reaction in vitro, by horseradish peroxidase in the presence of NADH or NADPH. Guaiacol oxidation was not apparent when peroxidase was inactivated by heating (30 min at 100 C) before it was added into the reaction mixture; guaiacol oxidation was markedly reduced when catalase was added. Guaiacol oxidation was also observed when NADH was replaced by NAD and malate in the presence of malate dehydrogenase or NADPH was replaced by NADP and glucose-6-phosphate in the presence of glucose-6-phosphate dehydrogenase. If the dehydrogenases or peroxidase was inactivated by heat, guaiacol oxidation was not evident.

Hydrogen peroxide at a very low concentration (2.61×10^{-5} M) prevented germination of *P. tabacina* sporangiospores and *C. cucumerinum* and *C. lagenarium* conidia in vitro (Table 1). The germination of sporangiospores of *P. tabacina* was also inhibited in the presence of peroxidase and NADH or NADPH in vitro (Table 2). When the mixture of sporangiospores with NADH or NADPH, peroxidase, and cofactors was applied to the upper surface of tobacco leaf disks, blue mold development on leaf disks was markedly inhibited. If peroxidase was inactivated by heating (30 min at 100 C) or catalase was added, the inhibitory effects on spore germination and disease development were abolished. Germination of sporangiospores of *P. tabacina* was not inhibited by NADH, NADPH, peroxidase, or cofactors. Peroxidase reduced disease severity of blue mold on tobacco leaf

TABLE 1. The inhibitory effect of hydrogen peroxide on the germination of sporangiospores of *Peronospora tabacina* and conidia of *Cladosporium cucumerinum* and *Colletotrichum lagenarium*

Hydrogen peroxide ($\times 10^{-5}$ M)	Germination[a] (%)		
	P. tabacina	*C. cucumerium*	*C. lagenarium*
0.00	54.7[a] ± 8.02[b]	92.0 ± 3.00	41.3 ± 4.73
0.03	57.7 ± 6.51	81.0 ± 15.87	35.7 ± 4.93
0.16	54.7 ± 5.50	88.3 ± 5.69	30.0 ± 3.61
0.33	39.3 ± 4.04	72.3 ± 8.62	8.33 ± 1.53
0.65	22.3 ± 5.86	37.7 ± 6.66	2.33 ± 2.08
1.31	11.7 ± 2.52	10.3 ± 2.52	1.00 ± 1.00
1.96	4.70 ± 4.16	1.70 ± 2.08	1.00 ± 1.73
2.61	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3.27	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[a] Means are based on three replicates. Each replicate included 100 spores.
[b] Standard deviation of the mean.

disks when applied alone or together with NADH or NADPH and cofactors. Germination of sporangiospores of *P. tabacina* was inhibited and disease severity was reduced on tobacco leaf disks when NADH was replaced by NAD and malate in the presence of malate dehydrogenase (Table 3).

DISCUSSION

Hydrogen peroxide was generated in vitro by horseradish peroxidase in the presence of NADH or NADPH and cofactors. NADH formed from NAD and malate by malate dehydrogenase also generated hydrogen peroxide. These results confirm earlier reports (10,13,14). In our experiments, we found that NADPH could also be used to generate hydrogen peroxide and that it was formed from NADP and glucose-6-phosphate by glucose-

TABLE 2. The inhibitory effects of NADH, NADPH, and horseradish peroxidase on the germination of sporangiospores of *Peronospora tabacina* in vitro and disease severity of blue mold on leaf disks of tobacco plants

Treatment	Germination[a] (%)	Disease scale[b] (1–4)
Buffer	64.0 ab[c]	3.58[c] a
NADH	62.7 ab	3.25 ab
NADPH	65.0 ab	3.42 a
Peroxidase	68.0 a	1.42 cd
Peroxidase (inactivated)[d]	61.0 ab	3.00 ab
Cofactors[e]	66.3 ab	3.17 ab
Catalase	59.7 ab	3.08 ab
Peroxidase + cofactors	64.3 ab	1.58 c
Peroxidase + NADH	51.7 ab	1.92 c
Peroxidase + cofactors + catalase	57.7 ab	2.83 ab
NADH + peroxidase + cofactors	18.3 c	0.83 d
NADH + peroxidase (inactivated)[d] + cofactors	55.0 ab	2.67 b
NADH + peroxidase + cofactors + catalase	50.3 ab	2.92 ab
NADPH + peroxidase + cofactors	15.0 c	0.67 d
NADPH + peroxidase (inactivated)[d] + cofactors	62.3 ab	2.83 ab
NADPH + peroxidase + cofactors + catalase	49.0 b	2.92 ab

[a] Means are based on three replicates. Each replicate included 100 spores.
[b] Data are the means of two experiments with 12 leaf disks per experiment.
[c] Numbers within a column followed by different letters are significantly different (*P* = 0.01) according to Duncan's multiple range test.
[d] Peroxidase was inactivated by heating (30 min at 100 C).
[e] Cofactors: *p*-coumaric acid and $MnCl_2$.

TABLE 3. The inhibitory effects of NAD, malate, and horseradish peroxidase in the presence of malate dehydrogenase on the germination of sporangiospores of *Peronospora tabacina* in vitro and disease severity of blue mold on leaf disks of tobacco plants

Treatment	Germination[a]	Disease scale[b]
Buffer	62.3[b] a[c]	3.50[c] a
NAD	56.7 ab	3.33 a
Malate	53.3 ab	3.17 a
Malate dehydrogenase	49.0 ab	2.83 a
Peroxidase	62.0 ab	1.50 b
Cofactors[d]	59.7 a	2.92 a
NAD + malate + malate dehydrogenase + cofactors	42.3 b	2.85 a
NAD + malate + malate Dehydrogenase + cofactors + peroxidase	25.3 c	1.17 b
NAD + malate + malate dehydrogenase + cofactors + peroxidase (inactivated)[e]	52.7 ab	2.92 a

[a] Means are based on three replicates. Each replicate included 100 spores.
[b] Data are the means from three experiments with 12 leaf disks per experiment.

6-phosphate dehydrogenase. This is of interest because oxidation of NADPH has an important regulatory role in the oxidative pentose pathway and the shikimate, malonate, and mevalonate pathways.

Hydrogen peroxide strongly inhibited fungal activity (Table 1). At a concentration 2.61×10^{-5} M, it totally inhibited the spore germination of *P. tabacina*, *C. cucumerinum*, and *C. lagenarium*. Peroxidase-generated hydrogen peroxide inhibited the germination of sporangiospores of *P. tabacina* in vitro and disease development of blue mold on tobacco leaf disks (Tables 2 and 3). The ability of peroxidase alone to reduce blue mold on leaf disks remains unexplained. It is possible that factors for the generation of hydrogen peroxide are on the leaf surface and/or that endogenous hydrogen peroxide oxidized leaf surface components to form antifungal compounds and polymeric barriers to fungal infection and development.

In many cases, a positive relationship between peroxidase activity and resistance has been reported (7). Enhanced peroxidase activity in disease development has been correlated with the expression of resistance in different host-pathogen systems (1,2,15,16,21).

Peroxidase-generated hydrogen peroxide may function as an antifungal agent in disease resistance. Reduced NAD and NADP in the presence of peroxidase and oxygen may generate antimicrobial quantities of hydrogen peroxide. Hydrogen peroxide inhibits pathogens directly, and/or it may generate other reactive free radicals that are antimicrobial. The rapid oxidation of reduced NAD and NADP would activate the oxidative pentose pathway, which requires oxidized NADP, to produce erythrose-4-phosphate and phosphoenolpyruvate. Both of the latter compounds are precursors to cinnamic acid-related phenols via the shikimate pathway. The cinnamic acid-related phenols may function as phytoalexins or phytoalexin precursors and may also be polymerized to lignin in a series of reactions that include H_2O_2 and peroxidase. Lignin could further restrict a pathogen within penetrated tissue that contains phytoalexins, hydrogen peroxide, and other antimicrobial compounds. Thus, peroxidase could not only participate in the biosynthesis of antimicrobial compounds and lignin but also serve as a regulator for the entire metabolic process. Detailed studies are necessary on the compartmentation of the components of this proposed scenario and their diffusibility and availability for reaction as well as contact with pathogens.

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