

Involvement of the Oxidative Burst in Phytoalexin Accumulation and the Hypersensitive Reaction¹

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ABSTRACT

The role of the oxidative burst, transient production of activated oxygen species such as H₂O₂ and superoxide (O₂⁻) in elicitation of phytoalexins and the hypersensitive reaction (HR) was investigated in white clover (*Trifolium repens* L.) and tobacco (*Nicotiana tabacum* L.). H₂O₂ and O₂⁻ production was measured as chemiluminescence (CL) mediated by luminol, which was added to suspension-cultured white clover just before measurement in an out-of-coincidence mode scintillation counter. Maximum CL occurred between 10 and 20 min after addition of 0.4 × 10⁸ colony-forming units/mL of incompatible *Pseudomonas corrugata* or 158 μM HgCl₂. Autoclaved *P. corrugata* produced a slightly higher response. Elicitation of cells with 25 μM HgCl₂ did not produce CL. Preincubation of plant cells in superoxide dismutase, which converts O₂⁻ to H₂O₂, for 2 min before addition of bacteria did not significantly increase maximum CL levels (P ≤ 0.05). Preincubation of plant cells with catalase for 2 min before addition of bacteria prevented the increase in CL, confirming that H₂O₂ is the substrate for the luminol reaction. Addition of live bacteria or HgCl₂ (25 and 158 μM) to white clover increased levels of the phytoalexin medicarpin during a 24-h period, but addition of autoclaved bacteria did not elicit formation of medicarpin. Preincubation of plant cells with catalase, which quenched the bacteria-induced oxidative burst, did not decrease phytoalexin accumulation. Live bacteria infiltrated into Havana 44 tobacco leaf panels induced development of the HR, but autoclaved bacteria did not. Incubation of live bacteria with superoxide dismutase and catalase before infiltration into tobacco leaves did not interfere with development of the HR. Tobacco leaf panels infiltrated with up to 158 μM HgCl₂ did not develop an HR. These results suggest that an oxidative burst consisting of H₂O₂ and O₂⁻ does occur during these two plant defense responses, but it may not be a necessary element of the signaling system for HR and phytoalexin formation.

H₂O₂ (2, 13, 18). In light of its similarity to the respiratory burst of activated phagocytes (4, 5), the oxidative burst may function as a first line of defense in resistant plants by directly attacking the pathogen during the earliest stages of infection.

It has also been postulated that highly reactive active oxygen molecules initiate a lipid peroxidation chain reaction in the plasmalemma (1, 20, 21, 23). The ensuing disruption of the membrane is proposed to have two major effects. One is increased cell leakiness, which constitutes the first distinctive sign of an HR (18, 21). The other effect of peroxidative membrane disruption is either the release of a signal from the membrane or an influx of signal molecules into the plant cell (2, 13, 23). The signal is postulated to be responsible for activating transcription of genes coding for phytoalexin biosynthetic enzymes.

A number of reports in the literature document the presence of the oxidative burst, but it is not clear whether this burst always occurs during plant-pathogen interactions, and if so, whether the oxidative burst is necessary for elicitation of plant defense responses. We undertook these studies to test the relationship between the occurrence of oxidative burst in two defense responses, phytoalexin elicitation and HR. An incompatible strain of the bacterium, *Pseudomonas corrugata*, and HgCl₂ were used as elicitors of the oxidative burst and phytoalexin accumulation in white clover (*Trifolium repens* L.) cultured cells and HR in tobacco (*Nicotiana tabacum* L.) leaf tissue. Exogenously introduced antioxidant enzymes, SOD and catalase, were used to examine the role of active oxygen in the development of these plant defense responses.

MATERIALS AND METHODS

Plant Material

The callus culture line was derived from white clover (*Trifolium repens*) hypocotyls and maintained on modified Gamborg's B5 agar medium as previously described (14). To initiate suspension cultures, callus clumps (1.5–2.0 g) were suspended in 30 mL of modified Gamborg's B5 liquid medium and incubated on an orbital shaker (New Brunswick Scientific Co., Inc., Edison, NJ³) at 125 rpm in a growth chamber at 25°C with a 16-h photoperiod (200–400 μmol·

Recent findings suggest the involvement of an oxidative burst in plant defense responses. Leaf tissue and cell cultures produce an oxidative burst within minutes of exposure to pathogenic bacteria (19), fungi (11), fungal cell wall components (2, 10), and abiotic elicitors (13), involving the generation of at least three activated oxygen species: O₂⁻,² ·OH, and

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² Abbreviations: O₂⁻, superoxide anion; ·OH, hydroxyl radical; CL, chemiluminescence; SOD, superoxide dismutase; HR, hypersensitive reaction; cfu, colony-forming unit.

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$\text{m}^{-2} \cdot \text{s}^{-1}$). After 9 to 13 d, the cells were subcultured into fresh Gamborg's medium and incubated for an additional 6 or 7 d. Cells from these flasks were filtered through plastic mesh (2-mm² perforations) to remove large clumps of cells. The resulting cell filtrate consisted of individual cells and cell aggregates of nonuniform size. The cells were collected on Miracloth and divided into two groups, one to be used in the oxidative burst assay and the other to be used in the phytoalexin assay.

Tobacco (*Nicotiana tabacum* L.) cv Havana 44 was grown under greenhouse conditions until the largest leaf was approximately 10 cm long. Panels of second, third, and fourth fully expanded leaves were infiltrated with bacterial suspensions or HgCl_2 .

Bacteria

Pseudomonas corrugata (strain RB15) was maintained as previously described (17). The rough colony type isolate was used for these experiments and gave the incompatible HR response on tobacco and white clover leaves. The smooth colony type, which did not produce an incompatible response, was not used. Bacteria were grown on yeast-dextrose-phytopeptone-agar (17) plates for 48 h in the dark at 25°C. Because the bacteria may produce active, water-soluble extracellular components, both washed and unwashed bacteria were used. Bacterial suspensions were scraped from the surface of three culture plates of *P. corrugata* and suspended in 10 mL of 0.7% saline. After the material was centrifuged, the bacterial pellet was suspended in 10 mL of buffer (5 mM Mes, pH 5.6) and centrifuged again. The bacterial pellet was then resuspended in 6 mL of buffer (washed bacteria). A second set of bacteria from two plates was suspended in 6 mL of buffer (unwashed bacteria). Washed and unwashed bacteria suspensions were adjusted to a final concentration of 5×10^8 cfu/mL, and half of each was autoclaved. These served as stock suspensions for experiments.

Reagents and Enzymes

A 1-mM luminol solution was prepared by addition of concentrated NH_4OH dropwise until the luminol dissolved, and then the volume was adjusted with Mes buffer to maintain ionic conditions similar to that of cultured cells during incubation. Just before measurement of CL, luminol was added to a 1-mL aliquot of suspension cells to give a final luminol concentration of 4 μM . The final pH of the medium for CL measurement was 9.0, which supported both optimum CL and highest ratio of CL in elicited to nonelicited cells. These parameters and those described below for oxidative burst measurements were optimized in preliminary experiments (data not shown). All chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Measurement of Oxidative Burst

Suspension cells collected on Miracloth (2 g of cells) were suspended in 40 mL of assay medium (175 mM mannitol, 0.5 mM CaCl_2 , and 0.5 mM K_2SO_4 in Mes buffer) as described by

Baker et al. (6). While being dispersed by a magnetic stir bar, 1-mL aliquots of cells were drawn into a pipet and transferred to 20-mL glass scintillation vials. The cells were left for 2 h in the dark at room temperature to reduce H_2O_2 levels elevated by handling. All subsequent operations were conducted under low levels of room light.

For experiments testing the effects of antioxidant enzymes, appropriate volumes of stock solutions of SOD (1.1×10^4 units/mL) and/or catalase (3.3×10^4 units/mL) in assay buffer were added to 1-mL aliquots of plant cells 2 min before addition of elicitor. To the nonelicited controls, either buffer or saline was added, or no addition was made. At each test time, three vials were placed in a scintillation counter (Packard 1500 Tri-Carb Liquid Scintillation Analyzer, Packard Instrument Co., Downers Grove, IL) operated in the out-of-coincidence mode. As each vial was entering the counting chamber, 40 μL of luminol was added. Light emissions were monitored for 10 s at 0-, 10-, 15-, 20-, and 25-min intervals. Nonelicited controls were monitored in parallel with treatment samples.

Each experiment was conducted a minimum of two times. Data were expressed as cpm and are reported as the average of three replicates in a given experiment. Values were not averaged across experiments because of the variability among cell cultures in their baseline CL activity. Those readings that exceeded the upper limit of the scintillation counter were reported as 10^7 cpm.

Phytoalexin Assay

Assay suspensions were prepared by adding 1.5 g of *T. repens* suspension cells to 20 mL of assay medium in a 50-mL beaker. SOD and/or catalase were added to the plant cell suspensions and allowed to stand for 15 min. Each elicitor was added at time zero, and the total volume was adjusted to 30 mL with Mes buffer. The assay beakers were then incubated on the rotary shaker (125 rpm) in a growth chamber for 24 h. Nonelicited control cells were tested in parallel with treatment samples.

Cells from each treatment were collected by vacuum filtration and extracted as described before (14). A 2-mL sample from each cell suspension filtrate was extracted twice with 2 mL of ethyl acetate. The combined organic phases were flash evaporated at 30°C, and the residue was dissolved in hexanes:ethyl acetate (8:2, v/v). Medicarpin concentrations were determined in cultured cell and culture medium extracts by HPLC (14). Peaks were detected and integrated by a Waters 990+ Photodiode Array Detector (Waters, Milford, MA). Medicarpin content per treatment was calculated as total amount of medicarpin isolated from suspension cells and medium per gram fresh weight of cells. Each experiment was conducted two or three times. Within an experiment, each sample was extracted in duplicate. Data are presented as the average of four values across two experiments.

HR Assay

Each test solution or suspension (1 mL) was infiltrated into a separate interveinal area (or "panel") of the leaf (22). Four to six infiltrations could be accommodated per leaf, depend-

ing on leaf size. After infiltration, plants were placed under fluorescent lights in the laboratory and observed 5, 8, 11, 14, and 20 h after inoculation.

The HR was evaluated according to rate and extent of response. Test solutions that induced visible symptoms of hypersensitivity (water-soaked spots, chlorosis) within 5 h after injection were rated as rapid. Those that elicited no visible response, a mottled and incomplete necrosis, or a solid patch of necrosis were rated "none," "partial," or "full," respectively.

Each test solution was tested in three separate experiments. Within an experiment, each suspension was injected into two leaf panels on separate leaves. Data are presented as the number of injections (of a total of six) per treatment inducing a given response.

RESULTS

Oxidative Burst

Bacterial suspensions were tested for the ability to elicit active oxygen-enhanced CL after their addition to *T. repens* suspension-cultured cells. All four *P. corrugata* treatments (washed or unwashed, live or autoclaved) elicited a sharp increase in CL within 10 min (Figs. 1 and 2A). Washed and unwashed autoclaved bacteria elicited the highest, most rapid CL response (approximately 10–15 min) in both experiments. Washed live bacteria elicited a consistently high, somewhat slower response (approximately 15–20 min). Unwashed live bacteria elicited a variable, although significant, peak at 15 to 20 min. Washed, autoclaved bacteria elicited a second, diminished peak of CL, significantly greater than the control level ($P \leq 0.05$), approximately 3 h after addition of *P. corrugata* (Fig. 1). Live or autoclaved washed bacteria did not produce increased CL levels at any other time from 30 min

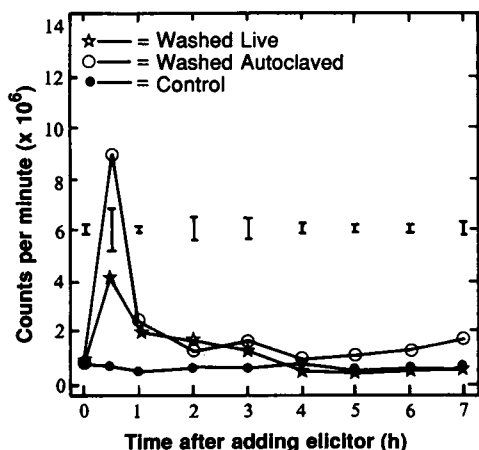


Figure 1. Long-term elicitation of oxidative burst. Cells of live and autoclaved *P. corrugata* and cell suspensions of *T. repens* were prepared as described in "Materials and Methods." Bacteria were added to the plant suspension cultures. CL was measured at the indicated times during a 7-h period. Nonelicited, control plant cultures in Mes buffer were observed in parallel with treated samples. Bacterial cell concentration, 0.4×10^8 cfu/mL. Bars represent the LSD at the 0.05 level of significance.

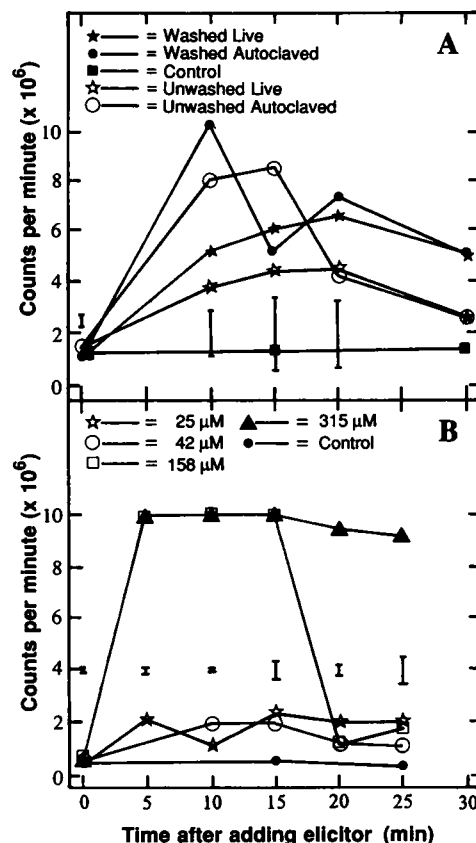


Figure 2. Short-term elicitation of oxidative burst. Following addition of elicitors (A, *P. corrugata*; or B, $HgCl_2$), CL was measured during a 30-min period. Experimental conditions and controls were otherwise the same as in Figure 1. Bars represent the LSD at the 0.05 level of confidence.

to 2 h when measured at 5-min intervals (data not shown). Nonelicited cultures in Mes buffer produced a constant, low level of CL between 0 and 7 h.

A series of $HgCl_2$ concentrations was tested for ability to elicit active oxygen after addition to *T. repens* cell suspensions. Lower concentrations of $HgCl_2$ (25 and 42 μM) induced only a slight increase in CL (Fig. 2B). At higher concentrations (158 and 315 μM), $HgCl_2$ was a strong elicitor of CL.

Under the conditions of the oxidative burst measurements used, addition of H_2O_2 in the absence of elicitor led to production of CL. The levels of CL in 0, 1, 2, and 5 μM H_2O_2 were 0.45, 0.94, 4.8, and 8.7×10^6 cpm, respectively. Thus, addition of SOD 2 min before elicitation of the cells would increase CL because SOD should increase the rate of O_2^- dismutation to H_2O_2 . Addition of increasing amounts of SOD led to increasing levels of CL that were significantly higher than control levels; however, the increases over no SOD (Fig. 3A) were not statistically significant ($P \leq 0.05$).

Addition of catalase 2 min before elicitation of cells would decrease CL if catalase increases the rate of disappearance of H_2O_2 . Catalase at 165 units/mL significantly decreased CL, but these levels were still significantly higher than nonelicited control levels (Fig. 3B). At 1320 and 2640 units/mL, catalase

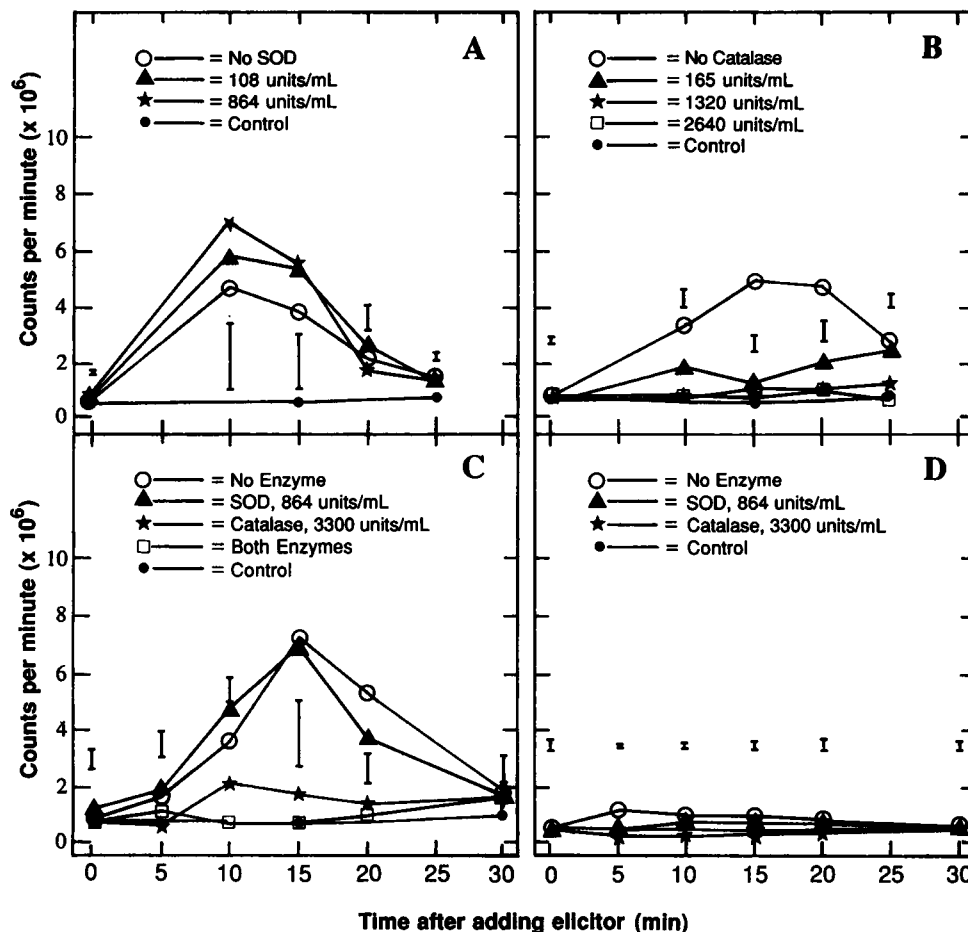


Figure 3. Effect of SOD and catalase on induced oxidative burst. Cells of *P. corrugata* and cell suspensions of *T. repens* were prepared as described in "Materials and Methods." Live, washed bacteria (A-C, 0.4×10^8 cfu/mL) or HgCl_2 (D, $25 \mu\text{M}$) were used to elicit CL in plant suspension cultures. Nonelicited, control plant cultures in Mes buffer were observed in parallel with treated samples. SOD and/or catalase were added at the indicated final concentrations 2 min before elicitation of plant cells. Bars represent the LSD at the 0.05 level of significance.

decreased CL to nonelicited control levels. At 25 min, the plant cells exposed to 1320 units/mL of catalase emitted CL that was slightly, although significantly, higher than that emitted by nonelicited controls.

Addition of both SOD and catalase 2 min before elicitation was expected to convert all generated O_2^- to H_2O_2 and all H_2O_2 to water and oxygen, thus quenching the oxidative burst. Addition of SOD and catalase at final concentrations of 864 and 3300 units/mL, respectively, to cell suspensions before addition of bacteria resulted in emission levels indistinguishable from nonelicited control levels (Fig. 3C). Pretreatment with only SOD enhanced CL slightly (although significantly at 10 min) above the CL of bacteria-induced cell suspensions. Plant cells pretreated with only catalase emitted CL that was significantly lower than that emitted by bacteria-induced cell suspensions. However, these low levels of CL were still significantly higher than that emitted by nonelicited control cells at 10 min.

Addition of SOD (864 units/mL) or catalase (3300 units/mL) before elicitation by HgCl_2 had less dramatic effects on CL in *T. repens* cell suspensions. This was partly because

treatment with $25 \mu\text{M}$ HgCl_2 elicited only small (although significant) levels of CL in the absence of SOD or catalase (Fig. 3D). Addition of SOD significantly reduced CL at 5 min but not at other times. Addition of catalase decreased the CL to control levels for the duration of the experiment.

Medicarpin Accumulation

Gustine (15) reported that only *P. corrugata*, one of four bacterial plant pathogenic species tested, elicited medicarpin in *T. repens* callus. This bacterium was tested for its ability to elicit phytoalexin accumulation in *T. repens* cell suspensions. Live *P. corrugata* (both washed and unwashed) elicited phytoalexin accumulation in the 24-h incubation (Fig. 4A). However, autoclaved bacteria did not elicit phytoalexin accumulation.

Mercuric chloride elicited medicarpin in suspension cultures as it did in callus (14) and did so in a concentration-dependent response (Fig. 4B). No combination of SOD (864 units/mL) or catalase (3300 units/mL), added before elicitor, significantly ($P \leq 0.05$) altered the levels of medicarpin

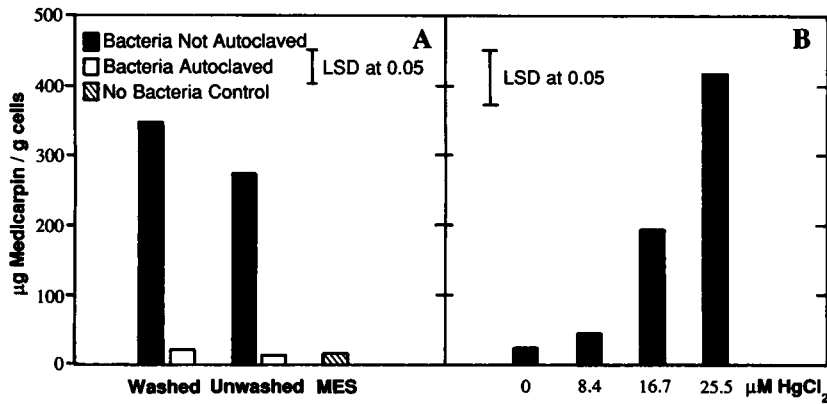


Figure 4. Elicitation of phytoalexin accumulation. Cells of *P. corrugata* and suspensions of *T. repens* cells were prepared as described in "Materials and Methods." Treated and nonelicited, control plant cultures in Mes buffer were incubated for 24 h. A, Washed and unwashed bacteria (0.4×10^8 cfu/mL); B, three concentrations of HgCl₂. Bars represent the LSD at the 0.05 level of significance.

elicited in *T. repens* cell-suspension cultures by *P. corrugata* (Fig. 5A) or by 25 µM HgCl₂ (Fig. 5B).

rugata (whether washed or not) or enzymes did not induce an HR reaction in any trial.

HR

Live bacteria (both washed and unwashed) at $>5 \times 10^8$ cfu/mL yielded a rapid, fully developed HR (Table I). At 5×10^7 cfu/mL live, washed bacteria did not induce HR when infiltrated into leaf panels of three plants. Autoclaved bacteria (whether washed or unwashed) yielded either no visible signs of HR or a slow, only partially developed response.

Only the highest concentration of HgCl₂ (315 µM) elicited a rapid, complete HR. At 158 µM, HgCl₂ did not induce HR, or it induced a slow, partial response. Lower concentrations did not elicit an HR. None of six leaf panels infiltrated with 0.7% saline control or with Mes buffer control had signs of HR.

Addition of combined SOD (864 units/mL) and catalase (3300 units/mL) delayed the initial development of bacteria-induced HR, which, nonetheless, reached completion in the six trials by 20 h after injection. Addition of an equal volume of Mes buffer also slowed the development of HR without interfering with complete development. Autoclaved *P. cor-*

DISCUSSION

These experiments established that, when incompatible bacteria were added to suspension cells, activated oxygen production increased sharply within 10 min. Production peaked between 10 and 20 min after addition of bacteria. That autoclaved *P. corrugata* elicited the oxidative burst indicated it was, indeed, the plants that produced the activated oxygen species.

Addition of catalase completely quenched the bacteria-induced CL peak (Fig. 3C), indicating a reaction of H₂O₂ with luminol to produce CL. SOD would be expected to increase CL in the presence of O₂⁻ by increasing the rate of O₂⁻ dismutation to H₂O₂ and its subsequent reaction with luminol. That SOD did not significantly increase CL suggests that O₂⁻ production was not a part of the oxidative burst (Fig. 3A) or that SOD activity within the bacteria and plant cells was sufficient to convert any O₂⁻ to H₂O₂. Because O₂⁻ in the plant cells may react with H₂O₂ produced during the oxidative

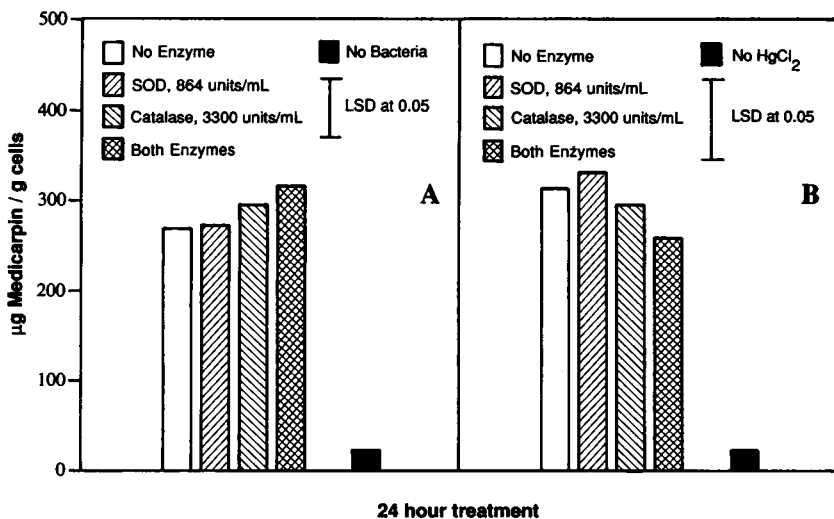


Figure 5. Effect of SOD and catalase on elicitor-induced phytoalexin accumulation. Cells of *P. corrugata* and suspensions of *T. repens* cells were prepared as described in "Materials and Methods." Treated and nonelicited, control plant cultures in Mes buffer were incubated for 24 h. A, Washed bacteria (0.4×10^8 cfu/mL); B, HgCl₂ (25 µM). Bars represent the LSD at the 0.05 level of significance.

Table I. Elicitation of HR in Tobacco

Treatments ^a	No. of Panels in Category ^b		
	No HR	Partial HR	Full HR
Bacteria, unwashed			
Live, 8×10^8 cfu/mL	0	0	6
Autoclaved, 8×10^8 cfu/mL	4	2	0
Bacteria, washed			
Live, 8×10^8 cfu/mL	0	0	6
Live, 5×10^7 cfu/mL	3 ^c	0	0
Autoclaved, 8×10^8 cfu/mL	5	1	0
Mercuric chloride (μM)			
8	6	0	0
17	6	0	0
25	6	0	0
158	4	2	0
315	0	0	6

^a Cells of *P. corrugata* were prepared as described in "Materials and Methods." Each infiltration treatment was replicated in six inoculated leaf panels of two tobacco (Havana 44) plants. ^b The extent of development of HR in a panel was evaluated in six plants 20 h after inoculation as none, partial (mottled, chlorotic), or full (confluent necrosis). Interactions designated full yielded a visible HR within 5 h; those designated partial yielded a visible HR later than 5 h. ^c The extent of development of HR was evaluated in three inoculated leaf panels of three plants.

burst to form $\cdot\text{OH}$ and $\text{}^-\text{OH}$ by the Haber-Weiss reaction (12), H_2O_2 , O_2^- , $\cdot\text{OH}$, and $\text{}^-\text{OH}$ all may be constituents of the oxidative burst.

In white clover callus, an induction period of up to 6 h was necessary before medicarpin levels increased (14, 16). Increased rate of K^+/H^+ exchange, an indicator of HR, is first detected in an incompatible interaction 2 to 6 h following addition of *P. syringae* pv *syringae* to tobacco suspension cells (7). Live *P. corrugata* added to *T. repens* suspension cells likely caused production of activated oxygen species before medicarpin levels increased and before development of external signs of HR were evident in tobacco (Table I). However, autoclaving *P. corrugata* eliminated its ability to elicit medicarpin accumulation and substantially reduced the extent of the HR without affecting its ability to produce activated oxygen species in the short term. The oxidative burst, therefore, was separated from these two well-established plant defense responses. These results indicate that the oxidative burst was not capable of causing the release of intracellular phytoalexin-eliciting signals, nor was it the only cause of the onset of HR.

The results from the HgCl_2 experiments cast further doubt on the involvement of the oxidative burst in phytoalexin elicitation. Concentrations of HgCl_2 at or below $42 \mu\text{M}$ elicited phytoalexin accumulation in plant suspension cells (Fig. 4B) but did not induce HR in leaf panels (Table I) or enhance production of activated oxygen species in plant suspension cells (Fig. 2B). Concentrations at 158 or $315 \mu\text{M}$ induced hypersensitivity and led to enhanced production of activated oxygen species. These results may indicate that the oxidative burst is involved in the development of HR in tobacco but not in phytoalexin elicitation in clover suspension cells.

Baker and colleagues (6) found that both incompatible and compatible bacteria caused an increased production of activated oxygen species within 30 min after addition to tobacco cell suspensions. However, only bacteria that elicited HR stimulated a second, long-term production, beginning at 3 h and continuing until at least 6 h after inoculation. Their findings were consistent with those of Duke (11) and may indicate that the prolonged, rather than the initial, oxidative burst is necessary for the development of HR. Results from our experiments in which we measured long-term (up to 7 h) production of activated oxygen species by white clover suspension-cultured cells following addition of incompatible bacteria indicated that a small prolonged oxidative increase was elicited 3 to 5 h after addition of autoclaved bacteria but not after addition of live bacteria (Fig. 1). This may indicate inactivation of bacterial SOD and catalase. Furthermore, infiltration of tobacco leaf panels with incompatible live bacteria produced HR, whereas treatment with incompatible autoclaved bacteria did not produce HR. Thus, because of the above, and because autoclaved bacteria did not elicit medicarpin production (Fig. 4A), the small prolonged increase in production of activated oxygen molecules did not correspond with the elicitation of phytoalexin accumulation or with development of the HR.

Addition of the antioxidant enzymes SOD and catalase neither decreased bacteria- or HgCl_2 -induced phytoalexin accumulation in white clover suspension-cultured cells nor deterred the development of HR in tobacco leaf tissue. Because addition of the enzymes at levels capable of quenching the oxidative burst had no measurable effect on these two plant defense responses, the oxidative burst does not appear to be a necessary component of elicitation. However, this conclusion may be premature for several reasons.

First, although results from the CL experiments indicate that catalase and SOD were functional for the first 30 min of the plant-microbe interaction, the enzymes may not have retained activity for the entire 24-h incubation period. Even if the enzymes were only temporarily functional and simply delayed elicitation by several hours, induction of phytoalexin accumulation or HR would still have been detected. Thus, we cannot be certain that the 24-h data for phytoalexin induction and HR reflect a lack of effect by catalase and SOD. In support of this possibility is the finding of Keppler and Baker (18). Addition of SOD at the onset of a hypersensitive interaction between tobacco suspension cells and *P. syringae* delayed lipid peroxidation for only 2 h, which suggests that the enzyme was inactivated after 2 h. Keppler and Baker (18) were able to detect these brief effects because they were measuring events occurring within 4 h after the onset of elicitation.

Second, we cannot be sure the catalase permeated the plasmalemma. If the oxidases responsible for producing activated oxygen species are located in the plasmalemma as suggested by Duke (11), then some of the activated oxygen may interact with membrane components rather than with the enzyme scavengers. For example, $\cdot\text{OH}$ is so reactive that it can diffuse only 5 to 10 molecular diameters from its site of formation before reacting with other molecules (9). Some active oxygen species initiate peroxidative chain reactions in the lipid bilayer of cell membranes (8, 23) and alter membrane

protein activity (24, 25). In that case, exogenously added catalase may interact only with activated oxygen species that diffuse beyond the plasmalemma, as is the case for SOD. Under these conditions, quenching the oxidative burst would not interfere significantly with membrane events leading to elicitation of defense responses.

Third, HgCl₂ and activated oxygen species can oxidize free sulfhydryl groups. Oxidation of key sulfhydryl groups alters the function of key membrane pumps (3, 21). Both sulfhydryl reagents (i.e. HgCl₂) and activated oxygen species may have this effect on plasmalemma proteins. Thus, very low concentrations of HgCl₂ may induce a Ca⁺² influx sufficient to cause phytoalexin elicitation without enhanced production of activated oxygen species. The crucial elicitation event may be a massive oxidation of plasma membrane proteins rather than the production of activated oxygen species per se.

Conflicting results concerning the role of an oxidative burst during elicitation of plant defense responses have also been reported elsewhere (1, 2, 13). Although many reports support the hypothesis that activated oxygen species are involved in elicitation, definitive results have not yet been obtained. The results reported here do not support the hypothesis that the pathogen-induced oxidative burst is a necessary element of the elicitation of either phytoalexin production or of HR. Rather, they suggest that, if the oxidative burst is involved in plant defense responses to pathogens, it is an independent event.

LITERATURE CITED

1. Adam A, Farkas T, Somlyai G, Hevesi M, Kiraly Z (1989) Consequence of O₂⁻ generation during a bacterially induced hypersensitive response in tobacco: deterioration of membrane lipids. *Physiol Mol Plant Pathol* **34**: 13–26
2. Apostol I, Heinstein PF, Low PS (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. *Plant Physiol* **90**: 109–116
3. Atkinson MM, Keppler LD, Orlandi EW, Baker CJ, Miscjke CF (1990) Involvement of plasma membrane Ca⁺⁺ influx in bacterial induction of the K⁺/H⁺ and hypersensitive responses in tobacco. *Plant Physiol* **92**: 215–221
4. Babior BM (1978) Oxygen-dependent microbial killing by phagocytes. *N Engl J Med* **298**: 659–668
5. Babior BM, Curnutte JT, Okamura N (1988) The respiratory burst oxidase of the Human Neutrophil, *In* B Halliwell, ed, Oxygen Radicals and Tissue Injury, Proceedings of the Upjohn Symposium. Upjohn, New York, pp 43–48
6. Baker CJ, Atkinson MM, Collmer A (1989) Active oxygen production during a bacteria-induced hypersensitive reaction in tobacco suspension cells. *Phytopathology* **79**: 974–978
7. Baker CJ, O'Neill NR, Keppler D, Orlandi EW (1991) Early responses during plant-bacteria interactions in tobacco cell suspensions. *Phytopathology* **81**: 1504–1507
8. Buege JA, Aust SD (1978) Microsomal lipid peroxidation. *Methods Enzymol* **52**: 302–310
9. Cadenas E (1989) Biochemistry of oxygen toxicity. *Annu Rev Biochem* **58**: 79–110
10. Doke N (1983) Generation of superoxide anion by potato tuber protoplasts during the hypersensitive response to hyphal wall components of *Phytophthora infestans* and specific inhibition of the reaction by suppressors of hypersensitivity. *Physiol Plant Pathol* **23**: 359–367
11. Doke N (1985) NADPH-dependent O₂⁻ generation in membrane fractions isolated from wounded potato tubers inoculated with *Phytophthora infestans*. *Physiol Plant Pathol* **27**: 311–322
12. Elstner EF (1982) Oxygen activation and oxygen toxicity. *Annu Rev Plant Physiol* **33**: 73–96
13. Epperlein MM, Noronha-Dutra AA, Strange RN (1986) Involvement of the hydroxyl radical in the abiotic elicitation of phytoalexins in legumes. *Physiol Mol Plant Pathol* **28**: 66–77
14. Gustine DL (1981) Evidence for sulfhydryl involvement in regulation of phytoalexin accumulation in *Trifolium repens* callus tissue cultures. *Plant Physiol* **68**: 1323–1326
15. Gustine DL (1986) Regulation of accumulated phytoalexin levels in Ladino clover callus. *In* MB Green, PA Hedin, eds, Natural Resistance of Plant to Pests: Roles of Allelochemicals. American Chemical Society, Washington, DC, pp 55–69
16. Gustine DL (1987) Induction of medicarpin biosynthesis in Ladino clover callus by *p*-chloromercuribenzoic acid is reversed by dithiothreitol. *Plant Physiol* **84**: 3–6
17. Gustine DL, Sherwood RT, Moyer BG, Lukezic FL (1990) Metabolites from *Pseudomonas corrugata* elicit phytoalexin biosynthesis in white clover. *Phytopathology* **80**: 1427–1432
18. Keppler LD, Baker JC (1989) O₂⁻-initiated lipid peroxidation in a bacteria-induced hypersensitive reaction in tobacco cell suspensions. *Phytopathology* **79**: 555–562
19. Keppler LD, Baker CJ, Atkinson MM (1989) Active oxygen production during a bacteria-induced hypersensitive response in tobacco suspension cells. *Phytopathology* **79**: 974–978
20. Keppler LD, Novacky A (1986) Involvement of membrane lipid peroxidation in the development of a bacterially induced hypersensitive reaction. *Phytopathology* **76**: 104–108
21. Keppler LD, Novacky A (1987) The initiation of membrane lipid peroxidation during bacteria-induced hypersensitive reaction. *Physiol Mol Plant Pathol* **30**: 233–245
22. Klement Z (1963) Rapid detection of pathogenicity of phytopathogenic Pseudomonads. *Nature* **199**: 299–300
23. Rogers KR, Albert A, Anderson AJ (1988) Lipid peroxidation is a consequence of elicitor activity. *Plant Physiol* **86**: 547–553
24. Slater TF (1984) Free-radical mechanisms in tissue injury. *Biochem J* **222**: 1–15
25. Wolff SP, Garner A, Dean RT (1986) Free radicals, lipids and protein degradation. *Trends Biochem Sci* **11**: 27–31