

## Active Oxygen Production During a Bacteria-Induced Hypersensitive Reaction in Tobacco Suspension Cells

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### ABSTRACT

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In tobacco cell suspensions, onset of the hypersensitive reaction induced by phytopathogenic bacteria can be monitored as increased extracellular pH. Previous reports suggested that active oxygen-initiated lipid peroxidation preceded this increased extracellular pH. Here, we investigated the timing of active oxygen production and increased extracellular pH during the onset of the hypersensitive reaction. Active oxygen levels were monitored by measuring light production (chemiluminescence), in the presence of luminol. Increase in active oxygen levels over time correlated with increased extracellular pH. *Pseudomonas syringae* pv.

*syringae* (wild-type, a pathogen of wheat) was used to induce a hypersensitive reaction in tobacco cell suspensions. *P. s. syringae* B7, a Tn5 insertion mutant that does not induce a hypersensitive reaction, and *P. s. tabaci*, a pathogen of tobacco, were used as controls. All bacterial treatments induced transient increases in both active oxygen and extracellular pH between 0 and 1 hr. Concomitant increases of extracellular pH and active oxygen levels were observed between 2 and 4 hr only in tobacco cell suspensions undergoing the hypersensitive reaction (inoculated with wild-type bacteria).

Active oxygen may be involved in the initiation of the hypersensitive reaction induced by phytopathogenic bacteria. Keppler and Novacky (27,28) reported that lipid peroxidation, initiated by the active oxygen species,  $O_2^-$ , was involved in the development of a bacteria-induced hypersensitive reaction. They reported that lipid peroxidation preceded altered membrane permeability (measured as electrolyte leakage) in cucumber cotyledons undergoing a bacteria-induced hypersensitive reaction. In addition the levels of unsaturated fatty acids (targets of lipid peroxidation) decreased in an enriched plasma membrane fraction from cucumber cotyledons undergoing the bacteria-induced hypersensitive reaction. Addition of the  $O_2^-$  scavenger, superoxide dismutase, inhibited development of the altered membrane permeability (28). Recently, Keppler and Baker (26) reported concurrent transient increases in  $O_2^-$  production and lipid peroxidation during the onset of a bacteria-induced hypersensitive reaction in tobacco cell suspensions, and that  $O_2^-$  scavengers inhibited the transient increase in lipid peroxidation and the development of hypersensitive reaction symptoms.

Involvement of  $O_2^-$  in other plant/pathogen interactions has been reported. Doke (12) and Doke and Chai (13) have reported that  $O_2^-$  radicals are produced by membrane fractions from potato tuber tissue undergoing a hypersensitive reaction induced during an incompatible reaction with *Phytophthora infestans*. More recently, Chai and Doke (11) have reported that  $O_2^-$  radicals are also produced in potato leaf tissue in response to *Phytophthora infestans*.  $O_2^-$  generation also has been reported to be an early phenomenon in a cascade of reactions during an induced defense mechanism of rice leaf to factor(s) from the blast fungus, *Pyricularia oryzae* Cav (32).

The generation of the active oxygen species,  $O_2^-$ , can proceed via transfer of an electron to groundstate molecular oxygen by enzymes such as oxidases or peroxidases (10). Other even more reactive "active oxygen" species can be generated from the  $O_2^-$

radicals, such as hydrogen peroxide ( $H_2O_2$ ), singlet oxygen ( $^1O_2$ ) and the hydroxyl radical ( $OH\cdot$ ).  $H_2O_2$  can be formed by a dismutation reaction between  $O_2^-$  radicals (19,20).



This reaction can proceed nonenzymatically or can be catalyzed by superoxide dismutase (18-20). The  $OH\cdot$  radical can be formed by reaction of  $O_2^-$  with  $H_2O_2$  (the metal mediated Haber-Weiss reaction) (15).



Singlet oxygen ( $^1O_2$ ) can also be formed by the Haber-Weiss reaction (24).



These "active oxygen" species have been described in plants and play important roles in such diverse processes as electron transport in photosynthesis (3,4), polymerization of lignin (21), and senescence (9,17), and can initiate lipid peroxidation (31,33).

The objective of this study was to characterize "active oxygen" ( $O_2^-$  and  $H_2O_2$ ) production during the onset of a bacteria-induced hypersensitive reaction. This was facilitated by using tobacco cell suspensions in which development of the bacteria-induced hypersensitive reaction is characterized by increased extracellular pH (5,6). Using these cell suspensions, we monitored active oxygen levels by measuring chemiluminescence produced by the reaction of active oxygen with luminol (1,22).

### MATERIALS AND METHODS

**Plant cells.** Tobacco suspension cells from cultures in log-phase growth were collected onto Miracloth (Calbiochem, LaJolla, CA) and washed with assay medium that contained 175 mM mannitol, 0.5 mM  $CaCl_2$ , 0.5 mM  $K_2SO_4$  in MES (2-[*N*-morpholino]ethanesulfonic acid) buffer. The MES buffer was 0.5 mM adjusted

to pH 6.00 for experiments requiring pH measurements; MES concentration was increased to 5.0 mM and adjusted to 5.60 for chemiluminescence experiments (to maintain pH between 5.5 and 6 throughout the experiment including subsequent additions of luminol or enzymes). The collected cells were suspended at a concentration of 0.1 g fresh weight ml<sup>-1</sup> of assay medium. Large cell aggregates were removed by filtering the cell suspension through one layer of cheesecloth. For pH assays 15-ml aliquots and for chemiluminescence assays 50-ml aliquots were transferred to 50- and 100-ml beakers, respectively, and allowed to equilibrate for 1.5 hr on a rotary shaker at 27 C and 180 rpm.

**Bacteria.** A nalidixic acid resistant strain (*P. s. syringae* Nal<sup>R</sup>) is incompatible in tobacco and induces a hypersensitive reaction. A transposon mutant, B7 (7), derived from *P. s. syringae* Nal<sup>R</sup> (produces no symptoms in tobacco) and *P. s. tabaci* (compatible, pathogen of tobacco) were used as controls. Bacterial inocula were prepared from 20–22-hr cultures incubated at 30 C on King's B nutrient agar plates. Bacteria were suspended in 0.5 mM MES adjusted to 6.0 with NaOH for pH assays and 5.0 mM MES adjusted to pH 5.60 for chemiluminescence assays. The bacteria were washed by centrifugation and bacterial density determined turbidimetrically. One milliliter of bacterial inoculum or buffer was added to 15 ml of tobacco suspension cells. The final bacterial concentration was 10<sup>8</sup> colony-forming units per milliliter, as checked periodically by dilution plating.

**Extracellular pH and chemiluminescence.** Extracellular pH was measured every 0.5 hr through 4 hr of incubation with a pH electrode and assay buffer containing 0.5mM MES.

Chemiluminescence in the presence of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, from Sigma, St. Louis, MO) was measured with a scintillation counter in the "out-of-coincidence" mode. One-milliliter aliquots of tobacco cell suspension in 5 mM assay buffer were transferred to glass scintillation vials. Vials were placed in the vial holder of the scintillation counter and 10 μl of luminol stock solution added (final concentration of 10 μM) immediately before counting. Luminol stock solution contained 5.0 mM MES buffer, pH 5.60, and enough NH<sub>4</sub>OH (0.58%) to bring the luminol into solution. The final pH of the cell suspension was about 5.75. Chemiluminescence in samples was measured for 1.0 min. Under these conditions, the pH of the assay medium remained between pH 5.5 and 6.0 throughout the 4-hr incubation period (WT 5.77 ± 0.02; *P. s. tabaci* 5.58 ± 0.02; B7 5.57 ± 0.03; buffer 5.55 ± 0.04; four experiments with two replicates per treatment). Preliminary tests showed that pH differences within this range had little effect on the chemiluminescence resulting from addition of luminol. Also tests without luminol showed negligible differences in chemiluminescence between treatments and controls at any time over the 4-hr incubation period.

It has been reported that chemiluminescence measured in the presence of luminol is produced from the reaction of H<sub>2</sub>O<sub>2</sub> and luminol (8). We tested this possibility in the tobacco cell suspensions by assaying the ability of added catalase (Sigma C-40, bovine liver purified powder, thymol-free, EC 1.11.1.6) to inhibit chemiluminescence in the presence of luminol. One-milliliter aliquots of cell suspension were transferred to two scintillation vials, and 10 μl of catalase (in 5.0 mM MES buffer, pH 5.60, containing about 1,000 units activity) was added (final concentration 100 μg of catalase preparation per milliliter) to one of the aliquots. The cells were incubated for 2 min on a rotary shaker (180 rpm) before transfer to the scintillation counter, where luminol was added (10 μM final concentration). Chemiluminescence in parallel samples with and without added catalase, were compared. Preliminary experiments with heat-killed catalase showed insignificant effects on chemiluminescence.

We followed O<sub>2</sub><sup>-</sup> radical production by measuring chemiluminescence in the presence of luminol. O<sub>2</sub><sup>-</sup> radicals in the cell suspensions were converted to H<sub>2</sub>O<sub>2</sub> by incubating with superoxide dismutase. One-milliliter aliquots of cell suspension were transferred to pairs of scintillation vials and superoxide dismutase (Sigma S-4636, EC 1.15.1.1) was added (10 μl) to give a final concentration of 100 μg ml<sup>-1</sup> (1 unit of activity μg<sup>-1</sup>). The cells were incubated for 2 min on a rotary shaker (180 rpm).

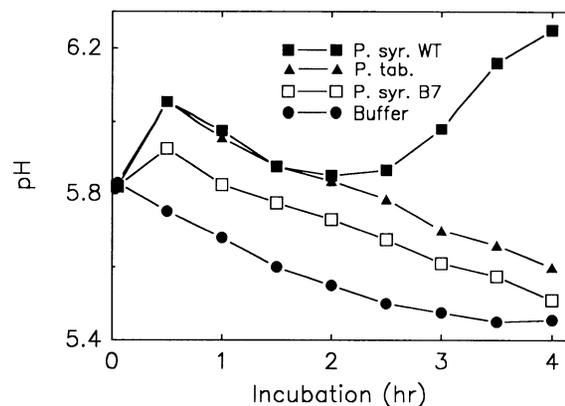
Chemiluminescence due to total H<sub>2</sub>O<sub>2</sub> was then measured as chemiluminescence in the presence of luminol (10 μM). Chemiluminescence in parallel aliquots (with and without added superoxide dismutase) was compared. Preliminary experiments with heat-killed superoxide dismutase showed insignificant effects on chemiluminescence.

H<sub>2</sub>O<sub>2</sub> scavenging in cell suspensions by endogenous catalase (bacterial or plant cell) was assayed. We tested the ability of the catalase inhibitor, aminotriazole (16) (3-amino-1,2,4-triazole; from Sigma), to increase chemiluminescence in the presence of luminol. One-milliliter aliquots of cell suspension were transferred to pairs of scintillation vials, and 10 μl of aminotriazole was added (final concentration of 100 μM) to one of the aliquots. The cells were incubated for 2 min on a rotary shaker (180 rpm). Total H<sub>2</sub>O<sub>2</sub> level was then determined by measuring chemiluminescence in the presence of luminol (10 μM). Chemiluminescence in parallel samples, with and without added aminotriazole, was compared.

## RESULTS

Extracellular pH was equivalent (mean range 5.82–5.83) in tobacco cell suspensions equilibrated for 1.5 hr and then exposed to either bacteria in buffer or buffer alone (Fig. 1). Extracellular pH declined through 4 hr in cell suspensions exposed to buffer alone. Tobacco cell suspensions inoculated with each of the three bacterial inocula induced an early, transient increase in pH between 0 and 1 hr of incubation (Fig. 1). Wild-type and *P. s. tabaci* induced larger pH transient increases than did mutant B7. After these transient increases, extracellular pH declined, through 4 hr, in cell suspensions exposed to B7 and *P. s. tabaci*. In cell suspensions exposed to wild-type bacteria, extracellular pH declined between 0.5 and 2 hr but then increased markedly between 2- and 4-hr incubation.

Tobacco cell suspensions exposed to each of the bacterial inocula resulted in transient increases of chemiluminescence (in the presence of luminol) between 0 and 1 hr of incubation (Fig. 2). Chemiluminescence increased dramatically by 0.5 hr of incubation and declined dramatically between 0.5 and 1 hr in all three bacterial treatments. Chemiluminescence continued to decline in cell suspensions exposed to *P. s. tabaci* and B7 bacteria through 4 hr. In cell suspensions exposed to wild-type bacteria, chemiluminescence continued to decline between 1 and 3.5 hr, but the decline was slower than in *P. s. tabaci* and B7 treatments (Fig. 2). Chemiluminescence increased in the wild-type treatment



**Fig. 1.** Extracellular pH in tobacco cell suspensions. Tobacco cell suspensions (~0.1 g fresh weight ml<sup>-1</sup>) were equilibrated in assay medium (see Materials and Methods) for 1.5 hr and then exposed to bacteria (final concentration of 10<sup>8</sup> cfu ml<sup>-1</sup>) in 0.5 mM MES buffer, pH 6.00. Bacteria used were: wild-type (*Pseudomonas syringae* pv. *syringae*, strain 61; induces a hypersensitive reaction); B7 (Tn5 insertion mutant of wild-type; does not induce a hypersensitive reaction); or *P. s. tabaci* (compatible pathogen on tobacco). Values are means of extracellular pH from a minimum of six experiments each with two replicates; standard errors <±0.025 pH units.

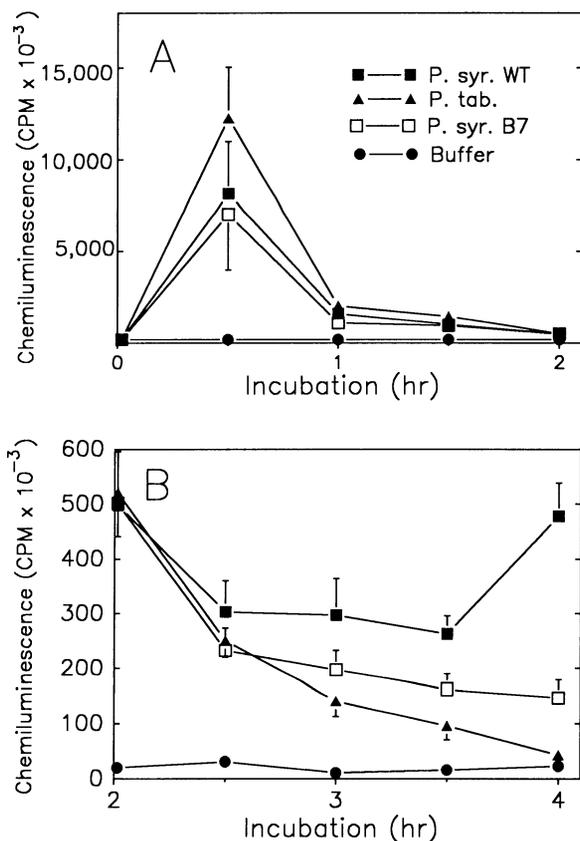
between 3.5 and 4 hr. No increase in the level of chemiluminescence was observed in cell suspensions exposed to buffer alone and the level was much lower than in cell suspensions exposed to bacteria throughout the 4-hr incubation (Fig. 2).

The bacteria-induced increase in tobacco cell suspension chemiluminescence, in the presence of luminol, was produced primarily by reaction between  $H_2O_2$  and luminol. When tobacco cell suspensions inoculated with each of the bacterial inocula were incubated for 2 min with catalase ( $100 \mu\text{g ml}^{-1}$ ), chemiluminescence (in the presence of luminol) was inhibited dramatically. After 0.5 hr of incubation (when chemiluminescence without added catalase was maximal) chemiluminescence was inhibited  $\geq 98\%$  in all bacteria treatments. Throughout the 4-hr incubation, chemiluminescence (in the presence of luminol and catalase) in all bacteria treatments was within 20,000 CPM of the values observed in buffer treated controls in Figure 2.

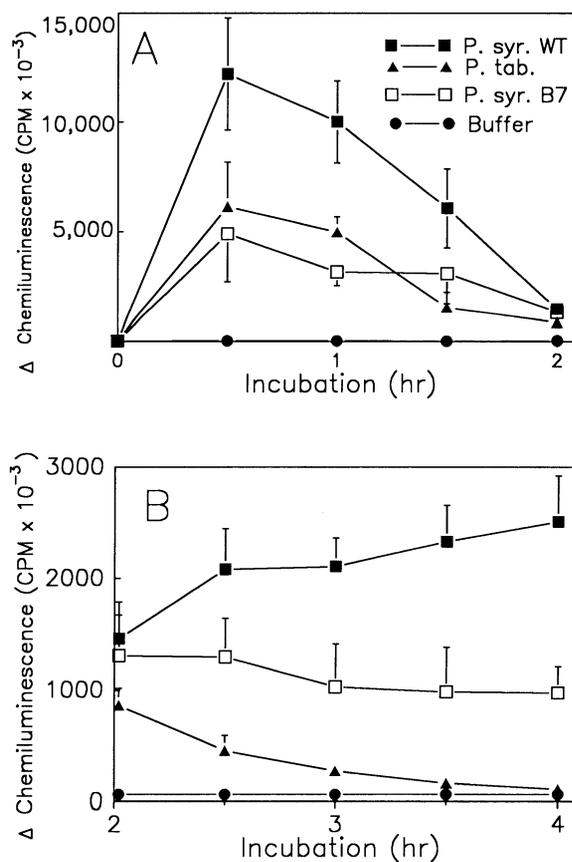
Superoxide,  $O_2^-$ , levels were estimated by conversion to  $H_2O_2$  by incubating with superoxide dismutase and measuring the increase in chemiluminescence. A transient burst of  $O_2^-$ , increased chemiluminescence due to superoxide dismutase, was observed between 0 and 1 hr of incubation in tobacco cell suspensions exposed to each of the bacteria (Fig. 3).  $O_2^-$  levels were maximal in all bacteria treatments between 0 and 1 hr, and  $O_2^-$  production was highest in cell suspensions exposed to wild-type bacteria.  $O_2^-$  levels declined dramatically between 0.5 and 2 hr in cell suspensions exposed to each of the bacteria. In cell suspensions

exposed to *P. s. tabaci* or B7 bacteria,  $O_2^-$  levels continued to decline through 4 hr. The levels of  $O_2^-$  increased between 2 and 4 hr of incubation in cell suspensions exposed to wild-type bacteria. Between 2.5 and 4 hr,  $O_2^-$  levels were greater in cell suspensions treated with wild-type bacteria than in the other treatments. No increase in the level of  $O_2^-$  was observed in cell suspensions exposed to buffer alone, and the level was much lower than in cell suspensions exposed to bacteria, throughout the 4 hr of incubation (Fig. 3).

The dramatic decrease of chemiluminescence (in the presence of luminol) observed in all bacteria treatments (Figs. 2 and 4) indicates that  $H_2O_2$  is rapidly scavenged in the tobacco cell suspensions. Therefore, we looked for scavenging of  $H_2O_2$  by endogenous catalase. Catalase activity was detected in cell suspensions exposed to each of the bacteria (Fig. 4). Incubation with the catalase inhibitor, aminotriazole, increased chemiluminescence (in the presence of luminol) in cell suspensions exposed to bacteria but not in cell suspensions exposed to buffer alone. Between 1 and 4 hr of incubation, scavenging of  $H_2O_2$  by catalase was highest in wild-type treated cell suspensions.



**Fig. 2.** Chemiluminescence (in the presence of luminol) in tobacco cell suspensions. Tobacco cell suspensions ( $\sim 0.1$  g fresh weight  $\text{ml}^{-1}$ ) were equilibrated in assay medium (see Materials and Methods) for 1.5 hr and then exposed to bacteria (final concentration of  $10^8$  cfu  $\text{ml}^{-1}$ ) in 5.0 mM MES buffer, pH 5.60. Bacteria used were: wild-type (*Pseudomonas syringae* pv. *syringae*, strain 61; induces a hypersensitive reaction); B7 (Tn5 insertion mutant of wild-type; does not induce a hypersensitive reaction); or *P. s. tabaci* (compatible pathogen on tobacco). Aliquots were transferred to scintillation vials and placed in a scintillation counter. Luminol was added (final concentration of  $10 \mu\text{M}$ ), and chemiluminescence measured (for 1.0 min) in the "out-of-coincidence" mode. Values are means  $\pm$  standard error from a minimum of four experiments each with two replicates.

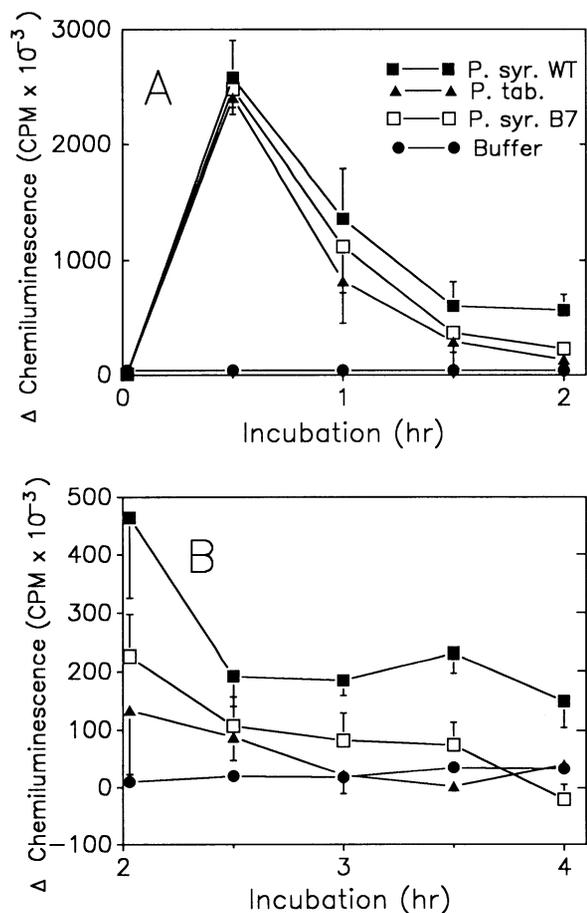


**Fig. 3.** Superoxide dismutase increased chemiluminescence (in the presence of luminol) in tobacco cell suspensions. Tobacco cell suspensions ( $\sim 0.1$  g fresh weight  $\text{ml}^{-1}$ ) were equilibrated in assay medium (see Materials and Methods) for 1.5 hr and then exposed to bacteria (final concentration of  $10^8$  cfu  $\text{ml}^{-1}$ ) in 5.0 mM MES buffer, pH 5.60. Bacteria used were: wild-type (*Pseudomonas syringae* pv. *syringae*, strain 61; induces a hypersensitive reaction); B7 (Tn5 insertion mutant of wild-type; does not induce a hypersensitive reaction); or *P. s. tabaci* (compatible pathogen on tobacco). Parallel 1.0-ml aliquots were transferred to scintillation vials. Superoxide dismutase (final concentration of  $100 \mu\text{g ml}^{-1}$ ) was added to one vial and incubated for 2 min. The scintillation vials were placed in the scintillation counter, luminol added (final concentration of  $10 \mu\text{M}$ ) and chemiluminescence measured (for 1.0 min) in the "out-of-coincidence" mode. Values are means  $\pm$  standard errors of increased chemiluminescence affected by addition of superoxide dismutase between parallel aliquots and are the result of a minimum of four experiments each with two replicates.

## DISCUSSION

Doke et al (14) have proposed that  $O_2^-$  levels increase in two steps during interactions between plants and phytopathogenic fungi. An increase of  $O_2^-$  (step 1) occurs early in both the compatible (normal disease development) and incompatible combinations (hypersensitive reaction produced). Later  $O_2^-$  production increases only in the incompatible combination (step 2). We observed a similar situation in a plant-bacteria interaction.

An early (step 1), transient increase in active oxygen levels (both  $O_2^-$  and  $H_2O_2$ ) occurs in both the compatible and incompatible combinations (Figs. 2 and 3). After this initial increase, levels of active oxygen compounds, both  $H_2O_2$  and  $O_2^-$ , continued to decline in the compatible combination (cell suspensions plus *P. s. tabaci*). Active oxygen levels also continued to decline in cell suspensions exposed to B7, a mutant of wild-type *P. s. syringae* (does not induce a hypersensitive reaction). In tobacco cell suspensions exposed to wild-type *P. s. syringae* (induces a hypersensitive reaction),  $O_2^-$  levels increased between 2 and 4 hr (step 2). In cell suspensions treated with wild-type



**Fig. 4.** Effect of added catalase inhibitor, aminotriazole, on chemiluminescence (in the presence of luminol) in tobacco cell suspensions. Tobacco cell suspensions ( $\sim 0.1$  g fresh weight  $ml^{-1}$ ) were equilibrated in assay medium (see Materials and Methods) for 1.5 hr and then exposed to bacteria (final concentration of  $10^8$  cfu  $ml^{-1}$ ) in 5.0 mM MES buffer, pH 5.60. Bacteria used were: wild-type (*Pseudomonas syringae* pv. *syringae*, strain 61; induces a hypersensitive reaction); B7 (Tn5 insertion mutant of wild-type; does not induce a hypersensitive reaction); or *P. s. tabaci* (compatible pathogen on tobacco). Parallel 1.0-ml aliquots were transferred to scintillation vials. Aminotriazole (final concentration of 100  $\mu M$ ) was added to one vial and incubated for 2 min. The scintillation vials were then placed in the scintillation counter, luminol added (final concentration of 10  $\mu M$ ) and chemiluminescence measured (for 1.0 min) in the "out-of-coincidence" mode. Values are means  $\pm$  standard error of the change in chemiluminescence affected by addition of aminotriazole between parallel aliquots, and are the result of a minimum of four experiments each with two replicates.

bacteria,  $H_2O_2$  levels did not increase until after 3.5 hr, but the levels of  $H_2O_2$  between 2 and 4 hr were higher than in cell suspensions exposed to the other bacteria. The elevated active oxygen levels between 2 and 4 hr in the incompatible combination are actually even greater because the levels of catalase-mediated scavenging of  $H_2O_2$  was highest in wild-type treated cell suspensions (Fig. 4).

Atkinson et al (5,6) previously reported that extracellular pH increased within 1.5–2 hr after tobacco cell suspensions were inoculated with *P. s. pisi* (which is capable of inducing a hypersensitive reaction in tobacco leaves). We have previously reported (7,25,26) that the model system used in this investigation produces a similar increase in extracellular pH after 2 hr and continues through 8 hr; this investigation followed only the first 4 hr (Fig. 1). Here we report for the first time an early transient pH increase that occurs within the first hour of exposure of tobacco cell suspensions to wild-type and B7, *P. s. syringae* and *P. s. tabaci* bacteria (Fig. 1).

Increases in extracellular pH and levels of active oxygen were positively correlated during the 4 hr of incubation for all treatments. Coincident, transient increases in extracellular pH and levels of  $H_2O_2$  and  $O_2^-$  occurred in cell suspensions within 1 hr after inoculation with bacteria. Concurrent increases in extracellular pH and levels of  $O_2^-$  and  $H_2O_2$  levels also occurred between 2 and 4 hr in cell suspensions exposed to wild-type bacteria. The correlation between increased extracellular pH and active oxygen levels could indicate that an increase in one affects an increase in the other. Increased chemiluminescence affected by increased extracellular pH can be ruled out because in all chemiluminescence measurements, cell suspensions were adequately buffered to prevent any increase in extracellular pH that could affect chemiluminescence. Therefore, it is possible that increased extracellular pH could be the result of active oxygen production.

We are currently investigating possible involvement of oxidase and/or peroxidase mediated active oxygen production in the development of the hypersensitive reaction. At least two mechanisms are possible. Reduction of extracellular  $O_2$  to  $O_2^-$  by a plasma membrane bound NAD(P)H oxidase to  $O_2^-$  and subsequent dismutation of  $O_2^-$  ( $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ ) to  $H_2O_2$  would increase extracellular pH. Also, removal of  $H^+$ 's from solution have been reported during the oxidation of NAD(P)H by peroxidase (2,23,29,30).  $O_2^-$  radicals are involved in this reaction because superoxide dismutase has been reported to inhibit the reaction (21).

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