

O₂⁻-Initiated Lipid Peroxidation in a Bacteria-Induced Hypersensitive Reaction in Tobacco Cell Suspensions

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ABSTRACT

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O₂⁻-Initiated lipid peroxidation was observed in a hypersensitive reaction induced in an incompatible plant/bacterium combination of tobacco cell suspension and the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae*. The earliest symptoms of this hypersensitive reaction are increases in extracellular pH and K⁺. A Tn5 insertion mutant of *P. s. syringae*, which does not induce the hypersensitive reaction, and *Pseudomonas syringae* pv. *tabaci*, a pathogen of tobacco, were used as controls. Transient increases in lipid peroxidation and O₂⁻ production

which preceded the initial increases in extracellular pH and K⁺ were observed. Recovery of *P. s. syringae* was lower from cell suspensions undergoing a hypersensitive reaction than from those treated with bacteria that did not induce a hypersensitive reaction. O₂⁻ scavengers added with *P. s. syringae* inhibited initial transient O₂⁻ production and lipid peroxidation and increased recovery of bacteria. O₂⁻-Initiated lipid peroxidation seems to be involved in the hypersensitive reaction, and O₂⁻ radicals may be involved in reduced recovery of bacteria during the reaction.

Normal plant cell membrane structure and function are altered during the hypersensitive reaction (HR) to phytopathogenic bacteria. Early investigators reported dramatic changes in leaf tissue membrane permeability, as measured by electrolyte leakage (8,20). More recently, a decline in membrane potential during bacteria-induced HR was reported (25,30,31). Effects on that part of the membrane potential controlled by diffusion rather than active transport were implicated in the decline. Alteration of the lipid phase was suggested (30,31).

A possible mechanism for the alteration of the membrane lipid phase is lipid peroxidation. During processes such as leaf senescence (11), ozone injury (29), anoxia (22), susceptibility to drought (10), herbicide injury (6), wounding (33), and virus-induced HR (23), a positive correlation between altered membrane permeability and increases in lipid peroxidation has been reported. Keppler and Novacky (25) investigated possible lipid peroxidation-mediated membrane lipid phase alteration during bacteria-induced HR and reported that a significant increase in lipid peroxidation (between 1.5 and 3 hr incubation) preceded membrane alteration (expressed as increased electrolyte leakage, which was initially observed between 3 and 4 hr of incubation). Also, application of superoxide dismutase (SOD), a specific scavenger of O₂⁻ radicals, decreased the rate of electrolyte leakage (26). They suggested that membrane lipid peroxidation, possibly initiated by O₂⁻ radicals, was the mechanism by which the plasma membrane was altered during bacteria-induced HR.

Lipid peroxidation may be initiated by production of active oxygen species by plant cells in response to the incompatible pathogen. In animals, polymorphonuclear lymphocytes generate superoxide radicals (O₂⁻) in the course of phagocytosis following recognition of foreign bacteria (3). In plants, Doke (13-15) and Doke and Chai (16) have reported that O₂⁻ radicals are produced by membrane fractions from potato tuber tissue undergoing HR induced by an incompatible race of *Phytophthora infestans* but are not produced in response to a compatible race of the pathogen. More recently, Chai and Doke (5) reported that O₂⁻ radicals are

produced in potato leaf tissue in response to the incompatible race of the pathogen. Also, O₂⁻ generation has been reported to be an early phenomenon in a cascade of reactions during an induced defense mechanism of rice leaves to factor(s) from blast fungus (*Pyricularia oryzae* Cav.) (32).

The objective of this study was to further evaluate possible involvement of O₂⁻-Initiated lipid peroxidation in HR initiation and to test for possible inhibitory effects of the putative O₂⁻ generation and/or subsequent lipid peroxidation on HR-inducing bacteria.

This investigation was facilitated by the use of tobacco suspension cells exposed to an HR-inducing bacterium, *Pseudomonas syringae* pv. *syringae*. HR development in this plant tissue/pathogen combination has been well characterized. Atkinson et al (1,2) have reported that a K⁺ efflux/H⁺ influx exchange is initiated by the addition of HR-inducing bacteria. This K⁺/H⁺ exchange can be monitored rapidly and accurately by measuring extracellular pH and conductivity. Thus, initiation of the HR can be followed with precision. Also, the use of tobacco suspension cells simplifies measurement of lipid peroxidation, O₂⁻ production, and bacterial growth and facilitates treatment of plant cells with reagents such as O₂⁻ scavengers.

MATERIALS AND METHODS

Plant cells. Tobacco suspension cells from cultures in log-phase growth were collected onto Miracloth (Calbiochem, LaJolla, CA) and washed with 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, and 1.0 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, adjusted to pH 6.00 with NaOH (assay medium). The collected cells were suspended at a concentration of 0.1 g fresh weight ml⁻¹ of assay medium. Large-cell aggregates were removed by filtering the cell suspension through one layer of cheesecloth. For all assays except measurement of ethylene and ethane evolution, 15-ml aliquots of collected cell suspension were transferred to 50-ml beakers and preincubated for 1 hr on a rotary shaker at 27 C and 180 rpm.

Bacteria. *P. s. syringae* strain 61 (wild type) was provided by M. Sasser, University of Delaware, Newark. In previous experiments (4), a nalidixic acid resistant strain, which gave wild-type symptoms, was selected (*P. s. syringae* NaI^R) for transposon

mutagenesis. Transposon mutants of the strain were selected that did not induce HR symptoms in tobacco cell suspensions or leaf tissue or induce K^+/H^+ exchange in tobacco cells. One of these Tn5 insertion mutants, B7, and the compatible bacterial pathogen *Pseudomonas syringae* pv. *tabaci* were used as controls. The Tn5 insertion mutant, B7, produces no symptoms in tobacco leaf tissue (observations in our laboratory). Bacterial inocula were prepared from 20–22 hr cultures incubated at 30 C on King's B nutrient agar plates. Bacteria were suspended in 1.0 mM MES adjusted to pH 6.00 with NaOH and washed by centrifugation. Bacterial density was determined spectrophotometrically ($\lambda = 500$ nm). One milliliter of bacterial inoculum or buffer was added to 15 ml of tobacco suspension cells. The final bacterial concentration was 10^8 colony-forming units (cfu) ml^{-1} .

Bacterial growth in cell suspensions. Bacteria were recovered from tobacco cell suspensions 0, 2, and 4 hr after introduction of bacteria. The level of bacteria in the complete cell suspension (bacteria free in the bathing medium and bacteria closely associated with tobacco cells) was monitored. Aliquots (0.1 ml) of complete cell suspensions were serially diluted, and 10 μ l of each dilution (two replicates/dilution) was spotted on King's B nutrient agar (2.0%) plates. Colonies in each spot were counted after incubation of plates for 24 hr at 30 C. The level of bacteria closely associated with tobacco cells (attached or in close proximity to tobacco cells) also was monitored. One-milliliter aliquots of tobacco cell suspensions were centrifuged (maximum speed for 1 min on a clinical tabletop centrifuge), and the supernatant solution was removed with a Pasteur pipet. The pellet was ground with a glass rod, diluted with 1 ml of sterilized, deionized water, and resuspended. Aliquots (0.1 ml) were serially diluted, and 10- μ l aliquots of the dilutions were spotted on King's B nutrient agar (2.0%) plates. Plates were incubated and colonies were counted as described above.

Extracellular pH and conductivity. Extracellular pH and conductivity were monitored for the first 4 hr of incubation. Extracellular conductivity and pH were measured with a conductivity meter and a pH meter, respectively. To eliminate possible changes in the initial extracellular pH by the addition of bacteria, O_2^- scavengers or other additives that might alter subsequent HR development, changes in extracellular pH also were monitored using a titration procedure. Tobacco suspension cells were incubated in an assay medium containing the same components described earlier, except 2.0 mM MES buffer was used. This assay medium was adjusted to pH 5.75 with NaOH. Upon addition of bacteria and every 30 min thereafter, suspensions were titrated to pH 5.75 with acid (24 mM HCl) or base (24 mM NaOH). Changes in extracellular pH were expressed as the nanomoles of acid or base ml^{-1} of cell suspension required to maintain the pH at 5.75.

Ethylene evolution. Tobacco cell samples (1.5 g fresh weight plus 0.9 ml of assay medium) were placed on top of a 5-ml layer of solidified assay medium (containing 2.0% agar) in a 25-ml Erlenmeyer flask. The cells were incubated at room temperature on a flatbed rotary shaker (150 rpm). After 1 hr of incubation, 0.1 ml of bacterial suspension in MES buffer (pH 6.00) at a final concentration of 10^8 cfu ml^{-1} or buffer alone was added. Flasks were sealed with a rubber cap 0, 1, 2, or 3 hr after inoculation. After 1 hr of incubation, 1 ml of the headspace gas was removed with a hypodermic syringe. The level of ethylene in the headspace gas was determined using a Poropak N column on a gas chromatograph (Perkin-Elmer Corp., Norwalk, CT). The retention time of the ethylene peak was verified using an ethylene standard and an ion trap detector (Finnigan Corp., San Jose, CA). The concentration of evolved ethylene was determined using a standard curve generated by injection of known concentrations of an ethylene standard.

Lipid peroxidation. Lipid peroxidation was monitored by measuring thiobarbituric acid reactivity of lipid peroxidation breakdown products (21), autofluorescence characteristic of Schiff's bases produced by reaction of aldehyde groups of lipid peroxidation breakdown products and amine groups of proteins and lipids (7,12,18,34), and production of the volatile lipid

peroxidation breakdown product ethane (28).

Thiobarbituric acid reactivity. Three-milliliter aliquots of tobacco suspension cells were collected by centrifugation. The cells were frozen with liquid N_2 and ground with a glass rod, and 4 ml of 0.5% thiobarbituric acid (w/v) and 0.01% (w/v) butylated hydroxytoluene was added. The solutions were incubated at 95 C for 30 min and cooled to room temperature, and absorbance at 532 and 600 nm was measured. Nonspecific absorbance at 600 nm was subtracted, and the extinction coefficient for malondialdehyde ($156 \times 10^{-3} M^{-1} \cdot cm^{-1}$) was used to estimate lipid peroxidation (11).

Autofluorescence. One-milliliter aliquots of tobacco cell suspensions were centrifuged and the supernatant solutions were removed. Cell pellets were extracted with 1 ml of 100% ethanol. Extracted cell debris was removed by centrifugation. Autofluorescence of the supernatant solution characteristic of Schiff's bases was measured at 350 and 420 nm, excitation and emission wavelengths, respectively (7,12,18,34).

Ethane evolution. Ethane evolution from tobacco cell suspensions was measured concurrently with ethylene evolution as described above. Retention time of the ethane peak was verified using an ethane standard and an ion trap detector (Finnigan Corp.). Ethane concentration was determined from a standard curve.

Addition of O_2^- scavengers. Tiron (35) has been reported to specifically react with O_2^- and to inhibit reactions requiring O_2^- (27). SOD can scavenge O_2^- by the following reaction: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (19). Tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid) (1 mM) and SOD (100 μ g ml^{-1} , 1 unit of activity μ g $^{-1}$) were added (separately and together) with wild-type bacteria to tobacco suspension cells. Tiron was obtained from Aldrich Chemical Co., Milwaukee, WI. SOD was obtained from Sigma Chemical Co., St. Louis, Mo. To rule out possible effect on bacterial growth rate by the radical scavengers, we verified that these concentrations of tiron and SOD alone or together did not affect the growth rate of the wild-type bacteria in a minimal medium. The minimal medium (9) contained K_2HPO_4 , 10.5 mg ml^{-1} ; KH_2PO_4 , 4.5 mg ml^{-1} ; $(NH_4)_2SO_4$, 1.0 mg ml^{-1} ; trisodium citrate $\cdot 2H_2O$, 0.5 mg ml^{-1} ; $MgSO_4 \cdot 7H_2O$, 0.25 mg ml^{-1} ; and sucrose, 1.0 mg ml^{-1} . The growth of bacteria in minimal medium was followed spectrophotometrically ($\lambda = 500$ nm) every 2 hr for 8 hr. The maximum concentration that did not affect bacterial growth on minimal medium was 1 mM tiron, and therefore it was used for subsequent experiments. A SOD concentration of 100 μ g ml^{-1} (1 unit of activity μ g $^{-1}$) alone or together with 1 mM tiron had no effect on bacterial growth rate on minimal medium.

O_2^- production. O_2^- production was determined using the method of Doke (14) with the following modifications. One-milliliter aliquots of tobacco cell suspension, treated with *P. s. syringae* (wild-type), B7, or bacteria of *P. s. tabaci* in pH 6.00 1.0 mM MES buffer or buffer alone, were incubated for 10 min with 0.05% nitroblue tetrazolium. Tobacco and bacterial cells were removed from samples by centrifugation in an Eppendorf microfuge, Brinkmann Instruments Co. Westbury, NY. (5 min). Supernatant solution (0.75 ml) was transferred to test tubes and heated for 15 min at 85 C. After the samples were allowed to cool to room temperature, reduced formazan was measured by recording absorbance at 580 nm. Reduction of nitroblue tetrazolium to formazan by O_2^- was verified by the ability of SOD to inhibit the reaction.

RESULTS

HR symptoms. Extracellular pH increased between 2 and 4 hr incubation in suspension cells treated with the HR-inducing bacterium (*P. s. syringae*, wild type) (Table 1). Also, when extracellular pH was followed by acid/base titration, only wild-type-treated cell suspensions required addition of acid between 2 and 4 hr to maintain the cell suspension pH at 5.75 (Table 1). Conductivity of tobacco cell suspensions also increased between 2 and 4 hr after addition of wild-type bacteria (Table 2). Conductivity also increased in cell suspensions treated with the compatible pathogen *P. s. tabaci* (Table 2) but to a much lower

TABLE 1. Changes in tobacco cell suspension pH^a

Treatments ^b	Change in pH incubation period (hr)			Change in pH (acid/base titration) ^c incubation period (hr)		
	1 to 2	2 to 3	3 to 4	1 to 2	2 to 3	3 to 4
WT	-0.09 ± 0.03	+0.27 ± 0.05	+0.19 ± 0.06	-24.0 ± 11.5	+129.6 ± 10.6	+101.3 ± 21.1
B7	-0.12 ± 0.03	-0.13 ± 0.03	-0.14 ± 0.05	-59.0 ± 6.7	-60.5 ± 14.9	-54.2 ± 14.9
TAB	-0.14 ± 0.02	-0.09 ± 0.02	-0.07 ± 0.03	-13.4 ± 10.1	-33.1 ± 12.0	-47.0 ± 11.5
Buffer	-0.12 ± 0.05	-0.05 ± 0.03	-0.03 ± 0.02	-30.2 ± 11.5	-31.2 ± 17.8	-61.0 ± 21.1

^a Values are means ± standard error of a minimum of four experiments with two replicates per treatment.

^b Buffer (1.0 mM 2-[N-morpholino]ethanesulfonic acid, pH 6.00) alone or bacteria in buffer (final concentration = 10⁸ colony-forming units ml⁻¹) was added to tobacco cell suspensions. Bacteria used were: WT (wild type; *Pseudomonas syringae* pv. *syringae*, strain 61; induces hypersensitive reaction); B7 (Tn5 insertion mutant of WT; does not induce hypersensitive reaction); or TAB (*Pseudomonas syringae* pv. *tabaci*; compatible pathogen of tobacco).

^c Tobacco cell suspensions were titrated to pH 5.75 every 30 min. Values are net nanomoles acid (+) or base (-) ml⁻¹ of cell suspension.

TABLE 2. Changes in tobacco cell suspension conductivity^a

Treatments ^c	Change in conductivity ^b incubation period (hr)		
	1 to 2	2 to 3	3 to 4
WT	-4.3 ± 1.8	+9.8 ± 1.0	+15.3 ± 2.8
B7	-7.3 ± 1.3	-8.3 ± 1.3	-5.5 ± 0.6
TAB	-1.8 ± 0.8	+2.1 ± 1.2	+2.5 ± 0.8
Buffer	-10.0 ± 0.7	-8.3 ± 2.1	-7.2 ± 0.7

^a Values are means ± standard error of a minimum of four experiments with two replicates per treatment.

^b Units are μS cm⁻¹.

^c Buffer (1.0 mM 2-[N-morpholino]ethanesulfonic acid, pH 6.00) alone or bacteria in buffer (final concentration = 10⁸ colony-forming units ml⁻¹) was added to tobacco cell suspensions. Bacteria used were: WT (wild type; *Pseudomonas syringae* pv. *syringae*, strain 61; induces hypersensitive reaction); B7 (Tn5 insertion mutant of WT; does not induce hypersensitive reaction); or TAB (*Pseudomonas syringae* pv. *tabaci*; compatible pathogen of tobacco).

level than in wild-type-treated cell suspensions.

Ethylene production was highest in suspension cells treated with wild-type bacteria (Fig. 1). Between 1 and 2 hr of incubation, ethylene evolution from cell suspensions exposed to wild-type bacteria exceeded the level produced by cell suspensions treated with B7, *P. s. tabaci*, or buffer. Ethylene evolution remained highest in wild-type-treated cell suspensions through 4 hr.

Bacterial growth in tobacco cell suspensions. Increased levels of wild-type, B7, and *P. s. tabaci* bacteria were recovered after 4 hr of incubation in tobacco cell suspensions. Increased levels of bacteria were recovered both from the complete cell suspension (Fig. 2A) and from close association with the tobacco cells (Fig. 2B). After 4 hr of incubation, recovery of viable wild-type bacteria from the complete cell suspension had increased 54 and 27% in the tobacco cell fraction. Levels of B7 and *P. s. tabaci* recovered increased to even higher levels: 160 and 251%, respectively, in the complete cell suspension and 185 and 141%, respectively, in the tobacco cell fraction. In addition, fewer wild-type bacteria were recovered (number of bacteria recovered was below the 0-hr level) in both the complete cell suspension and the tobacco cell fraction after 2 hr of incubation. Levels of *P. s. tabaci* and B7 did not decrease below the 0-hr level in either the complete cell suspension or the tobacco cell fraction.

Lipid peroxidation. Transient increases in lipid peroxidation measured as ethane evolution (Fig. 3A), thiobarbituric acid reactivity (Fig. 3B), and autofluorescence (Fig. 3C) were observed only in suspension cells treated with wild-type bacteria. Transient increases in thiobarbituric acid reactivity and ethane evolution were observed between 1 and 3 hr of incubation. Autofluorescence declined from the 1-hr level in all treatments except the wild-type cell suspension. Autofluorescence remained at the 1-hr level after 2 and 3 hr of incubation in wild-type cell suspensions and therefore was higher than in B7, *P. s. tabaci*, and buffer treatments after 2 and 3 hr of incubation.

Effect of O₂⁻ scavengers. Addition of O₂⁻ scavengers SOD and

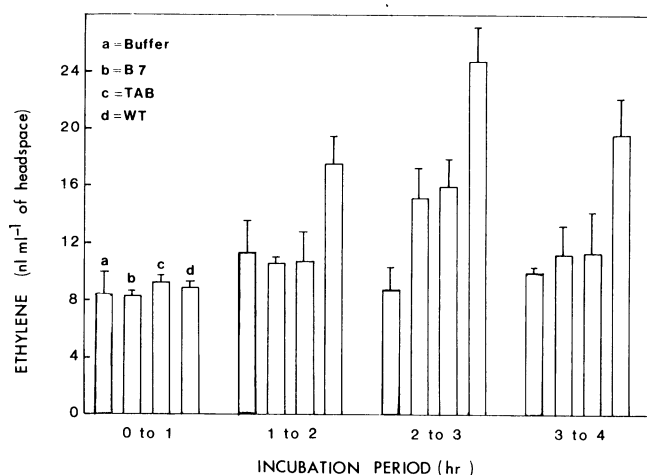


Fig. 1. Ethylene evolution from tobacco cell suspensions. Cell suspensions were exposed to bacteria (final concentration of 10⁸ colony-forming units ml⁻¹) in 2-(N-morpholino)ethanesulfonic acid buffer (1.0 mM, pH 6.00) or buffer alone (a). Bacteria used were B7 (Tn5 insertion mutant of wild type; does not induce hypersensitive reaction [HR]) (b); *Pseudomonas syringae* pv. *tabaci* (TAB) (compatible pathogen of tobacco) (c); or wild type (WT) (*Pseudomonas syringae* pv. *syringae* strain 61; induces HR) (d). Values are means ± standard error of nl ml⁻¹ of headspace from a minimum of four experiments (containing all treatments) with two replicates per experiment.

tiron (separately or together) delayed the transient increase in lipid peroxidation, measured as ethane evolution (Table 3), in suspension cells treated with wild-type bacteria. Lipid peroxidation was inhibited during the incubation periods of 0 to 1 and 1 to 2 hr. But lipid peroxidation was higher in cell suspensions treated with wild-type bacteria and O₂⁻ scavengers than in cell suspensions treated with wild-type bacteria alone during the incubation period of 2 to 3 hr. Tiron and SOD (separately and together) also decreased ethylene evolution in cell suspensions treated with wild-type bacteria (Table 3).

Increased extracellular pH (Table 4) and conductivity (Table 5) observed between 2 and 4 hr of incubation in tobacco suspension cells treated with wild-type bacteria was less when SOD was added alone or with tiron. When tiron was added alone, increased extracellular pH (Table 4) was lowered but the increased conductivity (Table 5) was not. Moderation of extracellular pH increase was not the result of a pH change produced by the addition of the O₂⁻ scavengers. When pH of wild-type-treated cell suspensions was maintained at 5.75 (by acid titration), addition of the O₂⁻ scavengers did reduce the nanomoles of acid required to maintain the pH at 5.75 (Table 4). Also, addition of the O₂⁻ scavengers reduced extracellular pH and conductivity differences between wild-type-treated and B7-treated cell suspensions (Table 6). Because the Tn5 insertion mutant B7 does not induce the HR, we suggest that mathematical differences of wild-type-treated and B7-treated tobacco cell suspension extracellular pH and conductivity may be used as measures of the HR.

Addition of tiron, SOD, or tiron and SOD together with wild-type bacteria increased the levels of wild-type bacteria recovered from tobacco cell suspensions throughout the 4-hr incubation

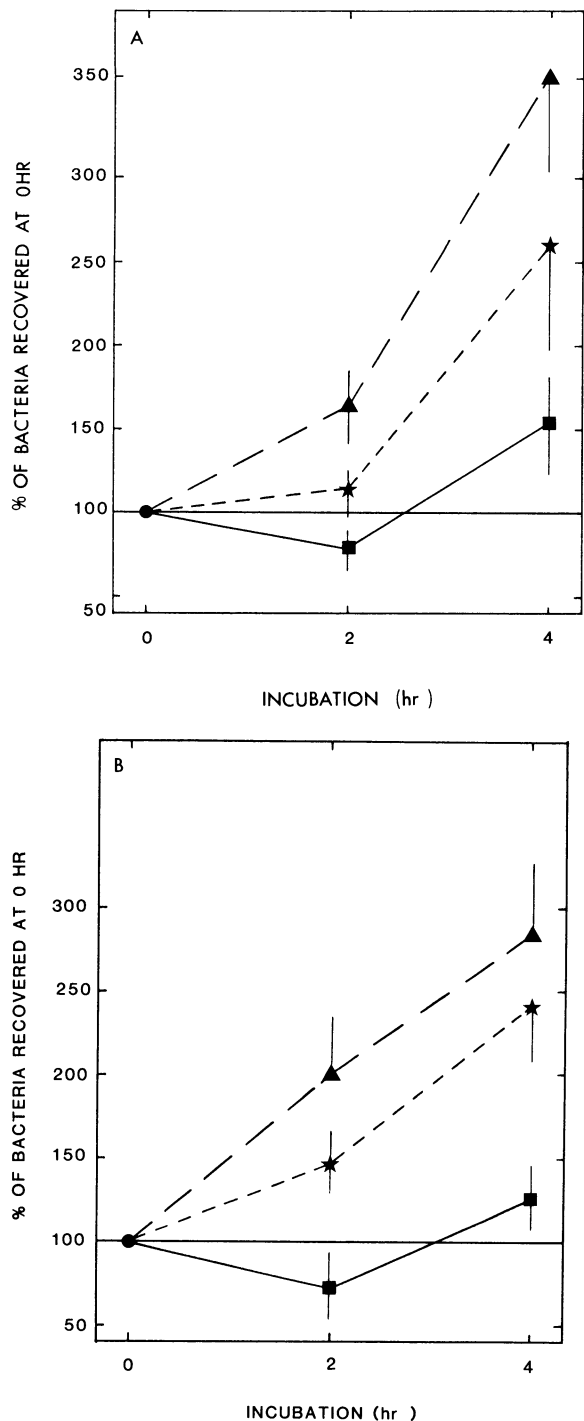


Fig. 2. Bacterial growth in tobacco cell suspensions. Tobacco cell suspensions were exposed to 10^8 colony-forming units (cfu) ml^{-1} (final concentration) of the following bacteria: wild type (*Pseudomonas syringae* pv. *syringae* strain 61; induces hypersensitive reaction [HR]) (▲); B7 (Tn5 insertion mutant of wild type; does not induce HR) (★); or *Pseudomonas syringae* pv. *tabaci* (compatible pathogen of tobacco) (■). Bacteria were recovered: **A**, from the complete cell suspension (bacteria free in the bathing medium and those closely associated with tobacco cells), and **B**, from close association with the tobacco cells. Data are plotted as mean of the percent of the number of bacteria recovered immediately after the addition of bacteria. Means \pm standard error ($n \geq 8$) of the number of bacteria ($\times 10^7$ cfu ml^{-1}), recovered immediately after addition, from the complete cell suspension and from close association with tobacco cells were: wild type (7.1 ± 0.73 , 2.6 ± 0.30); B7 (6.3 ± 0.63 , 1.3 ± 0.38); and *P. s. tabaci* (5.3 ± 0.59 , 1.7 ± 0.28).

period. After 4 hr of incubation, levels of wild-type bacteria recovered from the complete cell suspension were increased 25, 79, and 65% by the addition of tiron, SOD, and tiron plus SOD, respectively (Fig. 4A). Recovery of wild-type bacteria from close association with tobacco cells was increased 50, 52, and 73% after

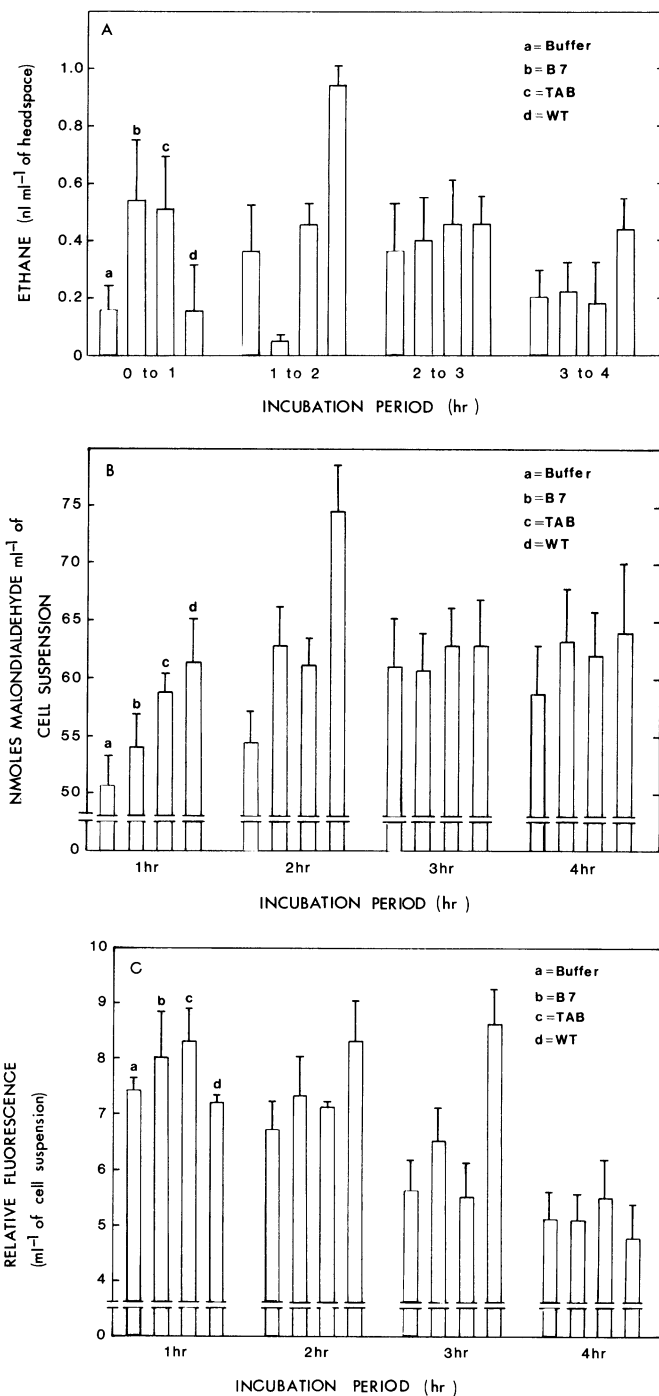


Fig. 3. Lipid peroxidation in tobacco cell suspensions. Cell suspensions were exposed to bacteria (final concentration of 10^8 colony-forming units ml^{-1}) in 2-(N-morpholino) ethanesulfonic acid buffer (1.0 mM, pH 6.00) or buffer alone (a). Bacteria used were: B7 (Tn5 insertion mutant of wild type; does not induce hypersensitive reaction [HR]) (b); *Pseudomonas syringae* pv. *tabaci* (TAB) (compatible pathogen of tobacco) (c); or wild type (WT) (*Pseudomonas syringae* pv. *syringae* strain 61, induces HR) (d). Lipid peroxidation was measured as: **A**, mean \pm standard error ethane evolution (nl ml^{-1} of headspace above cell suspension); **B**, mean \pm standard error of nanomoles malondialdehyde ml^{-1} of cell suspension; and **C**, mean \pm standard error of autofluorescence at excitation $\lambda = 350$ nm and emission $\lambda = 420$ nm. All data points are the means of a minimum of four experiments with two replicates per experiment.

TABLE 3. The effect of O₂⁻ scavengers on ethane and ethylene evolution from tobacco cell suspensions during the hypersensitive reaction (HR)^a

Treatments ^c	Ethane evolution ^b : percent of wild-type treatment incubation period (hr)				Ethylene evolution ^b : percent of wild-type treatment incubation period (hr)			
	0 to 1	1 to 2	2 to 3	3 to 4	0 to 1	1 to 2	2 to 3	3 to 4
WT + tiron	40 ± 21.0	75 ± 22.6	150 ± 27.1	38 ± 13.7	80 ± 11.6	82 ± 7.2	83 ± 9.3	81 ± 4.6
WT + SOD	88 ± 16.7	87 ± 22.6	171 ± 36.3	57 ± 13.8	85 ± 7.2	77 ± 10.2	70 ± 5.9	86 ± 16.4
WT + SOD and tiron	38 ± 8.4	57 ± 12.3	219 ± 18.4	220 ± 34.6	92 ± 0.8	84 ± 6.4	82 ± 1.2	82 ± 12.3

^a Values are means ± standard error of a minimum of four experiments with two replicates per treatment.

^b Changes in ethane and ethylene evolution were monitored as described in Materials and Methods.

^c The HR was initiated by adding 10⁸ cells ml⁻¹ of wild-type (WT) bacteria *Pseudomonas syringae* pv. *syringae*, strain 61). The O₂⁻ scavengers superoxide dismutase (SOD) (100 µg ml⁻¹, 1 unit of activity µg⁻¹) and tiron (1 mM) were added (separately or together) with WT bacteria to tobacco cell suspensions. Parallel samples of tobacco cell suspensions received WT bacteria or WT bacteria with O₂⁻ scavengers. Data are expressed as percents of WT-treated samples. The means ± standard error of the WT-treated samples are: ethylene evolution: 0 to 1 hr = 7.5 ± 0.8; 1 to 2 hr = 12.0 ± 2.5; 2 to 3 hr = 18.5 ± 2.6; 3 to 4 hr = 20.8 ± 2.9 nl ethylene ml⁻¹ of headspace; and ethane evolution: 0 to 1 hr = 0.18 ± 0.08; 1 to 2 hr = 0.45 ± 0.04; 2 to 3 hr = 0.26 ± 0.11; 3 to 4 hr = 0.30 ± 0.14 nl ethane ml⁻¹ of headspace.

TABLE 4. Effect of O₂⁻ scavengers on tobacco cell suspension pH during the hypersensitive reaction (HR)^a

Treatments	pH increase: percent of wild-type bacteria ^b incubation period (hr)			pH increase (acid titration): percent of wild-type bacteria incubation period (hr)		
	2 to 3	3 to 4	2 to 4 ^d	2 to 3	3 to 4	2 to 4 ^d
WT + tiron	61 ± 12.2	79 ± 18.4	70	71 ± 11.5	64 ± 11.4	68
WT + SOD	50 ± 2.0	62 ± 0.8	56	68 ± 6.0	73 ± 16.0	70
WT + SOD and tiron	18 ± 0.9	44 ± 3.5	31	40 ± 5.5	61 ± 6.3	49
WT + heat-killed SOD				120 ± 14.4	59 ± 6.6	93

^a Values are means ± standard error of a minimum of four experiments with two replicates per treatment. The means ± standard error of the wild-type (WT)-treated samples are: cell suspension pH increases: 2 to 3 hr = 0.25 ± 0.04; 3 to 4 hr = 0.35 ± 0.10; and increases in nanomoles of acid added ml⁻¹ of cell suspension: 2 to 3 hr = 99.8 ± 19.2; 3 to 4 hr = 84.0 ± 13.9.

^b Increased extracellular pH was measured as the increase in the pH of the cell suspension or nanomoles of acid ml⁻¹ of cell suspension required to maintain a pH of 5.75. Parallel samples of tobacco cell suspensions received WT bacteria or WT bacteria plus O₂⁻ scavengers.

^c The HR was initiated by adding 10⁸ cells ml⁻¹ of WT bacteria *Pseudomonas syringae* pv. *syringae*, strain 61). The O₂⁻ scavengers superoxide dismutase (SOD) (100 µg ml⁻¹, 1 unit of activity µg⁻¹) and tiron (1 mM) were added separately or together with WT bacteria to tobacco cell suspensions. Heat-killed SOD (autoclaved 20 min) also was added with WT bacteria as a control.

^d The 2- to 4-hr incubation period column is the average of the 2- to 3-hr and 3- to 4-hr incubation period columns.

TABLE 5. The effect of O₂⁻ scavengers on conductivity of tobacco cell suspension during the hypersensitive reaction (HR)^a

Treatments ^c	Conductivity increase: percent of wild-type bacteria ^b incubation period (hr)		
	2 to 3	3 to 4	2 to 4 ^d
WT + tiron	98 ± 26.2	100 ± 13.6	99
WT + SOD	66 ± 16.7	78 ± 18.4	72
WT + SOD and tiron	17 ± 0.7	51 ± 8.4	34

^a Values are means ± standard error of a minimum of four experiments with two replicates per treatment. The means ± standard error of the wild-type (WT) treated samples of cell suspension conductivity increases (µS cm⁻¹) are: 2 to 3 hr = 9.8 ± 1.0; 3 to 4 hr = 15.3 ± 2.8.

^b Parallel samples of tobacco cell suspensions received WT bacteria or WT bacteria plus O₂⁻ scavengers.

^c The HR was initiated by adding 10⁸ cells ml⁻¹ of WT bacteria (*Pseudomonas syringae* pv. *syringae*, strain 61). The O₂⁻ scavengers superoxide dismutase (SOD) (100 µg ml⁻¹, 1 unit of activity µg⁻¹) and tiron (1 mM) were added separately or together with WT bacteria to tobacco cell suspensions.

^d The 2- to 4-hr incubation period column is the average of the 2- to 3-hr and 3- to 4-hr incubation period columns.

4 hr of incubation by the addition of tiron, SOD, and tiron plus SOD, respectively (Fig. 4B). Only when tiron and SOD were added together was the recovery of wild-type bacteria increased after 2 hr incubation from the tobacco cell fraction.

O₂⁻ production. Nitroblue tetrazolium reduction to formazan was greater in tobacco cell suspensions treated with wild-type bacteria than in suspensions treated with buffer, B7, or *P. s. tabaci* (Fig. 5). Reduction of nitroblue tetrazolium in wild-type-treated cell suspensions was greatest after 2 hr. Nitroblue tetrazolium reduction was prevented when SOD was added to wild-type-treated cell suspensions.

TABLE 6. Comparison of the effect of added O₂⁻ scavengers on pH and conductivity change in tobacco cell suspensions treated with wild-type or B7 bacteria^a

Treatments ^b	Change in pH ^c	Change in conductivity ^d
Bacteria	+0.91 ± 0.03	+15.3 ± 1.3
Bacteria plus:		
SOD	+0.72 ± 0.04	+12.7 ± 0.6
Tiron	+0.48 ± 0.03	+ 7.7 ± 0.4
SOD and tiron	+0.47 ± 0.04	+ 6.4 ± 0.4

^a Values are means ± standard error of a minimum of four experiments with two replicates per treatment.

^b Bacteria (final concentration = 10⁸ colony-forming units ml⁻¹) in 1.0 mM 2-(N-morpholino) ethanesulfonic acid (pH 6.00) buffer was added to tobacco cell suspensions. Bacteria used were: WT (wild type; *Pseudomonas syringae* pv. *syringae*, strain 61; induces hypersensitive reaction) and B7 (Tn5 insertion mutant of WT; does not induce hypersensitive reaction). O₂⁻ scavengers superoxide dismutase (SOD) (100 µg ml⁻¹, 1 unit of activity µg⁻¹) and tiron (1 mM) were added (separately or together) with bacteria.

^c Change in pH is the change in pH between 2 and 4 hr of incubation of WT-treated cell suspensions minus the change in pH between 2 and 4 hr of incubation of B7-treated cell suspensions.

^d Change in conductivity is the change in conductivity between 2 and 4 hr of incubation of WT-treated cell suspensions minus the change in conductivity between 2 and 4 hr of incubation of B7-treated cell suspensions.

DISCUSSION

Development of HR symptoms, increases in extracellular pH, and increases in conductivity were observed only in tobacco suspension cells incubated with the HR-inducing bacterium (*P. s. syringae*, wild type). Also, increased ethylene production was

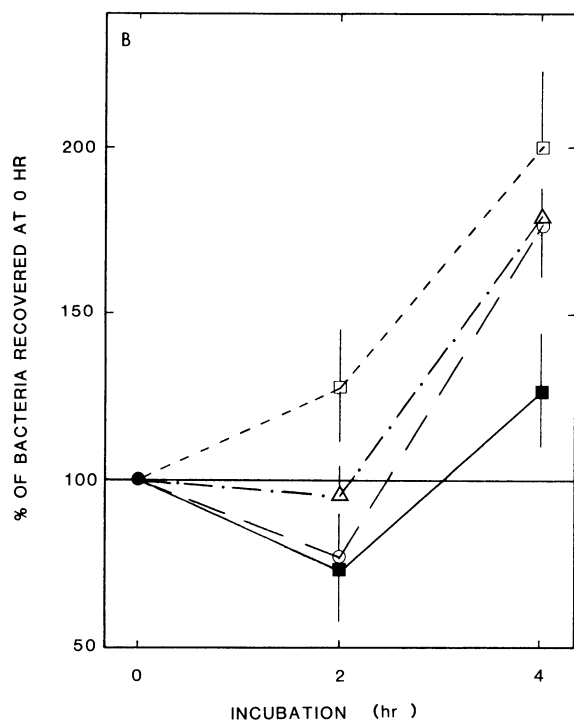
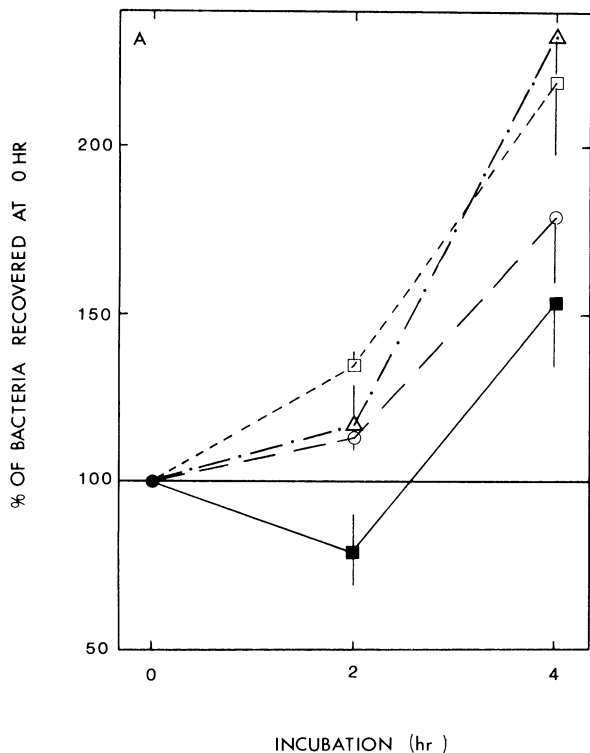


Fig. 4. The effect of O_2^- scavengers on the growth of wild-type (WT) bacteria in tobacco cell suspensions. The O_2^- scavengers superoxide dismutase (SOD) ($100 \mu\text{g ml}^{-1}$, 1 unit of activity μg^{-1}) and tiron (1 mM) were added (separately and together) with WT bacteria (final concentration of 10^8 colony-forming units [cfu ml^{-1}]). ■ = WT alone; ○ = WT + tiron; △ = WT + SOD; and □ = WT + SOD and tiron. Bacteria were recovered: **A**, from the complete cell suspension (bacteria free in the bathing medium and those closely associated with tobacco cells), and **B**, from close association with the tobacco cells. Data are plotted as a percent of the number of bacteria ml^{-1} recovered immediately after addition of bacteria. Means \pm standard error ($n \geq 8$) of the number of WT bacteria ($\times 10^7 \text{ cfu ml}^{-1}$), recovered immediately after addition, from the complete cell suspension and from close association with tobacco cells were: WT alone (7.1 ± 0.73 , 2.6 ± 0.30); WT + tiron (8.4 ± 1.09 , 2.2 ± 0.12); WT + SOD (6.3 ± 1.40 , 2.4 ± 0.03); and WT + SOD and tiron (8.0 ± 1.93 , 1.8 ± 0.40).

observed in cell suspensions treated with wild-type bacteria. Because plant cells produce ethylene in response to stress, the increased ethylene production associated with HR development also was used to follow the HR. All of these symptoms of the HR increased before 3 hr after adding wild-type bacteria. As reported previously (24), death of tobacco suspension cells does not increase until after 8 hr of incubation in the HR induced by *P. s. syringae* (wild-type bacteria). And tobacco cell death does not increase in cell suspensions exposed to the B7 mutant of *P. s. syringae* or *P. s. tabaci* through 12 hr.

Our results imply that lipid peroxidation may be involved in the initiation of the HR. Lipid peroxidation measured as thiobarbituric acid reactivity and ethane evolution increased (transiently) before the onset of the increased extracellular pH and conductivity and concurrent with increased ethylene production. Increased autofluorescence was observed coincident with increased extracellular pH and conductivity. Autofluorescence, at the excitation and emission wavelengths reported here, is consistent with fluorescence produced by the reaction of aldehyde and amine groups to form Schiff's bases which has been reported to occur during cell senescence (7,12,18,34). Thus, the increased autofluorescence could be due to fluorescence of Schiff's bases produced via reaction of the aldehyde groups of lipid peroxidation breakdown products with amine groups of proteins and/or lipid head groups. The later increase in autofluorescence (compared to increases of the other measures of lipid peroxidation) may reflect time required for reactions between aldehyde and amine groups to

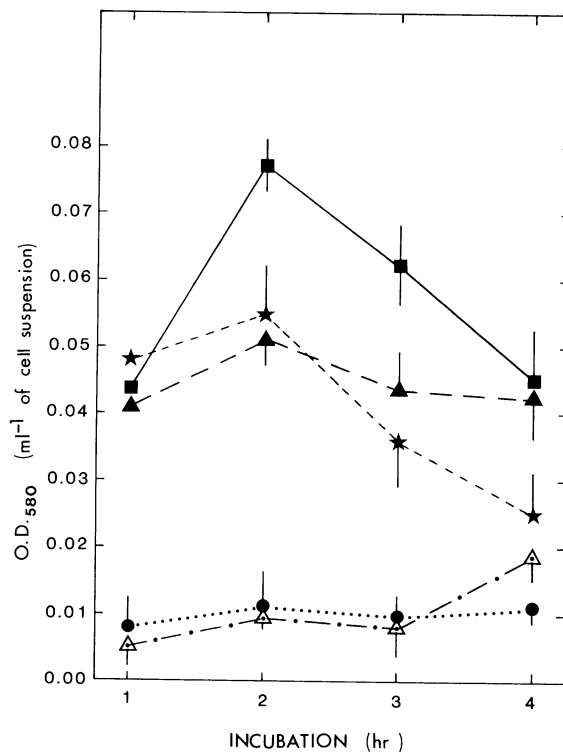


Fig. 5. O_2^- production, measured as nitroblue tetrazolium reduction, in tobacco cell suspensions. Tobacco cell suspensions were exposed to bacteria (final concentration of 10^8 colony-forming units ml^{-1}) in 1.0 mM 2-(N-morpholino) ethanesulfonic acid buffer, pH 6.00, or buffer alone (●). The bacteria used were: wild-type bacteria (WT) (*Pseudomonas syringae* pv. *syringae* strain 61; induces hypersensitive reaction [HR]) (■); B7 bacteria (Tn5 insertion mutant of WT bacteria; does not induce HR) (▲); or *Pseudomonas syringae* pv. *tabaci* (compatible pathogen of tobacco) (★). After 1, 2, 3, and 4 hr of incubation, nitroblue tetrazolium (0.05% final concentration) was added to 1-ml aliquots of cell suspensions and incubated for 10 min. Tobacco cells and bacteria were removed by centrifugation, and nitroblue tetrazolium reduction to formazan was measured as OD_{580} . Nitroblue tetrazolium reduction by O_2^- radicals was verified by the ability of superoxide dismutase ($100 \mu\text{g ml}^{-1}$, 1 unit of activity μg^{-1}) to inhibit nitroblue tetrazolium reduction to formazan (△).

build up measurable levels of Schiff's bases.

Involvement of O_2^- radicals in the initiation of lipid peroxidation in wild-type-treated cell suspensions is supported by the results reported here. Increased lipid peroxidation, measured as thiobarbituric acid reactivity and ethane evolution, correlated well with increased O_2^- production, measured as nitroblue tetrazolium reduction. Thiobarbituric acid reactivity, ethane evolution, and nitroblue tetrazolium reduction all increased to maximum levels after 2 hr, only, in suspension cells exposed to the wild-type bacterium. Also, the addition of the O_2^- scavengers (tiron and SOD) delayed lipid peroxidation and inhibited HR symptom development. The addition of SOD and/or tiron with wild-type bacteria to tobacco cell suspensions delayed lipid peroxidation (measured as ethane evolution). The transient increase in lipid peroxidation between 1 and 2 hr of incubation was eliminated, but lipid peroxidation between 2 and 3 hr of incubation was higher than lipid peroxidation in cell suspensions exposed to wild-type bacteria alone. Thus, the scavengers delayed the onset of lipid peroxidation but were unable to prevent its eventual initiation at the concentrations used. A similar pattern of inhibition of HR symptom development also was observed. Inhibition of increased extracellular pH and conductivity was greater between 2 and 3 hr than between 3 and 4 hr. Also, increased extracellular pH and conductivity and ethylene evolution were inhibited but not eliminated by the O_2^- scavengers.

Incomplete inhibition of lipid peroxidation and HR symptoms by the O_2^- scavengers may reflect inefficient delivery of the O_2^- scavengers to the site of O_2^- production. Because penetration of SOD through the cell wall to the plasma membrane is unlikely, complete scavenging of O_2^- radicals at a plasma membrane site of O_2^- production may not be possible. Also, scavenging of O_2^- radicals by SOD ($O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) (19) would decrease H^+ concentration, thereby increasing extracellular pH. Therefore, the lowering of increased extracellular pH by SOD (as reported here) would actually be greater. In addition, the scavenger concentrations used here may not be high enough to scavenge O_2^- radicals fast enough to prevent lipid peroxidation. Higher concentrations of the scavengers were not used because they altered bacterial growth rate on minimal medium.

Initiation of HR symptom development by O_2^- -initiated lipid peroxidation also is supported by positive correlation between decreased levels of wild-type bacteria recovered and transient increases in lipid peroxidation and O_2^- production. The levels of wild-type bacteria recovered from cell suspensions increased less than levels of B7 or *P. s. tabaci* bacteria throughout the 4-hr incubation period. After 2 hr of incubation, the level of wild-type bacteria was actually lower than the level recovered after 0 hr. The decreased level of wild-type bacteria recovered after 2 hr is coincident with the increased lipid peroxidation and O_2^- production. This decreased level of wild-type bacteria recovered may reflect bacterial injury by: 1) O_2^- radicals directly; 2) other toxic oxygen molecules generated by O_2^- radicals (hydroxyl radical, OH^{\cdot} ; hydrogen peroxide, H_2O_2 ; singlet oxygen, 1O_2) (17); or 3) toxic lipid peroxidation breakdown products produced as the result of O_2^- -initiated lipid peroxidation. These possibilities are supported by the ability of O_2^- scavengers to increase levels of wild-type bacteria recovered from cell suspensions.

The results suggest that lipid peroxidation, initiated by O_2^- radicals, may be involved in the initiation of bacteria-induced HR in tobacco suspension cells by the wild-type *P. s. syringae* bacterium.

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