

Use of Dimethyl Sulfoxide to Detect Hydroxyl Radical during Bacteria-Induced Hypersensitive Reaction¹

Phillip L. Popham and Anton Novacky*

Department of Plant Pathology, University of Missouri, Columbia, Missouri 65211

ABSTRACT

Excess active oxygen is generated during the hypersensitive reaction (HR), an incompatible reaction of plants to bacterial pathogens. During HR, lipid peroxidation correlates chronologically with production of the oxygen species, superoxide ($O_2^{\cdot-}$). However, $O_2^{\cdot-}$ may not be the active oxygen species that initiates lipid peroxidation. Evidence from other systems suggest that $O_2^{\cdot-}$ is converted to the hydroxyl radical (HO^{\cdot}) before lipid peroxidation is initiated. Until recently, HO^{\cdot} could not be detected directly *in vivo*. This study utilizes a newly reported method to directly detect and quantify the formation of HO^{\cdot} *in vivo*. Dimethyl sulfoxide (DMSO), used as a molecular probe, is oxidized by HO^{\cdot} , forming the stable compound methanesulfinic acid. The methanesulfinic acid can be easily extracted from plant tissues and measured with a colorimetric assay. This study demonstrates significant increases in HO^{\cdot} concentration after simultaneous infiltration of cucumber (*Cucumis sativa* L.) plants with paraquat and DMSO. The concentration of HO^{\cdot} did not increase significantly when cucumber plants were infiltrated simultaneously with the HR-inducing bacteria, *Pseudomonas syringae* pv. *pisi*, and with DMSO. Lipid peroxidation, however, could be measured at times when HO^{\cdot} was not detectable. It appears that HO^{\cdot} is not generated during bacteria-induced HR; therefore, HO^{\cdot} is not responsible for the initiation of lipid peroxidation.

The bacteria-induced HR² develops when bacteria are in contact with nonhost plant cells. HR is expressed as rapid cellular death and tissue necrosis. After the symptomless induction period, several physiological changes occur. Electrolyte leakage (6, 10, 11), cell membrane depolarization (21), and lipid peroxidation (15) are the earliest detectable changes. This evidence suggests that a major alteration of cellular membranes occurs during the initial phase of HR.

The mechanism responsible for membrane alterations has not been fully elucidated, but the production of active oxygen species correlates chronologically with lipid peroxidation. Ádám *et al.* (1) reported that the superoxide radical is produced during the early stages of bacteria-induced HR. Apostol *et al.* (2) demonstrated that excess hydrogen peroxide is produced within 5 min of elicitor addition; furthermore, hydrogen peroxide alone can stimulate the formation of phytoalexins. However, singlet oxygen does not seem to cause

the membrane symptoms of HR (23). Other active oxygen species, such as HO^{\cdot} , have been implicated as the cause of cellular damage (5). HO^{\cdot} attacks membrane lipids *in vitro*; this in turn initiates a chain reaction of lipid peroxidation caused by lipid radicals (13). Until recently, HO^{\cdot} could not be detected *in vivo*; however, a colorimetric assay that utilizes the ability of HO^{\cdot} to oxidize DMSO forming MSA has been reported (3). The present work uses a similar assay to elucidate a possible role for HO^{\cdot} in bacteria-induced HR.

MATERIALS AND METHODS

Plants

Cucumber (*Cucumis sativa* L. var Straight Eight, Royal Seeds, Kansas City, MO) seedlings were grown in a growth chamber at 23 to 27°C with a photoperiod of 14 h/10 h light/dark. The seedlings were grown in vermiculite in plastic trays with cheesecloth covering aeration holes in the bottom. The plants were watered daily from below with 1 mM $CaSO_4$. On days 3 and 5, the $CaSO_4$ was replaced with a nutrient solution consisting of the following: 4 mM $CaCl_2 \cdot 2H_2O$, 0.45 mM $K_2HPO_4 \cdot 3H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM K_2SO_4 , 10 mM NH_4NO_3 , 26 μ M Fe citrate, 2.3 μ M H_3BO_3 , 0.9 μ M $MnSO_4 \cdot H_2O$, 0.6 μ M $ZnSO_4 \cdot 7H_2O$, 0.15 μ M $CuSO_4 \cdot 5H_2O$, 0.1 μ M $Na_2MoO_4 \cdot 2H_2O$, 0.01 μ M $CoCl_2 \cdot 6H_2O$, and 0.11 μ M $NiCl_2 \cdot 6H_2O$ (D.G. Blevins, Department of Agronomy, University of Missouri, personal communication). Experiments were performed on 9- to 11-d-old plants.

Bacteria

Pseudomonas syringae pv. *pisi*, a bacterium that induces HR on cucumber, was used for all experiments. The bacteria were stored at $-70^\circ C$ in 12% glycerol. When needed, bacteria were removed from the freezer and streaked onto a nutrient agar plate. The bacteria from the plate were transferred to a nutrient agar slant 48 h before inoculation and then were transferred from the slant to nutrient broth 12 to 18 h before inoculation. Bacteria were incubated at 25°C on a reciprocal shaker to obtain a high concentration of cells in the log phase of growth. Preceding inoculation, bacteria were collected by centrifugation at 10,000g for 5 min. The bacteria were resuspended in distilled water and collected by centrifugation at 10,000g for 5 min. The bacteria were again resuspended in distilled water, and the suspension was adjusted spectrophotometrically to 10^8 colony forming units mL^{-1} . Plants were inoculated by piercing the abaxial epidermis of the cotyledons

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² Abbreviations: HR, hypersensitive reaction; HO^{\cdot} , hydroxyl radical; MSA, methanesulfinic acid; MDA, malondialdehyde.

with a needle, placing the syringe tip over the hole, and infiltrating the intercellular space.

Hydroxyl Radical

The HO \cdot was monitored with an assay similar to the one developed by Babbs *et al.* (4). DMSO was used as a molecular probe to trap HO \cdot . DMSO is oxidized to form a single stable product, MSA, which was assayed by a color reaction with the diazonium salt, fast blue BB dye, after the removal of interfering lipophilic compounds by extraction and filtration.

When the plants were inoculated with bacteria (10^8 colony forming units mL $^{-1}$) or treated with paraquat ($5 \mu\text{g mL}^{-1}$), DMSO was added to the inoculum at a final concentration of 3% (v/v). Control plants were inoculated with a 3% (v/v) DMSO solution only. Cotyledons were removed from the seedlings, weighed, and frozen in liquid nitrogen at 1.5, 3.5, 6.5, and 8 h after infiltration. Eight to 10 plants (1.5–3.5 g) were harvested from each treatment. The frozen tissue was pulverized with a mortar and pestle and then suspended in 15 to 20 mL of distilled water. Plant debris was removed by centrifugation at 10,000g, and the supernatant was lyophilized to dryness.

Lyophilized samples were resuspended in 2 mL of distilled water and subsequently were extracted twice with 2 mL of toluene:*n*-butanol (3:1). The organic (upper) phase was discarded. The aqueous (lower) phase was titrated to pH 2.5 with HCl, 0.1 mL of 30 mM fast blue BB salt (freshly prepared and stored in the dark) was added, and the sample was applied to a Sep-Pak C $_{18}$ column (Waters Associates, Milford, MA) to remove detergent-like interference. The column had been preeluted with 3 mL methanol, 3 mL isopropanol, and 3 mL distilled water. In addition to the sample liquid, 1.5 mL distilled water was used to elute the methanesulfinate anion. The first 1.1 mL of effluent was discarded, and the remaining aqueous effluent (1.5 mL) was collected for the colorimetric assay.

The 1.5 mL of column effluent was adjusted to pH 2.5 by the addition of HCl. The color reaction was initiated by the addition of 0.1 mL of 30 mM fast blue BB salt. The solution was mixed with a Vortex mixer for several seconds and then incubated at room temperature for 10 min in the dark. After incubation, 2 mL of toluene:*n*-butanol (3:1) were added and mixed for 60 s to remove the yellow hydrophobic reaction products (diazosulfones) from the aqueous phase. The phases were separated by centrifugation (500g for 3 min) and the aqueous phase was removed and discarded. Two milliliters of *n*-butanol-saturated water were added to the organic phase, and the solution was mixed for 30 s to remove remaining unreacted diazonium salt from the sample. The samples were centrifuged at 500g for 3 min, and the organic phase containing the unreacted diazosulfones was transferred to a cuvette. Pyridine (0.1 mL) was added to stabilize the color. The absorbance was measured on an LKB Biochrom Ultraspec 4050 spectrophotometer at 420 nm. The concentration of sulfonic acid was calculated from an MSA (Fairfield Chemical, Blythwood, SC) standard curve.

Three repetitions were performed on various days for each treatment. Means from these repetitions were analyzed by the

least squares means test from the SAS general linear models procedure (24).

Lipid Peroxidation

The method of Heath and Packer (14) as modified by Dhindsa *et al.* (8) was used to measure MDA, an early product of lipid peroxidation.

Cotyledons were removed from seedlings at 2, 4, 6, and 8 h after infiltration with water or a bacterial suspension of 10^8 colony-forming units. The midrib was removed from each cotyledon, and the tissue was weighed and cut into 5 mm segments. After harvest, the segments were frozen with liquid nitrogen and pulverized for 20 s with a Mikro-Dismembrator II (B. Braun Instruments). Two milliliters of 0.2 M citrate-phosphate buffer (pH 6.5) containing 0.5% Triton X-100 were added to each sample, which then was homogenized for 5 s with the Mikro-Dismembrator II. After centrifugation at 20,000g for 15 min, a 50 μL aliquot of supernatant was removed for determination of protein concentration by the bicinchonic acid:copper sulfate (50:1 v/v) method (Pierce, Rockford, IL), and 1 mL of the supernatant solution was removed for the MDA assay.

The 1 mL of supernatant was added to an equal volume of 20% (w/v) TCA containing 0.5% (w/v) 2-thiobarbituric acid. The solutions were mixed and incubated at 95°C for 40 min and then immediately cooled on ice for 15 min. After centrifugation at 10,000g for 15 min, the supernatant was removed and the absorbance was measured at 532 nm. Non-specific absorbance at 600 nm was measured and subtracted from that value measured at 532 nm. The difference was transformed with the MDA extinction coefficient of 155 mm $^{-1}$ cm $^{-1}$ (13) to obtain a final concentration. The concentration was expressed as nmol MDA mg $^{-1}$ protein.

Each replication contained two cotyledons from the same treated plant. Means of four replications were analyzed by a least squares means test in the SAS general linear models procedure and a regression analysis in the SAS regression procedure (24).

RESULTS

The addition of 3% (v/v) DMSO to the bacterial inoculum did not alter the wilting symptoms and the final tissue collapse during HR. However, the DMSO solutions, with bacteria or water alone, were more difficult to infiltrate into the cotyledons than solutions without DMSO. Water-treated control cotyledons remained watersoaked for 45 to 60 min after infiltration; however, cotyledons treated with DMSO solutions remained watersoaked for 70 to 90 min. No additional differences in appearance were observed for 24 h after infiltration. Chlorotic areas were often visible on water + DMSO control cotyledons 36 to 48 h after infiltration.

The concentration of MSA produced during the *P. syringae* pv. *pisii* + DMSO infection was not statistically different from the amount produced in water + DMSO control plants at 1.5, 3.5, 6.5, or 8 h after infiltration (Table I, Fig. 1). Paraquat was used as a positive control of MSA production. Paraquat + DMSO-treated cucumber cotyledons yielded a significantly higher concentration of MSA than cotyledons infiltrated with

Table 1. Mean Concentrations of MSA Produced as a Result of Infiltration with DMSO or DMSO + *P. syringae* pv. *pisi*.

Treatment	Time After Inoculation h	MSA	MSA
		Concentration nmol/plant	Concentration nmol/g fresh wt
DMSO + bacteria	1.5	0.60 ± 0.46	1.71 ± 1.05
DMSO + bacteria	3.5	0.77 ± 0.19	2.23 ± 0.56
DMSO + bacteria	6.5	0.78 ± 0.26	2.47 ± 0.86
DMSO + bacteria	8.0	0.79 ± 0.34	2.99 ± 1.27
DMSO	1.5	0.59 ± 0.51	1.56 ± 1.07
DMSO	3.5	0.66 ± 0.27	1.80 ± 0.65
DMSO	6.5	0.65 ± 0.33	1.96 ± 0.95
DMSO	8.0	0.90 ± 0.63	2.79 ± 2.08

water + DMSO (Fig. 1). The MSA concentrations for cucumber cotyledons treated with *P. syringae* pv. *pisi* + DMSO or water + DMSO ranged from 1 to 3 nmol g⁻¹ or 0.6 to 0.9 nmol per treated plant. Paraquat induced levels of 53 nmol MSA g⁻¹ or 18 nmol MSA per treated plant.

Paraquat-treated plants were harvested at 3.5 h after infiltration because macroscopic symptoms were present. The paraquat-treated cotyledons began collapsing 2.5 to 3 h after infiltration.

Lipid Peroxidation

The concentration of MDA (nmol MDA μg⁻¹ soluble protein) in *P. syringae* pv. *pisi*-inoculated cotyledons was significantly higher than the concentration detected in water-infiltrated control tissue 4 to 6 h after inoculation. In a representative experiment, 0.133 ± 0.017 nmol MDA μg⁻¹ soluble

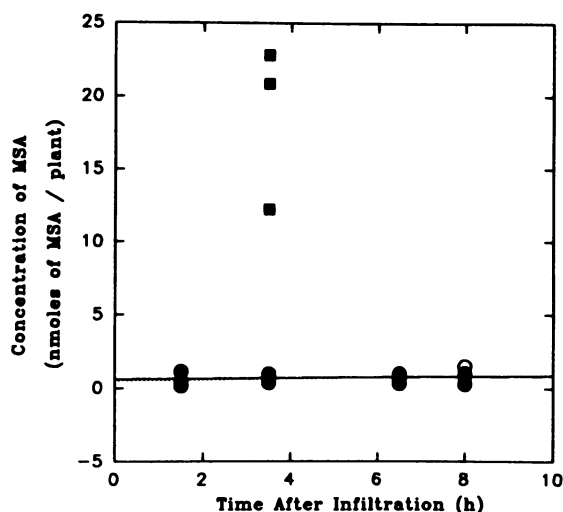


Figure 1. The concentration of MSA in cucumber plants treated with paraquat + 3% DMSO (■), 10⁸ colony-forming units mL⁻¹ of *P. syringae* pv. *pisi* + 3% DMSO (●), and 3% DMSO (○). Each symbol represents a single replication. The solid line represents the regression line for the bacteria + DMSO-treated plants, and the regression line for the DMSO-treated plants is portrayed as the dotted line. No regression was displayed for the paraquat + 3% DMSO-treated plants.

protein was measured in bacteria-inoculated tissue 4 h after infection, and water-infiltrated cotyledons contained 0.084 ± 0.016 nmol MDA μg⁻¹ soluble protein. By 8 h after infiltration, bacteria treatments contained 0.258 ± 0.028 nmol MDA μg⁻¹ soluble protein, and water treatments had 0.148 ± 0.034 nmol MDA μg⁻¹ soluble protein (Fig. 2). Regression analysis indicated that the slopes of the regression lines for the two treatments were greater than zero. This means that the MDA concentration increased over time in bacteria-inoculated and water-treated control tissue.

DISCUSSION

Hydroxyl radicals are probably not generated in significant amounts during the first 8 h of *P. syringae* pv. *pisi*-induced HR of cucumber. With DMSO as a probe, the concentration of HO· was measured directly during bacteria-induced HR. No significant increase in the accumulation of MSA was observed as a result of the infiltration of cucumber cotyledons with *P. syringae* pv. *pisi* + DMSO, but lipid peroxidation did occur well within this time period.

Significant increases in the accumulation of MSA were observed when cucumber cotyledons were subjected to treatment with paraquat. The results from these experiments support the conclusion of Babbs *et al.* (4) that large amounts of HO· are produced when plants are treated with paraquat.

Minotti and Aust (19) reported that HO· is not necessary for the initiation of lipid peroxidation. They demonstrated that HO· scavengers did not inhibit the *in vitro* lipid peroxidation initiated by iron and H₂O₂. Several investigators also suggest that HO· is an unlikely candidate for the initiation of lipid peroxidation *in vivo*, because the radical is a highly reactive species and, therefore, would not migrate from the site of its formation (20, 22, 25). This evidence supports the hypothesis derived from our present study that HO· is not

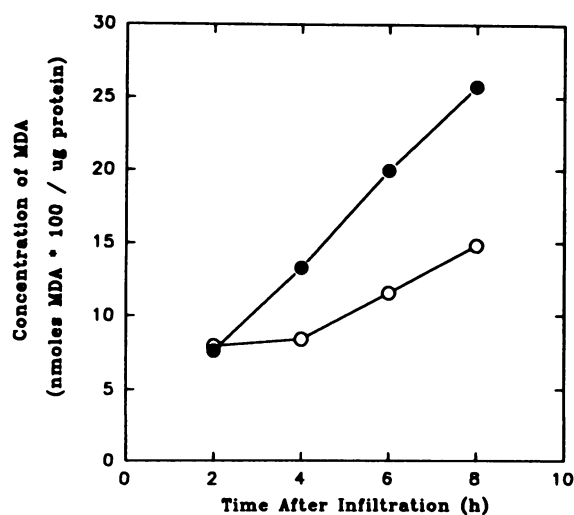


Figure 2. MDA concentrations in cucumber plants inoculated with 10⁸ colony-forming units mL⁻¹ of *P. syringae* pv. *pisi* (●) and water (○). Each point is the mean of four replications. The treated and control points at 4, 6, and 8 h after infiltration are significantly different from each other using a least squares means test at P > 0.05.

necessary for initiation of the lipid peroxidation observed during bacteria-induced HR.

Halliwell and Gutteridge (12) reported that HO scavengers could inhibit the initiation of lipid peroxidation in several *in vitro* superoxide-generating systems. Hydroxyl radical scavengers inhibit plant phytoalexin synthesis induced by treatments with abiotic factors (9). With this information, Klotz *et al.* (17) suggested that HO[•] could be responsible for the initiation of lipid peroxidation observed during bacteria-induced HR. However, none of these studies measured hydroxyl radicals directly. Our direct measurements indicate that alternative explanations are more likely.

Lipid peroxidation and excess superoxide generation have previously been reported during bacteria-induced HR (1, 15). Attempts have been made to correlate these events to propose a mechanism for lipid peroxidation initiation because, *in vitro*, superoxide initiates lipid peroxidation (25). Supporting this hypothesis, Ádám *et al.* (1) reported that excess superoxide is generated 3 h after tobacco tissue is inoculated with *P. syringae* pv. *syringae* but that detection of lipid peroxidation occurs from 3 to 4 h after inoculation. They concluded from this evidence that superoxide initiated lipid peroxidation during bacteria-induced HR.

Alternatively, incompatible bacteria could trigger an increase in host cell lipoxygenase activity which would be responsible for the initiation of lipid peroxidation. During senescence, lipoxygenase activity increases at the same time that lipid peroxidation is detected and that excess superoxide is generated (18, 26). Keppler and Novacky (16) demonstrated an increase in lipoxygenase activity during *P. syringae* pv. *pisi* infection of cucumber cotyledons. Croft *et al.* (7) reported that the increase in lipoxygenase activity preceded ethane evolution (a measure of lipid peroxidation) during *P. syringae* pv. *syringae* (an avirulent race) infection of *Phaseolus vulgaris*. In this scenario, superoxide and lipid radicals form during lipid reactions with lipoxygenase. These radicals would then perpetuate the lipid peroxidation.

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