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Communication

Effects of Singlet Oxygen Quenchers and pH on the Bacterially Induced Hypersensitive Reaction in Tobacco Suspension Cell Cultures[1]

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ABSTRACT

Suspension cell cultures of *Nicotiana tabacum* L. inoculated with the incompatible pathogen *Pseudomonas syringae* pv *pisi* undergo a hypersensitive reaction. Addition of the singlet oxygen quencher bixin to cell suspensions had no effect on hypersensitive cell death. Addition of the singlet oxygen quencher 1,4-diazabicyclo octane (DABCO) increased the medium pH and delayed the onset of cell death. This delay was eliminated when cell suspensions were buffered, and could also be induced by increasing medium pH with KOH. Bixin and DABCO also did not suppress the hypersensitive reaction in tobacco leaves. These data do not support a role for singlet oxygen in the hypersensitive reaction. Medium pH, however, appears to be a critical factor in cell suspension cultures.

Several reports have suggested that active oxygen-induced lipid peroxidation may play a role in the HR,[3] a defense reaction commonly induced by plant pathogens following inoculation of incompatible hosts. Keppler and Novacky (10) reported increases in lipid peroxidation during the HR in inoculated cucumber cotyledons and postulated that this increased lipid peroxidation leads to the typical loss of electrolytes during the HR. They further demonstrated that the HR-induced lipid peroxidation could be inhibited by the addition of SOD (11). Doke (4) and Doke and Chai (5) demonstrated activation of an O_2^- generating system in potato leaves and tuber tissue undergoing an HR, and that induction of O_2^- production by digitonin results in resistance of potato tuber slices to infection by compatible races of *Phytophthora infestans*. Haga *et al.* (8) demonstrated O_2^- accumulation in rice tissue treated with elicitors from the rice blast fungus (*Pyricularia oryzae*). Rogers *et al.* (14) demonstrated lipid peroxidation in bean cell suspension cultures treated with elicitor fractions from *Colletotrichum lindemuthianum*. They further demonstrated that the effects of the elicitors (lipid peroxidation, phytoalexin accumulation, browning) could also be induced by generators of active oxygen.

Singlet oxygen ($^1O_2$) is an excited state of molecular oxygen which can be generated in a number of ways including the spontaneous dismutation of two O_2^- radicals, and by the interaction of O_2 with the triplet state of photosensitizing compounds such as Chl (7, 13). $^1O_2$ is a very potent inducer of lipid peroxidation and membrane damage (13). There has been one brief report that β-carotene (a singlet oxygen quencher) increased bacterial populations in an incompatible host when coinjected into leaves, suggesting an involvement of $^1O_2$ in the HR (16). The purpose of this study was to further investigate a possible role of $^1O_2$ in the HR.

In this study we investigated the effects of two singlet oxygen quenchers, DABCO and the carotenoid carboxylic acid bixin, on the HR induced in leaves and in cell suspension cultures of tobacco following inoculation with the incompatible pathogen *Pseudomonas syringae* pv *pisi*. Both of these compounds are potent $^1O_2$ quenchers and have been shown to protect tobacco cell suspension cultures against photosensitizer-generated $^1O_2$ (3). Suspension cultured cells have been used in previous studies of bacterially induced HR (1, 2). In this study, changes occurring in cells undergoing the HR were followed by using the fluorescent vital stain FDA (15, 19). Decreased staining of cells by FDA has been correlated with progress of the HR in cell suspension cultures (9).

MATERIALS AND METHODS

Bacteria

Cultures of *Pseudomonas syringae* pv *pisi* Young, Dye, and Wilkie were originally obtained from R. N. Goodman, University of Missouri. Cultures were grown for 24 h at 28°C on nutrient agar (Difco) with 0.5% yeast extract and 1.0% dextrose (NYDA) at pH 7.0. Bacteria were suspended in sterile water or 50 mM Mes buffer (pH 6.0) and the concentration determined by measuring the optical density at 520 nm. Concentrations were confirmed by dilution plating.

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[3] Abbreviations: HR, hypersensitive reaction; SOD, superoxide dismutase, Sigma; O_2^-, superoxide; $^1O_2$, singlet oxygen; DABCO, 1,4-diazabicyclo[2.2.2]octane, Sigma; FDA, fluorescein diacetate, Sigma; NYDA, nutrient yeast dextrose agar; SH, Schenk and Hildebrandt medium; FCCP, (*p*-trifuoromethoxy)carbonyl cyanide phenylhydrazone; cfu, colony forming units.

Leaf Injection

Bacterial suspensions were infiltrated into the intercellular spaces of leaves of tobacco (cv 'Hicks') (abaxial surface) following the methods of Klement *et al.* (12). Plants were at the 8 to 10 leaf stage. DABCO was added as a powder directly to the bacterial suspensions. Bixin (Chemical Procurement Co., College Point, NY) was dissolved in 95% ethanol. The solution was then mixed with an equal volume of 0.05 N KOH, reduced to half-volume on a rotary evaporator, and mixed with the bacterial suspensions. DABCO and bixin were injected into leaves without bacteria as controls. In all cases, the presence or absence of confluent necrosis in the injected area was recorded after 24 h.

Tobacco Cells

Suspension cultured tobacco cells, originally derived from *Nicotiana tabacum* (L.) cv 'Hicks' callus as described by Atkinson *et al.* (1) were grown at room temperature in SH liquid medium (17) on a rotary shaker at 150 to 170 rpm. Cells were transferred by diluting cultures 1:1 (v/v) with fresh SH medium every 3 to 5 d. All tobacco suspensions used in experiments were transferred 48 h prior to the experiment. Cells were centrifuged in conical graduated tubes at 300*g* for 1 min to determine tobacco cell densities. Tobacco cell suspension densities ranged from 2.5 to 4.7 mL packed cells per 10 mL initial suspension.

Inoculation of Tobacco Cultures

Nine mL aliquots from tobacco cell suspension cultures were placed in sterile 50 mL Erlenmeyer flasks. Each flask was inoculated with either 1.0 mL of a bacterial suspension (7.4×10^6 to 4.3×10^7 cfu/mL) or 1.0 mL of a treatment solution plus 0.1 mL of a concentrated (10×) bacterial suspension. Flasks were returned to the shaker and tobacco cell samples removed for staining at 4, 6, 8, 10, and 12 h following inoculation unless otherwise noted.

Vital Staining

The stock solution of 0.5% (w/v) FDA in acetone was diluted 1:50 (v/v) with SH medium or water immediately before staining. One drop of diluted FDA stain was added to one drop of suspension cells on a glass slide (19). Fluorescence of cells was observed by illumination with light (incident wavelengths 420–485 nm) from a Nikon Inverted Microscope equipped with a blue filter cassette. For each cell sample, the entire microscope slide was viewed and assigned a rating for the number of brightly fluorescing tobacco cells at ×60 magnification within the first 5 min of observation. Rating criteria were as follows: 1 = 100% of cells bright, 2 = 75 to 95% of cells bright (other cells visible but less bright), 3 = 25 to 74% of cells bright, 4 = 0 to 24% of cells bright (all cells fluoresce slightly), and 5 = total lack of fluorescence (cells not visible) (Fig. 1). In all experiments, three cell samples per flask were taken at each time point and the mean cell death rating determined.

Treatments

DABCO was added as a powder to suspension cell aliquots and was used at final concentrations of 1.0, 5.0, 10.0, and 100.0 mM. Bixin was solubilized as described and added as 1 mL of a diluted solution followed by the addition of 0.1 mL of a 10× bacterial suspension; final bixin concentrations were 1.0, 0.5, and 1.0 mM. Similarly, 1.0 mL of stock KOH solutions were added to tobacco cell aliquots followed by 0.1 mL of 10× bacterial suspension for final KOH concentrations of 0.005 and 0.01 N. For buffered treatments, 0.1 g Mes was added to cells for a final Mes concentration of approximately 50 mM. Controls included 1.0 mL of water or water plus Mes added to inoculated tobacco suspensions as well as buffered and unbuffered chemical treatments added to uninoculated tobacco suspensions. All concentrations of chemicals used in experiments were tested for antibacterial activity by plating bacteria on NYDA in the presence of the chemicals. None of the treatments used inhibited bacterial multiplication. The number of bacteria present in inoculated tobacco suspensions was monitored by removing samples of medium at various intervals, diluting and plating on NYDA. The concentrations of bixin, DABCO, and KOH used did not decrease bacterial multiplication in the suspension cultures as compared to controls. The pH of tobacco suspensions was measured using a combination glass electrode.

RESULTS

Leaf Injection

Injecting *P. syringae* pv *pisi* into tobacco leaves at concentrations of 8.7×10^7, 1.0×10^8, and 1.6×10^8 cfu/mL resulted in confluent necrosis of the injected area by 24 h. Bixin at 0.1 mM was not toxic (did not induce necrosis) when injected alone into leaves and did not alter the development of the HR necrosis when mixed with the bacteria just prior to injection. Similarly, DABCO at 0.1 to 10 mM was not toxic to tobacco leaves and did not affect the appearance of the HR when mixed with the bacteria. This was true whether bacteria were suspended in water or in Mes buffer.

Cell Culture Assays

Healthy tobacco cells fluoresced bright yellow-green with numerous cytoplasmic strands extending from the tobacco cell wall to the central cytoplasm (Fig. 1). Dying cells did not fluoresce brightly and these cells gradually plasmolyzed with a loss of cytoplasmic strands and accumulation of cytoplasm appressed to one wall. Tobacco cell death was not synchronized, but rather cell populations contained individual cells in various stages of cell death.

In control tobacco suspensions inoculated with 4.3×10^6 to 1.3×10^7 cfu/mL bacteria, a decrease in FDA fluorescence (increase in cell death) was first observed 4 h after inoculation (rating 2) with a significant decrease in fluorescence (rating 3) 6 h after inoculation (Fig. 2A). Fluorescence progressively decreased over time, and a complete loss of fluorescence (rating 5) was seen by 12 and 15 h after inoculation, depending on the bacterial concentration. Results were similar when the medium was buffered with Mes, except that cell death was

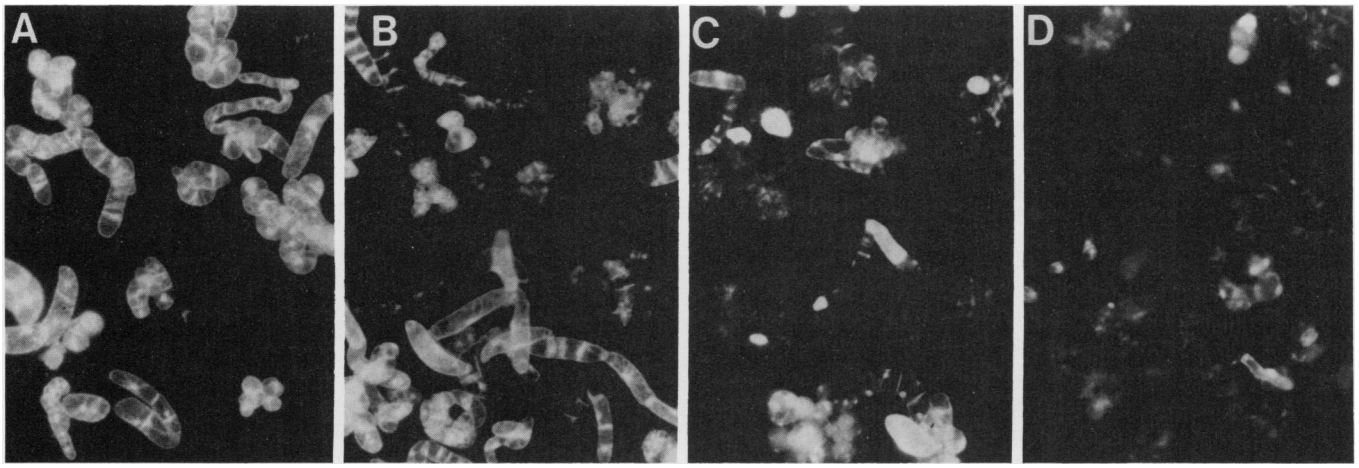

Figure 1. Rating system for FDA-stained tobacco cells undergoing HR cell death. Cells inoculated with *P. syringae* pv *pisi* suspended in sterile water at a concentration of 1.0×10^7 cfu/mL. A, Rating 1 (uninoculated cells); B, rating 2; C, rating 3; D, rating 4. Rating 5 (complete loss of fluorescence) not shown.

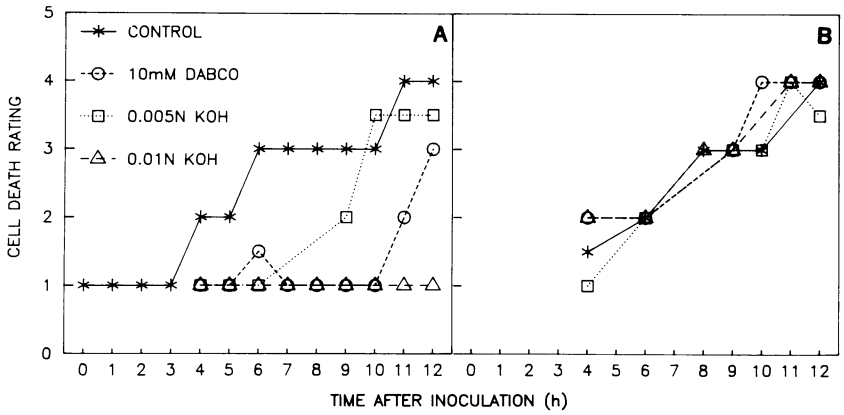

Figure 2. Cell death ratings over time for suspension-cultured tobacco cells inoculated with *P. syringae* pv *pisi* suspended in sterile water. A, Unbuffered treatments; B, treatments in the presence of 50 mM Mes. Control (*); 10 mM DABCO (○); 0.005 N KOH (□); 0.01 N KOH (△).

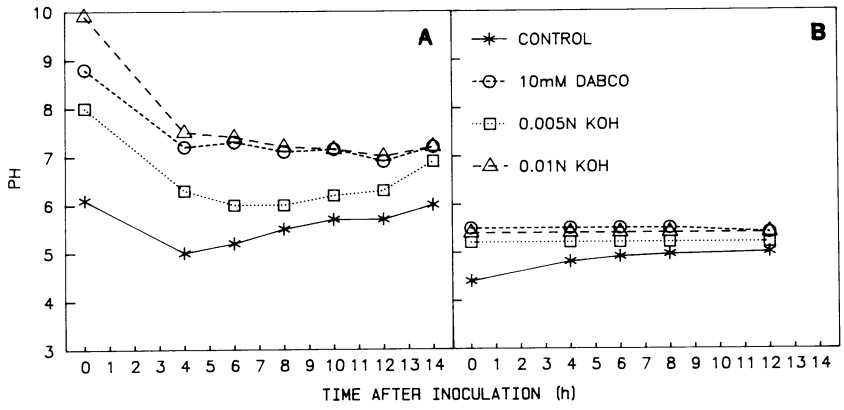

Figure 3. pH of tobacco cell suspensions inoculated with *P. syringae* pv *pisi*. A, Unbuffered; B, treatments in the presence of 50 mM Mes. Bacteria only control (*); 10 mM DABCO (○); 0.005 N KOH (□); 0.01 N KOH (△).

delayed slightly with a mean fluorescence rating of 1.5 at 4 h, 2 at 6 h, and 3 at 8 h (Fig. 2B).

The addition of bixin at a final concentration of 1 mM was toxic to tobacco cells; 0.5 and 0.1 mM bixin were not toxic and also had no effect on HR-induced cell death (data not shown). The onset of the loss of fluorescence was delayed for unbuffered 10 mM DABCO-treated cells until 11 h after inoculation (Fig. 2A). Five mM DABCO also delayed cell death, whereas 1 mM DABCO had no effect and 100 mM DABCO was toxic to uninoculated cells (data not shown). The addition of Mes buffer abolished the delay in cell death seen with 10 mM DABCO and resulted in HR development similar to that seen in the inoculated control (Fig. 2B). The addition of 10 mM DABCO to unbuffered suspension cultures raised their pH significantly (Fig. 3A); in the presence of 50 mM Mes, there were no differences in pH between control suspension cultures and those containing 10 mM DABCO (Fig. 3B).

In order to determine if the delay in HR-induced cell death by DABCO was due simply to a pH effect, the pH of the suspension cultures was altered by the addition of KOH (Fig. 3A). At 0.005 and 0.01 N KOH, the onset of the HR-induced cell death was delayed until 9 h and >12 h after inoculation, respectively (Fig. 2A). As with the DABCO treatment, this delay was abolished by the inclusion of Mes buffer (Fig. 2B). Mes buffer also eliminated the pH change (Fig. 3B).

DISCUSSION

In this study, changes occurring in cells undergoing the HR were followed by using the fluorescent vital stain, FDA (15, 19). This stain enters metabolically active cells where esterases cleave the hydrophobic moiety and render the stain molecule unable to recross the plasmalemma. FDA staining traditionally has been used to measure viability of cells, and we interpreted our results in this way, *i.e.* that decreased staining during progress of the HR represented an increase in cell death. Recently, Keppler *et al.* (9) studied the staining of cells by FDA during the HR induced in cell suspension cultures. They were unable to correlate decreased staining with FDA with cell death as measured by Evan's blue staining. They suggested that decreased FDA staining by cells during the HR was due to reduced FDA influx through a damaged plasma membrane. Although it is not clear which hypothesis is correct, Keppler *et al.* did show that a decrease in FDA staining parallels events which occur during the HR (such as the K^+/H^+ exchange); thus, FDA is a useful stain for monitoring progress of the HR.

The results of this study suggest that $^1O_2$ is not involved in the bacterially induced HR in tobacco leaves or suspension cultures. Neither of the $^1O_2$ quenchers used in this study inhibited symptoms of HR development in leaves. Bixin also had no effect on HR development in cell suspension cultures. DABCO did significantly delay hypersensitive cell death in cultures, but this effect was abolished when the cultures were buffered to prevent the increase in pH resulting from the addition of DABCO. A similar delay in hypersensitive cell death could be induced simply by raising the pH of the suspension culture medium with KOH. Thus, we conclude that the inhibitory effect of DABCO on the HR in suspension culture was due to an alteration of medium pH rather than quenching of $^1O_2$.

It is significant that a high medium pH (whether caused by the addition of DABCO or KOH) delayed the onset of HR cell death with the length of delay positively correlated with alkalinity. Atkinson *et al.* (2) found that excess K^+ external to the cells prevented the characteristic loss of K^+ during the tobacco suspension cell HR and that the ionophore, FCCP, also prevented the K^+/H^+ exchange. Thus, a simple explanation of high pH delaying HR may be that a high pH medium will have a low concentration of H^+ available for uptake by tobacco cells during the HR. On the other hand, the nature of bacterial binding to plant cells is not well understood and a high medium pH may alter possible ionic interactions. Duvick and Sequeira (6) suggested that binding between *Pseudomonas solanacearum* and tobacco suspension cells had an ionic component. Stall and Cook (18) reported that *Xanthomonas vesicatoria* must bind to plant cells before the HR is elicited.

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