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Rapid Stimulation of an Oxidative Burst during Elicitation of Cultured Plant Cells[1]

Role in Defense and Signal Transduction

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ABSTRACT

Stimulation of cultured plant cells with elicitors of the defense response leads to the rapid destruction of a variety of water-soluble compounds including indoleacetic acid and certain fluorescent dyes. This destructive activity, which is often vigorously manifested within 5 minutes of elicitor addition, is shown to derive from the rapid production of H_2O_2 and its use by extracellular peroxidases. Because of its speed of appearance, this oxidative burst may qualify as the first induced line of defense against invading pathogens. Since H_2O_2 has been implicated as a second messenger of hormone-stimulated metabolic changes in some animal cells, its possible role in transduction of the defense signal in plants was also examined. Not only did exogenous H_2O_2 alone stimulate phytoalexin production in the plant cell suspension, but inhibition of elicitor-stimulated phytoalexin production was observed upon addition of catalase and other inhibitors of the oxidative burst. Furthermore, for inhibition to occur, the presence of catalase was required during elicitor addition, since if introduction of the enzyme was delayed until 1 hour after addition of the elicitor, no inhibition resulted. These results suggest that H_2O_2 also plays an important role in inducing subsequent defense responses such as phytoalexin production.

In response to pathogenic attack, plants may mobilize a large variety of defense mechanisms designed either to strengthen barriers against invasion or to weaken and destroy the invading pathogen. Examples of defense strategies aimed at obstructing the pathogen's access to plant cell nutrients include lignification (22), suberization (14), hydroxyproline-rich protein biosynthesis (38), callose biosynthesis (21) and the hypersensitive response (12, 19, 35). Defense products thought to debilitate or injure the pathogen may include phytoalexins (18, 24), hydrolytic enzymes such as chitinase and β-glucanase (4), tannins and *o*-quinones (3), and proteinase inhibitors (36). Since genetic deficiencies in the plant's ability to activate the above pathways are commonly associated with enhanced susceptibility to disease, most of the above strategies are believed to be important in disease resistance.

Comparison of the kinetics of appearance of defense mechanisms of susceptible and resistant plant varieties suggests that a major determinant of the success of a resistance strategy lies in its speed of expression (18, 24). In general, if significant levels of defense products appear before pathogen colonization is achieved, the attack can be successfully repelled. However, if the resistance pathways are delayed until counteracting mechanisms have been activated by the pathogen, colony expansion will likely proceed.

Even in a successful resistance response, not all defense mechanisms are rapidly expressed. For example, quinones, tannins, phytoalexins, and proteinase inhibitors generally appear before the hydrolytic enzymes, and these in turn precede hydroxyproline-rich protein biosynthesis (3, 4, 9, 13, 15, 38). Other cell wall stabilizing mechanisms also commonly occur late in the chronology of defense product expression, usually 12 to 24 h after elicitation. The most rapidly appearing defense components (*e.g.* tannins and phytoalexins) are initially detected 2 to 4 h after stimulation with elicitor (13). It is conceivable that these more rapid responses serve to retard the pathogen's invasion until more potent or long-lasting defense mechanisms can be mobilized.

In our studies of elicitor signal transduction in cultured plant cells, we have observed that a variety of exogenously added compounds are destroyed within 0 to 8 min of elicitor addition, depending on the nature and concentration of the elicitor added. Because this burst of destructive activity could qualify as a new, extremely rapid mechanism of disease resistance, we have characterized its source and properties further. In this report, we demonstrate that the above destructive process derives from the rapid stimulation of H_2O_2 production and its subsequent use by endogenous cell wall peroxidases to oxidize susceptible substrates. We also partially characterize the peroxidase and propose a role for it and the H_2O_2 burst in the overall disease resistance mechanism. A less extensive study leading to similar conclusions appeared in press during preparation of this manuscript (26).

MATERIALS AND METHODS

Chemicals

The fluorescent molecular probes, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (pyranine; λ_{ex} 405 nm, λ_{em} 512 nm); bis-(3-propyl-5-oxoisoxazol-4-yl) pentamethine oxonol

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(oxonol VI; λ_{ex} 609 nm, λ_{em} 645 nm); 3,3′-diethylthiacarbocyanine iodide (carbocyanine; λ_{ex} 551 nm, λ_{em} 568 nm); 1,1′-diethyl-3,3,3′,3′-tetramethylindocarbocyanine iodide (indocarbocyanine; λ_{ex} 540 nm, λ_{em} 556 nm); and 5(and-6)-carboxy-4′,5′-dimethylfluorescein diacetate (carboxyfluorescein; λ_{ex} 495 nm, λ_{em}, 530 nm) were purchased from Molecular Probes, Eugene, OR. Stock solutions were prepared at a concentration of 1 mg/mL in the solvent specified by the distributor (Molecular Probes, Handbook of Fluorescent Probes and Research Chemicals). Chemicals and media ingredients were reagent grade and purchased from Sigma Chemical Co. and Mallinckrodt, Inc. Catalase (50,000 units/mg protein; bovine liver) and superoxide dismutase (3000 units/mg protein; bovine liver) were purchased from Sigma Chemical Co. Chemicals to be added to cell cultures were sterilized by membrane filtration (GS 0.2 μm, Millipore). Glyceollin was a generous gift from Dr. David Kuhn, Purdue University.

Plant Cell Cultures

Cell suspension cultures of *Glycine max* Merr var Kent were obtained from Dr. Mike Hasegawa, Purdue University, and were grown in W-38 medium (17). Cell cultures were transferred to fresh medium every 7 d.

Elicitor

The *Verticillium dahliae* 277 elicitor was prepared as previously described (2, 27). A typical elicitor preparation contained 70 μg of protein and 134 μg of glucose equivalents per mL. In general, 10 μL were used for a fluorescence transition assay (total suspension volume, 1.5 mL) and 0.2 mL were used for a phytoalexin assay (total suspension volume, 30 mL). The oligogalacturonide fraction used as elicitor was prepared from polygalacturonic acid as described (31). A typical preparation contained 500 μg of uronic acid equivalents per mL. For a typical fluorescence transition assay 10 μL was added.

Fluorescence Transition Assay

The assay has been described in detail (2, 27). Two μL of molecular probe stock solution were added to 1.5 mL of soybean cells (36 h old) suspended in a quartz cuvette. The cells were continuously stirred at a slow speed to prevent sedimentation of cells without mechanically disrupting or eliciting them. Carboxyfluorescein diacetate was preloaded for 20 min and the cells were washed three times with medium to remove any extracellular probe. The other probes were added directly to the cell suspension approximately 1 min prior to elicitation and no washing step was employed. All other reagents were added to the cell suspension at the times indicated in the text.

Fluorescence Reversibility Experiments

Fluorophores were added to the cell suspensions and allowed to equilibrate as described above. Part of the cells were immediately treated with 0.4% SDS and the emission spectrum was determined. The other part of the suspension was stimulated by the addition of elicitor and the fluorescence transition was allowed to proceed. After no further elicitor-induced fluorescence changes were observed, the 1.2-mL cell suspension was similarly treated with 0.4% (final concentration) SDS solution and the emission spectrum recorded.

Phytoalexin Assay

The formation of glyceollin (a mixture of glyceollin isomers and daidzein) in the cells of soybean cell suspension cultures (36 h old) was measured according to the procedure of Ebel *et al.* (13) as described previously (2, 27). Glyceollin was analyzed by TLC (13) or by high pressure liquid chromatography according to the method of Mieth *et al.* (30), except a linear elution gradient of 62 to 100% methanol was used.

RESULTS

Reversibility of Fluorescence Transitions

Fluorescent probes have proven useful in studies of hormone-stimulated changes of intracellular pH and membrane potential in cultured animal cells (8, 40). When these same fluorescent probes are equilibrated with cultured plant cells, the cells absorb the dyes and display a bright fluorescence which can remain unaltered for several hours. This fluorescence, however, suddenly changes when an elicitor is added to the cell suspension (2, 27), suggesting that stimulation of the defense response activates processes which modify dye properties. The kinetics of the fluorescent transitions of three such probes (*i.e.* oxonol VI, pyranine, and carbocyanine) after stimulation with low concentrations of a crude *Verticillium* elicitor have been described previously (27). Following a brief lag period of 0 to 8 min (which depends on the elicitor concentration added, ref. 27), all three dyes display a gradual fluorescence loss. Carboxyfluorescein, on the other hand, shows a steady fluorescence increase upon stimulation of the cells with the *Verticillium dahliae* extract. Similar results were also obtained with other elicitors (*e.g.* an oligogalacturonide fraction from citrus pectin) and with other cell lines (*e.g.* cotton and tobacco), suggesting the phenomenon is neither restricted to the cultured plant cell line nor to the elicitor component used (data not shown).

Because oxonol VI and the carbocyanine dyes commonly report on changes in membrane potential and since changes in membrane potential depend inextricably on membrane integrity, it seemed reasonable that the observed fluorescence quenching should at least be partially reversed by disrupting the cellular membranes and equilibrating the ions among the various intracellular compartments. To evaluate this possibility, SDS was added to the cell suspensions either before or approximately 10 min after addition of the elicitor, and the fluorescence of each probe was evaluated. Surprisingly, although the cells were completely permeabilized by the SDS, no return of probe fluorescence in either of the elicited samples was observed (Fig. 1, A and B). However, the fluorescence of both unelicited samples remained high following SDS solubilization, suggesting the SDS was not responsible for the permanent loss in fluorescence but instead that the elicitation process was.

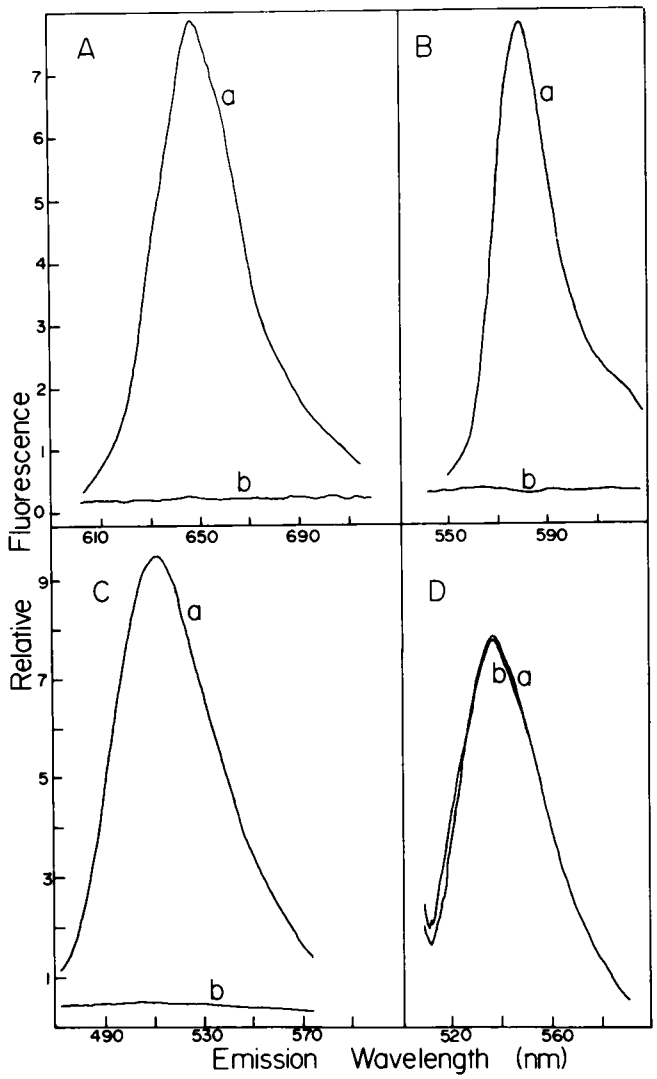

Figure 1. Fluorescence emission spectra of the molecular probes employed in Figure 1 scanned both (a) before, and (b) approximately 10 min after elicitation of the cultured soybean cells. To eliminate any contribution to the fluorescence change deriving from a change in the electrochemical properties of a cellular organelle or compartment, the labeled cells were partially dissolved in 0.4% SDS prior to each scan. A, oxonol VI (λ_{ex}, 609 nm); B, carbocyanine (λ_{ex}, 551 nm); C, pyranine (λ_{ex}, 405 nm); D, carboxyfluorescein (λ_{ex}, 495 nm).

In a similar manner, the reversibility of the fluorescence transitions of the pH-sensitive probes, pyranine and carboxyfluorescein, was examined by SDS solubilization (Fig. 1, C and D). Although SDS allowed complete pH equilibration throughout the sample, the fluorescence of the pyranine probe in the elicited sample was not restored even though the fluorescence of the similarly treated unelicited sample remained very intense (Fig. 1C). Furthermore, a scan of the entire emission spectrum of the elicited sample demonstrated that no shift in emission wavelength had occurred, but instead that the fluorescence of the probe had actually been destroyed. Carboxyfluorescein, on the other hand, showed the anticipated reversibility in emission properties following elicitation (Fig. 1D), demonstrating that no modification of the probe had occurred.

Because fluorophore destruction renders the probe useless as a reporter of changes in intracellular pH or membrane potential, it was of interest to determine which properties of a fluorescent dye rendered it susceptible to elicitor-stimulated bleaching. Therefore, a series of compounds which could be monitored fluorimetrically were examined in the above described reversibility assay. Although all water-soluble intracellularly trapped dyes were resistant to irreversible modification, virtually any extracellular or plasma membrane-associated probe with a phenolic, isoxazolium or thiazolium group was rapidly destroyed (data not shown). The auxin, IAA, was also found to be highly susceptible to elicitor-stimulated modification (Fig. 2), as was the common peroxidase and diphenlyoxidase substrate, L-DOPA[2] (not shown). Simple conjugated compounds (*e.g.* the membrane potential-sensitive dye, indocarbocyanine) were generally resistant to destruction.

Nature of Fluorescence Quenching Reactions

Since the bleached fluorescent probes all contained readily oxidizable functional groups, we decided to investigate whether an elicitor-activated oxidative process might be involved in probe modification. To resolve this issue, we treated the cell suspensions with superoxide dismutase, mannitol, or catalase prior to elicitation in order to destroy any elicitation-generated $O_2^{\cdot-}$, $OH^{\cdot}$, or H_2O_2, respectively. Figure 3 demonstrates that while neither 0.15 mg/mL superoxide dismutase nor 50 mM mannitol had any effect on the elicitor-induced fluorescence transition of pyranine, 0.15 mg/mL catalase nearly completely obliterated the quenching reaction. Furthermore, neither heat-denatured catalase nor heat-denatured superoxide dismutase altered the elicitor-stimulated fluores-

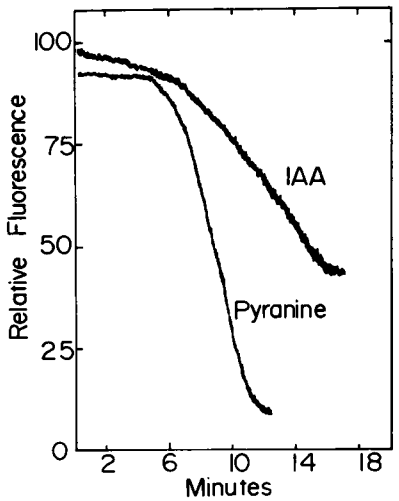

Figure 2. Effect of addition of elicitor to cultured soybean cells treated with 16 μM IAA on the fluorescence of the IAA. The excitation and emission wavelengths for monitoring the destruction of IAA were set at 302 and 360 nm, respectively. The fluorescence transition of pyranine in the same cell suspension is provided for comparison.

[2] Abbreviations: L-DOPA, L-β-3,4-dihydroxyphenylalanine: DEDTC, diethyldithiocarbamate; SHAM, salicylylhydroxamic acid.

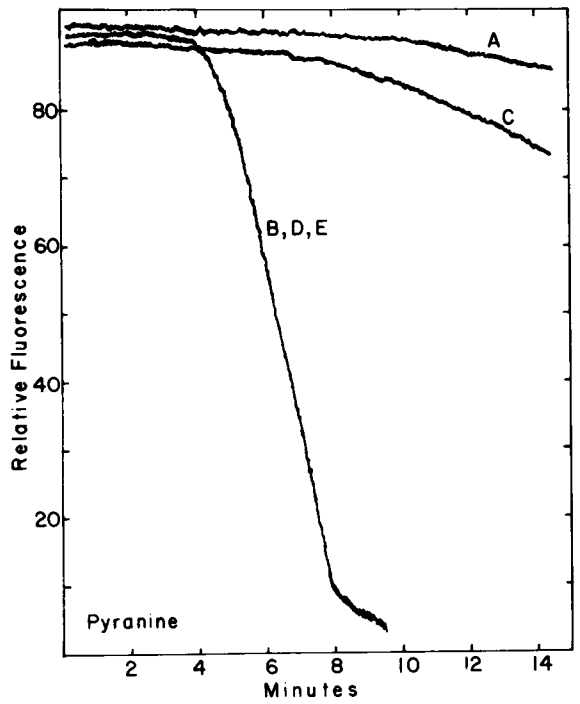

Figure 3. Effects of superoxide dismutase, mannitol, and catalase on the elicitor-stimulated fluorescence quenching of pyranine in cultured soybean cells. The cells were treated with (A) no elicitor, (B) 7 μL/mL elicitor, or 7 μL/mL elicitor plus (C) 0.15 mg/mL catalase, (D) 0.15 mg/mL superoxide dismutase, (E) 50 mM mannitol. Since neither superoxide dismutase nor mannitol modified the elicitor-induced quenching transition, curves B, D, and E are drawn superimposed.

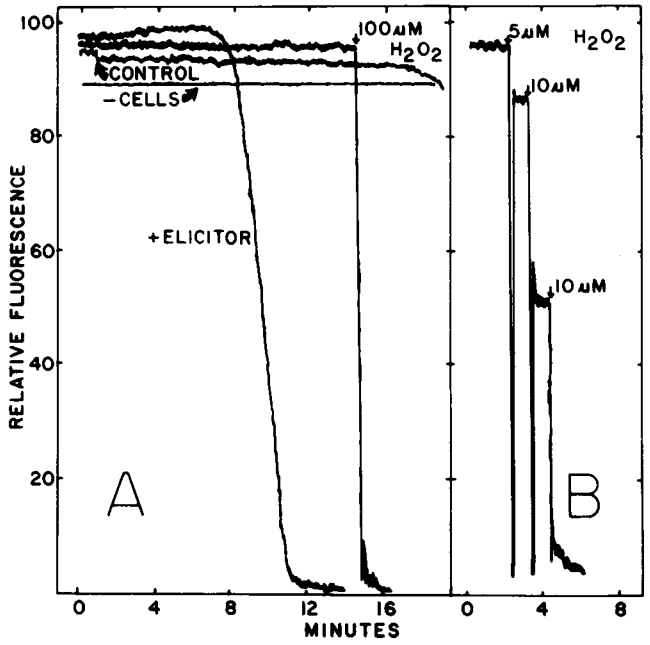

Figure 4. Effect of exogenous H_2O_2 on the fluorescence of pyranine in cell growth medium in the presence or absence of cultured soybean cells. A, In the absence of added H_2O_2 and elicitor, no fluorescence change is seen in the labeled cell suspension (control). In contrast, when 7 μL/mL elicitor is added to the labeled cell suspension, a sudden decrease in fluorescence intensity is seen after about 8 min (+ elicitor). Furthermore, when elicitor and 100 μM H_2O_2 are added to the cell growth medium containing pyranine but no cells, no fluorescence transition is observed (− cells). However, even in the absence of elicitor, when the labeled cell suspension is treated with 100 μM H_2O_2, a virtually instantaneous quenching is measured (100 μM H_2O_2). B, To estimate the amount of exogenous H_2O_2 needed to achieve the same degree of pyranine bleaching obtained upon addition of 7 μL/mL elicitor, aliquots of H_2O_2 were successively added to the same cell suspension and the pyranine fluorescence was monitored. As can be seen, full fluorescence quenching, similar to that obtained with the elicitor, required addition of a total of 25 μM H_2O_2.

cence quenching. These data demonstrate that H_2O_2 is required in the fluorescence bleaching process, either as a direct oxidant or as a substrate of an endogenous peroxidase, while superoxide and perhaps other free radicals are not.

To further define the role of H_2O_2 in the elicitor-stimulated oxidative burst, the series of experiments displayed in Figure 4 was conducted. In the first study, 100 μM H_2O_2 was added directly to cell-free growth medium containing both elicitor and pyranine (-cells) to learn whether the H_2O_2 directly or in combination with a component of the medium oxidized the fluorophores. As can be seen from the horizontal tracing in Figure 4A, no change in fluorescence was observed , suggesting the oxidative process requires the participation of enzymes from the plant cells. To evaluate whether elicitation triggers the oxidative burst by activating the peroxidase or by stimulating production of hydrogen peroxide, 100 μM H_2O_2 was added to the labeled cells in the absence of elicitor and the fluorescence response was monitored (Fig. 4A). Surprisingly, the dye was virtually instantaneously bleached, indicating that the oxidative enzymes are constitutively present and simply await the elicitor-stimulated production of H_2O_2 to destroy susceptible compounds. A dose-dependence study of this H_2O_2-mediated oxidation of pyranine by the cultured soybean cells is shown in Figure 5B, where the data demonstrate that a total of 25 μM H_2O_2 must be added to the suspension culture to achieve the same final peroxidation of pyranine observed upon elicitation. The observation that perfusing the cell suspension with argon prior to elicitation significantly inhibited the bleaching reaction (not shown) further supports the role of *de novo* H_2O_2 generation in initiating the oxidative response.

Properties of the Peroxidase(s)

Since intracellularly trapped dyes are not substrates of the elicitor-stimulated peroxidase activity and since catalase, an impermeable enzyme, is capable of totally blocking the oxidative burst, it can be concluded that the reactions involved in dye destruction take place outside the plasma membrane, possibly in the cell wall (39). To further characterize the nature of this oxidative activity, a series of known inhibitors of plant peroxidases were tested for their abilities to interfere with the elicitor-stimulated bleaching of both pyranine and IAA. Figure 5 and Table I display the results of several representative inhibition experiments. KCN, a known inhibitor of IAA oxidase and other enzymes involved in O_2 metabolism (16), inhibited both pyranine and IAA oxidation with a K_i of about 2 μM (Fig. 5, A and B). However, in neither case was the

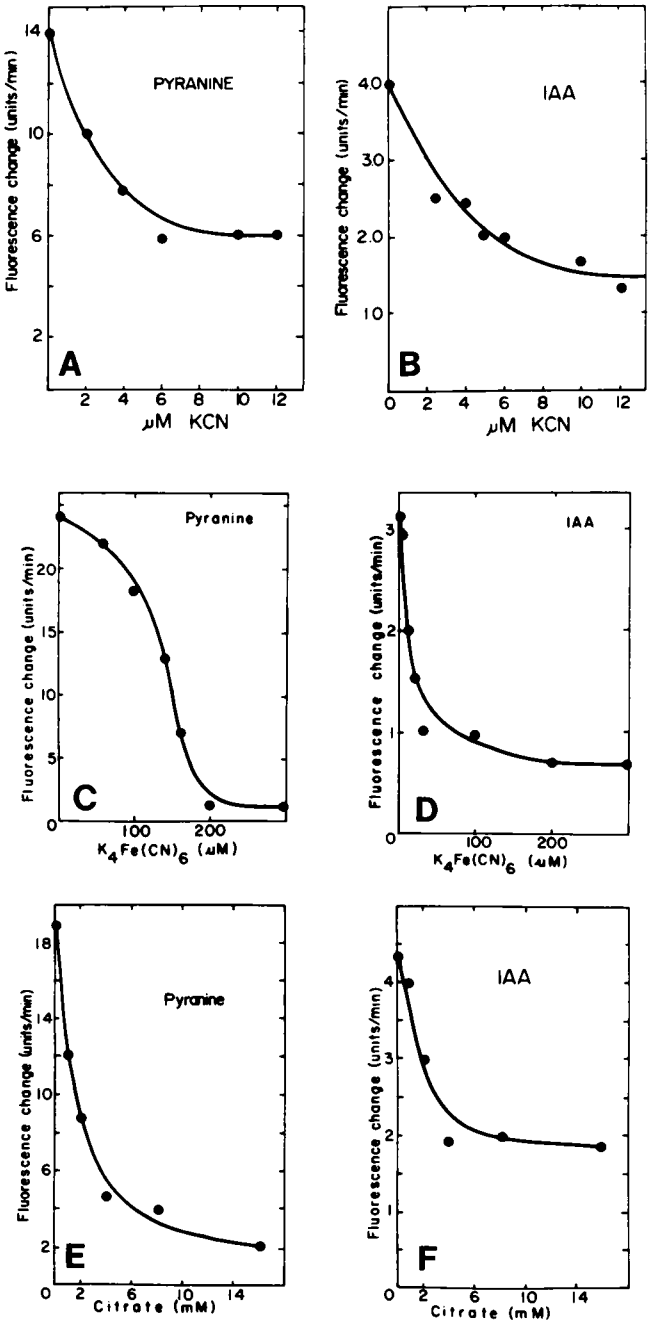

Figure 5. Effect of KCN, K_4 ($Fe(CN)_6$ and sodium citrate on the rate of elicitor-induced pyranine and IAA quenching in cultured soybean cells. The above inhibitors were added to the cell suspension directly prior to stimulation with 7 μL/mL elicitor.

inhibition complete, suggesting that while a KCN-sensitive enzyme may be a component of the oxidative burst, KCN-independent oxidation reactions also contribute to dye destruction. The apparent multiplicity of oxidative pathways was also manifested in the disparate effects of $Fe(CN)_6^{-4}$ on IAA and pyranine oxidation (Fig. 5, C and D). Whereas pyranine modification was nearly quantitatively inhibited by ferrocyanide with an apparent positive cooperativity, IAA oxidation was only partially inhibited, and the concentration

Table I. *Effect of Peroxidase Inhibitors on Elicitor-Stimulated Pyranine and IAA Oxidation and on Glyceollin Production in Cultured Soybean Cells*

Inhibitor	Concentration	Inhibition		
		IAA oxidation[a]	Pyranine oxidation[a]	Glyceollin formation[b]
	μM		%	
None[c]	0	0	0	0
Catechol	15	100	60	43
DEDTC	50	100	75	56
SHAM	40	100	75	41
KCN	6	55	60	26
$Fe(CN)_6^{-4}$	200	70	90	74
Citrate	10 mM	55	85	60
$FeSO_4$	10	85	100	50

[a] Soybean cells (1.5 mL, 36 h old) were stimulated with elicitor (10 μL) in the presence or absence of inhibitors and evaluated for elicitation by the fluorescence assay described in "Materials and Methods." [b] Glyceollin formation was determined 48 h after addition of elicitor (0.2 mL/30 mL cells) with or without inhibitors as described in "Materials and Methods." [c] In the absence of inhibitors, elicited cultures produced 86 μmol glyceollin per g dry weight of cells.

dependence was hyperbolic. Furthermore, the $Fe(CN)_6^{-4}$ concentration at 50% maximal inhibition was 140 μM for pyranine but only 15 μM for IAA. Citrate, a known inhibitor of elicitation in cultured cells (2), also blocked the oxidation of pyranine and IAA with a similar apparent K_i of 2 mM (Fig. 5, E and F). However, inhibition of IAA oxidation was never complete (Fig. 5F) in contrast to pyranine (Fig. 5E), and elicitor-stimulated oxonol VI destruction (not shown) was nearly unimpeded by citrate. Additional evidence for heterogeneity in elicitor-stimulated oxidative processes can be seen by comparing the abilities of other potential peroxidase and/or electron transport inhibitors to block IAA and pyranine oxidation (Table I).

Possible Involvement of Oxidative Burst in Elicitor Signal Transduction

As elaborated later in the "Discussion," multiple potential functions can be assigned to the elicitor-triggered oxidative burst. Because of the speed of the response and since H_2O_2 can rapidly diffuse across biological membranes, one such function was speculated to be that of a short-lived second messenger. To partially evaluate this hypothesis, a series of experiments was conducted to examine the role of H_2O_2 in elicitor signal transduction. In the first experiment, H_2O_2 was added directly to a flask of cultured soybean cells, and the levels of glyceollin, a phytoalexin, were measured after the usual induction period of 48 h. As seen in Table II, direct addition of H_2O_2 to the suspension culture stimulated a significant production of phytoalexins. This demonstrates that H_2O_2 can directly induce at least part of the defense response. To determine whether obstruction of normal H_2O_2 production can interfere with elicitation, catalase was added to the soybean cell culture, and the ability of the elicitor to promote phytoalexin production was reevaluated. When catalase was introduced prior to elicitation, glyceollin production was re-

Table II. *Role of H_2O_2 in Signal Transduction*

Addition to Soybean Suspension Culture	Glyceollin produced[a]
	μmol/g dry weight
None	0
H_2O_2 (0.5 mM)	68
H_2O_2 (1.0 mM)	117
Elicitor[b]	189
Catalase + elicitor[c]	40
Elicitor + catalase[d]	172
Boiled catalase + elicitor[e]	241

[a] Glyceollin content in the cell mass (excluding extracellular medium) was determined 48 h after the start of the incubation as described in "Materials and Methods" (14). [b] Ten μL crude elicitor preparation was added per mL of cells. [c] Elicitor was added 1 h after introduction of catalase. [d] Catalase was added 1 h after stimulation with elicitor. [e] Catalase was immersed in boiling water for 20 min prior to addition to the cell culture. Similar results were obtained with boiled superoxide dismutase.

duced nearly 80% (Table II). However, when addition of catalase was delayed until 1 h after elicitor addition or when boiled catalase was added, no significant inhibition of phytoalexin biosynthesis was observed. This strict dependence of catalase inhibition on its time of introduction demonstrates that catalase must interfere with one of the initial events of elicitation, a step or process that is complete within 1 h of the initial extracellular stimulus but considerably before any phytoalexins are produced. This behavior is clearly diagnostic of an inhibitor of signal transduction and points to the participation of H_2O_2 in this process. Obviously, confirmation of the role of H_2O_2 as a second messenger in elicitation must await more detailed experimentation. However, it is interesting to note that all of the inhibitors of the oxidative burst examined to date also block glyceollin production with roughly equal potency (Table I).

DISCUSSION

We have presented evidence that elicitation of cultured plant cells stimulates a burst of oxidative activity which can lead to the destruction of a variety of susceptible compounds. The oxidative burst, which derives predominantly from the rapid formation of H_2O_2 and its subsequent use by extracellular peroxidases, qualifies as the earliest defense product yet reported. While phytoalexins and other defense components are generally not detected for 2 h postelicitation (18, 36, 38), destruction of fluorescent dyes can be measured less than 1 min after elicitor addition (27). This response is, therefore, clearly distinct from the well-characterized peroxidase activities which first appear approximately 9 h after elicitation and probably derive from *de novo* synthesis of the enzymes (20, 35). Because of its speed of appearance, the oxidative activity may serve as a first line of defense against the invading pathogen.

It is difficult to avoid comparing the oxidative burst seen in this study with that observed in higher animals in response to infection. When a granulocyte encounters an antibody-coated bacterium, virus, or other foreign particle in the blood stream or interstitial spaces, the granulocyte is stimulated to generate superoxide which is rapidly converted to H_2O_2 and other reactive oxygen intermediates (33). These intermediates, which form the basis of the principal intraphagosomal killing mechanism as well as the basis of the extracellular cytotoxity of neutrophils and macrophages, cause membrane damage primarily through lipid peroxidation (25). In plants, lipid peroxidation is also known to be a consequence of peroxidase activation (19, 35) and may even form an essential element of the hypersensitive response (19). For example, superoxide radicals are generated during interaction of tobacco leaves with pathogens and have been implicated in the host's defense mechanism (12). Several workers have shown that the ensuing lipid peroxidation can lead to membrane leakiness and cell death, thus performing an essential step in the hypersensitive reaction (12, 129, 35). However, whether reactive oxygen intermediates act offensively in plants as they do in animals in directly attacking the pathogen is not currently known. Still, in considering the role of extracellular peroxidases in the overall defense strategy of plants, it is worth remembering that H_2O_2 is a powerful bacteriocidal agent (25, 33), and that alone or in combination with cell wall peroxidases it could contribute significantly to repulsion of pathogens, as recently suggested (20).

That the elicitor-stimulated oxidative activity could serve other functions besides those associated with oxygen toxicity should not be overlooked. Thus, lignin production is believed

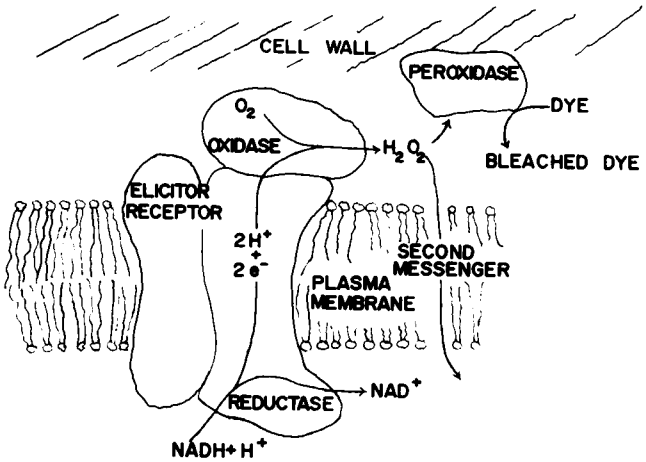

Figure 6. A hypothetical model of the arrangement of those redox components in the plasma membrane/cell wall which may be involved in the oxidative burst. An elicitor receptor is hypothesized to be linked in some manner to a redox chain which consumes NADH/NADPH to generate H_2O_2. The reductase is somewhat arbitrarily oriented across the membrane, since it likely employs cytosolic NADH/NADPH to reduce extracellular O_2 to H_2O_2. The peroxidase may be cell wall associated as suggested by many others. The target of the H_2O_2 in transmembrane signaling could be virtually any component, but since mRNA synthesis must occur in the nucleus, the signaling arrow has been sketched pointing toward the cell interior. Based on this model, any component which interferes with receptor-reductase coupling, or which consumes electrons (*i.e.* an oxidant such as $Fe(CN)_6^{-3}$) or reduces H_2O_2 (*e.g.* $Fe(CN)_6^{-4}$), or which blocks the oxidase (*e.g.* KCN) would be expected to inhibit both the bleaching and transmembrane signaling functions of the oxidative burst.

to require H_2O_2 and peroxidases (39), and lignification is a common response to pathogen attack (22). Hydrogen peroxide is also employed in the metabolism of IAA (16), and this hormone has been observed to inhibit the defense response (7, 37) and should probably be temporarily removed in a successful resistance mechanism. Production of hydroxyproline is also catalyzed by peroxidases, and this modification is essential for biosynthesis of hydroxyproline-rich proteins which may help stabilize cell walls against pathogen invasion (39). H_2O_2 may even be involved in the synthesis of toxic quinones which can react with and modify essential proteins in invading microorganisms (39). Thus, the spectrum of potential roles for the oxidative burst in the plant's defense response is large, and a complete explanation of its adaptive value in repelling a pathogen will likely be very complicated.

Although H_2O_2 has never been implicated as a component of a signal transduction pathway in plants, its probable role as a hormone second messenger in animal cells has received substantial attention (34). For example, H_2O_2 mimics many of the effects of insulin, including activation of glucose transport, stimulation of pyruvate dehydrogenase activity, enhancement of lipid synthesis, elevation of the hexose monophosphate shunt and gluconeogenesis, stimulation of receptor tyrosine kinase activity, and inhibition of lipolysis (11, 23, 29). Since insulin stimulates the rapid production of H_2O_2, it has been suggested that the peroxide may mediate many of the metabolic changes attributed to the hormone (28). H_2O_2 may similarly explain the capacitation of sperm, the process by which sperm is prepared for participation in the acrosome reaction (I. Bize, G. Sautander, P. Cabello, D. Driscoll, manuscript in preparation). During this period, sperm produce large amounts of H_2O_2 (1) which by itself has been found to stimulate *in vitro* many of the biochemical consequences of capacitation (I. Bize et al, manuscript in preparation). Likewise, small amounts of H_2O_2 are known to trigger the process of histamine secretion by mast cells, suggesting the oxidant may also play a role in initiating the inflammatory response (32). Since the interaction of several hormones with their receptors has been found to regulate transplasma membrane redox enzymes capable of oxidizing NADH to generate H_2O_2 [or to reduce $Fe(CN)_6^{-3}$], a possible mechanism explaining the coupling between hormone binding and plasma membrane redox reactions is already present (10, 34). Importantly, similar plasma membrane redox components have recently been described in plants (5, 10) and current evidence suggests they may even be tightly coupled to hormone receptors (6). The elicitor-stimulated production of H_2O_2 observed here may simply represent another class of such receptor-linked transplasma membrane redox components, as illustrated schematically in Figure 6 and described by others (5, 10). In this hypothetical model, the H_2O_2 produced would be available to serve any of the oxidative or second messenger functions described above. Thus, by promoting formation of a regulatory disulfide bond in a modulator of gene expression, or by displacing the ratio of reduced to oxidized glutathione or NADP, pathways leading to phytoalexin biosynthesis might be stimulated. Furthermore, inhibitors of any single component of the oxidative pathway, *i.e.* the receptor, the reductase, the oxidase, the peroxidase, or the H_2O_2 target in signal transduction, could significantly alter or inhibit the process.

In summary, elicitation of cultured plant cells triggers the rapid generation of H_2O_2, which can be employed by extracellular peroxidases to oxidize susceptible compounds. Some fluorescent dyes commonly used in other studies to monitor changes in pH and membrane potential are destroyed by this oxidative burst, suggesting caution must be exercised when selecting such dyes for plant studies. For example, the fluorescent transitions of pyranine and oxonol previously described by our group (27) can now be unequivocally attributed to oxidative quenching. However, other dyes, such as carboxyfluorescein, are not modified by the oxidative burst, but report on the ion movements to which they are normally sensitive (our manuscript in preparation). Despite the rapid bleaching of pyranine and carbocyanine, during elicitation these oxidizable substrates are very useful as probes of elicitation, since they report on one of the very initial events of the defense process. Although H_2O_2 may be directly employed in several pathogen resistance mechanisms, evidence also suggests that it may indirectly facilitate the process by serving as a second messenger. H_2O_2 has, in fact, many of the properties expected of a defense-related signal transducer, since (a) it is formed rapidly upon elicitor treatment, (b) it can be rapidly destroyed, (c) it can independently stimulate phytoalexin production, and (d) its removal by catalase or other inhibitors blocks the defense response.

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