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COMMENTARY

THE ORIGIN OF THE OXIDATIVE BURST IN PLANTS

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A large number of publications recently have drawn strong analogies between the production of active oxygen species in plant cells and the "oxidative burst" of the phagocyte, even to the point of constructing elaborate models involving receptor mediated G-protein activation of a plasmalemma NADPH oxidase in plant cells. However there are potentially other active oxygen species generating systems at the plant cell surface. The present work examines these alternatives with particular emphasis on the rapid production of active oxygen species, in common with a number of other systems, by suspension-cultured cells of French bean on exposure to an elicitor preparation from the fungal pathogen *Colletotrichum lindemuthianum*. The cells show a rapid increase in oxygen uptake which is followed shortly afterwards by the appearance of a burst of these active oxygen species, as measured by a luminescence assay, which is probably all accounted for by hydrogen peroxide. An essential factor in this production of H_2O_2 appears to be a transient alkalinization of the apoplast where the pH rises to 7.0–7.2. Dissipation of this pH change with a number of treatments, including ionophores and strong buffers, substantially inhibits the oxidative burst. Little evidence was found for enhanced activation of a membrane-bound NADPH oxidase. However the production of H_2O_2 under alkaline conditions can be modelled *in vitro* with a number of peroxidases, one of which, an M_r 46,000 wall-bound cationic peroxidase, is able to sustain H_2O_2 production at neutral pH unlike the other peroxidases which only show low levels of this reaction under such conditions and have pH optima at values greater than 8.0. On the basis of such comparative pH profiles between the cells and the purified peroxidase and further inhibition studies a direct production of H_2O_2 from the wall peroxidase in French bean cells is proposed. These experiments may mimic some of the responses to plant pathogens, particularly the hypersensitive response, which is an important feature of resistance. A cell wall peroxidase-origin for the oxidative burst is clearly different from a model consisting of receptor activation of a plasmalemma-localised NADPH oxidase generating superoxide. An alternative simple and rapid mechanism thus exists for the generation of H_2O_2 which does not require such multiple proteinaceous components.

KEY WORDS: Plants, French bean, oxidative burst, peroxidase, elicitor, pH change.

INTRODUCTION

The production of active oxygen species appears to be a ubiquitous defence mechanism of eukaryotes. It differs from the production of superoxide and other free radicals

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through side reactions of metabolism in that it is induced as a defence mechanism specifically for the destruction of invading pathogens. Normally, free radical production is at a level capable of detoxification by catalase, superoxide dismutase and peroxidases. Such detoxification systems are universal across the plant, animal and microbial kingdoms. Under stress conditions, this protective system is overridden by the rapid production of high levels of active oxygen species by systems that have evolved and are seen to be activated as an essential part of the overall defence mechanism.

The Oxidative Burst in Plants

An important feature of resistance responses of plant cells towards invading pathogens is thought to be the rapid production of hydrogen peroxide and other active oxygen species, collectively known as the "oxidative burst". A large number of publications recently have drawn strong analogies between the production of active oxygen species in plant cells and the oxidative burst of the phagocyte, even to the point of constructing elaborate models (e.g. refs 1–4) involving receptor mediated G-protein activation of a plasmalemma NADPH oxidase in plant cells. However an important difference is that the oxidative burst exhibited by phagocytes is lethal to the engulfed pathogen but not to the cell. Even in the case of apoptosis the role of any free radical production is debatable.[5] In contrast, successful resistance responses to pathogens in plants are very often manifested as the death of a small number of hypersensitive cells at the site of the interaction with the microbial pathogen. This immobilises the pathogen to expose it to the battery of defensive molecules. It is thought that the production of active oxygen species is involved with the dual role of direct toxic action and involvement in the construction of barriers by cross-linking of extracellular components. Furthermore, cells that undergo hypersensitive cell death show extensive membrane damage and the production of lipid derived signals may be involved in intercellular signalling.[6,7] However because of the highly localised reaction, the processes are difficult to follow. Recently, mutants compromised for the control of cellular damage during pathogenesis have been identified and offer a new way to study the genes involved.[8] However, the biochemistry of this process can be studied in suitable tissue culture systems developed to model plant pathogen interactions. Thus, production of potentially toxic oxygen species has been demonstrated in wounded plant cells and more recently in the response of plant cell suspension cultures to a variety of biotic elicitor molecules.[9–18] These elicitor molecules include both those derived from pathogens, as mixtures and relatively pure components intended to model the types of signals presented to plant tissues subjected to bacterial or fungal attack, and also host-derived oligosaccharides such as oligogalacturonides.

Alternative Models for the Origin of the Oxidative Burst in Plants

There has been much speculation as to the mechanism of the production of active oxygen species in plants[1–4,9–18] and in many cases this has invoked comparison with the plasmalemma-bound NADPH oxidase system.[19] Superficially, this response in cultured cells resembles the much better characterised oxidative burst in vertebrates, where the production of superoxide and hydrogen peroxide originates from a novel flavocytochrome b which is activated by a number of proteins including cytosolic factors and small GTP-binding proteins.[19–21] At present there is no conclusive indication that a similar NADPH oxidase system is responsible for the production of active oxygen species in plant cells or whether it could be linked to a receptor system. There

is some circumstantial evidence from work on soybean where the oxidative burst is inhibited by diphenylene iodonium and an antibody to a component of the human NADPH oxidase (p22) cross-reacts with a polypeptide of similar molecular weight from plant cell membranes. The involvement of protein phosphorylation is also indicated since the oxidative burst is blocked by protein kinase inhibitors and stimulated by an inhibitor of protein phosphatase 2A.[18] However there is some doubt as to the specificity of diphenylene iodonium and also protein phosphorylation is not exclusively involved in the regulation of NADPH oxidase. Furthermore, in the soybean system[18] there was no evidence for free extracellular superoxide production in contrast to the macrophage oxidative burst.

There is another potential source of H_2O_2; cell wall bound peroxidase has been shown to be responsible for the synthesis of H_2O_2 required in lignification in horseradish and tobacco.[22-26] Plant cell walls, in general, are quite rich in peroxidases. This is evident for peroxidases of suspension-cultured cells and intact tissues of French bean[27,28] and these peroxidases and other haem-containing proteins such as cytochrome P450 can also be found in membranes close to the inner surface of the wall. A requirement for the presence of a cell wall for the generation of an oxidative burst has also been indicated since carrot protoplasts showed no oxidative burst with elicitor treatment.[29] Furthermore, mechanisms to explain the generation of hydrogen peroxide from peroxidases exist. This can occur through reduction of compound III, the oxyperoxidase state formed by the addition of superoxide anion to the native enzyme to give Fe^{II}-O-O (ref. 30) and can be sustained with sulfhydryl compounds[31] or high concentrations of NADH or NADPH[32] with Mn^{2+} (ref. 24). However, there is little evidence for sufficient concentrations of these compounds in the wall to account for the magnitude of the oxidative burst.

A third possible mechanism involves oxalate oxidase and may operate in rather specialised cereal-pathogen interactions. It differs from the mechanisms above which require the plant cell to remain intact. However under conditions where cell rupture occurs, release of vacuolar contents probably leads to acidification and an alternative origin for H_2O_2 as a consequence of oxalate oxidase activity may function in those cells where oxalate is stored.[33]

The present work investigates the nature of the oxidative burst in intact suspension cultured cells of French bean in response to fungal elicitors and tests these hypotheses. It indicates how the various responses by plant cells to pathogen-derived signals may be coordinated.

The Oxidative Burst in Elicitor-treated French Bean Cells

Suspension-cultured cells of French bean produced an oxidative burst, as measured by luminol-dependent increases in luminescence, when stimulated by an elicitor preparation from *Colletotrichum lindemuthianum*.[34] Unlike the mammalian oxidative burst,[19] the increased oxygen uptake associated with the oxidative burst was cyanide-sensitive (Fig. 1) and totally inhibited by 5 mM sodium azide (data not shown). Cells not subjected to elicitor treatment showed a steady rate of oxygen uptake over a 30 min time period in the Clarke electrode.[34] The present study showed that *this* respiration was relatively insensitive to cyanide; inhibition in the presence of KCN was never greater than 10% (data not shown) indicating the operation of a cyanide-resistant alternative terminal oxidase system characteristic of plant cells. On addition of elicitor, the cells showed increased O_2 uptake which nearly doubled by eight minutes after addition of elicitor. In contrast, this additional oxygen uptake was almost totally

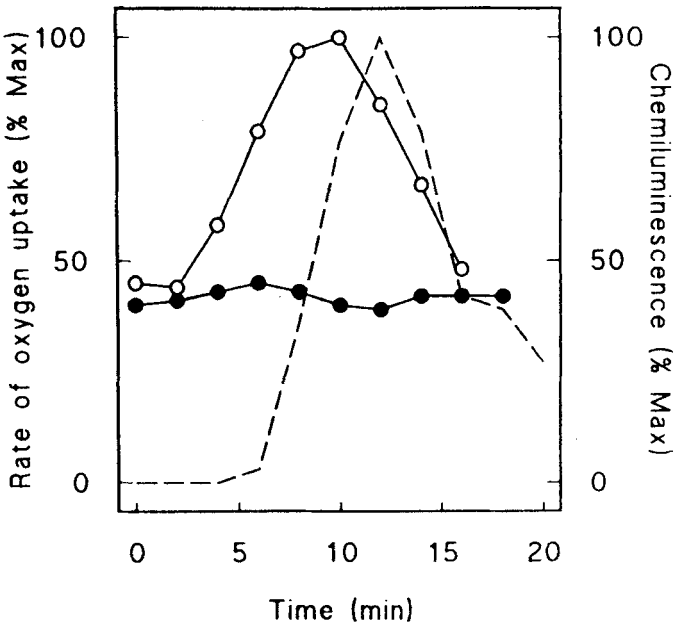

FIGURE 1 O_2 uptake and hydrogen peroxide production in cells following addition of elicitor in the presence or absence of cyanide. Cells were assayed for O_2 uptake in the presence (●) or absence (○) of 1 mM KCN and simultaneously for H_2O_2 production (- - - -) in the absence of 1 mM KCN. Typically, about 250 nmol extra O_2 was consumed in elicitor-treated cells between 6 and 12 min after stimulation. In all experiments, suspension-cultured cells of French bean were derived, maintained and subjected to treatment with elicitor from *Colletotrichum lindemuthianum* as described previously.[49] The standard experiment used cells between 4 and 6 days after subculture and treated with elicitor to a final concentration of 30 μg/ml (w/v) glucose equivalents of fungal cell wall extract in H_2O. O_2 uptake by cells was determined continuously using a Clarke oxygen electrode maintained at 22° as described previously.[34] Cells from the same batch and at the same packed volume were tested for hydrogen peroxide production simultaneously using the luminol assay using a luminometer fitted with a rotating cuvette holder and an injection port (model 1250, LKB Wallac, Broma Sweden). Cell suspensions (about 0.2g/ml medium) were taken from a batch of elicited cells every two minutes, added to the cuvette and loaded into the luminometer and continually stirred. 200 μl of luminol [0.1 $mgml^{-1}$5-amino-2,3-dihydro-1,4-phthalazinedione (Sigma, UK) in 99% H_2O/1% DMSO (v/v)] was then injected directly into the cuvette. Real time luminosity was recorded every second beginning from 10 s before injection and continuing after injecting the luminol until the level returned to background.

inhibited by the addition of KCN. This sensitivity to relatively low concentrations of KCN shows that there are some fundamental differences between this elicitor-stimulated oxygen uptake and the oxidative burst in mammalian phagocytes which is totally cyanide insensitive.[19] In this context, superoxide production by horseradish peroxidase in the presence of Mn^{2+} and reductant was inhibited by 1 mM KCN.[25] Furthermore, microsomal membranes from suspension cultures of bean, which will include plasmalemma,[35] were isolated from snap frozen elicitor-treated cells and showed no increase in extractable NADPH oxidation until nearly 4h after addition of the stimulus to the cells (Fig. 2). While this type of analysis might not duplicate a possible activation mechanism, the extractable NADPH content of the cells did not change significantly over the period of the oxidative burst (2.53 ± 0.36 at 0 time and 2.91 ± 0.78, 2.91 ± 0.81, and 2.97 ± 0.13 nmol/g fresh wt., Mean ± S.D., n = 3, at 10, 15 and 20 minutes respectively, after elicitation) in contrast to the ATP and NADH levels which did fall significantly.[34]

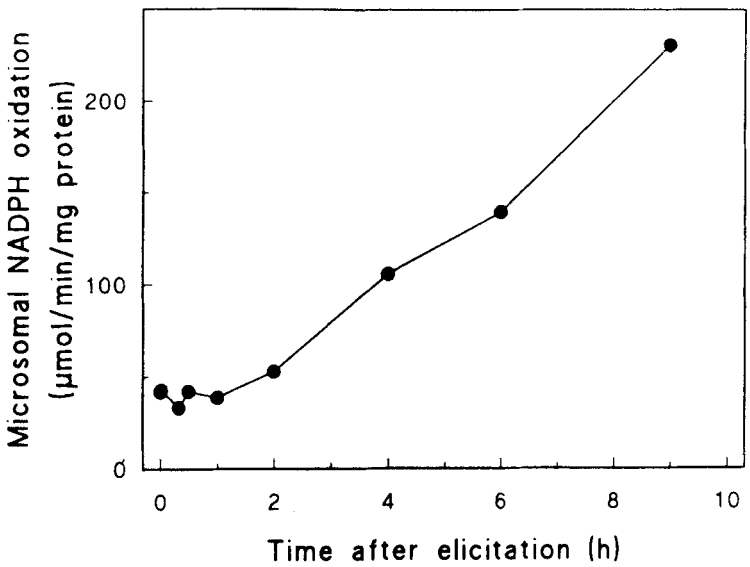

FIGURE 2 NADPH oxidation by isolated membranes from elicitor-treated French bean cells. Cells were treated with elicitor and harvested at different time points and snap frozen in lN_2. The preparation of isolated microsomal membranes has been described previously.[46] NADPH oxidation was measured spectrophotometrically at 340 nm in a mixture containing 50 mM glycyl-glycine buffer, pH 7.4, 100 mM KCl and 0.3 mM NADPH. The linear initial rate of reaction was measured after the addition of membrane preparation.

The production of active oxygen species in intact cells, as measured by the luminol assay, was related to the increased oxygen uptake in terms of the timing of their appearance, occurring within a minute of maximum oxygen uptake (Fig. 1). Their production was shown to be inhibited by KCN when measured in the presence of cells and also when assayed in aspirated medium using the ferricyanide method[36] which takes in to account any enzymatic background oxidation of luminol (Table 1). Evidence for a direct production of H_2O_2, which is not consistent with a production via superoxide characteristic of the NADPH oxidase, was also obtained. Use of enzymes and inhibitors (Table 1) showed that in the cases of both the peroxidases and cells that there was little evidence for the release of intermediate superoxide from either system in the presence of endogenous or added reductant; any superoxide must remain bound. Thus, catalase, but not superoxide dismutase (SOD), affected the luminol response (Table 1). Addition of cytochrome c to elicited cells had no effect on H_2O_2 production assayed by the ferricyanide method.[36] The hydrogen peroxide scavenger and thus peroxidase inhibitor, salicylate hydroxamate (SHAM) was also an effective inhibitor of the whole response but not tetcyclasis, a general cytochrome P450 inhibitor.[37] These results together with the demonstration that, in cell suspension cultures following addition of elicitor and using the cytochrome c reduction assay[38] no superoxide could be measured in the medium, indicate that the luminol-dependent chemiluminesence was wholly associated with the direct production of H_2O_2. This lack of detectable superoxide has also been demonstrated in soybean cell suspension cultures.[10,18]

TABLE 1
Characteristics of H_2O_2 production, as measured by luminol chemiluminescence, by isolated peroxidases and elicitor-treated suspension-cultured French bean cells

H_2O_2 Source-	M_r-46000	Cells	M_r-42000/HRP
Addition	(%untreated)	(%untreated)	(%untreated)
Catalase (50 U)	0	0	0
SOD (25 U)	100	100	100
Salicylyl hydroxamate (5 mM)	0	0	0
Tetcyclasis (50 μM)	100	82 ± 5	100
KCN (1 mM)	0	10 ± 3	0
MES (50 mM)	0	5 ± 3	0

Modulation of the oxidative burst was carried out using preincubation of cells or peroxidases for varying times before determining their capacity for hydrogen peroxide production using the luminol assay. For inhibitor studies on cells,[34] the data show the level of activity of the increased production of H_2O_2 relative to cells in the absence of inhibitor integrated over the whole time course. Inhibition for KCN was checked by the use of the ferricyanide assay[36] which is independent of the presence of peroxidase. For cells, aliquots of aspirated medium were assayed for H_2O_2 in reaction mixtures consisting of 50 μl medium, 50 μl luminol [0.1 $mg.ml^{-1}$ in 99% H_20/1% DMSO (v/v)], 800 μl Borate-KOH buffer pH 8.4 and 100 μl 14 mM freshly prepared $K_3Fe(CN)_6$ and chemiluminescence measured in the luminometer. This assay was used to confirm the action of the inhibitors on the peroxidases. The comparative effect of the prevention of the pH change is illustrated by use of medium, strongly buffered with MES pH 5.8 which is the usual starting pH upon subculture. Peroxidases were also preincubated and inhibition of the luminol reaction determined under the optimum conditions determined for each of them using cysteine as reductant. Catalase (50 units) or superoxide dismutase (25 units) were added 30 s before the luminol assay at each time point. The presence of H_2O_2 in the medium was confirmed by demonstrating that addition of medium to a standard peroxidase assay[27] could support guaiacol oxidation in the presence of any of the peroxidases, while using the cytochrome c reduction assay[38] no superoxide could be detected.

Production of H_2O_2 by Peroxidases

The production of H_2O_2 by peroxidases involving the oxidation of a reductant such as cysteine or NAD(P)H by molecular oxygen was characterised some years ago[22–26,31] but has received much less attention than their usual role in peroxidative activity and detoxification. In comparison with the bean cells, studies of the production of hydrogen peroxide from peroxidases were carried out using horseradish peroxidase (HRP) and the two cell wall peroxidases (M_r-46 000, FBP1 and M_r-42 000, FBP2) from French bean (Figs. 3,4). Using the luminol assay for active oxygen species, incubating the peroxidase alone at a higher pH than which it was routinely stored (pH 6.5) was sufficient to bring about a small brief transient (5–10 sec) production of hydrogen peroxide (Fig. 3). Addition of a reductant such as cysteine, glutathione, NADPH or ascorbate stimulated H_2O_2 production which was sustained. At concentrations that stimulated optimal production the thiols were approximately ten fold more effective than NAD(P)H or ascorbate. Luminol oxidation for both sets of conditions was totally inhibited in the presence of catalase (Table 1) indicating that it was dependent upon the production of hydrogen peroxide. The oxidation observed with peroxidase alone showed that some H_2O_2 is generated on storage and the changes in luminescence are not due to direct action of the peroxidase on luminol. This small transient production of H_2O_2, independent of added cysteine, may be derived by proton donation from a cysteine residue adjacent to the proximal haem binding site which is conserved in all plant peroxidases sequenced to date.[39] The reductant-dependent H_2O_2 production showed different pH optima for the three peroxidases (Fig. 4b). In the case of HRP and FBP2 this was 8.5–9.0. However for the cell wall-localised FBP1 this was pH 7.5

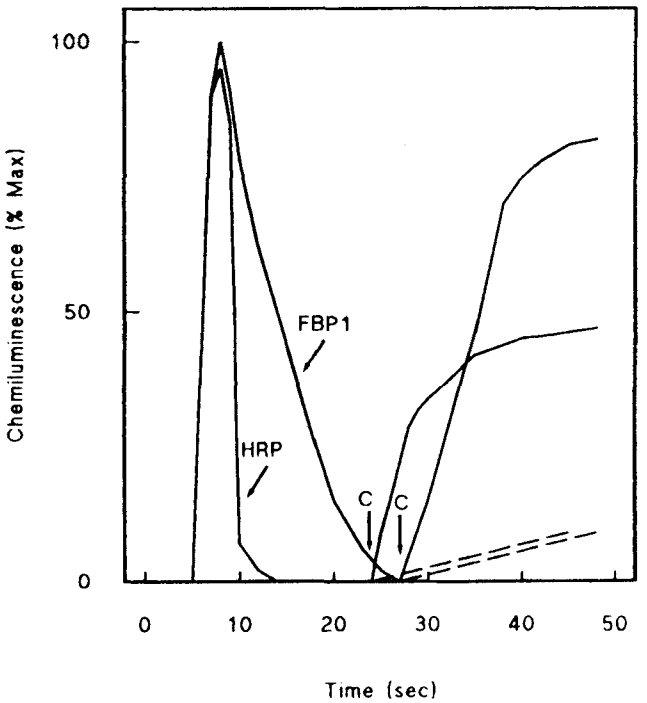

FIGURE 3 Production of H_2O_2 in vitro from plant peroxidases. Measurements of H_2O_2 production were determined for purified peroxidases in the luminometer, the operation of which was as described above. French bean peroxidases FBP1 and FBP2 were purified as described previously.[27,46] Horseradish peroxidase was obtained from Sigma, UK. Time courses for the production of hydrogen peroxide from FBP1 and HRP at pH 8.5 are shown. Enzyme stored at pH 6.5 was added to buffered growth medium at pH 8.5 to give 0.05 $A_{405\ nm}$units.ml^{-1} final concentration. The initial burst of H_2O_2 was allowed to subside, cysteine (500 μM) added where indicated (C) and the resultant sustained burst monitored. Similar results were obtained with reduced glutathione (250 μM). Maximum activities for NADH/NADPH were obtained at 2.5 mM (- - - -) and for ascorbate at 2 mM (data not shown) but were less than 10% of the relative activity obtained with thiols.

(Table 2). By comparison with the cysteine-dependent H_2O_2 production, the reaction of hydrogen peroxide with luminol alone over the same pH range showed no distinct optimum but increased especially between pH 7.5 and 8.5. The pH-dependent properties of the peroxidases are therefore not a function of the luminol assay but of the mechanism of production of H_2O_2.

Dependence of Extracellular Production of H_2O_2 on pH

H_2O_2 production could be stimulated even in the absence of elicitor treatment by simply transferring the cells to medium buffered at a higher pH (Fig. 4a). This production showed a similar time course to the production of H_2O_2 in cells exposed to elicitor and indicates a requirement for equilibration between the medium and wall irrespective of the direction of the pH change. The pH optimum for this response was found to be 7.2. Potentially therefore, hydrogen peroxide could be generated from the M_r 46 000 FBP1 peroxidase predominantly, with a lesser contribution from the other isoforms in the

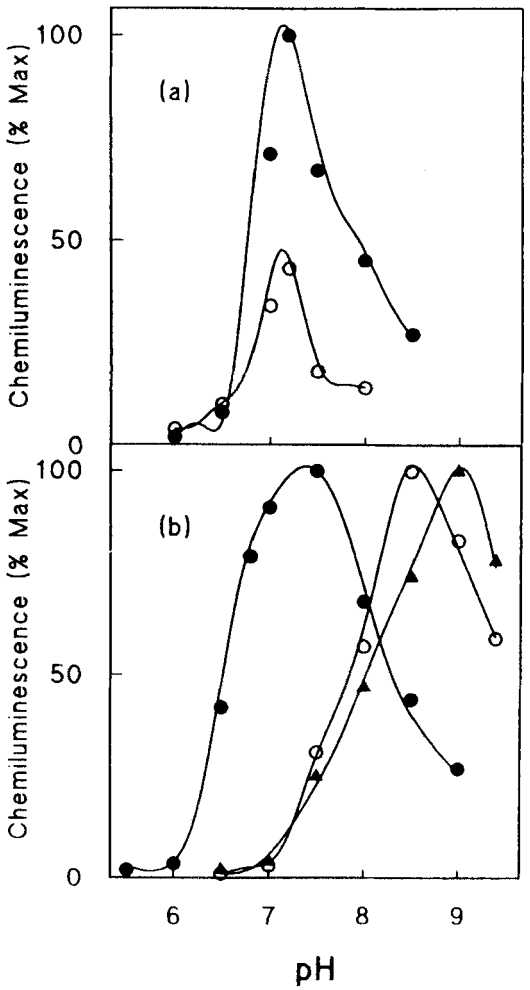

FIGURE 4 pH optima for the oxidative burst. The pH optima are shown for **a**, resuspending cells into medium at different pHs and subsequently measuring the oxidative burst with (●) or without (○) elicitor-treatment. **b**, The pH curves for the generation of H_2O_2 by peroxidases. FBP1 (●), Horseradish peroxidase (HRP; ▲) and a second French bean peroxidase FBP2 (○) in the presence of cysteine (500 μM) and determined under the same buffering conditions as for cells. Purified peroxidases or cells (0.2 g) were added to 1 ml of buffer or buffered growth medium[44] in the cuvette and loaded into the luminometer and continually stirred and luminol-dependent luminescence determined as described in Fig. 1. In some experiments, cysteine was added at this point (0.5 mM) and the time course of the additional production of luminosity followed to determine pH optima with or without added reductant (Table 2). The effect of pH on inducing the oxidative burst in cells with and without elicitation was determined by transferring cells batchwise to medium buffered with a range of sodium phosphate buffers (50 mM) and assaying chemiluminescence in the luminometer at subsequent time points after transfer to determine maximum H_2O_2 production at each pH.

bean cells which require a higher pH for the operation of the mechanism. The pH changes in the walls of the cells were measured by a simple assay system (Fig. 5), which showed that, following elicitor action, there is a rapid alkalinization of the medium which has been noted in the responses of plant cells to other elicitors.[29,40–43] Electrolyte

TABLE 2
A summary of the pH optima for hydrogen peroxide production

H_2O_2 Source	M_r -46 000	Cells	M_r -42 000/HRP
pH Optimum			
without Cys	9.0	7.2	9.0
+Cys (500 μM)	7.5	7.2	9.0

The effect of pH was determined in a range of sodium phosphate and Tris-HCl buffers (both at 50 mM) and in medium buffered similarly, and also in the presence or absence of cysteine (0.5 mM), using the luminol assay.

leakage has long been observed to be a primary effect of biotic stress on plant cells. However the wall appears to undergo a transient rise in pH which coincides with the window of peroxide production. This was demonstrated by direct measurement of the

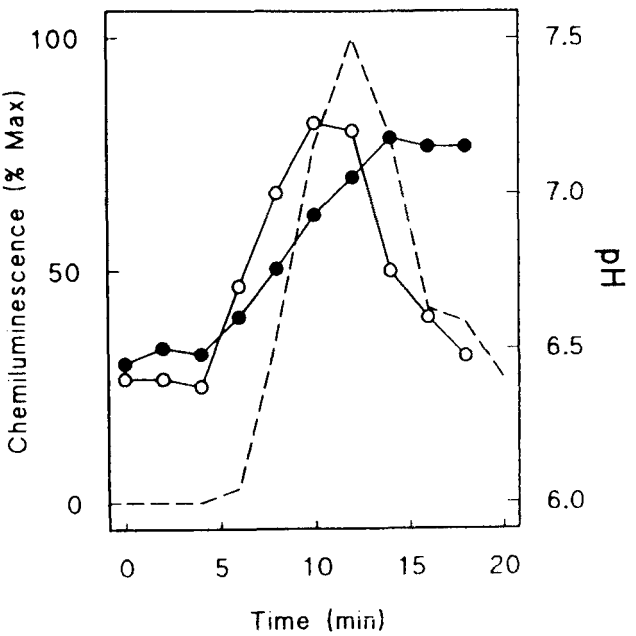

FIGURE 5 pH changes in cell walls, apoplastic fluid and culture medium in relation to H_2O_2 production. Changes in alkalinization of apoplastic (○) and culture (●) fluid with time in relation to H_2O_2 production (- - - -). pH changes were confirmed by staining (data not shown) with Universal indicator 4080 (BDH, Poole, UK) the culture filtrate and cell wall apoplastic fluid. Walls of the cells also showed a similar pattern of staining as the apoplastic fluid for all time points which was only maintained for 15 min since they appear to return to their initial pH of about 6.0 following recovery from the effect of the initial stress. External pH was determined as follows. A 1 ml sample of suspension culture was loaded into the luminometer and continually stirred until the time coincided with that for the simultaneous determination of H_2O_2 with the measurement of pH. At the same time points, 5 ml of cells were rapidly aspirated by gentle suction and the medium assayed for pH changes using a pH probe. 1 ml pure water was then added to the cells which was rapidly infiltrated by them. After the cells were allowed to equilibrate for 30 s, the apoplastic water solution was removed by suction and its pH measured by pH electrode. The validity of these methods was checked using staining with Universal indicator. The remaining cells were stained to check visually that they had the same external pH and the same pattern of changes as cells that had not undergone infiltration with water. The alkalinization was verified in this way.

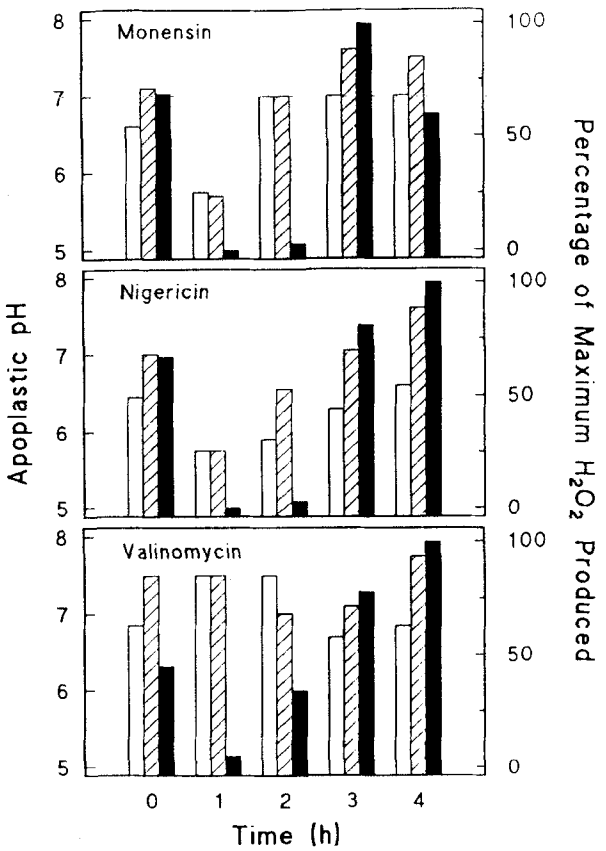

FIGURE 6 Effect of ionophores on the oxidative burst. Similar experiments to those described in Fig. 5 were performed with preincubation with the ionophores monensin (10 μM), nigericin (2 μM) and valinomycin (2 μM), concentrations previously shown to modulate the elicitation response.[44] The effect of monensin (10 μM), nigericin (2 μM) and valinomycin (2 μM) on the alkalinization of the extractable apoplastic fluid and the oxidative burst is shown. Cells were tested in the absence of ionophore (0 Time) or with ionophore for 1, 2, 3, or 4 h before elicitor was added. □ Initial pH of apoplastic fluid, ▨ pH at the height of the oxidative burst in control cells (10–12 min after addition of elicitor). Cells that underwent each treatment were assayed at this time. ■ Relative level of the oxidative burst compared with the maximum exhibited in each of the three experiments. The pH changes were confirmed visually by staining of cells and apoplastic fluid with universal indicator (data not shown).

pH of aspirated apoplastic fluid (Fig. 5) and staining of this material and cells with universal indicator (data not shown). In bean cells the apoplastic pH appears to reach pH 7.0–7.2. This is clearly compatible with the pH optimum for hydrogen peroxide production from the M_r 46 000 cell wall peroxidase. If the rise in pH is prevented by strongly buffering the medium with buffers such as 50 mM MES (2-[N-Morpholino]-ethanesulphonic acid) or potassium phosphate at the original pH of the medium

(pH 5.8), then the oxidative response to the fungal elicitor is more or less completely inhibited (Table 1).

The relationship between pH change and the elicitor-induced generation of H_2O_2 was further studied by treatment of cells with the ionophores monensin, nigericin and valinomycin for varying periods of time and comparing the effect with that shown by cells not treated with ionophore (Fig. 6). These ionophores have been previously shown to modulate the elicitor response in suspension-cultured cells of French bean in a dose-dependent way.[44] Pre-treatment of the cells with 10 μM monensin for 1 h completely abolished the oxidative burst. However, with prolonged incubation, the cells recovered and the release of the block resulted in the generation of hydrogen peroxide being somewhat amplified. Measurements of external pH, for both apoplast and medium, generated at the height of the burst clearly support the requirement for alkalinization. After 1 h preincubation the external environment proved to be too acidic in comparison with that observed in cells without pretreatment. Metabolism or sequestration of the ionophore with time resulted in increased alkalinization following elicitor-treatment and a concomitantly increased production of H_2O_2 upon addition of elicitor. Since monensin has an additional effect on endomembrane flow, the requirement for pH change was further investigated using the other ionophores. Nigericin (2 μM) completely inhibited the response in a similar way to monensin. Comparison of the wall pH before and at the time of the peak of the response in the control cells showed that there was no change in the relatively more acidic environment. Cells recovered with prolonged incubation and release of the block resulted in more alkaline conditions in response to elicitor and a consequently elevated production of H_2O_2. In contrast valinomycin permitted the alkalinization of the wall upon incubation for 1 h. However, in response to elicitor action, the pH did not change and there was no oxidative burst. As with the other ionophores, the cells recovered and showed an enhanced response corresponding to the high final pH in the wall. Recovery of the cells eliminates the possibility of a toxic mechanism for the effect of the ionophores in the short term incubations. It was checked that these ionophores did not interfere with H_2O_2 assay at the concentrations used. In summary, the generation of H_2O_2 clearly requires a pH change of about 0.5–1.0 units generally in the direction of alkalinization of the wall and in which the final pH attained is greater than 6.5.

The Origin of the Oxidative Burst and Relationship to Other Defence Processes

The advent of biotic stress brings about profound shifts in metabolism in plant cells. These responses can be identified as rapid events, such as ion fluxes and the production of active oxygen species and other free radicals followed by longer term effects requiring transcriptional activation. Although some evidence using different elicitors suggests that these events may not be causally related and can be separated,[15] other studies indicate that production of active oxygen species can influence the longer term responses involving activation, modification and turnover of products of increased transcription and translation.[2,12,13,18,45] Particularly prominent effects attributed to the production of hydrogen peroxide are the immobilisation of wall proteins[45] which in French bean is specific to a small subset of glycoproteins, most of which are hydroxyproline-rich (Wojtaszek P and Bolwell GP, submitted for publication) and, hypersensitive cell death[18] and the induction of glutathione S-transferase.

While there may be more than one origin for H_2O_2, evidence has been provided for the involvement of wall peroxidase in its generation in response to elicitor treatment of suspension-cultured cells of French bean. Two wall peroxidases which have been

isolated from endomembranes[46] and walls[27] of suspension cultured cells of French bean also show this phenomenon. However, one form M_r 46 000 FBP1 which has been immunolocalised to the wall[27,28] can generate H_2O_2 at neutral pH, since it has a pH optimum substantially lower than FBP2 and the model peroxidase from horseradish which show optima at pH 8.5–9.0 and relatively little activity at pH 7.2. Potentially the FBP1 could be the source of hydrogen peroxide. Furthermore, the pH curve for the generation of H_2O_2 by FBP1 with an optimum at pH 7.5 corresponds very closely with that observed for the cells. This pH dependence is very different for that seen *in vitro* for the reaction of H_2O_2 with luminol in the presence of the peroxidase alone indicating that the luminescence observed with the cells is not due to the reaction of the peroxidase and luminol with H_2O_2 derived from another source. It has also been demonstrated that transfer of the cells to a higher pH medium is sufficient to generate the oxidative burst. The requirement for pH change has been also demonstrated in response to elicitor and there is a remarkable correlation between the timing of the raising of the external pH and the generation of H_2O_2. Changing the external pH by use of monensin (an Na^+ ionophore), nigericin (a modulator of the K^+/H^+ antiporter) and valinomycin (a K^+ ionophore) eliminates the transient pH change and abolishes the generation of H_2O_2. More significantly, since the treatments may have multiple effects, the cells are able to recover and the restoration of the pH changes allows the oxidative burst to occur. All these experiments indicate that a requirement for the generation of hydrogen peroxide is a pH change of at least 0.5 units generally in the direction of alkalinization of the wall and in which the final pH attained is greater than 6.5.

A simple mechanism may therefore exist for the production of activated oxygen species from the cell wall of elicitor-treated plant cells. Elicitor molecules arrive at the plant surface where they bind or are endocytosed.[11,44,47] The unknown primary response leads to the influx of Ca^{2+} ions and a probable efflux of K^+ ions[4,29] and possibly other electrolytes and low molecular weight compounds. A number of these responses have been demonstrated in suspension-cultured cells of French bean.[44] These fluxes seem to be coupled with the movement of H^+ and lead to a transient alkalinization of the wall. A change in the binding of O_2 to the haem site of peroxidases occurs, leading to the formation of compound III (Fe^{II}-O-O) and subsequently superoxide, which can be initially reduced by oxidation of sulfhydryl groups on cysteines adjacent to the haem site. This model is compatible with the effect of the repeated addition of low amounts (1 μM) of cysteine to FBP2 or HRP which gives repeated transient bursts of H_2O_2 as opposed to the sustained burst in the presence of high concentrations of cysteine (data not shown). With a supply of reductant, which is at present uncharacterised, the generation of H_2O_2 can be sustained. However the pH change is transient and reacidification of the wall, as the cells recover from the initial stress, leads to a cessation of H_2O_2 production. This would constitute a rapid and regulated mechanism for the generation of peroxide as part of the early events during defence against pathogens in plants. It is however associated with electrolyte fluxes, the origin of which must now be considered amongst the initial events that remain obscure at the molecular level. These events are however probably additionally linked to gene activation which leads to the longer term production of antimicrobial materials. The work described indicates how all these events may be coordinated in the case in which cells and tissues retain their integrity.

There is another reason why the action of a superoxide generating NADPH oxidase is difficult to reconcile with the rapid production of H_2O_2 at the cell surface and in the wall. There is a conspicuous lack of evidence for extracellular SOD.[48] It may well be that the roles of intracellular SOD and catalase are to detoxify active oxygen species,

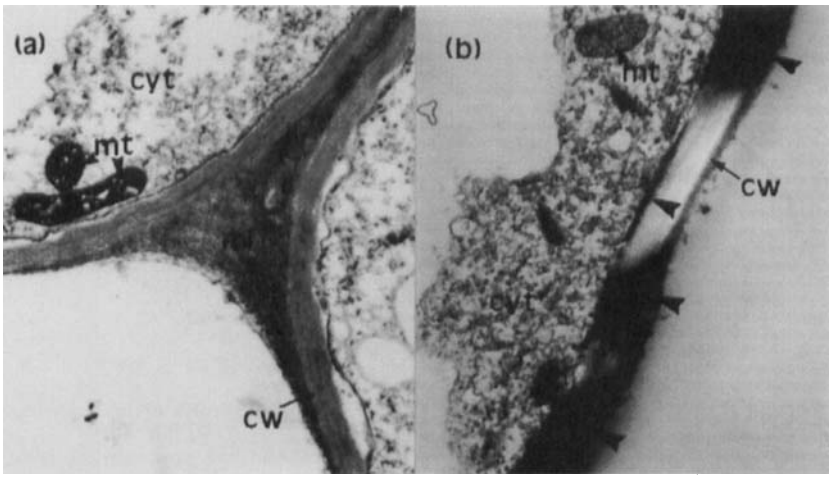

FIGURE 7 Comparison of the plasmalemma-wall interface in rapidly-fixed (a) unelicited cells and (b) cells 15 min after the addition of elicitor. Cells were harvested by gentle aspiration and rapidly fixed by vacuum infiltration with 4% (w/v) paraformaldehyde and 3% (v/v) glutaraldehyde in 50 mM Pipes/KOH buffer, pH 7.2 (1 h, RT), and post-fixed in 1% (w/v) OsO_4 in the same buffer for 1 h. Following dehydration in a graded series of ethanol, the cells were rinsed in propylene oxide, infiltrated for 16 h with propylene oxide:TAAB resin (TAAB Laboratories, UK) mixture (1:1; v/v), and with pure TAAB resin for 8 h. Finally, the cells were embedded in BEEM capsules, and polymerised for 24 h at 60°C. Sections were counterstained with uranyl acetate and lead citrate and observed on a Hitachi H 600 transmission electron microscope operating at 75 kV. Elicited cells show increased osmiophilicity of wall and plasmalemma interface (large bold arrows) at the height of the oxidative burst in comparison with control cells. cyt = cytoplasm; cw = cell wall; ml = middle lammella; mt = mitochondrion. (magnification = ×50 000).

while the production of these extracellularly, as part of the defence mechanism, results from the increase in pH and is allowed by the absence of SOD and catalase. The system is ultimately regulated by pH adjustment of the extracellular environment and a reversion of peroxidase to its more usual detoxifying role with respect to H_2O_2 and in production of cross linking in the wall. Membrane damage under these circumstances appears to be confined to the plasmalemma in cells producing H_2O_2 which becomes osmiophilic compared with other endomembranes when visualised in rapidly fixed cells (Fig. 7).

CONCLUSIONS

Study of the production of H_2O_2 in suspension cultured cells of French bean seems to favour a peroxidase origin rather than a plant equivalent of the phagocyte NADPH oxidase system. It has proved possible to demonstrate two of the components of the H_2O_2-generating system in French bean, a wall-bound peroxidase with the required properties and the transient alkalinisation of the wall. The third component, the reductant and its source remains obscure. However, we have recently developed methods which allow us to isolate the whole hydrogen peroxide generating system from the wall, including the reductant. We are currently attempting to stabilise and identify the reductant(s) responsible for sustaining the oxidative burst.

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