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A method to detect oxidative stress by monitoring changes in the extracellular antioxidant capacity in plant suspension cells☆

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Abstract

Detection of H_2O_2 in the supernatant of plant suspension cells is often used to indicate the time and extent of the oxidative burst during interactions with either bacteria or pathogen-related elicitors. We have found that suspensions of plant cells, depending on conditions, may produce considerable levels of extracellular phenolics that can function as antioxidants and prevent or suppress the detection of H_2O_2. These compounds can be used as substrates by extracellular peroxidases to scavenge stoichiometric amounts of H_2O_2. When this occurs during plant/pathogen interactions it can mask both the timing and extent of the oxidative burst if detection of free H_2O_2 is the only technique used. We have developed a chemiluminescent technique that will account for the H_2O_2 scavenged by these extracellular metabolites. A known quantity of H_2O_2 is added to samples and allowed to react with the extracellular antioxidants. The amount of H_2O_2 that remains is then determined by adding luminol to the sample and measuring luminol-dependent-chemiluminescence. The difference between treated and control samples represents the amount of H_2O_2 that has been produced by the cells in response to the treatment. We have found that this technique provides a better estimate of both the magnitude and timing of the oxidative burst in bacterial/suspension cell systems.
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Keywords: Reactive oxygen; Hydrogen peroxide; *Pseudomonas syringae*; *Nicotiana tabacum* L. cv. Hicks; *Solanum tuberosum* cv. Kennebec; Phenolics

1. Introduction

Plant/bacterial model systems incorporating suspension cells continue to provide unique insights into plant pathogenic interactions [4–6,8–10]. They have been especially valuable in gaining a better understanding of the role of oxidative metabolism in pathogenesis where reactive oxygen species and other metabolites are often transient and/or difficult to detect. For many years the oxidative burst has been detected by assaying for the appearance of H_2O_2 using sensitive chemiluminescent or fluorescent assays that linked directly to the free H_2O_2 present in the samples.[11–13,15]

Recently we found that suspensions of plant cells that are transferred to fresh assay buffer, produce substantial levels of extracellular antioxidants that can attenuate and often completely mask the oxidative burst in plant cells treated with bacterial pathogens [2]. It appears that as the oxidative burst occurs, the H_2O_2 associated with the event is continually scavenged by the cell wall peroxidases using the extracellular antioxidants as reductants. Unlike the cytoplasmic antioxidant mechanisms, which have considerable reserves of NADH or NADPH to regenerate antioxidants, the extracellular antioxidant mechanisms appear to be limited to the finite extracellular pool of ascorbic acid and phenolic antioxidants. The ascorbic acid in the cell wall region is capable of regenerating oxidized phenolics. There is evidence that the oxidized form of ascorbic acid, dehydroascorbate, is shuttled back to the cytoplasm and regenerated, however, this is a time dependent process [16,17,19,20].

We have developed a new technique that, (1) quantifies the constitutive and induced production of extracellular

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antioxidants by cell suspensions, and (2) provides a more complete estimation of oxidative events in suspension cells by accounting for reactive oxygen that has been scavenged by the extracellular antioxidants. In brief, a known amount of exogenous H_2O_2 is added to samples, allowed to react with the available extracellular antioxidant, and finally the amount of H_2O_2 that remains is determined. In untreated or control samples, the exogenous H_2O_2 consumed is directly related to the amount of extracellular antioxidant present. In treated samples that have undergone an oxidative burst, a portion of the extracellular antioxidants will have been oxidized and less exogenous H_2O_2 will be consumed by these samples. We have found this assay to give a more accurate estimation of the magnitude of the oxidative response of plants cells to pathogens and pathogen-related elicitors.

2. Materials and methods

2.1. Chemicals

Horseradish peroxidase (P-8250, purchased in units; one unit will oxidize 1 μmol of 2,2′azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) min^{-1} as described by Sigma), guaiacol (G-5502), hydrogen peroxide (H1009) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, A8511) were purchased from Sigma-Aldrich Chemicals Inc. (St Louis, MO, USA).

2.2. Plant material

Suspension cells of tobacco (*Nicotiana tabacum* L. cv. Hicks) and potato tuber (*Solanum tuberosum* cv. Kennebec) were maintained as previously described [2] except that tobacco callus and suspension cells were maintained on MS media (supplemented with 200 mg l^{-1} KH_2PO_4, 0.2 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin). Tobacco cultures, 10 ml, were transferred to 80 ml fresh media every 4 days. Potato cultures, 13 ml, were transferred to 80 ml fresh media every 7 days. Routinely, 2-day-old tobacco cells and 5-day-old potato cells were used for experiments. Cells were washed and suspended in assay buffer, containing 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 175 mM mannitol and 0.5 mM MES (pH 6) with a final cell density of 0.05 g ml^{-1}. Cell suspensions, 25 ml, contained in 50 ml beakers were equilibrated for 0.5 h in a rotary water bath shaker set at 27 °C and 180 rpm for tobacco cells or 200 rpm for potato cells. Treatments were added directly to the suspensions. All experiments were preformed twice with at least two replicates per treatment.

2.3. Bacterial preparations

Cultures of *Pseudomonas syringae* pv. *syringae* 61 were maintained as previously described [1]. Isolate WT (HR+) causes a hypersensitive reaction when infiltrated into tobacco and potato leafs. Isolate B7 (HR−) is a Tn5 insertion mutant that does not induce a hypersensitive response [3]. Bacterial cultures were grown for 20 h in Kings B broth, centrifuged, washed and suspended in deionized water. Based on optical density, the concentration of the suspension was adjusted with water so that addition of about 200 μl of the bacterial suspension to plant cell suspensions would result in 2×10^7 cfu ml^{-1}. Bacterial concentrations in cell suspensions were verified periodically by dilution plating.

2.4. Extracellular hydrogen peroxide assay

The luminol-dependent-chemiluminescent assay was used to detect extracellular H_2O_2 [11]. Using a wide-bore pipette, 0.45 ml samples of treated or untreated suspension cells were dispensed into tubes, and placed into a EG&G Berthold Autolumat 953 luminometer (Bad Wildbad, Germany). A stock solution was prepared containing horseradish peroxidase, 28.8 units ml^{-1}, and luminol, 1.7 mM, in 50 ml of 1 M sodium phosphate, pH 7. As each sample was ready to be assayed, the luminometer added 50 μl of the stock solution for a final concentration of 2.88 U ml^{-1} of peroxidase and 170 μM luminol. Chemiluminescence was recorded immediately as relative light units (RLU) every 0.1 s for 20 s. Under the standard conditions described here the peak height is proportional to the concentration of H_2O_2 in the sample. Standard curves were prepared with dilutions of H_2O_2 in assay buffer.

2.5. Extracellular antioxidant assay

The extracellular antioxidant capacity of cell suspensions was estimated by determining the amount of exogenous H_2O_2 consumed by samples. Because the antioxidant mechanism is rapid under the conditions described, it could be carried out using a modification of the luminol-dependent-chemiluminescent assay described above. A stock reagent of H_2O_2 contained 200 or 500 μM H_2O_2 in assay buffer for potato or tobacco samples, respectively. The H_2O_2 reagent, 50 μl, was automatically added to suspension samples, 0.4 ml, in the luminometer 4.5 s prior to the peroxidase/luminol reagent, 50 μl. This extra time was sufficient for the H_2O_2 to react with endogenous cell wall peroxidase and the extracellular antioxidant. The amount of extracellular antioxidant can be estimated by calculating the difference in RLU between controls and treatments. Standard curves were prepared with dilutions of H_2O_2 in assay buffer.

When this assay is applied to an untested cell line, preliminary assays with untreated control cells need to be conducted. First, the amount of exogenous H_2O_2 that is required to completely oxidize the extracellular antioxidants needs to be determined empirically. We have found it best to have at least 10 μM H_2O_2 in excess at the end of

the monitoring period. If the exogenous H_2O_2 is completely consumed the amount of antioxidant will be underestimated.

2.6. Endogenous extracellular peroxidase assay

Much of the extracellular peroxidase activity of these cell suspensions was associated with the cell wall. This assay attempts to estimate the total extracellular peroxidase activity in cell suspensions. Using a wide-bore pipette, 0.5 ml samples of cell suspensions were added to beakers containing: 9.1 ml sodium phosphate buffer, 50 mM pH 6; 0.2 ml guaiacol, 0.4 mM; 0.2 ml H_2O_2, 0.4 mM. The reaction mixtures were shaken in a water bath, 27 °C, for a 15 min period. Aliquots were removed periodically, filtered through miracloth to remove particles, and their absorbance determined on a Beckman DU 650 spectrophotometer at 470 nm. The linear rate of increase in absorbance, $OD_{470}\ min^{-1}$, was used to monitor changes in peroxidase activity over time.

3. Results and discussion

A previous study demonstrated that cell suspensions of tobacco and potato produced extracellular phenolics that were able to react with H_2O_2 using cell wall bound peroxidase [2]. We will first show examples of how the technique reported here can be used to quantify both the production of these phenolics as well as the oxidative events that oxidize these phenolics. Subsequently we describe the critical parameters that should be considered in using this technique or adapting it to other cell systems.

3.1. Monitoring antioxidant capacity during tobacco/bacterial interactions

Changes in the extracellular antioxidant capacity of tobacco suspension cells either untreated or treated with *P. syringae* pv. *syringae* strains were followed over a 6 h period (Fig. 1). Hydrogen peroxide, 50 μM, was added to samples to react with the extracellular antioxidants and subsequently, additional peroxidase and luminol were added to react with the remaining H_2O_2. The peak chemiluminescence was recorded and by comparison to a standard curve, the approximate amount of H_2O_2 that had reacted with the extracellular antioxidant was determined (Fig. 1A). Therefore, as the extracellular antioxidants increase and react with more of the added H_2O_2, the chemiluminescence decreases. Untreated tobacco cells began accumulating extracellular antioxidants after about 1.5 h and by 6 h the concentration was sufficient to react with nearly 35 μM H_2O_2. Treatment of the tobacco cells with strain B7 (HR−), 0.2×10^7 cfu ml^{-1}, increased the antioxidant capacity to nearly 40 μM H_2O_2 by 6 h, slightly more than untreated cells. We have found that this induction of extracellular phenolics varies with the bacterial

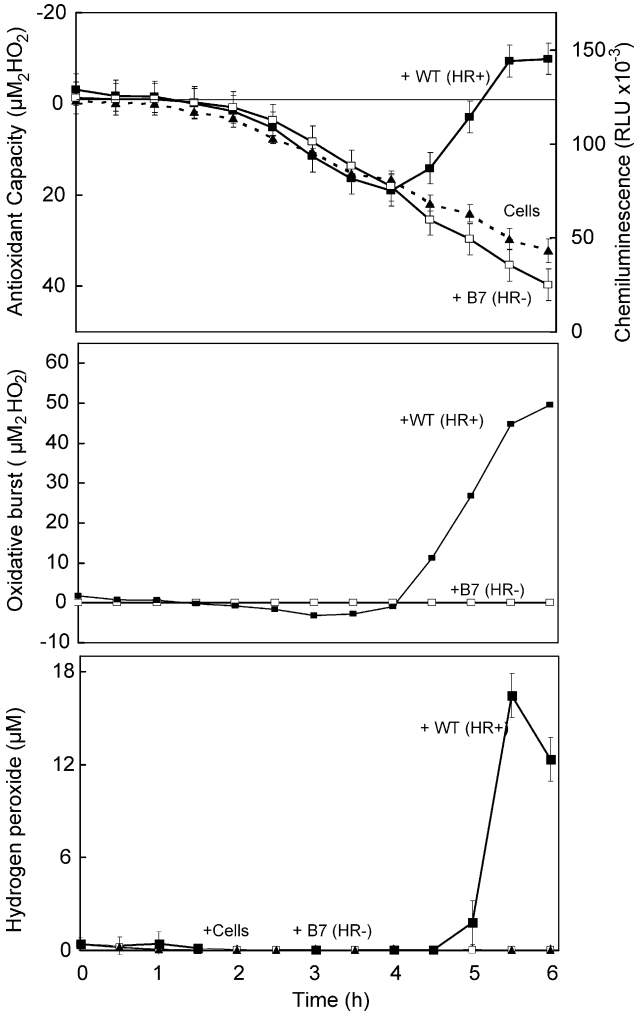

Fig. 1. Monitoring extracellular oxidative metabolism in tobacco suspension cells, 0.05 g ml^{-1}, treated with *Pseudomonas syringae* pv. *syringae* strains, 2×10^7 cfu ml^{-1}. (A) Determination of the extracellular antioxidant capacity in 0.4 ml samples by addition of 50 μM hydrogen peroxide to suspension samples and measurement of the unreacted H_2O_2 by luminol-dependent chemiluminescence. Chemiluminescence is expressed in relative light units (RLU). The concentration of H_2O_2 that remains is determined using a standard curve, and subtracted from the initial concentration of 50 μM to determine the antioxidative capacity of the sample. Antioxidant capacity is expressed in μM H_2O_2 equivalents. (B) Detection of the oxidative stress caused by the *P. syringae* pv. *syringae* strain WT (HR+) using strain B7 (HR−) as a control. The difference in antioxidant capacity between these treatments provides an estimate of the antioxidant concentration consumed by the oxidative burst in strain WT (HR+) treatments. Units are expressed in μM H_2O_2 equivalents. (C) Direct detection of free extracellular H_2O_2 in 0.45 ml samples using luminol-dependent-chemiluminescence. See Section 2 for details.

concentration (unpublished data). Cells treated with strain WT (HR+) behaved similarly to strain B7 (HR−) until about 4 h when the antioxidant capacity decreased and became negative (prooxidative) by 5–6 h. This indicated that H_2O_2 was being produced and by 5 h it reached a level greater than the extracellular antioxidants, therefore the amount of H_2O_2 detected was greater than the exogenous H_2O_2 added to the sample. Because bacteria-treated cells

produce higher levels of extracellular antioxidants, the difference in antioxidant capacity between the two bacterial strains, rather than untreated cells, is a better indication of the magnitude of the oxidative burst (Fig. 1B). By 6 h the oxidative burst reached the equivalent of 50 μM H_2O_2 per sample.

Free H_2O_2 was detected in samples after 5 h by a different chemiluminescent technique, which does not add exogenous H_2O_2 (Fig. 1C). This coincided with the approximate time where antioxidant capacity is zero (Fig. 1A) confirming that the extracellular antioxidants have been oxidized and any new production of extracellular phenolics is not able to completely scavenge newly produced H_2O_2.

3.2. Monitoring antioxidant capacity during potato/bacterial interactions

Changes in the extracellular antioxidant capacity of potato suspension cells treated with *P. syringae* pv. *syringae* strains were generally similar to the results of tobacco except that less antioxidant accumulated (Fig. 2). Because the antioxidant capacity was lower, only 20 μM H_2O_2 was added exogenously to potato samples. Untreated potato cells started accumulating extracellular antioxidants immediately and by 6 h produced levels sufficient to react with 10 μM H_2O_2 (Fig. 2A). Cells treated with either strain of *P. syringae* pv. *syringae* did not accumulate extracellular antioxidants until 1–1.5 h after treatment. Comparison of these samples with untreated cells suggested that a small oxidative event had occurred, coincident with the addition of the bacteria, and oxidized extracellular phenolics during the first 2 h period preventing their accumulation (Fig. 2 A and B). After about 2 h, cells treated with strain B7 (HR−) increased in antioxidant capacity at a faster rate than untreated cells and reached nearly 20 μM by 6 h. Cells treated with strain WT (HR+) underwent a second oxidative event equivalent to about 20 μM H_2O_2, after 5–6 h, when compared to B7 (HR−) treated cells.

Free H_2O_2 could be detected during the second oxidative burst using the luminol dependent direct detection technique without exogenous H_2O_2 (Fig. 2C). The H_2O_2 was detected over the entire period of the oxidative burst, whereas in tobacco, it was detected only after all the extracellular phenolics had been oxidized. Two factors may contribute to this earlier detection of free H_2O_2 in potato cells. Potato cells have lower endogenous peroxidase activity (discussed below) and lower concentrations of extracellular phenolics. These two factors directly contribute to a lower on-going in-situ rate of scavenging endogenous H_2O_2 by potato cells compared to tobacco cells. This lower rate of scavenging appears to increase the sensitivity of the H_2O_2 assay in potato by allowing the exogenous luminol and peroxidase to better compete for the endogenous H_2O_2.

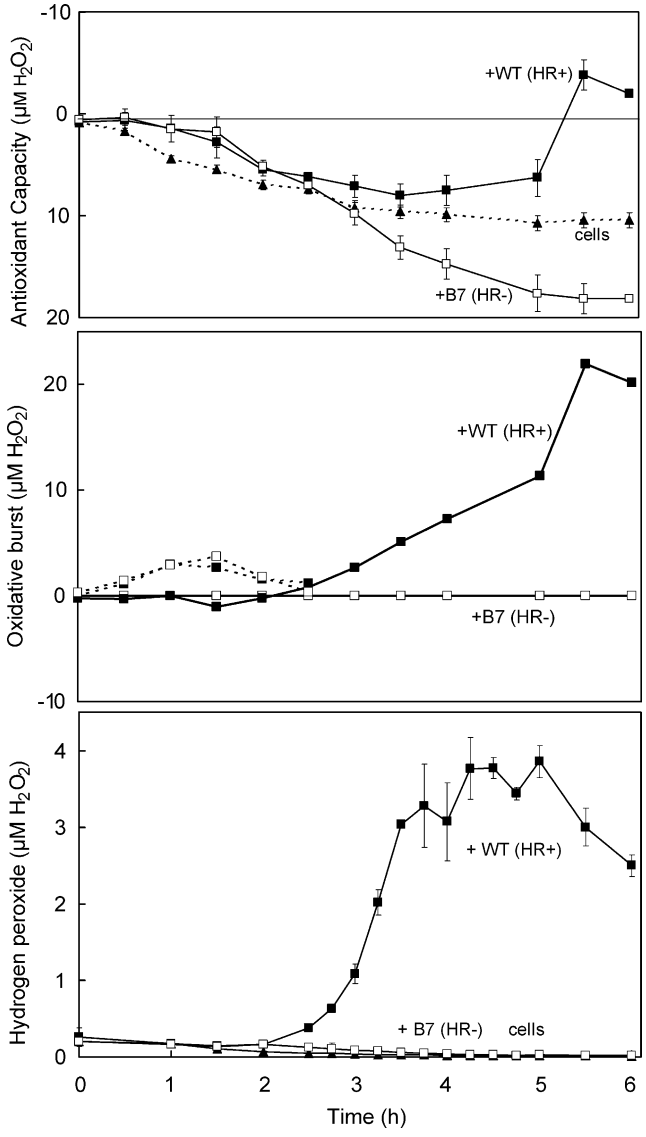

Fig. 2. Monitoring extracellular oxidative metabolism in potato suspension cells, 0.05 g ml^{-1}, treated with *P. syringae* pv. *syringae* strains, 2×10^7 cfu ml^{-1}. (A) Determination of extracellular antioxidant capacity in 0.4 ml samples by addition of 20 μM hydrogen peroxide to suspension samples and measurement of the unreacted H_2O_2 by luminol-dependent chemiluminescence. Units are expressed in μM H_2O_2 equivalents. (B) Detection of the oxidative stress in WT (HR+) treated cells by comparison to B7 (HR−) treated cells (solid lines). Detection of the oxidative stress during the first 2 h by comparing both bacteria treatments to untreated cells (dotted line; ■, WT (HR+); □, B7 (HR−)). Units are expressed in μM H_2O_2 equivalents. (C) Detection of free extracellular H_2O_2 in 0.45 ml samples using luminol-dependent-chemiluminescence. See Section 2 for details.

3.3. Monitoring antioxidant capacity during plant/heat-killed bacterial interactions

Fig. 3 shows the results of tobacco cells treated with heat-killed strain WT (HR+) bacteria, which previous studies [4] have shown elicits an oxidative burst. When heat-killed bacteria were added to cells at either 0 or 4.5 h, an oxidative burst could be detected. The first burst, 0 h, occurred

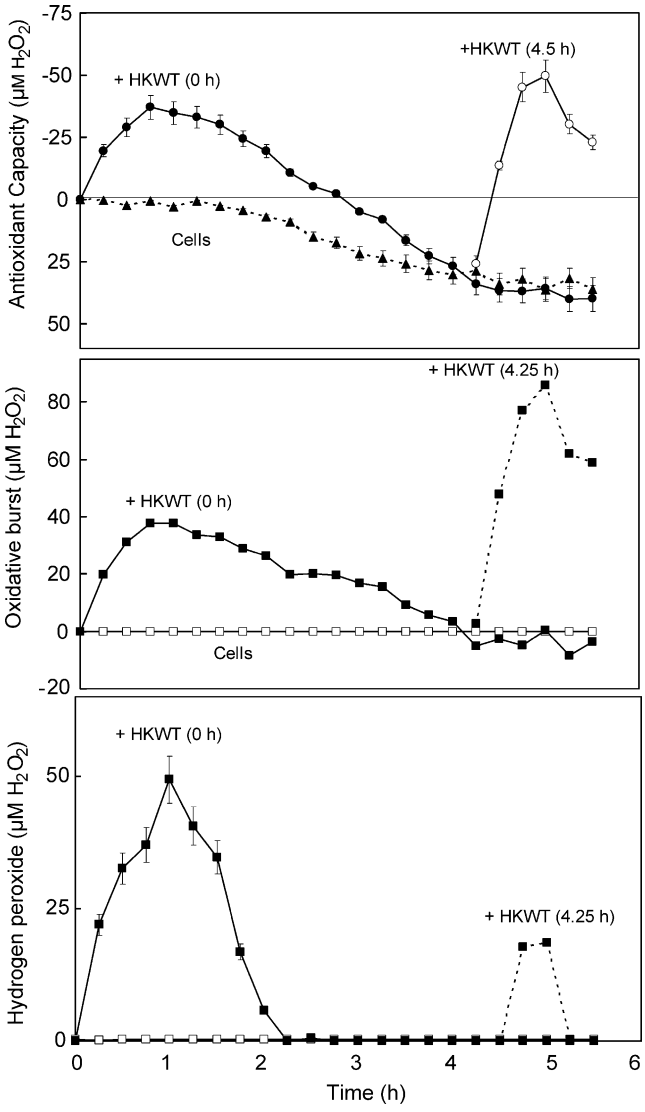

Fig. 3. Monitoring extracellular oxidative metabolism in tobacco suspension cells, 0.05 g ml^{-1}, treated with heat-killed *P. syringae* pv. *syringae* strain WT (HR+) added at 0 or 4.25 h. (A) Determination of extracellular antioxidant capacity by addition of 50 μM hydrogen peroxide to suspension samples and measurement of the unreacted H_2O_2 by luminol-dependent chemiluminescence. (B) Detection of the oxidative stress in HKWT-treated cell suspensions using untreated cells as a control. (C) Detection of free extracellular H_2O_2 in the suspension cells. Units are expressed in μM H_2O_2 equivalents.

immediately and peaked within an hour, after which the antioxidant capacity began to increase as extracellular antioxidants began to accumulate (Fig. 3A). The rate of accumulation was faster than untreated cells and by 4 h both untreated and treated cells had similar antioxidant capacities. Addition of heat-killed bacteria to untreated cells after 4.5 h also caused an immediate oxidative burst that peaked within an hour (Fig. 3A). Comparison of the 0 and 4.5 h treatments to untreated cells suggests that more H_2O_2 was produced by the 4.5 h response, 90 μM, than the burst at 0 h, 40 μM (Fig. 3B).

In contrast, direct detection of free H_2O_2 suggests that the oxidative burst at 0 h, 45 μm, was greater than the 4.5 h burst, 18 μM (Fig. 3C). This can be explained by the lower concentration of extracellular antioxidants at 0 h and therefore, the lower rates of scavenging during the first burst. The higher concentrations of extracellular phenolics at 4.5 h scavenge the H_2O_2 as it is being produced so that the concentration of free H_2O_2 remains lower. In addition as mentioned above, it appears that the luminol-dependent detection of H_2O_2 requires the luminol to compete with extracellular phenolics if present.

3.4. Parameters affecting the assay

The assay is based upon the peroxidase-dependent chemiluminescent reaction of luminol with H_2O_2. Fig. 4A shows a diagrammatic simplification of the enzyme reaction, which is complex and the subject of many mechanistic studies.[7,14,18] In reaction a, peroxidase (POX) is oxidized by H_2O_2 and loses two electrons. In each of the next two reactions, peroxidase gains back one electron from a donor substrate, which in this study would be either extracellular antioxidant or luminol. If the electron donor is luminol, light is generally produced from the unstable oxidized product (L*). We used a luminometer to measure the chemiluminescence, which corresponds to the near-steady state concentration of oxidized luminol, produced during the first 20 s of the reaction (Fig. 4B insert).

Under the conditions described here with H_2O_2 concentrations generally less than 100 μM and other assay reactants constant, the peak or maximum value of chemiluminescence is directly proportional to the H_2O_2 concentration (Fig. 4B). The extracellular antioxidant in suspension cells will reduce the chemiluminescence by scavenging of H_2O_2 (Fig. 4C). A final concentration of 50 μM H_2O_2 was added to tobacco suspensions that had been incubated for increased lengths of time and therefore contained increased amounts of antioxidant. The antioxidants, via endogenous peroxidase, reduce the hydrogen peroxide available for reaction with the luminol (Fig. 4C).

Slight variations in the concentration of luminol used in the present study, 170 μM, had minimal effect on the peak value of chemiluminescence under the current assay conditions (Fig. 4D).

The amount of chemiluminescence produced by this assay is also proportional to the peroxidase concentration as shown here with luminol, 170 μM, and H_2O_2, 10 μM, held constant (Fig. 4E). Because suspension cells contain endogenous cell wall peroxidase this must be considered for each cell line. Ideally the exogenous peroxidase should provide the majority of the enzyme for luminol oxidation to over shadow changes in endogenous peroxidase levels that may occur (Fig. 5). The traditional guaiacol-peroxidase reaction was adapted as described in the Section 2 to provide an estimation of the total extracellular peroxidase in cell suspensions including both soluble and cell wall bound enzyme. Using

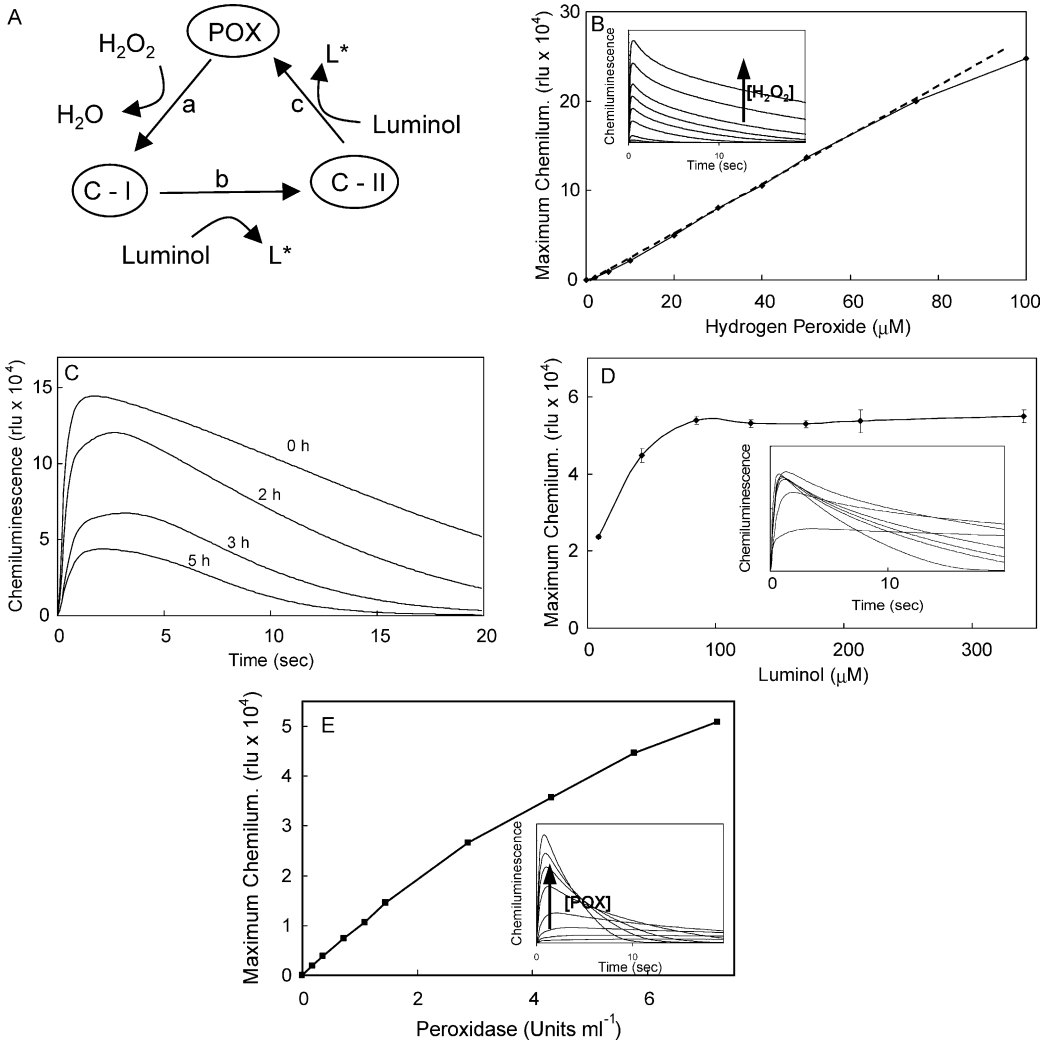

Fig. 4. The effect of various parameters on the luminol-dependent chemiluminescence reaction. (A) The three step peroxidase reaction: a, with the loss of 2 electrons, the native enzyme (POX) is oxidized to Compound I (C-I); b and c, C-I is reduced to Compound II (C-II) and subsequently back to POX, when it receives electrons from luminol. The loss of an electron by luminol produces an exited state (L*) that ultimately produces light. (B) The hydrogen peroxide concentration is varied in the presence of 170 μM luminol and 2.88 U ml^{-1} peroxidase per sample. The peak value produced during a 20 s reaction (insert) is plotted. (C) Chemiluminescence production when 50 μM H_2O_2, plus 170 μM luminol and 2.88 U ml^{-1} peroxidase, are added to tobacco cells that have been incubated in assay buffer for 0–5 h. (D) The luminol concentration is varied in the presence of hydrogen peroxide, 20 μM, and peroxidase, 2.88 U ml^{-1}. The peak value produced during a 20 s reaction (insert) is plotted. (E) The peroxidase concentration is varied in the presence of 170 μM luminol and 10 μM hydrogen peroxide. The peak value produced during a 20 s reaction (insert) is plotted.

potato and tobacco suspension cells it was apparent that peroxidase activity more than doubled over the 5–6 h monitoring period (Fig. 5). The increase in peroxidase was similar in untreated or bacteria treated cells for both potato and tobacco. The final endogenous peroxidase levels for potato and tobacco cells accounted for 5 and 10%, respectively, of the total peroxidase activity utilized in the luminol dependent assay. Bacterial pathogens at concentrations used in these experiments contributed less than 1% of the peroxidase activity. Therefore, when comparing various treatments, the slight changes in endogenous peroxidase should not greatly influence the results.

The production of phenolics and other antioxidants such as ascorbic acid in suspension cells can substantially affect the redox status of the extracellular region. The method described here provides a relatively simple and non-invasive means to quantify the extracellular antioxidants present in cell suspensions. In addition, by accounting for changes in the concentration of these antioxidants, this method can provide a better estimate of the timing and magnitude of oxidative bursts that may occur when cell suspensions are treated with pathogens or pathogen-related elicitors. The technique avoids the need to directly detect reactive oxygen species and accounts for scavenging that may have occurred. Variations of this concept can be used to detect oxidative events in other scenarios such as infection drops or intracellular fluid.

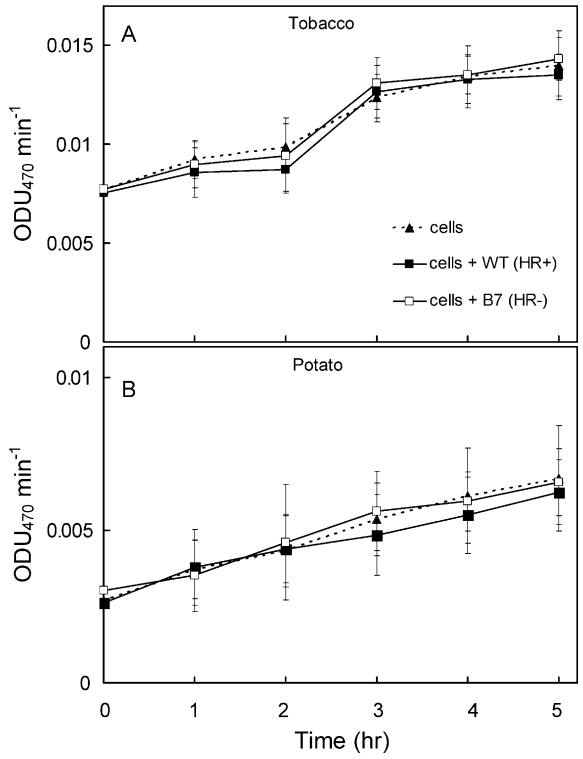

Fig. 5. Monitoring extracellular peroxidase activity in tobacco and potato suspension cells treated with with *P syringae* pv. *syringae* strains, 2×10^7 cfu ml^{-1} samples were incubated with guaiacol and H_2O_2 for 15 min, pH 6. The OD_{470} of the supernatant was determined every 2 min and the OD_{470} min^{-1} determined See Section 2 for details.

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