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A Noninvasive Technique for Monitoring Peroxidative and H_2O_2-Scavenging Activities during Interactions between Bacterial Plant Pathogens and Suspension Cells[1]

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Stimulation of active oxygen metabolism occurs during the early stages of interactions involving bacteria and plant cell suspensions. Although many cellular processes are known to affect active oxygen metabolism in plants, it is not known which of these factors affect active oxygen levels during plant-bacteria interactions. Extracellular peroxidases have been shown to participate in both the production and utilization of active oxygen species such as H_2O_2 and superoxide. Catalase and other scavenging mechanisms also affect the overall level of active oxygen. In this study the luminol-dependent chemiluminescent reaction previously used to measure H_2O_2 levels in suspension cells was modified to allow the assay of both peroxidase and H_2O_2-scavenging activity. The early stages of the interactions between tobacco (*Nicotiana tabacum*) and *Pseudomonas syringae* pv *syringae*, as well as between soybean (*Glycine max*) and *P. syringae* pv *glycinea*, were investigated. This method of monitoring peroxidase and H_2O_2-scavenging activity proved to be rapid, sensitive, and nonintrusive, allowing the processing of multiple samples using intact cells or cell-free preparations. The results from the study demonstrate that the scavenging activities can be significant and must be considered when studying active oxygen production in biological interactions.

As the study of plant-microbe interactions has become more molecular, there has been an increased use of specialized model systems to allow the study of specific phenomena. Studies of the bacteria-induced HR found that inoculation with incompatible species produces rapid plant cell death in cell suspensions similar to that found during the HR in whole plant tissues (Baker et al., 1993a; Baker and Mock, 1994). The use of suspension cells in addition to whole plants has allowed identification of early plant responses, which precede hypersensitive cell death (Atkinson et al., 1985b; Keppler et al., 1989; Baker et al., 1991, 1993a). One of these responses is the production of active oxygen. The response consists of a rapid, transient production a few minutes after addition of the incompatible bacteria followed by a second response 1.5 to 2.5 h later (Baker et al., 1991, 1993a; Orlandi et al., 1992). An immediate active oxygen burst is also produced when various pathogen-related "elicitors" are added to cells (Lindner et al., 1988; Apostol et al., 1989; Baker et al., 1993b). The mechanism of production and disappearance of this active oxygen and the parameters that affect it remain unclear.

Several species of active oxygen (O_2^-, H_2O_2, and $OH^\cdot$) result from the reduction of molecular oxygen, and there are numerous reactions possible that allow these species to interconvert (Elstner, 1987). H_2O_2, which has the longest half-life, provides a good estimate of the relative active oxygen level in the system. However, the level of H_2O_2 detected at a particular time can fluctuate dramatically, depending on its net rate of production versus its rate of degradation. Two factors that are critical to the immediate level of H_2O_2 are (a) peroxidase activity, which has been attributed with both production and utilization of H_2O_2, and (b) H_2O_2-scavenging mechanisms of both the plant and pathogen.

Plant peroxidases are known to play a number of roles in the plant cell. Cell-wall peroxidases have long been known to utilize H_2O_2 to oxidize phenolic alcohols into their free radical forms, which subsequently form lignin, often in response to pathogen ingress (Gross, 1977; Halliwell, 1978; Smith et al., 1991). These same extracellular peroxidases are also thought to mediate the formation of H_2O_2 under certain conditions through the oxidation of extracellular NAD(P)H (Elstner and Heupel, 1976; Halliwell, 1978; Mader and Amberg-Fisher, 1982; Ishida et al., 1987). This mechanism has recently been suggested to play a role in plant responses to polysaccharide elicitors (Lesney, 1990) and induced resistance (Peng and Kúc, 1992).

Numerous studies have associated changes in peroxidase activity with pathogenesis. Enhanced peroxidase activity associated with bacterial or fungal infection has been demonstrated to occur several hours or days after inoculation (Hammerschmidt et al., 1982; Kerby and Somerville, 1989) and was generally related to resistance and the development of lignin, phenolics, or the HR. Several studies investigated earlier changes in peroxidase using various elicitors. Changes in peroxidase isozymes have been noted

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Abbreviations: cfu, colony forming units; HR, hypersensitive response; LDC, luminol-dependent chemiluminescence.

in conjunction with increased phenolic metabolism within 12 h of adding pectic fragments to castor bean suspension cultures (Bruce and West, 1989) and within 4 to 8 h of adding *Phytophthora megasperma* f. sp. *glycinea* wall glucan to soybean cotyledons (Graham and Graham, 1991).

Fewer studies have monitored changes in H_2O_2-scavenging activity. Due to the ability of the neutral H_2O_2 molecule to pass across membranes, it is feasible that intracellular scavengers in either the plant or the pathogen may affect the active oxygen levels detected during plant-bacteria interactions. Catalase or ascorbate peroxidase have been reported to fluctuate in response to external stress conditions such as chilling, CO_2:O_2 ratios, or growth factors (Omran, 1980; Havir and McHale, 1989; Asada, 1992; Takahama, 1992). In a few cases it was shown that these changes occur over several hours or days. Bacterial catalase has also been shown to increase upon exposure to high concentrations of H_2O_2 (Klotz and Hutcheson, 1992) and has been implicated in facilitating colonization of plants by both soil and leaf pseudomonads (Katsuwon and Anderson, 1990, 1991).

In this study, we have adapted an LDC technique to monitor changes in peroxidative and H_2O_2-scavenging activities during the early stages of plant-bacteria interactions in suspension cells. Activity is quantified relative to light intensity rather than by relying on the oxidation of peroxidase substrates to colored products (i.e. guaiacol and pyrogallol), which can be unstable and form precipitates. Additionally, whole cells can be used and multiple samples can be processed in a relatively short time, in contrast to techniques that require tissue homogenization (Omran, 1980; Smith et al., 1991). The LDC assays are highly sensitive, allowing the detection of very low levels of activity with nonintrusive measurements that are completed within seconds.

MATERIALS AND METHODS

Chemicals

Peroxidase (EC 1.11.1.7) from horseradish, type II (approximately 220 units/mg), catalase (EC 1.11.1.6) from bovine liver (approximately 18,000 units/mg), H_2O_2 (30% solution, approximately 8.8 M), and luminol (5 amino-2,3-dihydro-1,4-phthalazine dione) were purchased from Sigma. H_2O_2 stock solution concentrations were periodically verified using $E_{240nm} = 43.6\ M^{-1}\ cm^{-1}$.

Bacteria

Pseudomonas syringae pv *glycinea* races 4 and 6 were maintained and prepared for assays as previously described (Orlandi et al., 1992). *P. syringae* pv *syringae* 61 (WT) and a Tn5 insertion mutant (B7) (Baker et al., 1987) were grown on Kings B agar with the antibiotics naladixic acid (25 μg/mL) or naladixic acid (25 μg/mL) plus streptomycin sulfate (40 μg/mL), respectively. For assays, these bacteria were prepared using the same method as for the *P. syringae* pv *glycinea* bacteria. One milliliter of bacteria was added to suspension cells for a final concentration of 1×10^7 or 1×10^8 cfu/mL.

Plant Cells

Soybean (*Glycine max* L. Merr. cv Mandarin) suspension cells were obtained and maintained as previously described (Orlandi et al., 1992). Tobacco (*Nicotiana tabacum* L. cv Hicks) suspension cells were derived from pith callus and grown as previously described (Atkinson et al., 1985a), with the exception of being transferred into fresh medium every 4 d. Both plant suspension cell types were prepared for assays as previously described (Glazener et al., 1991), using media adjusted to pH 5.7 for soybean and pH 6.1 for tobacco. Treated suspension cells (0.05 g/mL) were incubated on a rotary water bath shaker at 27°C and 150 rpm.

Peroxidase Activity

Peroxidase activity was monitored using LDC. Luminol stock solution (32.2 mM) was prepared by dissolving 5.7 mg/mL of luminol in 1 N NaOH. A working stock was prepared daily by adding 0.1 mL of luminol stock to 19.9 mL of 1 M sodium phosphate buffer, pH 7.0; 0.1 mL of working solution was added to 0.5-mL aliquots of the sample to be assayed for a final concentration of 23 μM luminol. H_2O_2 (88 μM in 0.2 M sodium phosphate, pH 7) was prepared from concentrated (30%) H_2O_2 daily; 100 μL was added to 0.5-mL aliquots to be assayed for a final concentration of approximately 15 μM. For peroxidase assays the luminol and H_2O_2 were added to samples simultaneously and the chemiluminescence was measured in an automated LKB 1251 luminometer (Glazener et al., 1991). The maximum chemiluminescence reading (mV) during a 30-s period was recorded. Changes in peroxidase activity could easily be detected using this technique, and actual peroxidase levels could be estimated by comparison to standard curves prepared with horseradish peroxidase (see Fig. 2).

H_2O_2-Scavenging Assays

H_2O_2-scavenging activity was estimated by measuring the loss of added H_2O_2 during a 1-min period. H_2O_2 was prepared as described above and was added to two 0.5-mL sample aliquots. Although this concentration generally provided an acceptable chemiluminescence output (about 5 V), the concentration used in a particular experiment occasionally varied somewhat, depending on the H_2O_2-scavenging activities of the plant-bacteria system. Horseradish peroxidase was prepared daily as a 100 μg/mL solution in water. Luminol and peroxidase (0.1 mL each) were added to aliquots either simultaneously with the H_2O_2 (0 min reading) or after a 1-min incubation. Catalase ranging from 0 to 1 μg/mL in assay medium was used for a standard curve. The maximum chemiluminescent reading over a 10-s period was recorded for both the 0- and 1-min readings and the difference reflected the H_2O_2-scavenging activity (see Fig. 3A).

The expected range of endogenous H_2O_2 during interactions between plant and bacterial cells is less than 2 to 3 μM (see Figs. 4 and 5). Although transient peaks, mainly in the HR-causing interaction, exceed this during the monitoring period, there is minimal effect on the overall peroxidase or

scavenging assays using the concentrations of H_2O_2, luminol, and peroxidase indicated above.

Active Oxygen Production

Active oxygen production was monitored by adding luminol and horseradish peroxidase to aliquots as previously described (Orlandi et al., 1992) to determine the level of H_2O_2 present in samples. The parameters vary slightly to maximize sensitivity to H_2O_2 production.

RESULTS AND DISCUSSION

Peroxidase

The chemical basis for the methods used in this study involves a complex LDC reaction summarized in Equation 1 (Cormier and Prichard, 1968).

$$\text{luminol} + H_2O_2 \xrightarrow{\text{peroxidase}} \alpha\text{-aminophthalate} + N_2 + \text{light (425 nm)} \quad (1)$$

Luminol in the presence of H_2O_2 and peroxidase at neutral pH is converted into α-aminophthalate, releasing nitrogen and a photon. The light produced from this reaction is easily measured by a luminometer that is capable of measuring the light radiating at all angles from the sample in the chamber. This mechanism was previously adapted for estimating H_2O_2 concentrations in suspension cells (Glazener et al., 1991) by adding a constant amount of peroxidase and luminol. By properly manipulating the parameters, this same mechanism can be used to estimate peroxidase activity by adding luminol and H_2O_2. To estimate the net H_2O_2-scavenging activity, a known amount of H_2O_2 was added and its degradation after 1 min was determined by adding luminol and peroxidase. The chemiluminescent nature of the reaction and the ability of the luminometer to measure light production from all directions allow these measurements to be done on intact suspension cells, thus providing a nonintrusive technique with excellent reproducibility. However, although luminol chemiluminescent assays have been used for many years in different capacities, it is very complex and any adaptations must be fully tested for anomalies such as those discussed below.

Peroxidase Assay Development

Since H_2O_2 must be added to samples to estimate peroxidase and H_2O_2-scavenging activity, preliminary in vitro studies were carried out to determine the saturating concentration of H_2O_2 and whether these higher concentrations might have significant oxidative effects on the enzyme and the luminol. An additional constraint in developing the assay was that the amount of H_2O_2 and/or peroxidase added to samples had to be balanced to prevent overloading of the luminometer. Using 0.02 μg/mL of horseradish peroxidase, which is within the range of peroxidase in the suspension cells, the enzyme became saturated at about 50 mM H_2O_2 (Fig. 1A). The control, containing no peroxidase, had a low level of chemiluminescence, which saturated at about 36 mM H_2O_2 and may reflect the nonenzymatic oxidation of luminol.

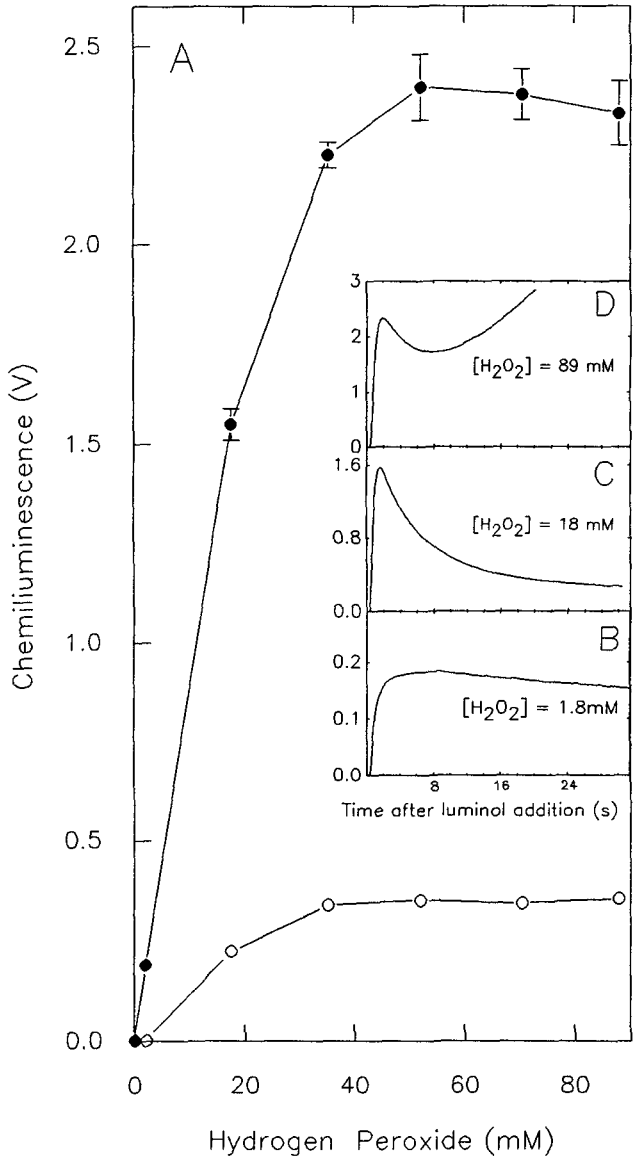

Figure 1. Effect of $[H_2O_2]$ on LDC in the presence (●) and absence (○) of peroxidase. The maximum output during the first 15 s after addition of the luminol was recorded for different H_2O_2 concentrations (A). The insets (B–D) indicate the actual luminometer output after luminol addition for selected H_2O_2 concentrations in the presence of peroxidase. The peroxidase concentration was kept constant at 0.02 μg/mL.

When adapting chemiluminescent techniques that utilize luminol, it is important to realize that the kinetics of the assay in the presence of peroxidase will differ at various concentrations of H_2O_2 (Fig. 1, B–D). At lower concentrations (1.8 mM), chemiluminescence increased over the first few seconds and then slowly declined at a fairly steady rate (Fig. 1B). However, at concentrations around 18 mM H_2O_2, the chemiluminescence increased more rapidly to a higher

level and then decayed more rapidly to background levels (Fig. 1C). The decrease in chemiluminescence was not due to limitations in luminol, which was saturating, and additional luminol had no effect on the reaction. One factor that is likely to be responsible for the decreasing chemiluminescence in the presence of higher H_2O_2 concentrations is the formation of the inactive form of peroxidase, compound III (Hayashi and Yamazaki, 1979; Adedrian and Lambier, 1989), as depicted in Equation 2.

(Peroxidase) compound II

$$+ H_2O_2 \rightarrow \text{compound III (active)} \quad (2)$$

$$\text{compound III} \rightarrow \text{compound I} + O_2^- \quad (3)$$

The reaction occurs when H_2O_2 is in great excess to peroxidase at neutral pH. At still higher levels, near 90 mM H_2O_2 (Fig. 1D), after the initial increase and decay, a subsequent increase occurs; this increase can be blocked by the addition of 0.1 mg/mL of superoxide dismutase (data not shown). At these higher levels of H_2O_2 it is feasible that superoxide is produced by release from compound III (Eqs. 2 and 3) or through further reactions with the heme group of the peroxidase (Cai and Tien, 1989). Superoxide can then react with the luminol nonenzymatically, which is a relatively slow reaction at neutral pH. The remainder of this study used H_2O_2 concentrations near or below 1 mM to minimize the formation of compound III and background chemiluminescence.

In the presence of 15 μM H_2O_2, horseradish peroxidase activity in the range of 0.1 to 10.0 μg/mL could be estimated by LDC (Fig. 2A). The standard curve of maximum chemiluminescence for this range of concentrations was linear and had very high reproducibility. The graph of the actual chemiluminescence output (Fig. 2B) indicated that the maximum level of chemiluminescence after luminol addition developed slowly at the lowest concentrations of peroxidase tested. By increasing the concentration of H_2O_2 to 1.25 mM and thereby increasing the rate of the reaction, the linearity between maximum chemiluminescence and peroxidase could be extended down to 0.02 μg/mL of peroxidase (Fig. 2C).

This assay does not attempt to measure all peroxidative activity of the sample but should provide estimates of and indicate changes in the nonspecific guaiacol-type peroxidative activity located in the cell wall region. Also, it must be kept in mind that the peroxidative activity measured here is a subset of the total scavenging activity, and therefore, both utilize H_2O_2. Although in the plant systems used in this study there was little or no interference, it is possible that in plant systems in which the scavenging activity is exceptionally high and the peroxidase activity is very low, interference caused by total scavenging of the added H_2O_2 could significantly underestimate the peroxidase component of the activity.

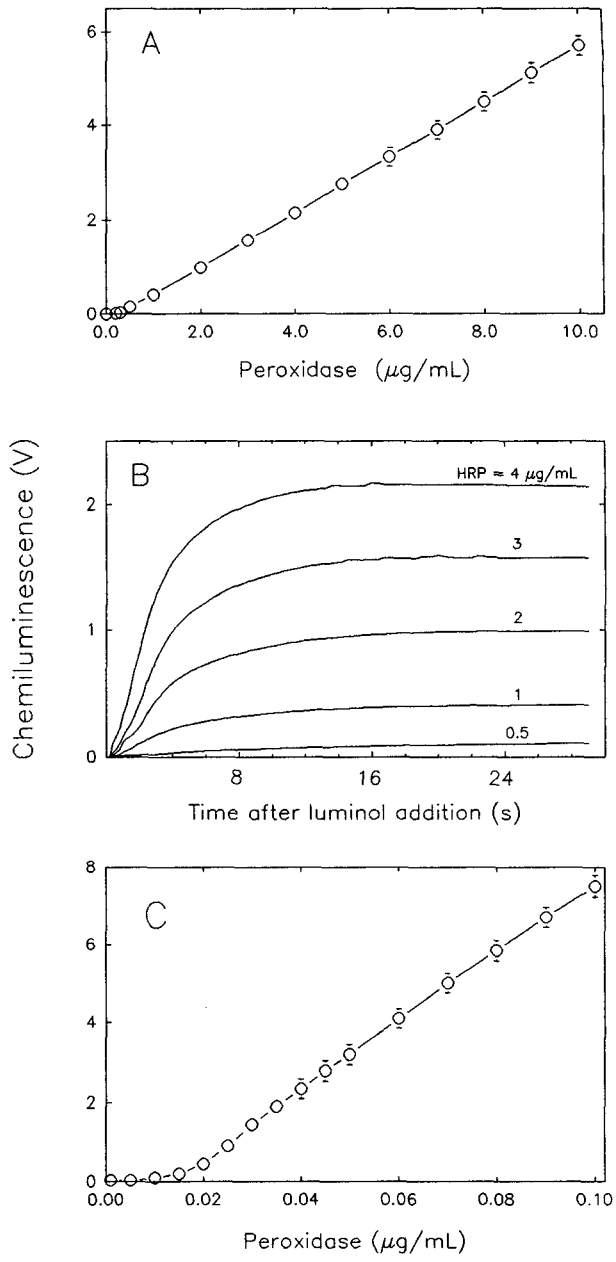

Figure 2. Standard curve for peroxidase activity as measured by LDC. The peak chemiluminescence for different amounts of horseradish peroxidase was plotted using a $[H_2O_2]$ of either 15 μM (A) or 1.25 mM (C). The actual luminometer output (B) is plotted for different amounts of horseradish peroxidase used in A.

H_2O_2-Scavenging Assay Development

An assay for monitoring H_2O_2-scavenging activity was developed by adding a known amount of H_2O_2 and monitoring the decrease after 1 min using LDC. By adding known amounts of catalase to assay medium, we found that the decrease in $[H_2O_2]$ between 0 and 1 min (Fig. 3, A and B) was linearly proportional to the amount of catalase present in the standards (Fig. 3B). When used with biological samples, the net "scavenging activity" estimated by this procedure would include all physiological activities that consume significant amounts of H_2O_2. Also, it is important to be aware that when developing a standard curve using catalase, which has a very high K_m, the in vivo levels

of substrate used to develop the assay are much below the concentrations normally used to determine enzyme activity. Therefore, the actual in vivo activity measured by this assay is much lower than the maximum in vitro activity. For example, the 1 μg of catalase used for the standard curve in Figure 3B had an activity of 20 μmol/min under in vitro conditions, but only 16 nmol/min under the conditions of the assay. The latter measurement would approximate the actual scavenging activity in vivo.

Monitoring Tobacco/*P. syringae* pv *syringae* Interactions

As previously reported, there are two phases of active oxygen production upon addition of incompatible (HR-causing) pathogens (Baker et al., 1991, 1993a). Phase I is nonspecific and occurs a few minutes after addition of either HR-causing (*P. syringae* pv *syringae*, WT) or non-HR-causing strains (*P. syringae* pv *syringae*, B7). Phase II occurs only with the HR-causing strains about 1.5 to 2 h after addition to the suspension cells (Fig. 4A). Interestingly, we have noticed that using a lower inoculum level (1×10^7 cfu/mL compared to 1×10^8 cfu/mL) elicits a much lower phase I response but a much larger phase II response (Fig. 4B). The increased phase I response with increased inoculum can be explained due to the presence of increased preformed elicitor (E.W. Orlandi, personal communication). However, the reason for the increase of the phase II response with lower inoculum was not so readily apparent. For this reason, peroxidase activity and H_2O_2-scavenging activity were monitored during this time period to assess their impact on active oxygen levels.

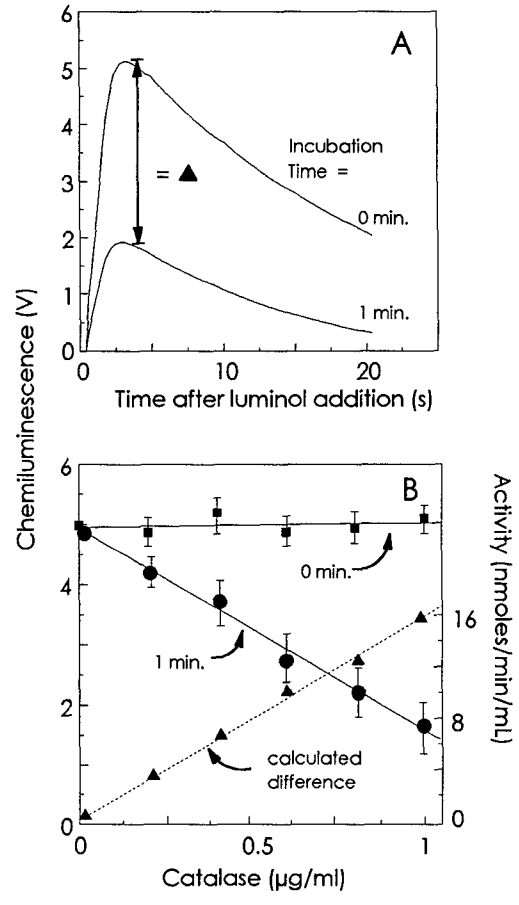

Figure 3. Standard curve for H_2O_2-scavenging activity by catalase. The continuous chemiluminescence (A) is shown for 1 μg/mL of catalase in 15 μM H_2O_2. Luminol and peroxidase (see "Materials and Methods") were added either immediately after addition of the H_2O_2 (upper trace) or 1 min later (lower trace). B, The change (▲) in the peak chemiluminescence at 0 min (■) and 1 min (●) after addition of H_2O_2 is plotted versus the catalase concentration.

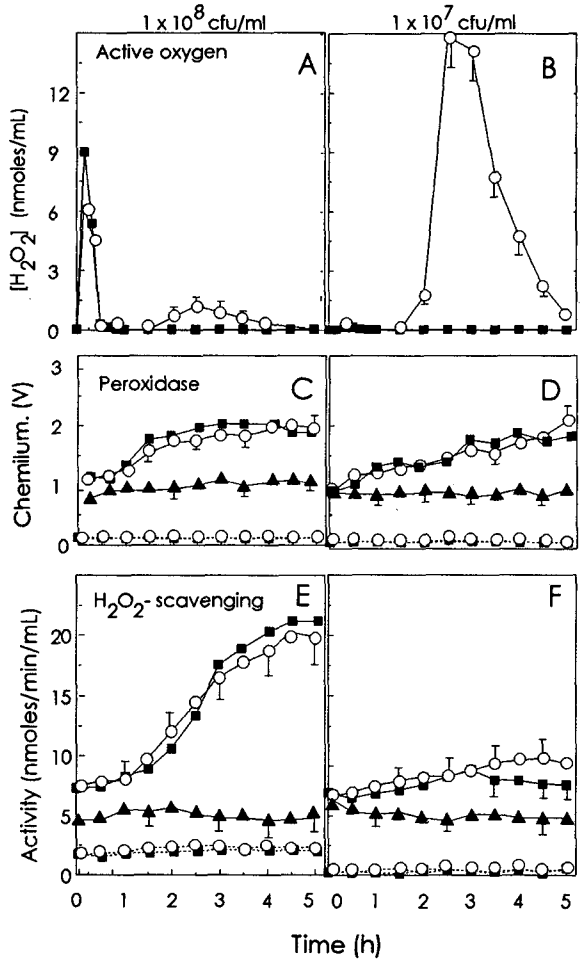

Figure 4. Monitoring tobacco suspension cells inoculated with *P. syringae* pv *syringae* (1×10^8 cfu/mL [A, C, and E]; 1×10^7 cfu/mL [B, D, and F]). Aliquots were monitored for active oxygen, peroxidase, and H_2O_2-scavenging activity as indicated. Suspension cells (—) were inoculated with the WT HR-causing isolate (○), the B7 non-HR-causing mutant (■), or buffer (▲). Bacteria in assay medium without suspension cells were also monitored (- - -) for H_2O_2-scavenging and peroxidase activity.

A comparable increase in peroxidase activity was observed in both the WT and B7 treatments at both inoculum concentrations (Fig. 4, B and C). The control cells did not show this increase and the bacteria controls showed negligible peroxidase activity.

The H_2O_2-scavenging activity assays, however, showed significant differences between the two inoculum concen-

trations tested (Fig. 4, E and F). Scavenging activity increased significantly after about 1.5 h in treatments with 1×10^8 cfu/mL of either WT or B7. Activities reached more than 16 units/mL. Increases in H_2O_2-scavenging activity after treatment with 1×10^7 cfu/mL bacteria were significantly less. Bacteria controls incubated without plant cells showed slightly elevated levels of H_2O_2-scavenging activity at the higher concentration; however, the level was not comparable to that seen in the plant-bacteria treatment. Therefore, the increased phase II response with lower inoculum appears to be due to the lower level of H_2O_2-scavenging activity induced by treatment with the lower concentration of inoculum.

The increased H_2O_2-scavenging activity observed with higher levels of bacterial inoculum could be due to increased scavenging activity in the bacteria and/or the plant. Because the higher inoculum levels induce a larger phase I response, it is conceivable that the increased H_2O_2-scavenging activity is in response to phase I active oxygen production. To determine whether the increased levels of H_2O_2 during phase I might induce this H_2O_2 scavenging in either source, we added 100 μM H_2O_2 to beakers of bacteria or suspension cells. This treatment did not induce increased H_2O_2 scavenging (data not shown). It is likely that the induction of increased H_2O_2 scavenging requires the presence of both bacteria and plant cells and does not appear to be elicited by phase I active oxygen production alone.

Monitoring Soybean-*P. syringae* pv *glycinea* Interactions

As was seen in the tobacco system, the phase II response in soybean cv Mandarin suspension cells was greater at 1×10^7 cfu/mL of the incompatible bacteria, *P. syringae* pv *glycinea* race 6 (Fig. 5B). The phase I response was significantly higher with 1×10^8 cfu/mL of either *P. syringae* pv *glycinea* race 4 or race 6 (Fig. 5A). Unlike the tobacco suspensions, however, the peroxidase activity decreased slightly in soybean cell suspensions for all interactions (Fig. 5, C and D). The increases in H_2O_2-scavenging activity were not as substantial as those observed in the tobacco system (Fig. 5, E and F). However, the increase was significantly greater in suspension cell cultures treated with 1×10^8 cfu/mL than in those treated with 1×10^7 cfu/mL *P. syringae* pv *glycinea* race 6 and could account for the lower phase II response detected.

In light of these estimates of scavenging activity for both the tobacco and soybean system, it is interesting to speculate on the actual production rate of H_2O_2. The peak rate of production, at about 2.5 h, must be greater than the scavenging activity of the 10^7 cfu/mL treatment, yet about equal to or slightly greater than the scavenging activity of the 10^8 cfu/mL treatment. This would be about 8 to 12 nmol min^{-1} mL^{-1} or 160 to 240 nmol min^{-1} g^{-1} for tobacco cells, and 2 to 6 nmol min^{-1} mL^{-1} or 40 to 120 nmol min^{-1} g^{-1} for soybean cells.

Using these assays we demonstrated that increases in peroxidase activity were independent of inoculum concentration in tobacco cells treated with *P. syringae* pv *syringae*. No increases in peroxidase activity were detected in any of

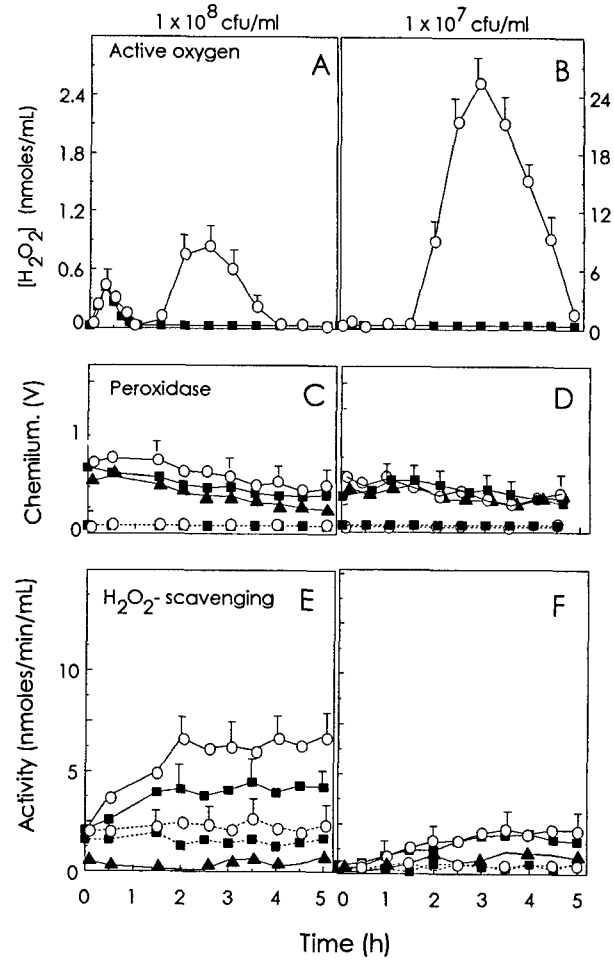

Figure 5. Monitoring soybean suspension cells inoculated with *P. syringae* pv *glycinea* (1×10^8 cfu/mL [A, C, and E]; and 1×10^7 cfu/mL [B, D, and F]). Aliquots were monitored for active oxygen, peroxidase, and H_2O_2-scavenging activity as indicated. Suspension cells (—) were inoculated with the *P. syringae* pv *glycinea* race 6 HR-causing isolate (○), the *P. syringae* pv *glycinea* race 4 non-HR-causing isolate (■), or buffer (▲). Bacteria in assay medium without suspension cells were also monitored (- - -) for H_2O_2-scavenging and peroxidase activity.

the interactions monitored in soybean suspension cells. However, H_2O_2-scavenging activity increased during the first few hours of the interaction of bacteria with both tobacco and soybean cells. The magnitude of the increase was greater with higher inoculum, which helps to explain why the phase II active oxygen response is lower after treatment of suspension cells with higher inoculum levels. These results demonstrate the necessity for careful monitoring of enzyme activities that affect active oxygen levels before reaching final conclusions concerning the production and role of active oxygen in plant-pathogen interactions. Increased scavenging activity might explain why phase II is not detected in studies in which high levels of inoculum are used (Devlin et al., 1992).

In summary, the methods described here involve adaptations of the LDC assay that allow the estimation of peroxidase and H_2O_2-scavenging activity in vitro as well as in

cell suspensions. These assays are rapid, highly sensitive, and nonintrusive, allowing us to monitor changes in these activities during plant-bacteria interactions.

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