

Phosphate Induces Rapid H₂O₂ Generation in Soybean Suspension Cells

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Abstract: Involvement of reactive oxygen species has been implicated in plant defence against pathogens. We report here a novel pathway of H₂O₂ generation induced by the addition of phosphate in soybean (*Glycine max* L.) cell suspension cultures. This H₂O₂ generation was initiated shortly after the addition of phosphate, and lasted only approximately one hour, as opposed to several hours observed during an attack by an avirulent strain of the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* (Psg). In addition, when cell cultures were treated with both phosphate and the avirulent pathogen, two distinct oxidative burst events were observed. In contrast to DPI-sensitive Psg-induced H₂O₂ generation, phosphate-induced H₂O₂ generation was insensitive to this NADPH oxidase inhibitor. This suggests that an NADPH oxidase-independent pathway may be involved in the phosphate-induced H₂O₂ accumulation, which could be involved in sensing of phosphate availability in the environment.

Key words: *Glycine*, hydrogen peroxide, oxidative burst, phosphate, *Pseudomonas syringae* pv. *glycinea*, soybean.

Abbreviations:

DPI: diphenylene iodonium

Introduction

Generation of reactive oxygen species in plants was first reported by Doke (1983^[4]) in aged potato tuber tissue inoculated with an avirulent race of the fungal pathogen *Phytophthora infestans*. Since then, this phenomenon of an oxidative burst has been reported in hypersensitive responses in many pathosystems (Lamb and Dixon, 1997^[7]; Wojtaszek, 1997^[13]). Some suggested cellular functions for the oxidative burst are oxidative cross-linking in the cell wall (Bradley et al., 1992^[2]) and activation of defence-related genes (Mehdy, 1994^[10]). In soybean cells inoculated with *Pseudomonas syringae* pv. *glycinea* (Psg) containing an avirulence gene *avrA*, a strong oxidative burst started 3–4 h after inoculation and was sustained for several hours, and hypersensitive cell death was attributed to the oxidative burst (Levine et al., 1994^[8]). In addition to this late-

phase (Phase II) oxidative burst, a weaker early-phase (Phase I) oxidative burst is often observed when cells are inoculated with either avirulent or virulent races of Psg. Phase I oxidative burst is not involved in race-cultivar specific resistance (Lamb and Dixon, 1997^[7]).

In our investigation, we detected the generation of H₂O₂ in soybean suspension cells following the addition of inorganic phosphate in the medium. Many molecules derived from invading pathogens or released from plant cell walls (elicitors) are known to cause an oxidative burst without pathogen infection (Wojtaszek, 1997^[13]). Oligogalacturonide is one such example and has been shown to trigger rapid release of H₂O₂ in soybean cells (Apostol et al., 1989^[1]). However, elicitation by inorganic phosphate has not been reported before to the best of our knowledge.

Materials and Methods

A suspension cell culture of soybean (*Glycine max* L.) cultivar Williams 82 was maintained at 25 °C in the dark on an orbital shaker (130 rpm) in Murashige–Skoog (MS) medium (Murashige and Skoog, 1962^[9]) supplemented with 2.22 μM 6-benzylaminopurine and 3 mg/L picloram. MS medium consisted of the following 13 salts and one sugar: KNO₃, 1900.00 mg/l; NH₄NO₃, 1650.00 mg/l; CaCl₂·2H₂O, 439.80 mg/l; MgSO₄·7H₂O, 370.60 mg/l; KH₂PO₄, 170.00 mg/l; FeNaEDTA, 36.70 mg/l; MnSO₄·4H₂O, 22.30 mg/l; ZnSO₄·7H₂O, 8.60 mg/l; H₃BO₃, 6.20 mg/l; KI, 0.83 mg/l; NaMoO₄·2H₂O, 0.25 mg/l; CoCl₂·6H₂O, 0.025 mg/l; CuSO₄·5H₂O, 0.025 mg/l; Sucrose, 30 000.00 mg/l. Cultures were transferred every 7 days by diluting five-fold in fresh MS medium, and experiments were performed 4–5 days after transfer.

Psg race 4, containing the avirulence gene *avrA* (Keen and Buzzell, 1991^[5]), was maintained at 28 °C on King's B medium (King et al., 1954^[6]) plates with 50 μg/ml streptomycin. Liquid cultures were prepared by inoculating King's B medium broth containing streptomycin with a single colony of plate cultures, and grown for 18 h at 28 °C with shaking. The overnight culture was centrifuged, and resuspended in sterile water for inoculation of soybean cell cultures. The final concentration of the bacteria in inoculated suspension cultures was adjusted to optical density A₆₀₀ = 0.1.

Treatments were performed on 900 μ l of soybean cell cultures grown in 12-well tissue culture plates with shaking at 70 rpm. One hundred microliters of various nutrients, 10 times higher in concentration than used in the regular MS medium, were added to the cell cultures (final concentrations equal to those used in the regular MS medium). DPI, K-252a, and staurosporine were purchased from Calbiochem (San Diego, California), and dissolved in DMSO and the final concentration was adjusted to 5, 1, and 2 μ M, respectively.

H_2O_2 generation by the cells was measured by the quenching of scopoletin fluorescence at 460 nm following excitation at 350 nm. In this highly sensitive and specific assay, fluorescing scopoletin is oxidized by H_2O_2 to non-fluorescing scopoletin (Root et al., 1975^[12]). Therefore, in this assay, we observe a loss of fluorescence with the generation of H_2O_2 . Briefly, 5 μ l of 2 mM scopoletin were added to the cell culture at various times, incubated for 5 min, and fluorescence was determined spectrophotometrically. This is a destructive sampling procedure, and scopoletin was added to the 1 ml samples each time the measurements were made. The scopoletin quenching was linear up to 80 μ M of H_2O_2 , tested in the present study.

Results and Discussion

We initially found that a common plant growth medium, MS, is a strong inducer of H_2O_2 generation in soybean suspension cells. MS medium contains sucrose and 13 salts. We observed that, despite the high concentration, sucrose does not contribute to H_2O_2 generation. Therefore, we removed one salt at a time from MS to identify the causal component(s) of H_2O_2 generation. When the total MS salts were added to the cell culture, H_2O_2 generation started in less than 30 min, whereas when the cells were inoculated with *Psg(avrA)* alone, the Phase II H_2O_2 generation started much later (Fig. 1). A Phase I oxidative burst was also observed when the cells were inoculated with *Psg*. When the cells were treated with both the MS salts and the pathogen, two distinct H_2O_2 accumulation patterns were observed. However, when KH_2PO_4 was omitted from MS salts, the H_2O_2 accumulation pattern was comparable to that of *Psg(avrA)* alone (Fig. 1). All of the other 12 salts in MS medium were also tested by omitting each individually, and we observed that the time courses of H_2O_2 generations were essentially the same as that of MS. H_2O_2 generation at 30 min for all omission treatments are shown in Fig. 2. We observed a reduction in the *Psg(avrA)*-induced oxidative burst when the cells were co-treated with salts (Fig. 1, between 2 h and 5 h). This might be due to the osmotic repression of bacterial *hrp* genes necessary for delivery of *avr* gene products (Rahme et al., 1992^[11]).

Each component was also tested as a single salt for the ability to cause an oxidative burst at the concentration prescribed for the MS medium. Only KH_2PO_4 (1.25 mM, pH 4.8) caused H_2O_2 generation with identical kinetics as observed for total MS salts (KH_2PO_4 in Fig. 3, data for other components not shown). Another phosphate, K_2HPO_4 (1.25 mM, pH 7.9), which is not a component of MS salts, caused similar H_2O_2 generation (Fig. 3). The quenching of scopoletin is not the result of a chemical interaction between phosphate and scopoletin because the incubation of scopoletin with phosphate without cells did not reduce fluorescence (data not shown). In addition, the time course of quenching of scopoletin fluorescence shows

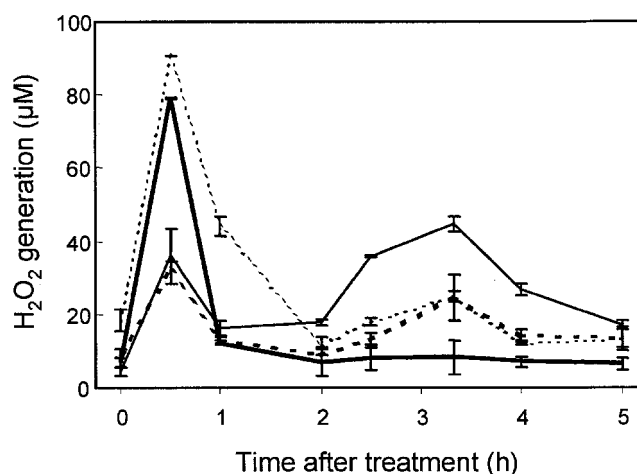


Fig. 1 H_2O_2 generation in soybean cells with *Psg(avrA)* and/or MS salts. H_2O_2 production was measured by quenching of scopoletin fluorescence over the background of the water control. H_2O_2 generations were even observed at time zero, because the measurements were taken immediately after, rather than before the treatments. Bold solid line, total MS salts; Regular solid line, *Psg(avrA)*; Regular broken line, total MS salts and *Psg(avrA)*; Bold broken line, MS salts minus KH_2PO_4 plus *Psg(avrA)*. The data represent the means of three replications, and bars indicate standard errors of the mean.

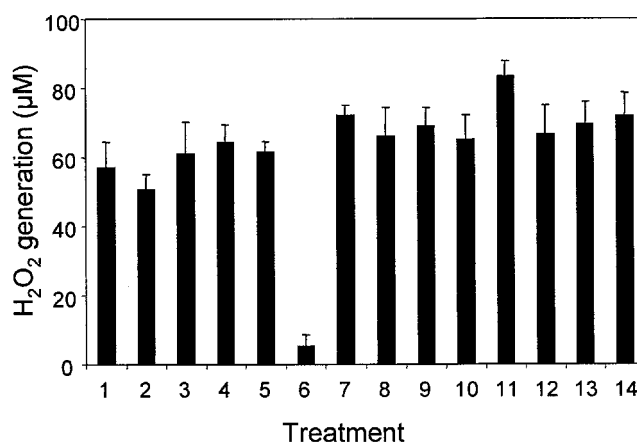


Fig. 2 Identification of the component responsible for MS salts-induced H_2O_2 generation in soybean cells by omission of one salt at a time. The measurement was made 30 min after the treatments. The data are the means of three replications and bars represent standard errors of the mean. 1, Complete MS; 2, MS - KNO_3 ; 3, MS - NH_4NO_3 ; 4, MS - $CaCl_2 \cdot 2H_2O$; 5, MS - $MgSO_4 \cdot 7H_2O$; 6, MS - KH_2PO_4 ; 7, MS - $FeNaEDTA$; 8, MS - $MnSO_4 \cdot 4H_2O$; 9, MS - $ZnSO_4 \cdot 7H_2O$; 10, MS - H_3BO_3 ; 11, MS - KI ; 12, MS - $NaMoO_4 \cdot 2H_2O$; 13, MS - $CoCl_2 \cdot 6H_2O$; 14, MS - $CuSO_4 \cdot 5H_2O$.

a peak in phosphate-treated cells, indicating the biological nature of the phenomenon. The change in pH of the medium as the cause of H_2O_2 generation or scopoletin quenching is precluded because both acidic and alkaline phosphate solutions were able to cause similar kinetics of H_2O_2 generation. Moreover, the suspension culture is well buffered, and when monitored immediately after the phosphate addition, pH change was minimal.

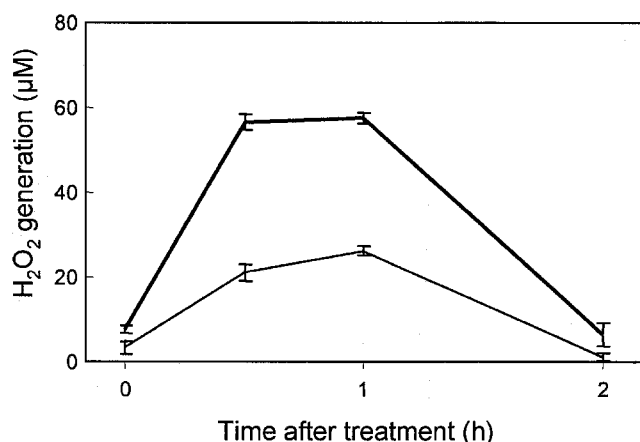


Fig. 3 H₂O₂ generation by addition of two different phosphate solutions in soybean suspension cell cultures. Soybean cells were treated with KH₂PO₄ (1.25 mM) or K₂HPO₄ (1.25 mM) solutions, and H₂O₂ production was measured by quenching of scopoletin fluorescence over the background levels of the water control. H₂O₂ generations were even observed at time zero, because the measurements were taken immediately after, rather than before the treatments. Data are means of three replications and bars represent standard errors of the mean. Bold line, KH₂PO₄; regular line, K₂HPO₄.

Table 1 Effects of the NADPH oxidase inhibitor DPI, and protein kinase inhibitors K-252a and staurosporine on phosphate- and *Psg(avrA)*-induced H₂O₂ generation. Samples for phosphate- and *Psg(avrA)*-induced H₂O₂ generation were collected 30 min and 3 h after treatment, respectively. Data are expressed as a percent of the control and represent means of three replications

Treatment	Scopoletin fluorescence (Percent of control ± SE)
Control	100.00
KH ₂ PO ₄	3.09 ± 0.04
KH ₂ PO ₄ + DPI	4.60 ± 0.54
KH ₂ PO ₄ + K-252a	106.64 ± 3.35
KH ₂ PO ₄ + staurosporine	119.68 ± 2.32
<i>Psg(avrA)</i>	21.60 ± 4.38
<i>Psg(avrA)</i> + DPI	146.51 ± 1.12
<i>Psg(avrA)</i> + K-252a	139.84 ± 1.72
<i>Psg(avrA)</i> + staurosporine	74.91 ± 2.42

Since the time courses of H₂O₂ generation were different in phosphate treated and infected cells, we were interested in learning if two independent pathways are involved in generating H₂O₂. It has been reported that the induction of an oxidative burst in soybean requires NADPH oxidase and protein phosphorylation (Chandra and Low, 1995^[3]; Lamb and Dixon, 1997^[7]). DPI, a suicide substrate inhibitor of NADPH oxidase (5 µM), or protein kinase inhibitors K-252a (1 µM) and staurosporine (2 µM) completely abolished a *Psg(avrA)*-induced oxidative burst in cultured soybean cells (Levine et al., 1994^[8]). These results were confirmed in our experiments (Table 1). However, phosphate-induced H₂O₂ generation was only marginally inhibited by 5 µM DPI (Table 1), indicating that there may be a DPI-insensitive mechanism that leads to H₂O₂ generation in response to the increase in phosphate concentration in the medium. As in the pathogen-induced oxidative burst, phosphate-induced H₂O₂ generation was completely abolished by K-252a and staurosporine (Table 1), suggesting the involvement of protein phosphorylation.

Phosphate plays critical roles in many physiological functions in plant cells, and thus phosphate availability must be monitored by the cells. It is conceivable that H₂O₂ is a signal for gene regulation in response to the change in phosphate concentration in the environment. Further work is necessary in determining the possible functions of the phosphate-induced H₂O₂.

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References

- 1 Apostol, I., Heinsteins, P. F., and Low, P. S. (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells: role in defense and signal transduction. *Plant Physiol.* 90, 109–116.
- 2 Bradley, D. J., Kjellbom, P., and Lamb, C. J. (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* 70, 21–30.
- 3 Chandra, S. and Low, P. S. (1995) Role of phosphorylation in elicitation of the oxidative burst in cultured soybean cells. *Proc. Natl. Acad. Sci. USA* 92, 4120–4123.
- 4 Doke, N. (1983) Involvement of superoxide anion generation in hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans*. *Physiol. Plant Pathol.* 23, 345–357.
- 5 Keen, N. T. and Buzzell, R. I. (1991) New disease resistance genes in soybean against *Pseudomonas syringae* pv *glycinea*: evidence that one of them interacts with a bacterial elicitor. *Theor. Appl. Genet.* 81, 133–138.
- 6 King, E. O., Ward, M. K., and Raney, D. E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44, 301–307.
- 7 Lamb, C. and Dixon, R. A. (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 251–275.
- 8 Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994) H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79, 583–593.
- 9 Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497.
- 10 Mehdy, M. C. (1994) Active oxygen species in plant defense against pathogens. *Plant Physiol.* 105, 467–472.
- 11 Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. (1992) Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 174, 3499–3507.
- 12 Root, R. K., Metcalf, J., Oshino, N., and Chance, B. (1975) H₂O₂ release from human granulocytes during phagocytosis: documentation, quantitation and some regulating factors. *J. Clin. Invest.* 55, 945–955.
- 13 Wojtaszek, P. (1997) Oxidative burst: an early plant response to pathogen infection. *Biochem. J.* 322, 681–692.

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